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Dissertation

Establishment of a High-Yield Phosphorylation Protocol
for Hsc70 and Analysis of the Structural Consequences
of Thr495 Phosphorylation

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III. Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AppNHp	Adenosine 5'-(β,γ -imido)triphosphate
aSEC	analytical size-exclusion chromatography
ATP	adenosine triphosphate
BiP	binding immunoglobulin protein
BLI	bio-layer interferometry
CCV	clathrin-coated vesicle
CK2	protein kinase 2
CK2 α'	protein kinase 2 alpha prime
CMA	chaperone-mediated autophagy
Da	Dalton
EDTA	ethylenediaminetetraacetic acid
EEF1D	Eukaryotic translation elongation factor 1 delta
ELISA	enzyme-linked immunosorbent assay
EPK	eukaryotic protein kinase
ER	endoplasmic reticulum
eSTKs	eukaryotic-like serine/threonine kinases
Fic	filamentation induced by cyclic-AMP
FicD	filamentation induced by cAMP domain-containing protein D
FRET	fluorescence resonance energy transfer
GFP-tag	green fluorescent protein tag
HAP28	Huntingtin-associated protein 28
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
high-MW	high-molecular-weight
His ₆ -tag	histidine-tag
HMW	high-molecular-weight
Hsc70	heat shock cognate 70
Hsp70	heat shock protein 70
HYPE	Huntingtin yeast interacting protein E
IDT	Integrated DNA Technologies
IEX	ion-exchange chromatography
IMAC	immobilized metal affinity chromatography
iMS	intact mass spectrometry
IPTG	isopropyl- β -D-thiogalactopyranoside
ITC	isothermal titration calorimetry
JDP/ J-proteins	J-domain proteins
KLD	(Kinase, Ligase and DpnI
LB	Luria-Bertani
LCV	Legionella-containing vacuole
LegK	Legionella kinase

methyl-TROSY	methyl transverse relaxation-optimized spectroscopy
MS	mass spectrometry
MW	molecular weight
MWCO	molecular weight cut-off
nanoDSF	nano differential scanning fluorimetry
NBD	nucleotide-binding domain
NCBI	National Center for Biotechnology Information
NEF	nucleotide exchange factor
NMR	nuclear magnetic resonance spectroscopy
NTA	Ni ²⁺ -nitrilotriacetic acid
OD ₆₀₀	optical density at 600 nm wavelength
p(r)	distance distribution
PCR	polymerase chain reaction
PDB	Protein Data Bank
pg	prepacked gravity column
pHsc70	T495 phosphorylated Hsc70
PMSF	phenylmethanesulfonyl fluoride
PTM	post-translational modification
R _g	radius of gyration
R _{max}	maximum intraparticle distance
RMSD	root-mean-square deviation
rpm	revolutions per minute
RT	room temperature
SAXS	small-angle X-ray scattering
SBD	substrate-binding domain
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
Ser/Thr kinase	serine/threonine kinase
SLIC	sequence- and ligation-independent cloning
SOC	super optimal broth with catabolite repression
Strep-tag	Strep-tag II (streptavidin-binding peptide tag)
SUMO	Small ubiquitin-like modifier
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEV	Tobacco Etch Virus
Thermofluor/ DSF	differential scanning fluorimetry
T _m	melting temperature
TReND	thiol-reactive nucleotide derivatives
Tris	tris(hydroxymethyl)aminomethane
Twin-Strep-tag	tandem Strep-tag II streptavidin-binding peptide tag
ULP1	ubiquitin-like protease 1
UV	ultraviolet
WT	wild type

1 Zusammenfassung

Da Kinase-Substrat-Interaktionen transient sind, wurden bislang nur wenige Strukturen von Kinase-Substrat-Komplexen aufgeklärt. Daher wurden γ -phosphatmodifizierte ATP-Analoga als kovalente *Capture*-Reagenzien zur Stabilisierung transienter Kinase-Substrat-Komplexe untersucht. Eine effiziente Bildung binärer Addukte mit den Kinasen LegK4 und CK2 α' konnte mittels Kristallstrukturanalyse und intakter Massenspektrometrie nachgewiesen werden. Es konnte jedoch weder ein Phosphotransfer noch die Bildung eines ternären Komplexes nachgewiesen werden.

Die Phosphorylierung von Hsc70 an Thr495 durch LegK4 reduziert ATPase-Aktivität und Substratprozessierung, während die entsprechende AMPylierung in BiP vergleichbare Effekte ausübt. Die strukturelle Charakterisierung von an Thr495 modifiziertem Hsc70 war bislang jedoch aufgrund fehlender robuster präparativer Methoden zur Gewinnung ausreichend modifizierten und stabilen Proteins eingeschränkt. Im Rahmen dieser Arbeit wurde ein robustes präparatives Phosphorylierungsprotokoll entwickelt, das Milligramm-Mengen stabil phosphorylierten Hsc70 mit Modifikationsgraden von etwa 70 % für den Wildtyp und bis zu etwa 86 % für die ATPase-defiziente Mutante Hsc70^{T204A} liefert. Entscheidend waren die Minimierung der Kinasekonzentration, ein hohes ATP/ADP-Verhältnis sowie die effiziente Entfernung von LegK4, um eine kinasevermittelte Dephosphorylierung zu verhindern. Parallel dazu wurde ein präparatives AMPylierungsprotokoll mit hoher Ausbeute etabliert, das AMPylierungsgrade von etwa 63 % erreichte, wobei sich die effektive Entfernung von FicD als entscheidend für die Stabilität der Modifikation erwies. *Small-angle X-ray scattering*, analytische Größenausschlusschromatographie und limitierte Proteolyse zeigen konsistent, dass die Phosphorylierung an Thr495 die Oligomerisierung reduziert und das Konformationsensemble selbst unter ADP-gebundenen und nukleotidfreien Bedingungen in Richtung eines kompakteren, domänen-gedockten, ATP-ähnlichen Zustands verschiebt. *Nano differential scanning fluorimetry* zeigt nur geringe Auswirkungen der Phosphorylierung auf die globale thermische Stabilität. Insgesamt legen diese Ergebnisse nahe, dass die Phosphorylierung die Funktion von Hsc70 primär durch die Verschiebung von Konformationsgleichgewichten und Oligomerverteilungen moduliert und nicht durch eine Destabilisierung des Proteins.

2 Abstract

Because kinase-substrate interactions are transient, only a few kinase-substrate complex structures have been solved. Therefore, γ -phosphate-modified ATP analogs were explored as covalent capture reagents for stabilizing transient kinase-substrate complexes. Efficient binary adduct formation with the kinases LegK4 and CK2 α' was confirmed by crystal structure and intact mass spectrometry. However, no detectable phosphotransfer or ternary complex formation was observed.

In human Hsc70, phosphorylation at Thr495 by the *Legionella* effector kinase LegK4 has been reported to reduce ATPase activity and client processing, while AMPylation at the homologous site in the ER-resident Hsp70 BiP results in similar effects on chaperone function. Nevertheless, structural characterization of Hsc70 modified at Thr495 has been limited, largely due to the lack of robust preparative methods yielding sufficiently modified and stable protein. In this work, a robust preparative phosphorylation protocol was developed that yields milligram quantities of stably phosphorylated Hsc70 (pHsc70) with modification levels of ~70% (wild-type) and up to ~86% for the ATPase-deficient mutant Hsc70^{T204A}. Critical parameters included minimizing kinase concentration, maintaining high ATP:ADP ratios, and efficient removal of LegK4 by affinity chromatography to prevent kinase-mediated dephosphorylation. In parallel, a high-yield AMPylation protocol was established, achieving ~63% AMPylation. Here, efficient depletion of FicD proved essential to maintain modification stability. Structural and biophysical characterization focused on pHsc70. Small-angle X-ray scattering, analytical size-exclusion chromatography, and limited proteolysis consistently demonstrate that phosphorylation at Thr495 reduces oligomerization and shifts the conformational ensemble toward a more compact, domain-docked, ATP-like state, even under ADP-bound and nucleotide-free conditions. Nano differential scanning fluorimetry measurements indicate that phosphorylation has only a minor effect on global thermal stability. Together, these results suggest that phosphorylation modulates Hsc70 function primarily by altering conformational equilibria and oligomeric distributions rather than by destabilizing the protein.

3 Introduction

3.1 Hsc70 and the Hsp70 family of chaperones

Proteins are essential for virtually all aspects of cellular structure and function. Their activity depends on acquiring and maintaining a defined three-dimensional structure. However, the crowded and often stressful cellular environment, together with the vast conformational space accessible to polypeptides, makes newly synthesized and pre-existing proteins inherently prone to misfolding and aggregation.

To mitigate this problem, cells employ a network of molecular chaperones and proteostasis factors that assist *de novo* folding, prevent aggregation, remodel partially unfolded or misfolded proteins, and, if necessary, target irreversibly damaged species for degradation [1]. Members of the (heat shock protein 70) Hsp70 family form a central hub within this chaperone network and will be introduced in more detail in the following sections.

Hsp70 homologues are conserved evolutionary. *Escherichia coli* encodes three Hsp70-like proteins, *Saccharomyces cerevisiae* (*S. cerevisiae*) contains 14 Hsp70 family members, and humans express 13 Hsp70 homologues [2]. In human cells, Hsp70 chaperones are distributed across different cellular compartments, and their sequences are highly conserved. For example, the stress-inducible Hsp70 and the constitutively expressed heat shock cognate 70 (Hsc70) are both cytosolic and share ~85 % sequence identity, whereas the endoplasmic reticulum (ER)-resident chaperone binding immunoglobulin protein (BiP) shares ~65 % sequence identity with the cytosolic Hsp70 family members. To date, no high-resolution structure of full-length human Hsc70 is available. However, numerous structural studies of other Hsp70 family members and homologous Hsc70 constructs indicate that the overall architecture and domain organization of Hsp70 chaperones are highly conserved [3]. Hsp70 chaperones consist of two major domains, an N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD), which are connected by a flexible linker region (Figure 1) [4]. The SBD itself is subdivided into the β -SBD, a β -sandwich structure that forms the client protein binding pocket, and an α -helical lid that covers this site. The NBD is responsible for binding and hydrolyzing adenosine triphosphate (ATP). When ATP is bound, Hsc70 interacts only

weakly with client proteins, corresponding to a low-affinity state [5]. Engagement of a client polypeptide stimulates the intrinsic ATPase activity of Hsc70, leading to hydrolysis of ATP and formation of the adenosine diphosphate (ADP)-bound state [4]. This nucleotide conversion triggers an allosteric rearrangement that is transmitted through the interdomain linker to the SBD, and the chaperone switches into a high-affinity conformation for its client [5]. Subsequent release of ADP, facilitated by nucleotide exchange factors (NEFs), and rebinding of ATP reset Hsc70 to the low-affinity state, which promotes dissociation of the bound client protein [4]. This nucleotide-driven allosteric cycle is a hallmark of Hsp70 chaperones and underlies their regulated activity [6].

Modulation of affinity during the allosteric cycle is mediated by conformational changes in the protein (Figure 1). In the ADP-bound state, the protein tends to be elongated with the linker region and SBD being detached from the NBD and the α -helical lid being closed on the β -SBD. Upon ADP release and ATP binding the protein adopts a domain docked conformation in which the linker and SBD make close contact with the NBD and the α -helical lid is detached from the β -SBD.

Although the overall structure and basic mechanism of Hsp70 chaperones are conserved and all rely on this allosteric cycle, different isoforms differ in their population of the individual conformational states, i.e. in their allosteric landscape [7]. For example, the human cytosolic isoforms Hsp70 and Hsc70 populate the domain-docked state almost completely in the ATP-bound form, whereas BiP occupies docked and undocked states more evenly and therefore shows a substantially lower fraction in the docked state [6, 8-10]. Conversely, in the ADP-bound state, BiP predominately adopts the domain-undocked conformation, whereas Hsc70 is roughly evenly distributed between docked and undocked states, and Hsp70 populates the undocked conformation only to a small extent [6, 8-10].

The differences in the allosteric landscape can be attributed, at least in part, to evolutionary divergence in amino acid sequence. For example, although the interdomain linker is a conserved structural element and is crucial for allosteric communication between the NBD and SBD, its exact amino-acid composition varies between isoforms, thereby modulating the allosteric behavior of individual Hsp70 family members [11]. Furthermore, so-called allosteric hotspots, residues that play a critical role in transmitting allosteric signals, have been identified within Hsp70s. Evolutionary divergence also

results in differences in substrate affinity among Hsp70s. While all Hsp70 chaperones preferentially bind to short, extended hydrophobic segments flanked by basic residues, their preferred targeted amino-acid types differ. For example, the cytosolic mammalian Hsp70 family members preferentially recognize aliphatic residues, whereas BiP appears to favor aromatic residues [12].

Another important regulatory layer is provided by co-chaperones. J-domain proteins (JDPs, or J-proteins) stimulate the basal ATP hydrolysis rate of Hsp70s and recruit them to specific client proteins or cellular processes, thereby allowing the same chaperone to participate in distinct functions (e.g. folding of nascent chains versus uncoating of clathrin-coated vesicles) [13]. NEFs represent a second major group of co-chaperones and accelerate ADP release from Hsp70 [2]. Given the high ATP/ADP ratio in the cell, this promotes ATP binding to the apo form of Hsp70 and thereby facilitates substrate release. Hsp70 proteins are also regulated at the level of gene expression. Cellular stresses that increase the load of unfolded or misfolded proteins (such as elevated temperature, infection, oxidative stress, dysregulated translation and protein aggregation) induce transcription and translation of Hsp70 family members. In the cytosol, for example, stress activates the heat shock response: under non-stress conditions, the transcription factor Hsf1 is kept in an inactive state through association with Hsp70, whereas upon stress Hsf1 is released and activates the expression of chaperone genes, including Hsp70s [14]. When proteostasis is restored, Hsp70 can rebind Hsf1 and thereby attenuate its transcriptional activity. A similar principle operates in the ER.

For both Hsc70 and BiP, higher-order oligomeric assemblies have been described, and these species have been suggested to act as inactive storage reservoirs that can be converted back into active monomeric chaperones when needed [15, 16]. Nucleotide-dependent effects on Hsp70 self-association are not uniform but differ between individual homologues and depend on the experimental conditions used. In the case of full-length Hsc70, the ADP-bound form populates a slowly exchanging mixture of monomers and higher-order oligomers, whereas ATP binding and the presence of peptides or unfolded client proteins shift this equilibrium towards smaller species *in vitro* [17]. Under nucleotide-free (apo) conditions, both Hsp70 and Hsc70 form heterogeneous mixtures of oligomeric species, showing that self-association in the absence of nucleotide is a common feature *in vitro* [18, 19]. In contrast, a more recent comparative study of several Hsp70

isoforms, including Hsc70, found that ATP promotes the formation of well-defined dimers and suggested that the tendency to form dimers is isoform-specific, with the stress-inducible Hsp70 displaying the largest dimer fraction under the tested conditions [20].

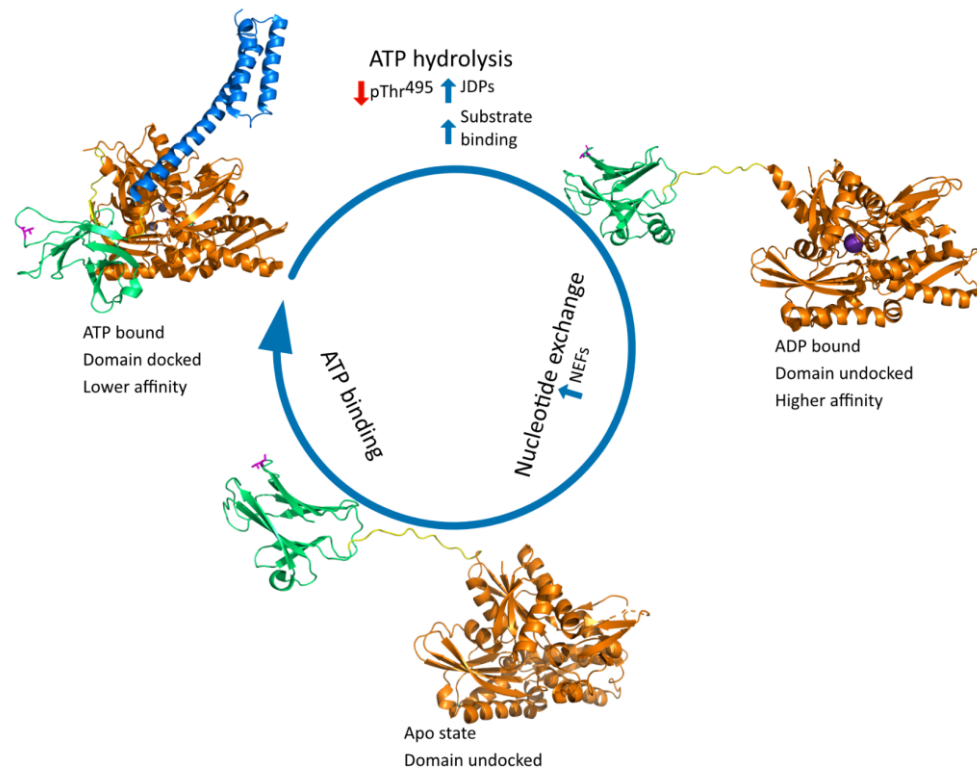


Figure 1: Allosteric cycle of HSP70 molecules

In the ATP-bound state, Hsp70 molecules generally adopt a domain-docked conformation with low substrate affinity (left panel). Upon ATP hydrolysis, the SBD and the interdomain linker detach from the NBD, resulting in a domain-undocked conformation with high substrate affinity (right panel). Hydrolysis is stimulated by J-domain proteins (JDs) and substrate binding. Depending on the modified residue and context, post-translational modifications (PTMs) such as phosphorylation at Thr495 (Hsc70 annotation) can reduce ATP hydrolysis. After nucleotide release, which is accelerated by nucleotide exchange factors (NEFs), the protein remains in the undocked state (bottom panel) until ATP binding restarts the cycle. Due to the lack of Hsc70 crystal structures in different nucleotide states, the crystal structures of the Hsc70 homolog BiP were used to illustrate these conformational states, including an ATP-bound full-length structure and ADP-bound and nucleotide-free lid-truncated structures that capture domain-undocked conformations. Top left: ATP-bound BiP (PDB entry 5E84). Top right: ADP-bound, lid-truncated BiP (PDB entry 7A4V). Bottom: lid-truncated, apo BiP (PDB entry 6HAB). The NBD is shown in orange, the β -SBD in green, the α -helical lid in blue, and linker regions in yellow. Coordinating ions are indicated in purple. Thr518 (BiP; corresponding to Thr495 in Hsc70) is highlighted in magenta. This figure and its caption were adapted from the cited publication [21].

3.2 Hsc70

Hsc70 is a constitutively expressed protein with important functions in unstressed cells. Under stress conditions, it cooperates with inducible Hsp70 to help restore cellular homeostasis. The functions of Hsc70 are manifold, and only selected key examples are highlighted here [22].

Hsc70 associates with ribosomes at the exit tunnel and binds nascent polypeptide chains via exposed hydrophobic segments [12, 23]. This interaction promotes correct folding and proper localization of newly synthesized proteins.

In addition, Hsc70 participates in the disassembly of protein aggregates as part of the cellular protein quality control system and is therefore implicated in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease [24].

Chaperone-mediated autophagy (CMA) is a selective pathway in which individual proteins are specifically recognized and imported to the lysosome for degradation [25]. In CMA, Hsc70 plays a central role by identifying CMA substrates via exposed sequence motifs related to the canonical KFERQ motif and by interacting with lysosome-associated membrane protein 2A at the lysosomal membrane [25].

Hsc70 is also a key factor in Clathrin-mediated endocytosis, a trafficking pathway in which cargo-containing vesicles are internalized from the plasma membrane into the cell [26]. Clathrin is important for forming the characteristic polyhedral coat using triskelions as the basic structural unit. Hsc70 functions in clathrin uncoating, with J-domain proteins such as auxilin recruiting and activating the chaperone [26, 27].

3.3 Protein phosphorylation and kinases

Protein phosphorylation is a process in which the γ -phosphate of ATP is transferred to a nucleophilic side chain (most commonly serine, threonine or tyrosine), forming a phosphoester bond. Comparable to phosphate monoesters, the phosphoanhydride bonds between the β - and γ -phosphates of ATP are kinetically stable in aqueous solution, so that efficient phosphoryl transfer to proteins requires enzyme-mediated catalysis by phosphotransferases such as protein kinases [28]. In line with that, the phosphoester bond is chemically inert at neutral pH, making phosphorylation a relatively long-lived signal that must be actively reversed by phosphatases, which hydrolyze the ester bond and thereby terminate the signal [28]. PhosphoSitePlus, a database that compiles experimentally observed post-translational modifications in mammalian proteins, currently reports phosphorylation for more than 20,000 distinct protein entries [29]. Phosphorylation of proteins can modulate protein stability, alter conformational equilibria, and affect interaction interfaces or ligand-binding sites [30]. As such, phosphorylation and its

regulation play a central role in cell signaling. The impact of phosphorylation on Hsp70 chaperones will be discussed in the following chapters.

3.3.1 Eukaryotic protein kinases

Eukaryotic protein kinases (EPKs) form one of the largest and best-characterized phosphotransferase families in eukaryotes. EPKs can be broadly divided into serine/threonine kinases, tyrosine kinases, and dual-specificity kinases based on the amino acid residues they phosphorylate. In the human genome, roughly 500 genes are predicted to encode protein kinases, corresponding to about 2 % of all protein-coding genes [31-33]. Of these genes, at least 303 have been shown to encode serine/threonine kinases (Ser/Thr kinases) and 90 to encode tyrosine kinases [33, 34].

Before structures of EPKs were available, a set of conserved sequence regions within the catalytic domain was defined, now commonly referred to as the Hanks subdomains [35]. These subdomains were later shown to play key roles in the structure and function of protein kinases, as became apparent when the first kinase structures were solved [36-40]. These structures revealed a conserved bi-lobed kinase core consisting of a smaller N-lobe, primarily involved in ATP binding, and a larger C-lobe that contributes mainly to substrate binding and catalysis [41]. The active site lies in the cleft between both lobes in which the adenine ring occupies the base of the cleft. Most residues that are conserved across the protein kinase family are concentrated in and around the active-site cleft.

ATP binding within the N-lobe involves several conserved structural elements. Among these, the glycine-rich loop (GxGxxG), which connects $\beta 1$ and $\beta 2$, forms part of the phosphate-binding pocket and helps clamp the β - and γ -phosphates together with a conserved lysine in the AxK motif on $\beta 3$ and catalytic Mg^{2+} ions [36, 42]. Furthermore, a hydrophobic residue in or immediately following the glycine-rich loop, often a valine (e.g. Val57 in PKA), makes hydrophobic contacts with the adenine base of ATP. The lysine in the AxK motif links the bound phosphates to the αC -helix, which plays a regulatory role by contacting the C-lobe and the activation loop/ activation segment [43, 44]. Many EPKs require activation-associated structural rearrangements to adopt a catalytically competent conformation, often promoted by phosphorylation within the activation segment. In contrast, the catalytic subunits of protein kinase CK2 (CK2), including protein kinase 2

alpha prime (CK2 α'), are constitutively active and do not depend on activation-loop phosphorylation for basal catalytic activity [45].

Canonical eukaryotic protein kinases share a conserved catalytic loop in subdomain VIb that contains an HRD-based motif. In Ser/Thr kinases this region typically conforms to an HRDLKPXN consensus, with the aspartate acting as the catalytic base and the downstream Lys/Arg contributing to substrate positioning [32].

Structures of kinases bound to short substrate peptides have been solved by X-ray crystallography [46-48]. In addition, mass spectrometry, protein microarrays, *in silico* approaches, live-cell imaging and FRET (fluorescence resonance energy transfer) have been used to probe kinase-substrate interactions [34, 49-51]. However, because kinases generally have relatively low affinity for their substrates and kinase-substrate complexes are transient, only a limited number of structures of kinases in complex with their protein substrates or adaptor proteins is available, and much of our knowledge of kinase-substrate recognition therefore is derived from indirect approaches rather than from structural biology methods at atomic resolution [49, 52].

Conserved features within the active site of Ser/Thr and Tyr kinases largely determine the preferred sequence context surrounding the phosphoacceptor residue [31]. The amino acid positions surrounding a phosphorylated residue are commonly denoted relative to the modification site, for example -3 for the third residue N-terminal and +1 for the first residue C-terminal to the phosphorylated residue. Together, these positions define the consensus sequence that determines substrate recognition by a given kinase [53-55]. Based on their primary sequence preferences around the phosphoacceptor, Ser/Thr kinases can be grouped into several subfamilies. Structural studies of peptide-bound kinases have revealed both shared principles and distinct modes of peptide binding among these groups, with kinases within a given subgroup generally displaying similar P+1 loop properties [48]. Thus, although the overall architecture of the P+1 pocket is conserved, it supports different sequence preferences towards peptide substrates in different kinase families [46-48]. For example, in the CK2 subgroup the P+1 loop contributes relatively little to overall substrate specificity but can favor negatively charged residues, whereas cyclin-dependent kinases often prefer a proline at the P+1 position [48, 54].

While the P+1 loop acts as an important specificity determinant, it usually does not provide sufficient interaction surface to mediate high-affinity binding on its own. Relatively

tight binding of substrates is typically achieved through additional contacts outside the catalytic cleft [48, 56]. These contacts can be formed either by auxiliary regions of the kinase (docking sites) or by adaptor proteins that bind simultaneously to the kinase and to its substrate [48, 57-59].

In summary, genome analyses and crystal structures of eukaryotic protein kinases have provided substantial insight into their overall architecture, conservation and modes of regulation. Despite considerable progress in understanding kinase structure and regulation, structural information on conventional kinases in complex with full-length protein substrates and/or adaptor proteins remains scarce. This is a major limitation, as interactions outside the catalytic cleft can be critical for substrate recognition and binding affinity.

3.3.2 Eukaryotic-like protein kinases in bacteria with focus on *Legionella pneumophila*

Genomic analyses have identified a group of bacterial kinases known as eukaryotic-like serine/threonine kinases (eSTKs) [60]. These kinases share most of the conserved Hanks motifs with EPKs as well as the overall bilobal architecture of the kinase core and key catalytic residues [60-62]. In many bacteria, eSTKs appear to represent relatively minimal kinase cores, in which the activation segment is shorter or less elaborated than in canonical EPKs and regulatory features such as activation-loop phosphorylation or extended docking elements are less prominent [63]. Consequently, their regulatory mechanisms are thought to be less elaborate than those of canonical EPKs [64-67].

One bacterium encoding eSTKs is *Legionella pneumophila*. *L. pneumophila* is a Gram-negative bacterium and the causative agent of Legionnaires' disease [68]. While the natural hosts are amoebae, human alveolar macrophages represent an accidental host [69, 70]. After uptake by phagocytosis, internalized bacteria are normally routed through a maturation pathway in which the nascent phagosome sequentially fuses with early and late endosomes and finally with lysosomes, leading to acidification and degradation of the ingested microbes [71]. This phagosome-lysosome fusion pathway is a central antimicrobial defense mechanism that allows the host cell to efficiently kill engulfed pathogens. *L. pneumophila* evades this fate by injecting more than 300 effector proteins into the host cell cytosol via its Dot/Icm type IV secretion system, thereby remodeling the

phagosome into a replication-permissive Legionella-containing vacuole (LCV) [72-76]. From the LCV, *L. pneumophila* manipulates the host cell and influences cell fate via its effector proteins. For example, it interferes with host autophagy [77], inhibits host protein translation [78], and alters ubiquitination pathways [79].

L. pneumophila encodes a family of eukaryotic-like serine/threonine kinases termed Legionella kinases (LegKs) [62, 80-83]. Of the known LegKs, the eSTK LegK4 most closely resembles canonical EPKs: its kinase domain contains all Hanks subdomains as well as the activation loop and a so called GHI subdomain [62]. In *L. pneumophila* strain Corby, LegK4 is a large 961-residue protein. In structural studies, N-terminally truncated constructs encompassing the kinase domain and flanking regions (e.g. residues 1-445) revealed a eukaryotic-like kinase domain flanked by a cap domain and a four-helix bundle [62]. The kinase domain adopts the typical bilobal fold with a well-ordered activation segment even in the absence of activation-loop phosphorylation. Binding of the non-hydrolyzable ATP analog Adenosine 5'-(β,γ -imido)triphosphate (AppNHp) to the active site is largely canonical, despite some unusual active-site residues [62]. When crystallized, LegK4 forms a dimer in which the two monomers interact via their kinase domains. Size-exclusion chromatography (SEC)-small-angle X-ray scattering (SAXS) experiments support that a similar dimer is present in solution, and the authors proposed that stabilization of the activation segment by this dimer interface contributes to constitutive LegK4 activity [62].

During *L. pneumophila* infection, LegK4 phosphorylates human Hsc70 on a conserved threonine residue in the substrate-binding domain, thereby reducing its refolding capacity and impacting host translation, as described in more detail in the following sections [84].

3.4 Regulation of the Hsp70 family by post-translational modifications

3.4.1 The chaperone code

With the improvement of methods to detect post-translational modifications (PTMs), their importance for regulating the Hsp70 family has become increasingly clear. By analogy to the histone code, this regulatory layer has been referred to as the chaperone

code, describing how distinct PTMs and their combinations shape chaperone function [85].

PTMs constitute an additional, often highly dynamic regulatory layer of chaperone activity that acts in concert with the mechanisms discussed above. In general, PTMs are reversible: dedicated enzymes write the modification (e.g. kinases, methyltransferases, acetyltransferases), whereas others erase it (e.g. phosphatases, demethylases, deacetylases) [86].

Hundreds of different PTMs have been identified on Hsp70 and related chaperones, including phosphorylation, AMPylation, methylation, acetylation, and ubiquitination [86]. These modifications can act in combination to tune the chaperone and co-chaperone response to cellular challenges, for example by changing subcellular localization, modulating the interaction of chaperones with co-chaperones and substrates, altering ATPase activity, or affecting chaperone stability [86].

In the following sections, phosphorylation and AMPylation of Hsp70 family members will be discussed in more detail, focusing on the examples of Hsc70, inducible Hsp70, and the ER-resident chaperone BiP.

3.4.2 Phosphorylation of the Hsp70 family

According to The Global Proteome Machine Organisation databank, ~95 serine, threonine, or tyrosine residues in Hsc70 have been reported as phosphorylated in aggregated mass spectrometry (MS) datasets (visited January 21, 2026) [87], suggesting that phosphorylation is a prevalent modification of Hsc70 and likely plays an important role in its regulation.

Metformin-mediated activation of the inhibitor of nuclear factor kappa B kinases (IKK α/β) leads to phosphorylation of Hsc70 at S85 [88]. This modification promotes activation of Hsc70 in the context of CMA. Based on their biochemical data, the authors propose that phosphorylation at S85 enhances the ATPase activity of Hsc70 and thereby favors conformational states with increased substrate binding. Consistent with this model, mutation of S85 to alanine (S85A), which prevents phosphorylation at this site, reduces the interaction of Hsc70 with CMA substrates, and the authors speculate that this reduction is due to a decrease in ATPase activity of the S85A mutant compared with the wild-type protein [88].

The important function of Hsc70 in dissociating clathrin-coated vesicles (CCVs) also appears to be regulated by phosphorylation. In mouse synapses, both unstable and stable CCVs were detected, with unstable CCVs containing more highly phosphorylated Hsc70 compared with stable CCVs [86, 89]. The exact target residue(s) are not known to date, but elevated levels of the kinases dual specificity tyrosine-phosphorylation-regulated kinase 1A and calcium/calmodulin-dependent protein kinase II delta suggest a role for these enzymes in hyperphosphorylating Hsc70 [89]. One possible target is T265. This residue maps to the CaM/Ca²⁺ binding region of Hsc70 [90]. In a working theory, phosphorylation of Hsc70 at T265 in CCVs acts as a switch that antagonizes CaM/Ca²⁺ binding to Hsc70. This is suggested to lift a CaM-dependent block on Hsc70 dimerization and vesicle association, thereby favoring clathrin-coat disassembly [89].

The stress-inducible cytosolic Hsp70 (HSPA1A) is phosphorylated at T504, a residue that is conserved in Hsc70 and BiP [91]. Phosphorylation at this site contributes to the stabilization of an antiparallel Hsp70 dimer. Treatment of Sf9 cell-derived Hsp70 with phosphatase, which removes these PTMs, shifts the equilibrium toward predominantly monomeric Hsp70. A T504E phosphomimetic mutant, in contrast, promotes dimer formation, supporting a role for phosphorylation at T504 in Hsp70 dimerization [91]. Hsp70 dimers are incorporated into a heterohexameric complex containing Hsp90, Hsp70, Hop and a fragment of the glucocorticoid receptor, which is important for loading the client onto Hsp90. Based on these data, Morgner et al. proposed that dimerization of Hsp70, in part regulated by phosphorylation, helps to position client proteins for transfer from Hsp70 to Hsp90 [91]. Interestingly, T504 lies within a sequence that is conserved among Hsp70, Hsc70, the yeast homologue Ssa1 and BiP, whereas this residue is absent in the *E. coli* Hsp70 homolog DnaK (Figure 2). This raises the possibility that antiparallel dimerization, regulated by phosphorylation at this threonine, may also play a role in these Hsp70 family members. In addition, another threonine residue, T495, has been identified as a regulatory site in Hsc70, where it is specifically phosphorylated by the eukaryotic-like *Legionella* kinase LegK4 [84]. The phosphorylated residue is conserved not only in Hsc70 and BiP, both of which can be phosphorylated by LegK4 *in vitro*, but also in Ssa1, for which LegK4-mediated phosphorylation has not been investigated to date (Figure 2). T495 is located in the β 7- β 8 loop of the β -SBD, whereas T504 lies in the hinge region between the β -SBD and the α -helical lid [91, 92]. Phosphorylation of Hsc70 by LegK4 has

been shown to inhibit host protein synthesis by trapping Hsc70 on ribosomes, interfere with the unfolded protein response, and reduce both basal and J-protein-stimulated ATPase activity as well as substrate folding capacity [84]. In that study, LegK4-mediated phosphorylation of Hsc70 reached only partial stoichiometry (~53 %) and the modified protein was primarily analyzed in biochemical assays that require only small amounts of protein and were performed in the presence of ATP. However, the temporal stability of the phosphorylation state was not examined in that work. Since the partial stoichiometry achieved by *in vitro* phosphorylation of Hsc70 by LegK4 limits direct biochemical and structural analysis of the modified protein, a follow-up study used the phosphomimetic Hsc70^{T495E} mutant in place of enzymatically phosphorylated Hsc70 [93].

Using this phosphomimetic approach, the follow-up study showed that modification at this site stabilizes an ATP-like, domain-docked conformation that is largely independent of the bound nucleotide. At the same time, ATPase activity is reduced, while substrate binding remains possible, suggesting a pseudo-open state that does not undergo normal nucleotide-dependent switching [93]. Consistent with a conserved regulatory role at this position, the homologous residue in yeast Ssa1 (T492) is phosphorylated in distinct cellular contexts, including DNA damage responses and heat shock adaptation. In both systems, phosphomimetic and phospho-null mutants exhibit pronounced phenotypes, including altered stress responses and context-dependent effects on cell growth, highlighting that phosphorylation at this conserved site represents a recurrent regulatory mechanism across Hsp70 homologues [93, 94].

Taken together, proteomic studies have identified numerous phosphorylation sites on Hsp70-family proteins, yet most remain functionally uncharacterized [95]. In the cases examined so far, phosphorylation has been shown to modulate ATPase activity, oligomerization, substrate binding, and interactions with co-chaperones [95]. Only in rare cases, such as phosphorylation of Hsc70 at T495, has the modified protein itself been directly analyzed. Structural studies typically require substantial amounts of stably phosphorylated protein and have not yet been reported. Existing studies have therefore largely relied on cellular and biochemical assays, including phosphomimetic substitutions, small-scale *in vitro* phosphorylation, or genetic code expansion approaches, underscoring the difficulty of obtaining enzymatically phosphorylated protein in sufficient quantity and homogeneity for structural analysis [84, 88, 89, 91, 93, 94, 96-99].

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DnaK   : 489 - DKNSGKEQKITIKAS - 504
Hsc70  : 491 - DKSTGKENKITITND - 506
BiP    : 515 - DKGTGNKNKITITND - 529
HSPA1A: 491 - DKSTGKANKITITND - 506
Ssa1   : 489 - EKGTKSNKITITND - 503

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Figure 2: Amino acid sequence alignment of a conserved Hsp70 region in the beta SBD

T504 in HSPA1A (Hsp70) is a phosphorylation site that stabilizes an Hsp70 dimer. This residue is conserved in Hsc70, BiP and the yeast homologue Ssa1. Hsc70 is phosphorylated at T495, and the corresponding residue in BiP can be both phosphorylated and AMPylated. Likewise, this position is conserved in Hsp70 and Ssa1. T504 and the homologous positions are highlighted in blue, whereas T495 and the corresponding positions are shown in red. Alignments were performed using UniProt [100].

3.4.3 AMPylation of Hsp70 molecules

AMPylation is a post-translational modification in which adenosine monophosphate (AMP) from ATP is covalently attached to the hydroxyl side chain of amino acids such as serine, threonine or tyrosine [101]. It has emerged as an important regulatory modification in both prokaryotic and eukaryotic systems and can strongly alter the activity of its target proteins [101-107].

Filamentation induced by cAMP (Fic) proteins are a major group of enzymes that catalyze AMPylation [108, 109]. Although this diverse protein family can contain a variety of additional domains, its members share a conserved Fic domain with a canonical Fic motif (HxFx(D/E)GN(G/K)R). The catalytic histidine within this motif is essential for deprotonating the target hydroxyl group and thereby increasing its nucleophilicity. Many Fic enzymes are autoinhibited by an α -inhibitory helix that carries a conserved glutamate residue [108, 110]. The glutamate is able to compete with ATP for the ATP-binding pocket, effectively inhibiting the enzyme.

Many AMPylating Fic enzymes are bifunctional in that they can both attach AMP to their targets and catalyze removal of the modification in a process termed deAMPylation [111-113]. One such enzyme is the only metazoan Fic protein: filamentation induced by cAMP domain-containing protein D (FicD) [114]. FicD specifically AMPylates and deAMPylates the ER chaperone BiP at T518 [111, 115]. Residue E234 in the α -inhibitory helix plays a central role in inhibiting AMPylation and in modulating the switch between AMPylation and deAMPylation at T518 [111, 112, 116]. Mutation of E234, for example to glycine, results in constitutively AMPylating FicD variants [110, 111]. Dimerization of FicD is

another determinant of this functional switch, with dimeric FicD favoring deAMPylation and monomeric FicD favoring AMPylation [117]. Consequently, E234 and L258 (which affects FicD dimerization) are commonly targeted in single or double mutants to obtain variants optimized for high-yield AMPylation of BiP [92, 117, 118]. Because such AMPylation-enhancing and deAMPylation-limiting mutants are available, protocols for generating stably AMPylated protein substrates are now well established [118-120].

Regardless of the nucleotide state, AMPylated BiP is biased toward an ATP-like, domain-docked conformation, which disrupts the normal progression of the Hsp70 allosteric cycle [10, 92]. AMPylation does not measurably alter binding of BiP to its J-domain cofactors, but it appears to allosterically dampen J-protein-stimulated ATP hydrolysis, so that modified BiP fails to reach a high-affinity client-binding state. The preferential adoption of an ATP-like conformation, largely independent of nucleotide, is a feature that AMPylated BiP shares with AMPylated Rab1.

As discussed above, BiP can assemble into higher-order oligomers that are hypothesized to serve as a storage form [15, 115]. AMPylation decreases BiP oligomerization and shifts the equilibrium toward lower-molecular-weight species [92]. This effect is particularly pronounced in the ADP-bound and apo states of the protein and in the presence of J-proteins. In current models, the resulting AMPylated, predominantly monomeric pool is considered an additional regulatory layer [111, 116]. When full BiP activity is not required, an inactive reservoir of AMPylated BiP is maintained, which can be rapidly converted into active chaperone by deAMPylation during periods of ER stress.

Interestingly, and as mentioned above, T518 in BiP is homologous to T495 in Hsp70 and Hsc70, and all three proteins can be modified by LegK4 [84]. While the consequences of BiP AMPylation are relatively well understood, owing to robust protocols for generating AMPylated BiP, the effect of phosphorylation at T495 on Hsc70 remain undercharacterized. What is known so far, largely based on biochemical data from the phosphomimetic Hsc70 T495E mutant [93], is broadly consistent with the effects of BiP AMPylation.

3.5 Structural and biophysical approaches to study Hsc70

Structural and mechanistic insights into Hsp70 chaperones have been obtained using a wide range of experimental techniques, including X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR), SAXS, single-molecule fluorescence and

ATPase/activity assays. In the following, selected methods that are particularly relevant for this thesis will be introduced in more detail.

3.5.1 Intact mass spectrometry

In intact mass spectrometry (iMS), the mass of the intact protein chain is measured. That is, the primary structure is preserved [121]. During iMS, the protein sample is denatured using organic solvent, desalted by liquid chromatography, and analyzed on high-resolution mass spectrometers such as quadrupole-time of flight instruments or Orbitraps. Because the protein remains intact and covalent bonds are largely preserved, the measured mass of the analyte reports on whether the protein matches the theoretical sequence and whether it carries additional modifications, such as PTMs [121]. This makes iMS a powerful tool for quality control, for example for recombinant proteins produced in *E. coli*, as it allows direct comparison of the measured and expected molecular mass [122]. Furthermore, if the ionization efficiencies of the modified and unmodified protein species are comparable, iMS can be used to quantitatively determine the stoichiometry of a modification. A prominent example is protein monophosphorylation. Addition of a single phosphate group does not substantially alter the ionization behavior of the protein, which makes iMS a suitable tool for detecting and quantifying the relative abundance of phosphorylated versus unmodified species (e.g. to state that a given preparation is ~70 % phosphorylated) [123]. At the same time, phosphorylation also illustrates the limitations of such quantitative comparisons, because multiple phosphorylation events can change ionization properties and thereby distort the apparent ratios of differently phosphorylated species.

3.5.2 Solution-State Nuclear Magnetic Resonance

Solution-state NMR probes atomic nuclei such as ^1H , ^{13}C , ^{15}N in a magnetic field. Because the resonance frequency of each nucleus depends sensitively on its local chemical environment, nuclei in different structural or conformational settings give rise to distinct signals. Solution-state NMR can therefore be used to monitor ligand binding, conformational changes, and structural dynamics in solution. If, for instance, ATP binding drives an Hsp70 molecule into a domain-docked conformation, residues at the interdomain interface will experience a changed environment and show chemical shift perturbations. Because NMR observes molecules in solution and can report on both structure and

dynamics, it has been widely used to dissect the allosteric cycle of different Hsp70 homologs and is now an established method in this field [6, 10, 124]. Because of their size (~71 kDa), Hsp70 molecules contain a large number of proton-bearing residues, which results in crowded spectra with many overlapping signals in conventional NMR experiments. Therefore, NMR analyses are performed on isotopically labeled protein using advanced pulse sequences. One of the most widely used approaches is methyl transverse relaxation-optimized spectroscopy (methyl-TROSY). The proteins are expressed under specialized labeling conditions, and a variety of protocols are available. In the case of Hsp70 family members, selective ^{13}C methyl labeling is used. ^{13}C is introduced into the terminal methyl groups, primarily in the side chains of valine, leucine, and isoleucine (ILV labeling), enabling selective detection of these sites by NMR [122]. In addition, the expression cultures are grown in D_2O so that NMR-inactive deuterium is incorporated into the backbone amides and most side chains. The proteins can be further uniformly labeled with ^{15}N in the backbone. The resulting samples are thus largely deuterated and contain NMR-active ^1H - ^{13}C methyl groups primarily on the ILV side chains (apart from the natural ^{13}C abundance), which enables high-resolution NMR studies of Hsp70 by methyl-TROSY.

3.5.3 SAXS

In SAXS, a protein sample in solution is irradiated with a collimated X-ray beam, and the X-rays are elastically scattered by the electrons in the particles [125]. The scattered intensity is recorded on a 2D detector and radially averaged to yield the one-dimensional scattering profile $I(q)$, where q (sometimes also denoted s) is the magnitude of the scattering vector. This profile can be plotted and analyzed to obtain low-resolution structural information about the protein.

A key global parameter is the radius of gyration (R_g), which describes the distribution of scattering mass around the molecule's center and is commonly used as an overall size/compactness parameter [126]. More extended conformations yield larger R_g values. R_g can be determined from the linear low- q (Guinier) regime by fitting $\ln I(q)$ as a function of q^2 . Alternatively, R_g can be obtained from the real-space distance distribution function $p(r)$, which reflects the relative frequency of intramolecular distances within the

particle and makes use of essentially the full small-angle scattering range. From $p(r)$ the maximum intraparticle distance (R_{max}) can also be estimated.

The shape of the distance distribution ($p(r)$) function is informative about overall architecture. A single, symmetric peak is characteristic of compact, roughly globular particles, whereas a broader, more asymmetric distribution indicates an elongated species. Bimodal or broad, shoulder-like distributions can reflect multidomain proteins with flexible linkers, where the shorter distances correspond to intradomain contacts and the longer distances to interdomain separations [127], or they may arise from overlapping contributions of different oligomeric species in the sample [128]. SAXS has been applied to several Hsp70 family members. For example, SAXS on a concentration series of DnaK revealed buffer-dependent oligomerization as well as ADP-induced domain undocking [129]. Similarly, SAXS analysis of a monomeric Hsc70^{E543} variant indicated an elongated but domain-docked conformation in the ATP-bound state and a domain-undocked conformation in the ADP-bound state [130].

3.6 Thiol-reactive nucleotide derivatives

Due to the transient nature of kinase-substrate interactions only a few kinase-substrate complex structures have been solved crystallographically at high resolution [49, 52]. The HPr kinase/phosphorylase (HPrK/P)-HPr system is one of the few examples where the complex structure could be determined by X-ray crystallography [131]. With the continuous improvement of cryo-EM resolution, there is now an additional method to study kinase-substrate interactions [132, 133]. However, the transient character of the complexes, together with the heterogeneity of kinase-substrate assemblies, can limit the achievable resolution. For future structural studies of kinase-substrate pairs, strategies to stabilize these complexes are therefore required. One possible approach can be borrowed from work on Fic enzymes, where ATP analogs are used to stabilize enzyme-substrate complexes. Like kinases, some Fic enzymes only form transient interactions with their targets. ATP analogs modified on the adenine base, termed TReNDs (thiol-reactive nucleotide derivatives), have been used to stabilize such complexes, allowing their structures to be determined by X-ray crystallography with great success [118, 119, 134]. In this thesis, ATP analogs referred to here as TReND-K, synthesized and generously provided by the group of Prof. Dr. Christian Hedberg (Umeå University, Sweden), were tested

(Figure 3A, C-D and Table S11). In contrast to the TReNDs developed for AMPylases, which carry the linker on the adenine base, the TReND-K analogs used here are modified at the γ -phosphate with a polycarbon linker that terminates in the reactive group (Figure 3A, C-D). Both classes of analogs contain a chloroacetamide moiety as the thiol-reactive warhead (Figure 3A). Figure 3B schematically compares the TReND-K analogs tested in this work with a previously described Fic-type TReND and with an ATP analog that has been established for covalent crosslinking of kinase-substrate complexes for target screening.

Like the TReNDs developed for AMPylases, the chloroacetamide group in the TReND-K analogs used for covalent capture of kinase-substrate interactions is designed to undergo a nucleophilic substitution with the sulfhydryl (SH) group of cysteine of the target protein (here, the kinase), forming a binary adduct (Figure 3D). Nucleotide and kinase are thereby covalently, and thus stably, linked. If the γ -phosphate together with the linker is subsequently transferred in the kinase reaction, a ternary complex consisting of kinase, analog, and substrate can be formed. As long as the kinase does not cleave off the transferred phosphate group, this complex is expected to be maintained and could, in principle, be used for subsequent crystallization and structure-determination experiments.

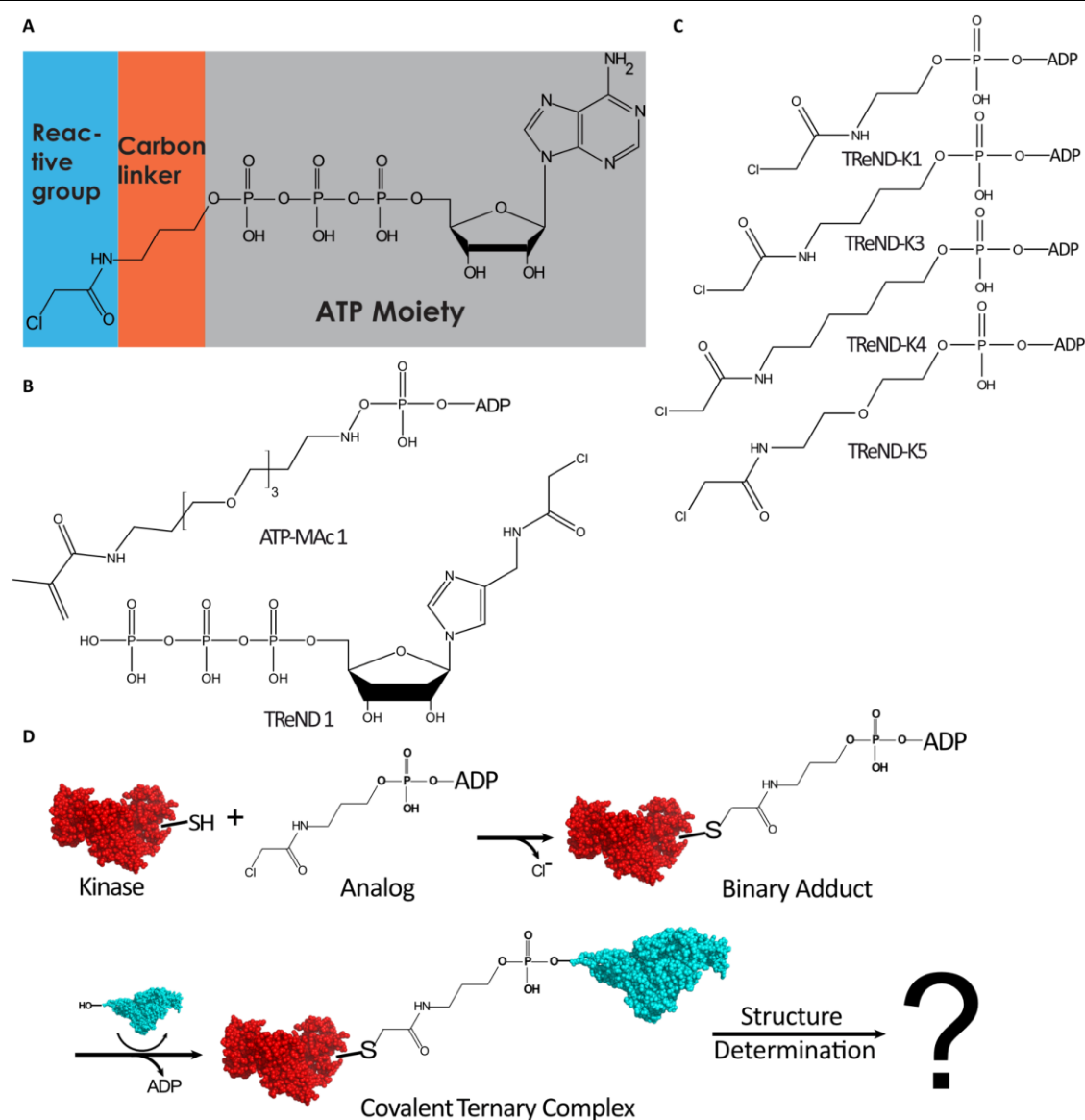


Figure 3: Workflow of ternary complex formation as tested with the TReND-K analogs used in this thesis (A) Schematic representation of the different structural elements of a TReND-K analog, illustrated using TReND-K2 as an example. (B) Comparison of an ATP analog previously used for kinase-substrate crosslinking for target identification (ATP-Mac1) [135] with a TReND designed for covalent capture of AMPylases and their targets (TReND1) [119]. (C) Chemical structures of TReND-K1 and TReND-K3-5, which were tested in this thesis for their ability to support binary adduct formation and ternary complex formation. (D) Workflow of complex formation as applied in this thesis. A kinase bearing a sulfhydryl group in proximity to the ATP-binding pocket (either endogenous or introduced by mutagenesis) is incubated with a TReND-K analog. Upon nucleophilic attack by the sulfhydryl group, a covalent binary adduct between kinase and nucleotide analog is formed. This binary adduct is then incubated with a protein substrate, allowing transfer of the modified γ -phosphate and formation of a covalent ternary complex (kinase-TReND-K-substrate). The resulting ternary complex can in principle be isolated, purified, and subjected to structural analysis.

4 Aim of this work

Phosphorylation of Hsc70 at T495 by the *Legionella* effector kinase LegK4 reduces Hsc70s ATPase activity and client folding capacity. In addition, data obtained with a phosphomimetic mutant suggest that phosphorylation promotes a more domain-docked conformation. To obtain a more complete understanding of how T495 phosphorylation affects Hsc70, further biochemical assays and structural studies on enzyme-phosphorylated Hsc70 are required. Progress in this direction has been limited by the lack of a robust phosphorylation protocol that yields highly and stably phosphorylated Hsc70 in preparative quantities.

Beyond phosphorylation, the corresponding residue in BiP, an Hsp70 homolog, is AMPylated in metazoans. AMPylation has been shown to reduce ATPase activity, client processing, and oligomerization of BiP while promoting domain docking. The existence of two chemically distinct modifications, a relatively bulky AMP moiety and a phosphate group, at the same homologous position in different Hsp70 family members raises the question of why evolution has selected these two regulatory strategies. Addressing this question ultimately requires direct comparison of phosphorylated and AMPylated forms of the same protein. The aim of this thesis was therefore to establish high-yield protocols for both phosphorylation and AMPylation of Hsc70 and to investigate the structural consequences of T495 modification.

While structural characterization of modified Hsc70 can provide mechanistic insight into how T495 phosphorylation and AMPylation reshape Hsc70 conformational equilibria, a structure of the LegK4-Hsc70 complex would additionally be informative to define the molecular basis of substrate recognition. However, kinase-substrate complexes are inherently transient, complicating structure determination. Therefore, in collaboration with the group of Prof. Christian Hedberg, ATP analogs designed for covalent capture were evaluated for their ability to stabilize kinase-substrate complexes. This strategy was tested using the LegK-Hsc70 system and, in parallel, the model kinase CK2 α' with its substrates EEf1D and HAP28.

5 Results

To investigate the effects of Hsc70 phosphorylation and AMPylation at T495, phosphorylation and AMPylation protocols first had to be established, as described in chapters 5.1 and 5.2 Chapter 5.3 then focuses on the structural consequences of T495 phosphorylation in Hsc70. In addition, ATP analogs synthesized by the Hedberg group were evaluated for their ability to enable stable, covalently trapped complexes between kinases and their substrates; these experiments are presented in Chapter 5.4.

5.1 Optimized production of stably phosphorylated Hsc70 (T495)¹

For structural studies, phosphorylation must be present at a sufficiently high level to enable, for example, structural investigations. Therefore, the protein must be produced in sufficient quantity, as many structural methods require milligram amounts of material. To date, a phosphorylation level of approximately 53% has been reported, while the yield and temporal stability of phosphorylation were not described. Therefore, to enable future structural analyses of T495 phosphorylated Hsc70 (pHsc70) under different nucleotide conditions and with different structural techniques, a robust preparative phosphorylation protocol was established as described in the following section.

5.1.1 Rationale design and establishment of an initial phosphorylation protocol

In previously reported phosphorylation workflows, hexahistidine (His₆)-tagged LegK4 and nickel affinity chromatography were used, and the kinase was added directly to the cell lysate [84]. This setup has the practical advantage that LegK4 can be removed efficiently after the reaction, as the kinase remains His₆-tagged and the workflow includes two consecutive nickel-based immobilized metal affinity chromatography (IMAC) steps. A potential drawback is that phosphorylation in crude lysate is less defined than in

¹ Parts of the following sections are adapted and substantially expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.jep.2026.106923., in which these findings were originally reported.

reactions with purified components carried out *in vitro*, as additional cellular factors and varying nucleotide levels may influence the reaction conditions. Therefore, in this work, LegK4 was added after the first IMAC step, i.e. to partially purified Hsc70, to perform phosphorylation under more controlled conditions while retaining the possibility to remove LegK4 by a subsequent IMAC step.

First, Hsc70 was heterologously expressed in *E. coli* in order to obtain high protein yields. Following cell harvest and lysis, wild-type Hsc70 and the ATPase-deficient mutant Hsc70^{T204A} were initially enriched by a first IMAC step. Because ATP-bound Hsc70 has low affinity for client proteins, an on-column wash with 2 mM ATP was performed to displace residual *E. coli* proteins bound to Hsc70. After elution, the protein solution was adjusted to a 30 ml reaction and supplemented with ATP (5 mM final concentration) and His₁₀-tagged full-length LegK4₅₉₋₁₀₁₉ (700 nM) to initiate enzymatic phosphorylation (Figure S1A). Phosphorylation was proceeded during overnight dialysis at 4 °C against buffer containing 5 mM ATP, maintaining a high ATP:ADP ratio and thereby limiting reversal of phosphorylation by ADP, the reaction byproduct. Subsequently, the reaction mixture was applied to a second IMAC column to remove LegK4 and was then further purified by SEC. The stability of the phosphorylation state was then assessed quantitatively by iMS. However, Hsc70^{T204A} phosphorylation declined from 73.4 % to 58 % over 5 h at 22 °C (Figure 4A), indicating that the phosphorylation state was not stable under these conditions. Reversal of phosphorylation by kinases *in vitro* has previously been described in the presence of ADP or under nucleotide-free conditions [136, 137]. Given that milligram quantities of full-length LegK4 were employed for phosphorylation, it was hypothesized that residual LegK4 in the preparation dephosphorylated pHsc70.

Consequently, additional high-yield experiments were performed as individual optimization trials to identify conditions under which high phosphorylation levels could be achieved with substantially lower amounts of kinase while maintaining phosphorylation stability. To enable the use of reduced LegK4 concentrations, the initial phosphorylation protocol was modified in several steps. First, the single overnight dialysis at 4 °C was replaced by a two-step dialysis procedure. The sample was dialyzed at room temperature or 37 °C for variable time periods against buffer containing 5 mM ATP, followed by overnight dialysis at 4 °C against buffer containing 2 mM ATP. For the overnight dialysis step, a sodium phosphate buffer was introduced, because Thermofluor analysis indicated that

Hsc70 is more stable in this buffer than in the other buffer systems tested (Table S1, Figure S2). These optimization experiments showed that relatively high phosphorylation levels could already be obtained with 1.5 nM LegK4 (approximately 0.22% of the kinase amount used in the initial protocol) (Table 1; Figure S3A-B) at 37 °C. Longer incubation times (e.g., 5.25 h compared with 3.25 h) and higher LegK4 concentrations further improved phosphorylation levels (Figure S3B-C).

In these preliminary optimization experiments, phosphorylation levels were measured as soon as possible after the 2nd IMAC step to obtain an initial estimate of reaction efficiency. Because phosphorylation was comparatively high with 3 nM LegK4 for 5.25 h at 37 °C, this condition was additionally tested for stability. Under these conditions, phosphorylation remained stable (Figure S3E), indicating that stable phosphorylation is achievable and likely depends on minimizing residual LegK4 after the reaction. Reactions performed at room temperature (RT) also yielded lower phosphorylation levels than those obtained at 37 °C (Figure S3D).

During protocol development, an additional LegK4-independent mass shift of approximately +274.7 to +276.4 Dalton (Da) was detected, which was markedly more pronounced at 37 °C. Therefore, the final protocol was set up using slightly higher LegK4 concentrations and incubation at room temperature. In the finalized workflow, 6.72 nM LegK4 is used in a 50 ml reaction and phosphorylation is carried out for 6.5 h (Figure S1B).

Table 1: Phosphorylation stoichiometry of Hsc70 after bulk phosphorylation and removal of LegK4 by 2nd IMAC, obtained under different LegK4 concentrations, incubation times, and temperatures in the modified two-step dialysis protocol

Condition	Level of phosphorylation [%]
1.5 nM LegK4, 3.25 h at 37 °C	68.4
1.5 nM LegK4, 5.25 h at 37 °C	74.6
3 nM LegK4, 5.25 h at 37 °C	81
3 nM LegK4, 6.15 h at RT	70.5

5.1.2 Validation of the final phosphorylation protocol and identification of LegK4-dependent dephosphorylation

Based on these results, the final phosphorylation protocol was established and subsequently validated with regard to phosphorylation stability, yield, and protein functionality. Despite the extended incubation times, Hsc70 remained stable, and no obvious

protein degradation was detected by denaturing polyacrylamide gel electrophoresis (Figure 4B).

Next, the stability of the phosphorylation state was examined to determine whether the earlier instability resulted from residual LegK4 in the sample. pHsc70 generated with the optimized protocol was incubated either in the absence of LegK4 or in the presence of 0.4 μ M of the truncated construct LegK4₅₉₋₄₆₂ or the kinase-dead mutant LegK4₅₉₋₄₆₂^{D253A}. As expected, in the absence of LegK4 the phosphorylation level remained constant over time (Figure 1C). In contrast, addition of LegK4₅₉₋₄₆₂ caused an approximately 56 % decrease in phosphorylation between 20 min (98.8 ± 0.57 %) and 20 h (43.35 ± 13.65 %) (Figure 4C). By comparison, incubation with kinase-dead LegK4₅₉₋₄₆₂^{D253A} led to only a minor reduction of about 3.5 % in Hsc70 phosphorylation between 20 min (100.27 ± 0.91 %) and 20 h (96.73 ± 1 %) (Figure 1C). Thus, residual LegK4 remaining from the initial phosphorylation reaction is responsible for reversing Hsc70 phosphorylation. This reversal clearly depends on LegK4 catalytic activity, as the kinase-dead mutant LegK4₅₉₋₄₆₂^{D253A} does not significantly reduce the phosphorylation level, indicating that the effect is not due to other contaminants such as phosphatases.

Upon scale-up, the optimized protocol produced roughly 10 mg of pHsc70 per liter of *E. coli* culture while it yielded predominantly phosphorylated protein, with 70 % of Hsc70 and 86 % of Hsc70^{T204A} carrying a phosphate at T495 (Figure 4D-E). Importantly, these phosphorylation levels remained stable during 20 h of incubation at room temperature. For Hsc70^{T204A}, an additional SEC step was carried out to remove high-molecular-weight (HMW) species (Figure 4F) for subsequent analytical SEC (aSEC) and SAXS measurements. Both Hsc70^{T204A} and pHsc70^{T204A} exist as mixtures of monomers, dimers and trimers, with additional higher-order oligomers and HMW species present. pHsc70^{T204A} eluted at a lower apparent molecular weight than Hsc70^{T204A}, consistent with phosphorylation reducing the extent of Hsc70 oligomerization.

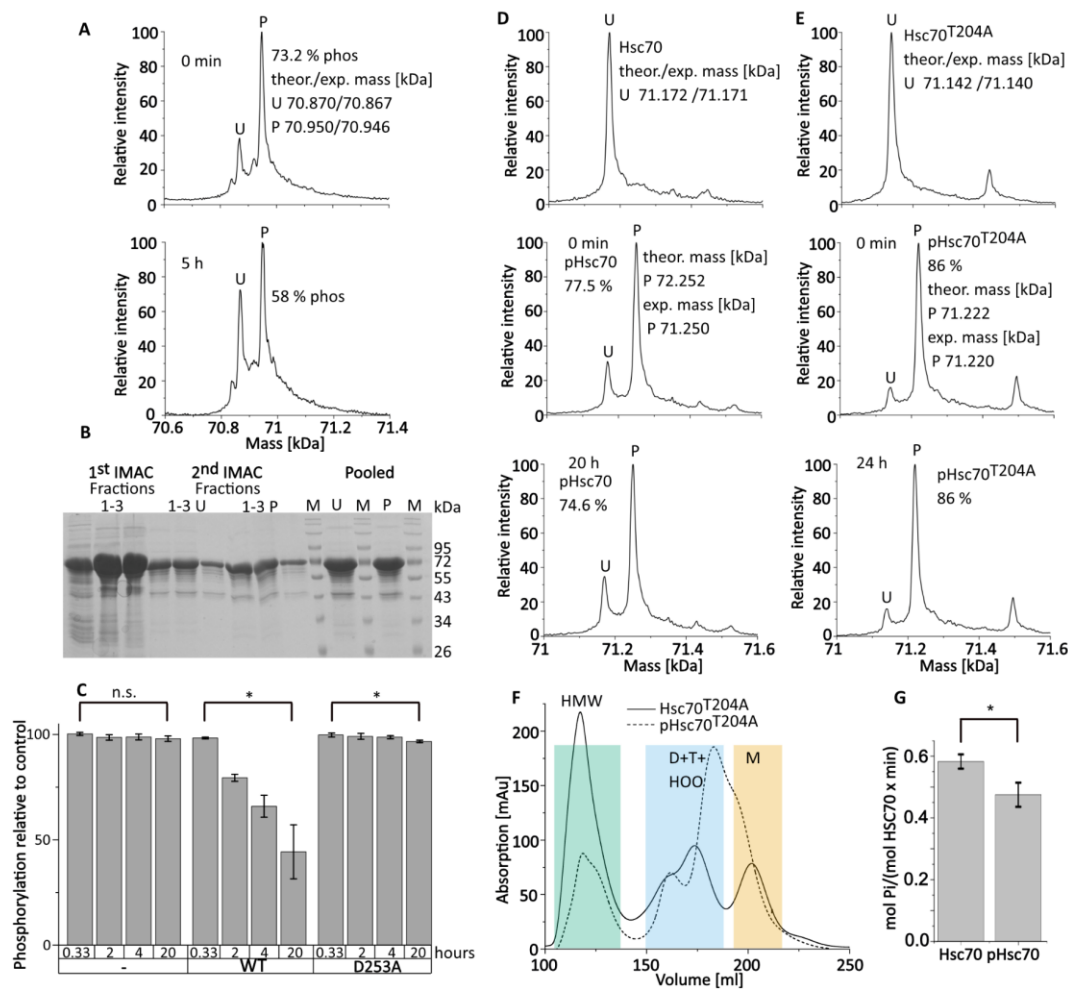


Figure 4: Purification and phosphorylation of Hsc70

(A) Intact mass spectrometry (iMS) spectra of Hsc70^{T204A} after phosphorylation with an excess of full-length LegK4 (700 nM in a 30 ml reaction). Top, protein measured directly after thawing (73.4 % phosphorylated); bottom, protein measured after 5 h incubation at 22 °C (58 % phosphorylated). Hsc70^{T204A} theoretical/experimental mass 70,870/70,867 Da, phosphorylated theoretical/experimental mass 70,950/70,946 Da. **(B)** SDS-PAGE analysis of Hsc70. Representative fractions after 1st and 2nd IMAC. Pooled corresponds to the stored protein and represents the final state of purification for wild-type Hsc70. Dialysis between the first and second IMAC steps did not result in any detectable protein fragmentation. U = Hsc70, P = pHsc70, M = marker. **(C)** Results of iMS analysis of purified pHsc70 incubated with or without LegK4₅₉₋₄₆₂ or LegK4₅₉₋₄₆₂^{D253A}. Data represent mean ± SD (n = 3, biological replicates). Time-dependent effects were assessed by one-way repeated-measures ANOVA (LegK4₅₉₋₄₆₂: Greenhouse-Geisser-corrected p = 0.019; LegK4₅₉₋₄₆₂^{D253A}: p = 0.021; no-enzyme control: p = 0.10). In addition, significance between the 20 min and 20 h time points was assessed by paired t-test (LegK4₅₉₋₄₆₂: p = 0.018; LegK4₅₉₋₄₆₂^{D253A}: p = 0.0055; no-enzyme control: p = 0.057); p < 0.05 was considered significant. No statistical outliers were detected by Grubbs' test (α = 0.05). **(D)** iMS spectra of Hsc70 (top) and pHsc70 (middle, no incubation; bottom, 20 h) after purification with the established protocol. Hsc70 theor. mass 71.172 kDa, phosphorylated theor. mass 72.252 kDa. U = unmodified protein, P = phosphorylated protein. **(E)** iMS spectra of Hsc70^{T204A} (top) and pHsc70^{T204A} (middle no incubation; bottom 24 h) after purification with the established protocol and size-exclusion chromatography (SEC). Hsc70^{T204A} theor. mass 71.142 kDa, phosphorylated theor. mass 72.222 kDa. U = unmodified protein, P = phosphorylated protein. **(F)** SEC of Hsc70^{T204A} (unphosphorylated and phosphorylated) prepared for SAXS measurements. (M, monomer (amber area); D, dimer; T, trimer; HOO, higher-order oligomers (blue area); HMW, high molecular weight species (green area)). **(G)** ATPase assay of Hsc70 and pHsc70. Data represent mean ± SD (n = 8, technical replicates). Significance was assessed by independent-samples t-test (p = 2.09 × 10⁻⁵); p < 0.05 considered significant. No statistical outliers were detected by Grubbs' test (α = 0.05).

This figure and its caption were adapted from the cited publication [21].

Next, the functional activity of Hsc70 and its phosphorylated variants following the preparation procedure was assessed. To this end, the intrinsic ATPase activity was used as an indicator of Hsc70 activity and structural integrity. Therefore, the ATPase activity was quantified using a Malachite Green assay, which measures inorganic phosphate released during ATP hydrolysis. Hsc70 and pHsc70 displayed measurable ATPase activity, suggesting that both Hsc70 and Hsc70^{T204A} are functionally intact. However, phosphorylation at T495 significantly lowered the basal ATPase activity of Hsc70 (Figure 4G), consistent with earlier reports [84].

In summary, stringent removal of LegK4 and ADP enabled the production of stably phosphorylated, active Hsc70 in quantities sufficient for structural studies.

5.2 Development of an AMPylation Protocol for Hsc70

After establishing the high-yield phosphorylation protocol, an AMPylation protocol was developed. To this end, preliminary small-scale AMPylation reactions were performed and analyzed by iMS, and the resulting data were used as the basis for developing a high-yield AMPylation protocol.

5.2.1 Factors determining AMPylation efficiency

First, the activity of purified FicD was tested, and AMPylation of Hsc70 was compared with that of BiP after 3 h of incubation in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-based buffer using a 1:20 FicD/chaperone ratio and the FicD^{102-458 E234G L258D} variant (Figure 5A). In this variant, the E234G substitution reduces autoinhibition, whereas L258D decreases dimerization and thereby reduces de-AMPylation [110, 116]. BiP AMPylation levels were markedly higher than those of Hsc70, suggesting that under the chosen conditions FicD is active but may preferentially AMPylate BiP. In contrast to BiP, the Hsc70 sample used in this experiment had not been purified by SEC, so an Hsc70 sample purified by SEC was tested next (Figure 5B). AMPylation levels were higher for the SEC-purified sample. This difference could be due to differences in nucleotide content after SEC, as observed during protocol implementation. In a representative HPLC analysis, the total nucleotide concentration (ATP, ADP, and AMP) was substantially lower after SEC purification (18.4 μ M) than when SEC was omitted and the sample was buffer-exchanged using concentrators after the second IMAC step (131 μ M) (Figure S4). It

should be noted that for the 2nd IMAC-purified Hsc70 used in the optimization experiments, buffer exchange was performed with concentrators rather than with the four-step dialysis protocol used in the final phosphorylation procedure presented in this thesis. Because AMPylation levels were still modest overall, the effect of a 1:5 FicD/Hsc70 ratio on AMPylation was examined using SEC-purified Hsc70. Indeed, increasing the ratio from 1:20 to 1:5 led to a pronounced increase in AMPylation (average increase 34.9 %). Given the limited number of biological replicates and the exploratory nature of the comparison between purification strategies, these results should be regarded with caution. Nevertheless, for the rational development of a high-yield AMPylation protocol, it was concluded that protocol efficiency may be improved when using SEC-purified Hsc70 and relatively high FicD/Hsc70 ratios (e.g. 1:5 instead of 1:20).

Because HEPES- and NaH₂PO₄-based buffers are used in the phosphorylation protocol established in this thesis, their effect on the extent of Hsc70 AMPylation was subsequently tested.

To further optimize the AMPylation conditions for Hsc70, an exploratory experiment was designed to address two related questions: (i) whether AMPylation in a HEPES-based buffer continues to increase between 3 h and 6 h, and (ii) whether a HEPES- or a NaH₂PO₄-based buffer is the more suitable buffer system within a 3-6 h incubation window. The 3-6 h window was chosen based on previous experience with Hsc70 phosphorylation. In the HEPES-based buffer, AMPylation levels increased modestly but consistently between 3 h and 6 h in both biological replicates (mean increase ≈ 7.7 %) (Figure 5C). Although AMPylation continues to rise over time, the additional gain between 3 h and 6 h is relatively small compared with the overall AMPylation level (from $73.6 \pm 0.5\%$ to $81.3 \pm 0.1\%$). These data suggest that incubation times within the 3-6 h window are suitable for a high-yield AMPylation protocol.

In the same experiment, AMPylation was also monitored in NaH₂PO₄ at 3 h and 6 h, allowing a direct comparison with the HEPES-based buffer condition. At 3 h, the HEPES-based buffer increased AMPylation by approximately 20 % compared to NaH₂PO₄ (Figure 5C). A similar effect was observed at 6 h, where HEPES-based buffer again yielded higher AMPylation levels than NaH₂PO₄ (mean difference ≈ 22.0 %). In all cases, values represent the mean of technical duplicates for each biological replicate. Taken together, these exploratory data show that a HEPES-based buffer consistently produces higher levels of

Hsc70 AMPylation than NaH_2PO_4 at both 3 h and 6 h, while AMPylation already approaches high levels by 3 h. Based on these findings, SEC-purified Hsc70, a higher FicD/Hsc70 ratio (e.g. 1:5), and a HEPES-based buffer with an incubation time of 3-6 h were selected as the starting conditions for the subsequent high-yield AMPylation protocol.

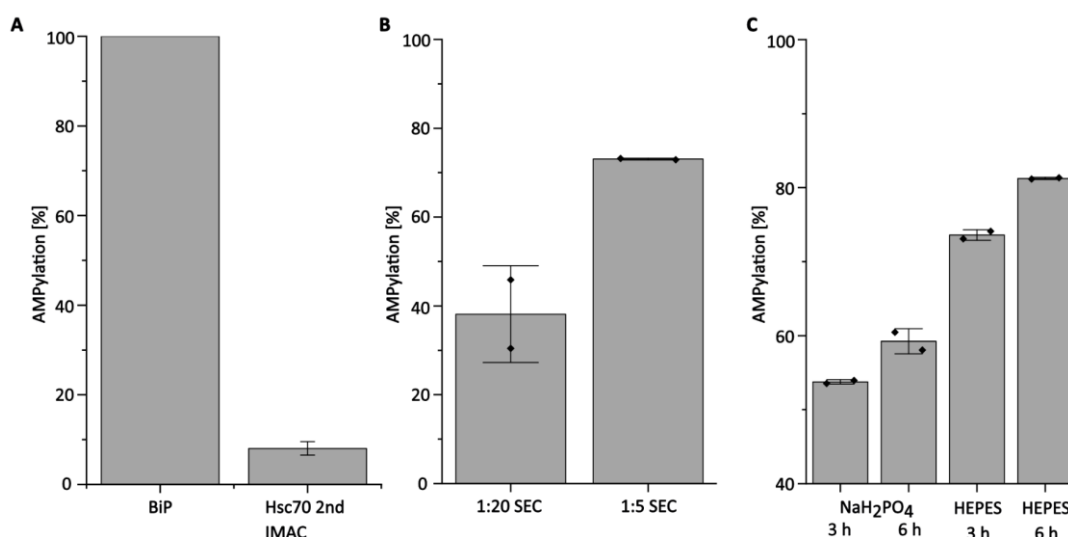


Figure 5: Optimization of Hsc70 AMPylation conditions across purification strategy, FicD/Hsc70 ratio, buffer composition, and incubation time

AMPylation levels were determined by intact mass spectrometry (iMS) after incubation of 10 μM chaperone with FicD₁₀₂₋₄₅₈^{E234G L258D} at 22 °C.

(A) AMPylation of BiP (SEC-purified) and Hsc70 (after 2nd IMAC) after 3 h incubation in HEPES-based buffer with 0.5 μM FicD (FicD/chaperone ratio 1:20). Bars show the mean of technical duplicates. (B) AMPylation of SEC-purified Hsc70 after 3 h incubation in HEPES-based buffer with 0.5 μM FicD (1:20 FicD/Hsc70) or 2 μM FicD (1:5 FicD/Hsc70). Bars show the mean of two independent biological replicates ($n = 2$); points indicate individual biological replicates (each the mean of technical duplicates); error bars represent the SD across biological replicates. (C) AMPylation of SEC-purified Hsc70 after incubation with 2 μM FicD (1:5 FicD/Hsc70) in NaH_2PO_4 -based buffer or HEPES-based buffer for 3 h or 6 h, as indicated. For each condition, two independent biological replicates were measured in technical duplicates. Bars show the mean across biological replicates; points indicate individual biological replicates (each the mean of technical duplicates); error bars represent the SD across biological replicates. Taken together, SEC-purified Hsc70, a higher FicD/Hsc70 ratio (1:5), HEPES-based buffer conditions, and longer incubation times (6 h) yielded the highest AMPylation levels within the 3-6 h incubation window.

5.2.2 Establishment of a high-yield and stable AMPylation protocol for Hsc70

Based on the insights gained from the preliminary experiments, an initial preparative AMPylation protocol was established (Figure 6A). In this protocol, IMAC purification steps were combined with SEC to obtain nucleotide-poor Hsc70 suitable for the subsequent AMPylation reaction. AMPylation was carried out in 100 ml buffer supplemented to 5 mM ATP for 4.25 h using 1.15 μM FicD₁₀₂₋₄₅₈^{E234G L258D} and dialyzing against buffer

with 5 mM ATP. In the initial protocol, FicD depletion after the AMPylation reaction was achieved solely by a second SEC step. The resulting AMPylated Hsc70 showed an AMPylation level of 52.9 % when measured before the second SEC (Figure 6B). However, after the second SEC and subsequent freezing and thawing, the AMPylation level had decreased to only 18.8 %. Similar to the phosphorylation of Hsc70 by LegK4, it was assumed that the deAMPylation activity was due to residual FicD₁₀₂₋₄₅₈^{E234G L258D} in the sample. This was unexpected, as the literature indicates that AMPylation is stable in the presence of the FicD^{E234G} mutant [92, 118]. In addition, previous work in this laboratory indicates stability of BiP AMPylation in the presence of the double mutant FicD₁₀₂₋₄₅₈^{E234G L258D}. To test the hypothesis, the protocol was therefore extended by introducing a third IMAC step after the AMPylation reaction in order to deplete FicD in addition to the 2nd SEC step (Figure 6A highlighted by the cyan rectangle). FicD₁₀₂₋₄₅₈^{E234G L258D} with a non-cleavable His₁₀-tag was added to an 80 ml reaction at a final concentration of 1.14 μ M and incubated for 4.5 h. This final protocol yielded ~2.8 mg of AMPylated Hsc70 per liter of *E. coli* culture, which remained stable under the conditions tested and reached an AMPylation level of approximately 63% (Figure 6C,D). AMPylated Hsc70 was then used to evaluate whether addition of FicD₁₀₂₋₄₅₈^{E234G L258D} renders AMPylation unstable again. Upon addition of 0.4 μ M FicD₁₀₂₋₄₅₈^{E234G L258D} to the purified protein, the AMPylation level decreased significantly by about 37 % (mean of three technical replicates), indicating that residual FicD activity is sufficient to de-AMPylation a substantial fraction of Hsc70. On this basis, the final high-yield protocol provides a robust source of AMPylated Hsc70 for subsequent structural and functional analyses. While the current workflow yields AMPylation levels of approximately 63%, further increases appear feasible, and potential routes for optimization are discussed in the Discussion section. An AMPylation level of ~63% may be sufficient for certain downstream applications, but suitability depends on the readout and on the magnitude of the expected effect. While this thesis initially aimed to compare phosphorylation and AMPylation at T495, the structural analysis was ultimately focused on phosphorylation-dependent changes in Hsc70^{T204A}. Subsequent efforts therefore prioritized implementing structural methods for pHsc70^{T204A} and characterizing pHsc70^{T204A}, rather than pursuing additional rounds of AMPylation optimization.

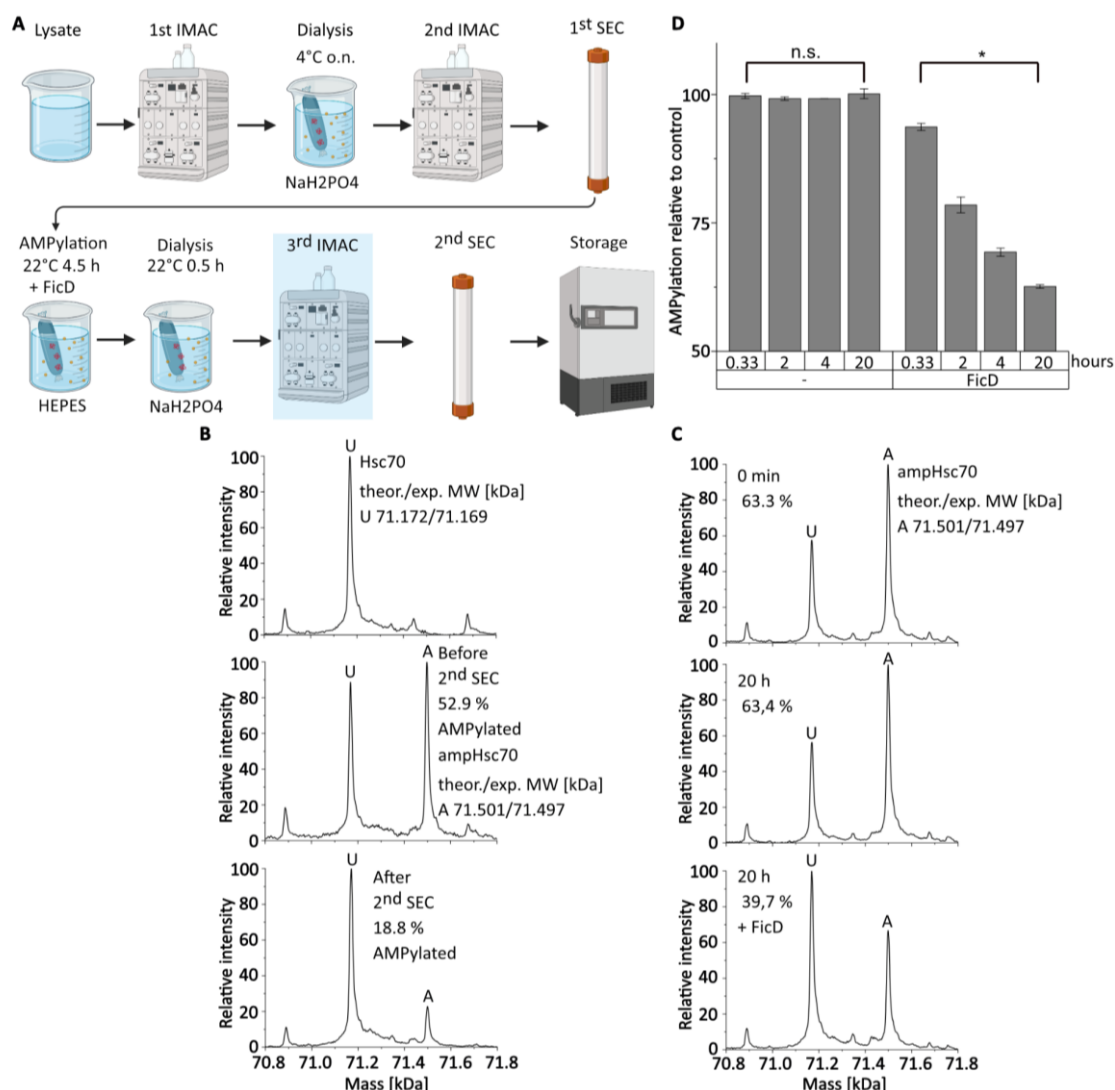


Figure 6: High yield protocol for AMPylated Hsc70 and AMPylation stability test

(A) Schematic overview of the high-yield workflows for obtaining AMPylated Hsc70. Recombinant Hsc70 is purified by affinity chromatography (IMAC) and size-exclusion chromatography (SEC), incubated with FicD₁₀₂₋₄₅₈^{E234G L258D} under AMPylation conditions, and subsequently repurified by SEC alone (initial protocol) or by a 3rd IMAC step followed by SEC (final protocol). The additional 3rd IMAC step (highlighted by the cyan rectangle) was introduced after the AMPylation reaction to further deplete FicD₁₀₂₋₄₅₈^{E234G L258D}, as AMPylation proved unstable when it was removed by SEC alone. The AMPylated protein is stored at -70 °C. Created in BioRender. Kley, N. (2026) <https://BioRender.com/kjc1ian>. (B) Intact mass spectrometry (iMS) spectra of Hsc70 and AMPylated Hsc70 obtained with the initial protocol. From top to bottom: unmodified Hsc70 (reference spectrum shown for comparison), AMPylated Hsc70 before and after the 2nd SEC step. AMPylation levels are lower after 2nd SEC step, indicating that AMPylation is unstable under these conditions. (C) Representative iMS spectra of Hsc70 and AMPylated Hsc70 obtained with the final protocol and after incubation at 22 °C. From top to bottom: AMPylated Hsc70 after purification and storage with the final protocol (0 min), AMPylated Hsc70 after 20 h at 22 °C without additional FicD, and AMPylated Hsc70 after 20 h at 22 °C in the presence of 0.4 μM FicD₁₀₂₋₄₅₈^{E234G L258D}. (D) Quantification of AMPylation levels from iMS measurements corresponding to the final protocol shown in (C). Bars show AMPylation levels at 0.33, 2, 4 and 20 h at 22 °C, normalized to the 0 min value (AMPylated Hsc70 directly after purification). Values represent the mean of three independent technical replicates performed in separate assay setups using the same purified proteins. Error bars indicate the standard deviation. Significance was assessed by paired t-tests. In the absence of FicD, no significant difference was observed ($p = 0.318$), whereas in the presence of FicD, the difference was significant ($p = 1.50 \times 10^{-4}$). U, unmodified protein; A, AMPylated protein; ampHsc70, AMPylated Hsc70. AMPylation leads to a +329 Da shift.

5.3 Structural and biophysical characterization of pHsc70

5.3.1 nanoDSF indicates similar thermal stability of Hsc70^{T204A} and pHsc70^{T204A}

After establishing the phosphorylation protocol and confirming that the protein preparations are functional, nano differential scanning fluorimetry (nanoDSF) was used to determine the melting temperature (T_m) and its changes upon nucleotide binding, in order to assess whether phosphorylation affects thermal stability. Changes in T_m can in turn be used as an indirect readout for nucleotide binding [138]. To this end, Hsc70 and pHsc70 were measured in the apo-, ATP- and ADP-bound states (Figure 7, Figure S5). The measurements and data analysis were performed with scientific advice from Dr. Vivian Pogenberg.

Both Hsc70 and pHsc70 exhibit similar melting temperatures, differing across nucleotide states only by about 0.75-1.5 °C. Because the T_m of pHsc70 is consistently slightly lower than that of Hsc70, this shift is unlikely to arise from experimental errors alone and probably reflects a modest destabilizing effect of phosphorylation. However, the absolute differences are small relative to the overall T_m , so both proteins can be considered similarly stable towards thermal denaturation.

For both Hsc70^{T204A} and pHsc70^{T204A}, nucleotide binding leads to a pronounced stabilization to a comparable extent (average temperature shifts were: Hsc70, +8.5 °C / +7.1 °C for ATP/ADP; pHsc70, +9.2 °C / +6.2 °C for ATP/ADP). This suggests that phosphorylation neither impairs nucleotide binding nor diminishes the stabilizing effect conferred by ATP or ADP. The overall comparable stability of Hsc70^{T204A} and pHsc70^{T204A} further supports the notion that the reduced basal ATPase activity of pHsc70 is not primarily due to an increased fraction of unstable/inactive protein but rather reflects a decrease in catalytic efficiency.

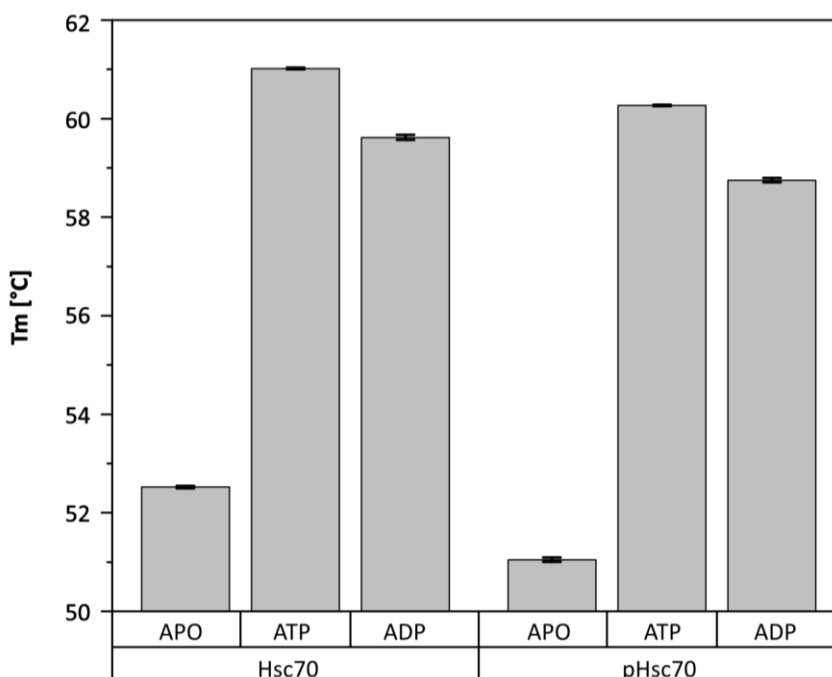


Figure 7: nanoDSF analysis of Hsc70^{T204A} and pHsc70^{T204A} (86 % phosphorylated)

Samples were measured in three nucleotide states (apo, ATP-bound, ADP-bound). Melting temperatures (T_m) were determined from the maximum of the first derivative of the intrinsic tryptophan/tyrosine fluorescence signal at 330 nm. Values represent technical triplicates.

5.3.2 Protocol for isotopic labeling of pHsc70

While nanoDSF provided an initial assessment of the thermal stability of Hsc70 and pHsc70 under different nucleotide conditions, it does not provide detailed structural information. To obtain more direct insight into phosphorylation-induced conformational changes, methyl-TROSY NMR was intended as the next structural approach. Solution-state NMR is a well-established method to investigate the allosteric cycle of Hsp70 proteins and has, for example, been used to probe potential allosteric effects of BiP T518 AMPylation [10].

To enable phosphorylation-dependent structural studies by methyl-TROSY NMR, my aim was to combine the phosphorylation protocol developed in this work with an established ILV labeling protocol for Hsc70^{T204A} (see 8.2.6).

To implement the ILV labeling workflow for Hsc70 in our laboratory, Hsc70^{T204A} was first produced in *E. coli* grown in M9 medium without isotopic supplementation. In initial test expressions, no clear increase in Hsc70^{T204A} expression was observed when comparing samples before and after induction (Figure S6A). However, protein was enriched in fractions from the first and second IMAC steps and after SEC in subsequent purifications, indicating successful expression and purification, as confirmed by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the corresponding fractions (Figure 8A-C). To assess whether Hsc70^{T204A} synthesized in M9-grown cells remains a substrate of LegK4, the established phosphorylation protocol was applied (Chapter 5.2.2 and Chapter 8.2.3). Successful modification showed that Hsc70^{T204A} produced under these conditions can still be phosphorylated by LegK4 (Figure 8D).

Hsc70^{T204A} produced in M9-grown cells eluted across multiple fractions during SEC and thus behaves similarly to Hsc70^{T204A} produced in Luria-Bertani (LB) medium-grown cells, consistent with the presence of multiple oligomeric states (Figure 8B and Figure S6B). The apparent purity was comparable (Figure 8C and Figure S6C). The yield was approximately 4 mg of protein per liter of expression culture.

After establishing the M9 expression protocol in our laboratory, a one-time comparison was performed to test whether uniform ¹⁵N labeling substantially reduces protein yield. Two expression cultures were grown in parallel using either ¹⁴NH₄⁺ or ¹⁵NH₄⁺ as the nitrogen source, and proteins were purified after expression. Based on Bradford assay quantification, the yield of ¹⁵N-labeled protein was approximately 20% lower than that of ¹⁴N-labeled protein, but this difference was considered sufficiently small to proceed with ¹⁵N labeling in subsequent experiments. After these preliminary experiments, the complete set of labeling reagents, including D₂O, was used for ILV labeling of Hsc70^{T204A}. Apart from these additions, cells were cultured as in the preliminary experiments, and the protein was purified using the established final phosphorylation protocol (Chapter 5.1.2 and Chapter 8.2.3). SDS-PAGE after SEC confirmed the presence of the target protein and showed a purity comparable to previously purified unlabeled Hsc70 preparations (Figure 8C and Figure S6C), with full-length protein typically accounting for 65-75 % of the total signal, depending on the preparation (densitometric analysis using Fiji). At 0.4 mg per liter expression culture the yield was very low and approximately 10x lower than for protein purification after expression in LB medium.

To test whether phosphorylation was successful for the labeled protein and to assess whether isotope incorporation was consistent with the labeling strategy, iMS was performed. A mass difference of 83 Da between pHsc70^{T204A} and Hsc70^{T204A} was observed, consistent with monophosphorylation (Figure 8E); the phosphorylation state remained stable over 25 h. Thus, the objective of producing stably phosphorylated protein using the labeling workflow was achieved.

Because methyl-TROSY spectra were not acquired during this study, the presence of the intended ^{13}C -labeled ILV methyl probes cannot be confirmed directly by NMR. However, the measured intact masses can be compared with theoretical expectations (see 8.5.1). Assuming 100% deuteration as defined in the Methods, the theoretical mass is $m_{\text{theor.}} = 75542.2$ Da. The measured mass of ILV-labeled Hsc70^{T204A} was 75662 Da, i.e., 119.8 Da higher than the theoretical value.

The reproducible shift between phosphorylated and unmodified protein indicates that both samples share the same isotope-related baseline and that the phosphorylation difference is genuine rather than driven by differential H/D exchange. In a recently published framework, exchangeable protons (-NH, NH₂, -OH, -SH, -COOH) were assumed to be fully back-exchanged/protonated and therefore treated as ^1H in the theoretical calculation for intact LC-ESI-MS [122]. A plausible explanation for the + ~120 Da offset is incomplete back-exchange at a subset of these exchangeable sites under the actual measurement conditions (such as 0.1 % formic acid used). Residual deuterium at exchangeable positions would increase the intact mass by ~ 1.006 Da per site; an offset of ~120 Da corresponds to ~119 remaining D atoms, i.e., on the order of 10 % of the ~1200 exchangeable sites estimated from the sequence. Because this residual D would affect both phosphorylated and unmodified samples similarly, it would preserve the ~ 80 Da phosphorylation difference, consistent with the observations.

In summary, an established protocol for producing NMR-active (methyl-TROSY, ILV-labeled) Hsc70^{T204A} was combined with the stable phosphorylation protocol developed in this work. Although the yield was very low (0.4 mg per liter of expression culture), stably phosphorylated protein could be produced. NMR activity (i.e., the expected methyl-TROSY labeling pattern) was not verified due to limited sample availability. Based on iMS measurements and theoretical calculations, the samples are consistent with a high degree of deuteration and incorporation of ^{13}C and ^{15}N , although the labeled proteins were heavier than predicted by the theoretical mass calculation.

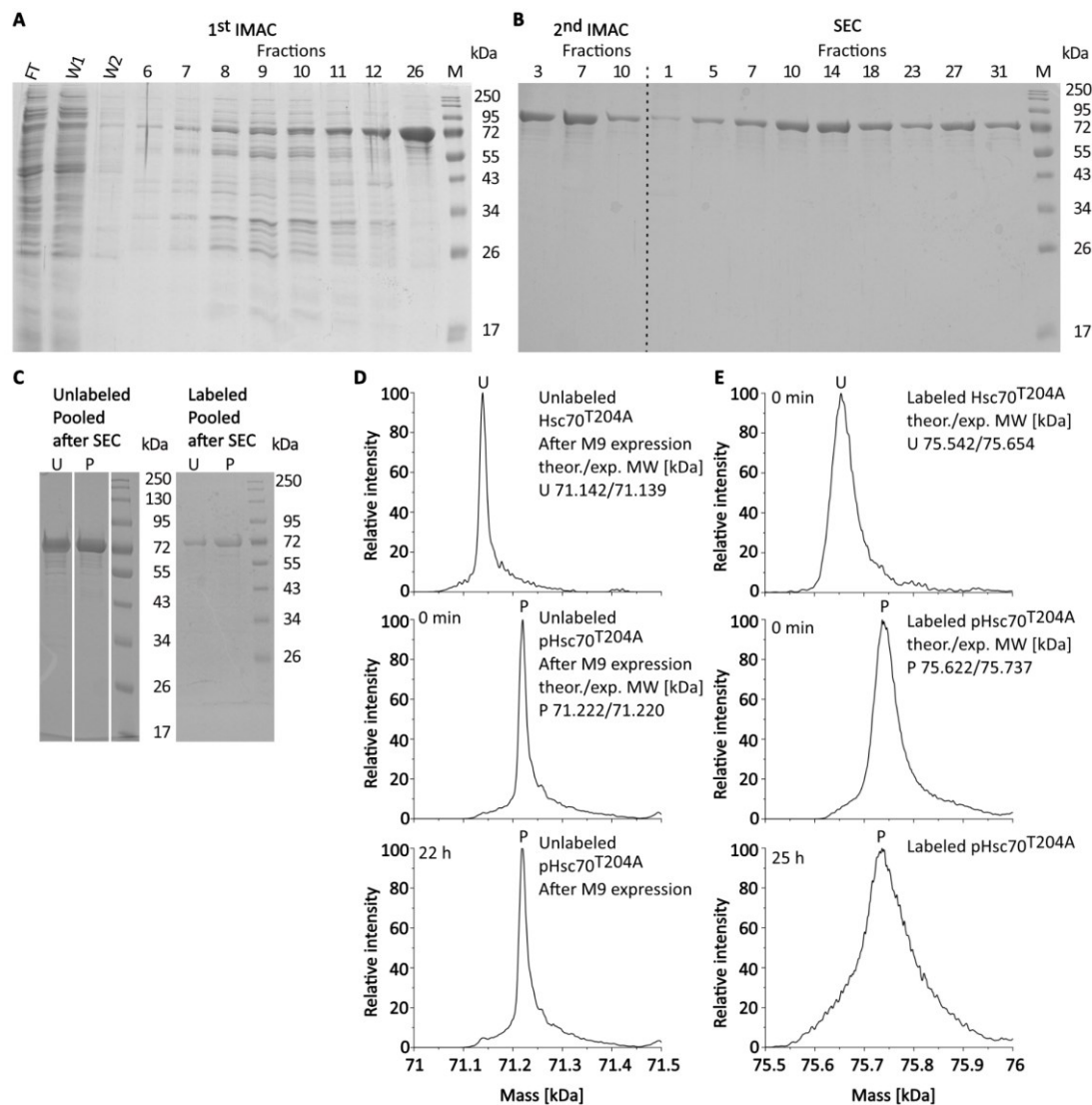


Figure 8: Development of an ILV-labeling and phosphorylation workflow for methyl-TROSY NMR analysis of Hsc70^{T204A} and pHsc70^{T204A}

(A) SDS-PAGE analysis of the first IMAC step for Hsc70^{T204A} expressed in M9 medium without isotopic supplementation. FT, flow-through; W1/W2, wash fractions; numbered lanes indicate elution fractions. Fractions 13-26 were pooled. (B) SDS-PAGE analysis of subsequent purification steps, with the dashed line separating fractions from the 2nd IMAC step performed after phosphorylation for kinase depletion (left) and fractions from the final SEC step (right). (C) SDS-PAGE of pooled fractions after SEC for unlabeled Hsc70^{T204A}/pHsc70^{T204A} (left) and ILV-labeled Hsc70^{T204A}/pHsc70^{T204A} (right). U, unmodified; P, phosphorylated. The overall purity is similar under both conditions. (D) Intact mass spectrometry (iMS) spectra of unlabeled Hsc70^{T204A} (U) and pHsc70^{T204A} (P) after expression in M9 medium, including a phosphorylation-stability test at 22 °C showing Hsc70^{T204A} (top), pHsc70^{T204A} at 0 min (middle), and pHsc70^{T204A} after 22 h (bottom). Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. The spectra indicate near-complete phosphorylation and stability over time. (E) iMS spectra as in (D), but for isotopically labeled Hsc70^{T204A} (top), pHsc70^{T204A} at 0 min (middle), and pHsc70^{T204A} after 25 h (bottom). Phosphorylation is near 100 % and stable. The spectra shown in (D) and (E) are representative of measurements performed in technical duplicates.

Even though the expression yield was low, the amount of protein obtained from 2 L of culture would, in principle, still allow preparation of a minimally concentrated NMR sample (for example, ~50 μ M protein in ~200 μ L) [6].

Based on the current yield, approximately 12 L of expression culture would be required to perform the planned measurements: Hsc70^{T204A} and pHsc70^{T204A}, each in the ATP-bound, ADP-bound and apo states. Because this would entail substantial financial cost and the protocol could not be sufficiently optimized within the available timeframe, SAXS was chosen as an alternative biophysical method to demonstrate that the phosphorylation workflow established here can produce the desired protein with stable phosphorylation.

5.3.3 Solution-state SAXS reveals structural consequences of Hsc70 phosphorylation at T495²

To demonstrate that the established phosphorylation protocol yields pHsc70 in quantities suitable for structural investigations, the structural consequences of phosphorylation at T495 were examined. The SAXS measurements and data analysis were performed with scientific advice from Dr. Aleksi Sutenin and Dr. Michael-Hecht Buchner, and with additional scientific advice on data analysis from Dr. Daniel Franke. Batch SAXS was selected as a solution-state method, as it allows characterization of global size, shape, and oligomerization.

The conformational dynamics and self-association of Hsc70 depend strongly on nucleotide state. In the ATP-bound condition, Hsc70 predominantly adopts a domain-docked conformation and typically shows reduced oligomerization, whereas ADP-bound and nucleotide-free (apo) conditions favor more extended, domain-undocked conformations accompanied by increased oligomer formation [6, 17, 19, 139]. Hsc70^{T204A} retains these nucleotide-dependent structural properties and is therefore suitable for probing phosphorylation-dependent effects [6, 140, 141]. To assess how phosphorylation at T495 influences these nucleotide-controlled ensembles, SAXS measurements were performed under ATP-bound, ADP-bound and nucleotide-free conditions. In the following, the nucleotide-free forms of Hsc70^{T204A} and pHsc70^{T204A} are referred to as Hsc70^{T204A}:apo and pHsc70^{T204A}:apo, respectively.

² Parts of the following sections are adapted and substantially expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, Advances on the large-scale bacterial production and stable phosphorylation of Hsc70. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923., in which these findings were originally reported.

5.3.3.1 Concentration-dependent oligomerization analyzed by Guinier plots and OLIGOMER

To obtain a size parameter, Guinier analysis of concentration-normalized SAXS curves was used to determine the R_g . As a first step, the dependence of R_g on protein concentration was examined (Figure 9A-C): For all nucleotide states except Hsc70^{T204A}:apo, R_g increases approximately linearly with protein concentration ($R^2 > 0.82$), consistent with attractive interparticle interactions and concentration-dependent oligomer formation in both Hsc70^{T204A} and pHsc70^{T204A}. Due to the common y-axis scaling in Figure 9A-C, the Hsc70^{T204A}:apo data appears linear at first glance (Figure S7A), but a rescaled view reveals marked deviations from linearity. Across all nucleotide conditions, pHsc70^{T204A} exhibits smaller R_g values than the corresponding unphosphorylated Hsc70^{T204A}. This difference suggests that phosphorylation at T495 alters the overall size and/or shape of the predominant scattering species, in line with changes in oligomeric state and/or conformation. To determine whether the observed R_g changes are attributable, at least in part, to altered oligomerization, the oligomeric composition of Hsc70^{T204A} and pHsc70^{T204A} was modeled with OLIGOMER, and volume fractions were calculated for several concentrations (Figure 9 D-F). Here, the volume fraction denotes the relative contribution of each species to the total scattering, with larger particles contributing disproportionately to the overall signal. For both phosphorylated and unmodified samples, the quality of OLIGOMER fits increased at lower concentrations, as reflected by reduced χ^2 values (Table S2). Although satisfactory fits could be obtained for only three concentrations in the pHsc70^{T204A}:ADP, Hsc70^{T204A}:ADP and Hsc70^{T204A}:apo series, the extracted oligomer distributions were internally consistent for these states. Overall, both Hsc70^{T204A} and pHsc70^{T204A} form oligomeric assemblies under all nucleotide conditions, with oligomer content increasing from the ATP-bound state to the ADP- and apo-states. In the ADP- and apo-states, monomeric species were generally not detected in the OLIGOMER fits, except for pHsc70^{T204A}:ADP at 1 mg/ml, where a small monomeric fraction (~23 %) was predicted together with a matching decrease in the dimer fraction. Across all nucleotide conditions, pHsc70^{T204A} consistently populates smaller oligomeric species than Hsc70^{T204A}. In summary, Guinier and OLIGOMER analyses reveal concentration-dependent oligomerization of both proteins and a reduced tendency to oligomerize upon T495 phosphorylation.

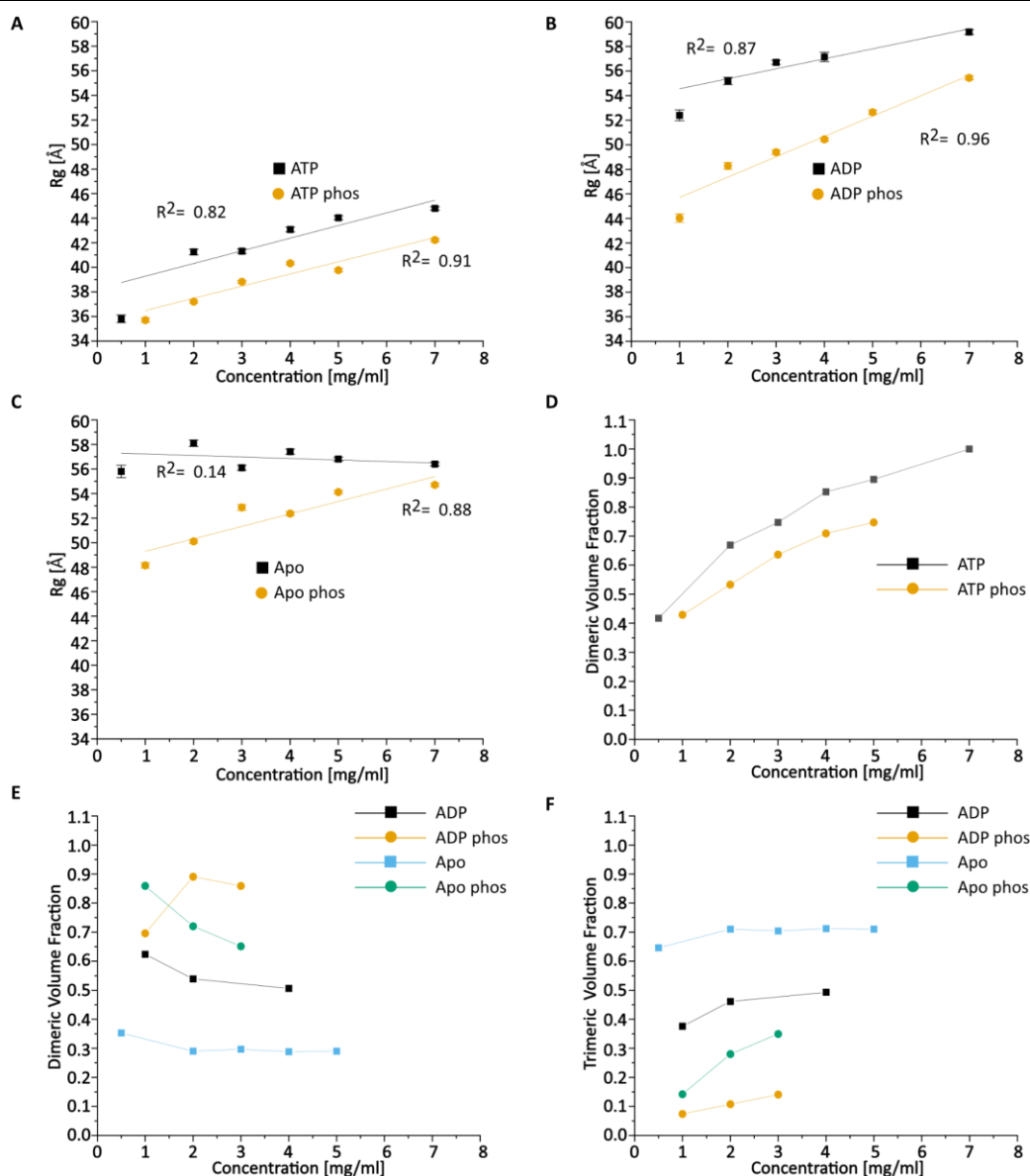


Figure 9: SAXS analysis reveals reduced oligomerization and smaller apparent particle size of pHsc70^{T204A}
 (A-C) Plots of the radius of gyration (Rg) versus concentration of Hsc70^{T204A}/pHsc70^{T204A} in different nucleotide states: ATP (A), ADP (B), and apo (C) with R^2 of linear fits indicated. Except for Hsc70^{T204A}:apo, the fits show a linear increase of Rg with increasing concentration. Note that for Hsc70^{T204A}:apo, the apparent linearity in panel C is influenced by the shared y-axis scale; see Figure S7A for a rescaled plot. The Rg for pHsc70^{T204A} is smaller than that of Hsc70^{T204A}. (D-F) Oligomeric volume fractions of Hsc70^{T204A} and pHsc70^{T204A} computed with OLIGOMER. Dimeric volume fraction in the ATP-state (D), dimeric (E) and trimeric (F) volume fractions in the ADP- and apo-states. Fit quality was assessed by the χ^2 values of the OLIGOMER fits. For pHsc70^{T204A}:ADP, pHsc70^{T204A}:apo, and Hsc70^{T204A}:ADP, only three concentrations yielded acceptable fits ($\chi^2 < 5.2$; Table S2). All datasets with higher χ^2 values were excluded from further analysis. According to OLIGOMER, there is no condition in which a 100 % monomeric population is sufficient to describe the data; for all nucleotide states of Hsc70^{T204A} and pHsc70^{T204A}, oligomeric species contribute appreciably to the scattering. Except for Hsc70^{T204A}:apo, the oligomerization behavior is concentration-dependent. Monomers are not observed in the ADP- and apo-states, except for pHsc70^{T204A}:ADP at 1 mg/ml, for which a monomeric volume fraction of 23 % is predicted, accompanied by a reduced dimeric volume fraction (E). In the ATP-states, only monomeric and dimeric volume fractions are detected. This figure and its caption were adapted from the cited publication [21].

5.3.3.2 *Real-space $p(r)$ analysis indicates reduced oligomerization and altered domain docking*

Complementary structural information was derived from the pair-distance distribution function $p(r)$, which provides real-space parameters including the R_g and the maximum particle dimension (R_{max}). Beyond these global parameters, the shape of the $p(r)$ distribution reflects overall conformation and domain organization and can also indicate contributions from different oligomeric species. $p(r)$ analysis was therefore used to compare Hsc70^{T204A} and pHsc70^{T204A} across the different nucleotide states. For all nucleotide conditions of Hsc70^{T204A} and pHsc70^{T204A}, apart from Hsc70^{T204A}:apo, R_{max} shows a linear increase with protein concentration (Figure S8). As observed for R_g , the shared y-axis in Figure S8C visually compresses the Hsc70^{T204A}:apo data; when plotted on an expanded scale (Figure S7B), clear deviations from linear behavior are evident. In addition, pHsc70^{T204A} consistently exhibits smaller R_{max} values than Hsc70^{T204A} in all nucleotide states. These findings mirror the trends observed for R_g and the OLIGOMER-derived volume fractions and further support the idea that phosphorylation diminishes the oligomerization propensity of Hsc70. In the ATP-bound state, both Hsc70^{T204A} and pHsc70^{T204A} show a single broadened $p(r)$ peak, consistent with elongated particles, a heterogeneous mixture of oligomers [128], or a combination of these cases (Figure 10A-B). The maxima of these peaks lie between approximately 34 and 37 Å. In the ADP- and apo-states of Hsc70^{T204A}, an additional, partially unresolved peak appears at larger distances, which can be interpreted in two ways: either as evidence of a heterogeneous mixture of oligomeric species, or as an indication that Hsc70^{T204A}:ADP/Hsc70^{T204A}:apo adopts a two-domain architecture connected by a flexible linker (Figure 10C-F). In oligomeric mixtures, such a second peak arises when the scattering contributions of the individual species are sufficiently distinct and show limited overlap [128]. For multidomain proteins, the first peak primarily represents intradomain distances, whereas a second peak at larger r values typically reports on the separation between domains [127]. Notably, the amplitude of this second peak is reduced in pHsc70^{T204A}:ADP and pHsc70^{T204A}:apo compared with their unphosphorylated counterparts.

Taken together with the R_g trends and OLIGOMER volume fractions, these $p(r)$ changes suggest that phosphorylation biases the conformational ensemble toward an ATP-like

state, likely via reduced oligomerization, increased domain docking, or a combination of both.

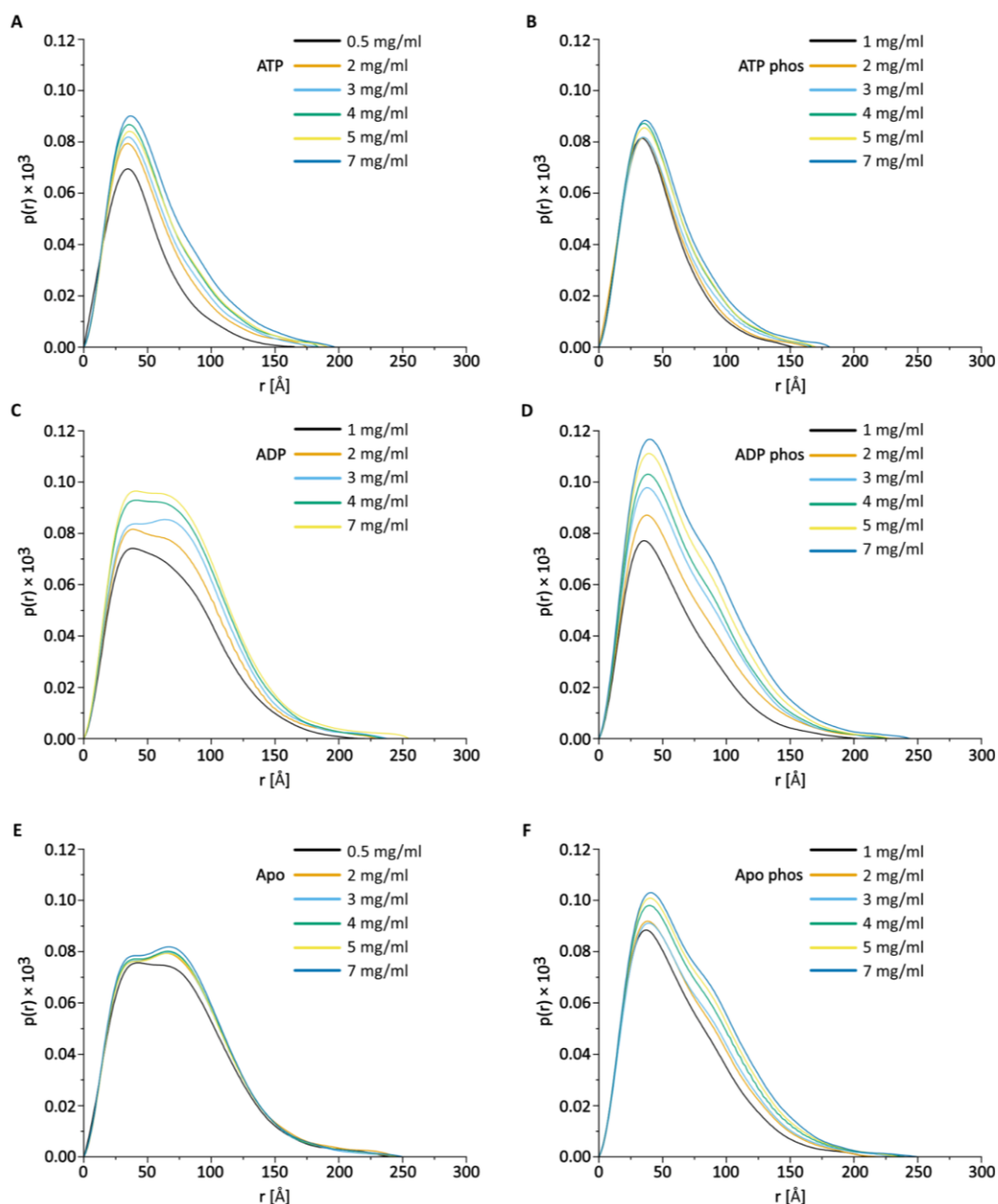


Figure 10: Distance distribution analysis indicates reduced long-distance contributions and altered conformational ensemble of pHsc70^{T204A} compared to Hsc70^{T204A}

(A-F) $p(r)$ distance distribution functions of Hsc70^{T204A} and pHsc70^{T204A} in different nucleotide states as analyzed using GNOM. Guinier ranges used in the analysis were determined with AUTORG. ATP-bound Hsc70^{T204A} (A) and pHsc70^{T204A} (B) display elongated curves that broaden with increasing concentration, accompanied by an increase in R_{max} (165-197 and 153-181 Å, respectively). Hsc70^{T204A}:ADP and Hsc70^{T204A}:apo (C,E) display a concentration-dependent second peak with R_{max} increasing with concentration in the ADP-bound state and remaining relatively constant in the apo state (220-255 and 240-250 Å, respectively). pHsc70^{T204A}:ADP and pHsc70^{T204A}:apo (D,F) also display a concentration-dependent second peak, which is smaller compared to Hsc70^{T204A}. R_{max} shows a concentration-dependent increase (203-244 Å and 210-250 Å, respectively). This figure and its caption were adapted from the cited publication [21].

5.3.3.3 *Dimensionless Kratky plots are consistent with increased domain docking of pHsc70*

Dimensionless Kratky plots are widely used to evaluate overall compactness and flexibility from SAXS data and can help distinguish compact particles from more extended, multi-domain conformations [142, 143]. They are therefore well suited to probe phosphorylation-dependent changes in domain organization of Hsc70^{T204A}, which transitions between domain-docked and domain-undocked states in a nucleotide-dependent manner. To reduce potential concentration-dependent interparticle contributions, scattering curves were extrapolated to zero concentration before calculating dimensionless Kratky plots (Figure 11).

In the ATP-bound state, both Hsc70^{T204A} and pHsc70^{T204A} display a single prominent maximum at $qR_g \approx 2.1$ (amplitude ~ 1.3), followed by an extended high- qR_g tail that levels off well below 2. Such a profile is typical of folded yet elongated particles rather than compact globular proteins [142, 143] and is consistent with a largely domain-docked conformation. This interpretation is supported by the corresponding $p(r)$ distributions, which likewise point to elongated conformations in the ATP-bound condition.

By contrast, Hsc70^{T204A}:apo and Hsc70^{T204A}:ADP exhibit two partially unresolved maxima, consistent with a flexible multi-domain architecture with linker regions and/or an overall dumbbell-like shape [144]. This is again in line with the $p(r)$ functions, which contain features compatible with a domain-undocked conformation.

In the ADP-bound and nucleotide-free (apo) states, pHsc70^{T204A} shows a single broadened maximum and lacks the bimodal pattern observed for unphosphorylated Hsc70^{T204A}. This shift toward a more compact Kratky profile is consistent with phosphorylation increasing the fraction of domain-docked conformations. Consistent with this view, phosphorylation has only a modest effect in the ATP-bound state, where Hsc70^{T204A} is already predominantly domain-docked.

As for the $p(r)$ analysis, differences in oligomerization between pHsc70^{T204A} and Hsc70^{T204A} are also expected to affect the Kratky profiles. However, the marked changes in Kratky curve shape, together with the Guinier trends and the $p(r)$ results, argue that oligomerization alone is insufficient to explain the data and that phosphorylation induces substantial changes in intramolecular organization consistent with enhanced domain docking.

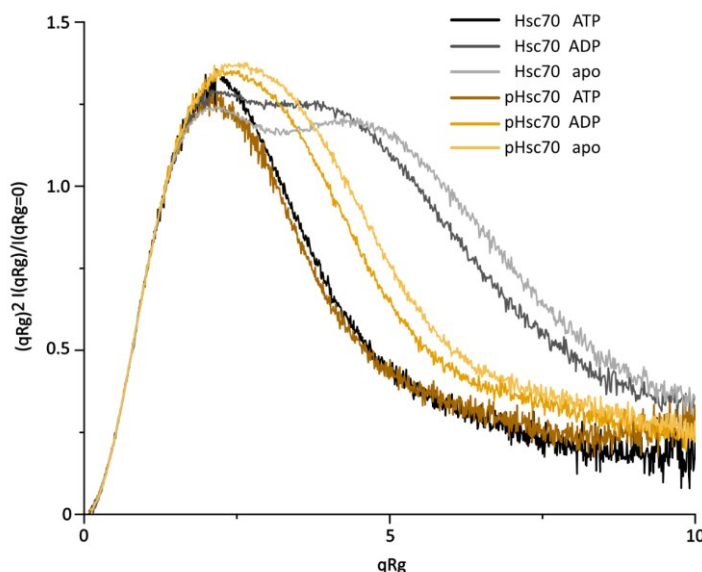


Figure 11: Dimensionless Kratky analysis indicates phosphorylation-induced stabilization of domain-docked conformations in Hsc70^{T204A}

Dimensionless Kratky plots calculated from zero-concentration-extrapolated scattering curves of Hsc70^{T204A} and pHsc70^{T204A} in the ATP-bound, ADP-bound and apo states. In the ATP-bound state, both proteins display an elongated curve with a single maximum at ~ 1.3 and $qRg \approx 2.2$, followed by a plateau below 2 at higher qRg values. For Hsc70^{T204A} in the ADP- and apo-bound states, the first peak is retained but a second, partially unresolved peak appears at larger qRg . In these two nucleotide states, pHsc70^{T204A} shows a mixed behavior: it exhibits only a single peak, but this peak is broader than in ATP-bound Hsc70^{T204A} and lacks the bimodal distribution seen for Hsc70^{T204A} in the ADP-bound and apo states. These differences are consistent with a shift toward more domain-docked conformations upon phosphorylation. This figure and its caption were adapted from the cited publication [21].

5.3.4 Analytical SEC of Hsc70^{T204A} and pHsc70^{T204A} supports phosphorylation induced oligomerization attenuation

SAXS indicated concentration-dependent changes in R_g , distance distribution, and OLIGOMER analysis. To complement the SAXS results and further evaluate whether these effects reflect changes in Hsc70^{T204A} oligomerization, and whether phosphorylation modulates this behavior, aSEC was performed. Separation by aSEC is based on the hydrodynamic properties of particles, which depend on both molecular mass and three-dimensional shape.

In the ATP state (Figure 12A-B), both proteins elute predominantly as a major peak (M). The mass corresponding to the peak center (84.81 kDa) is higher than the theoretical monomer mass of ~ 71 kDa (Table S3). This is likely due to the elongated shape of Hsc70^{T204A}, even in the domain-docked state, which results in altered elution behavior

compared to globular proteins. The peak therefore appears to consist predominantly of monomeric species with varying conformations, with a possible contribution from dimeric species.

In contrast to the ATP state, ADP and apo conditions promote oligomer formation in Hsc70^{T204A}, reflected by pronounced additional peaks and shoulders at lower elution volumes consistent with dimer/trimer and tetramer/higher-order assemblies (Figure 12C, E and Table S3). These oligomeric features are suppressed upon phosphorylation in the ADP and apo states, shifting the distribution toward the peak M across the concentration range (Figure 12D, F).

Notably, while the elution behavior and intensity of the oligomer-associated peaks remain largely unchanged across the concentration range, the position of peak M shows a reproducible, concentration-dependent shift (across technical duplicates). This shift is observed under all conditions tested, but is most pronounced for Hsc70^{T204A}:ATP and for pHsc70^{T204A} in the ADP and apo states, consistent with an increase in apparent hydrodynamic size, which may reflect conformational changes and/or a shift in the relative contributions of monomeric and dimeric species within peak M.

Considering both the magnitude of the monomer-peak shift and the accompanying early-eluting species, pHsc70^{T204A} in the ADP and apo states resembles unphosphorylated Hsc70^{T204A}:ATP. This suggests that phosphorylation biases Hsc70 toward an ATP-like solution-state distribution even under nucleotide-free or ADP-bound conditions, in line with the SAXS measurements.

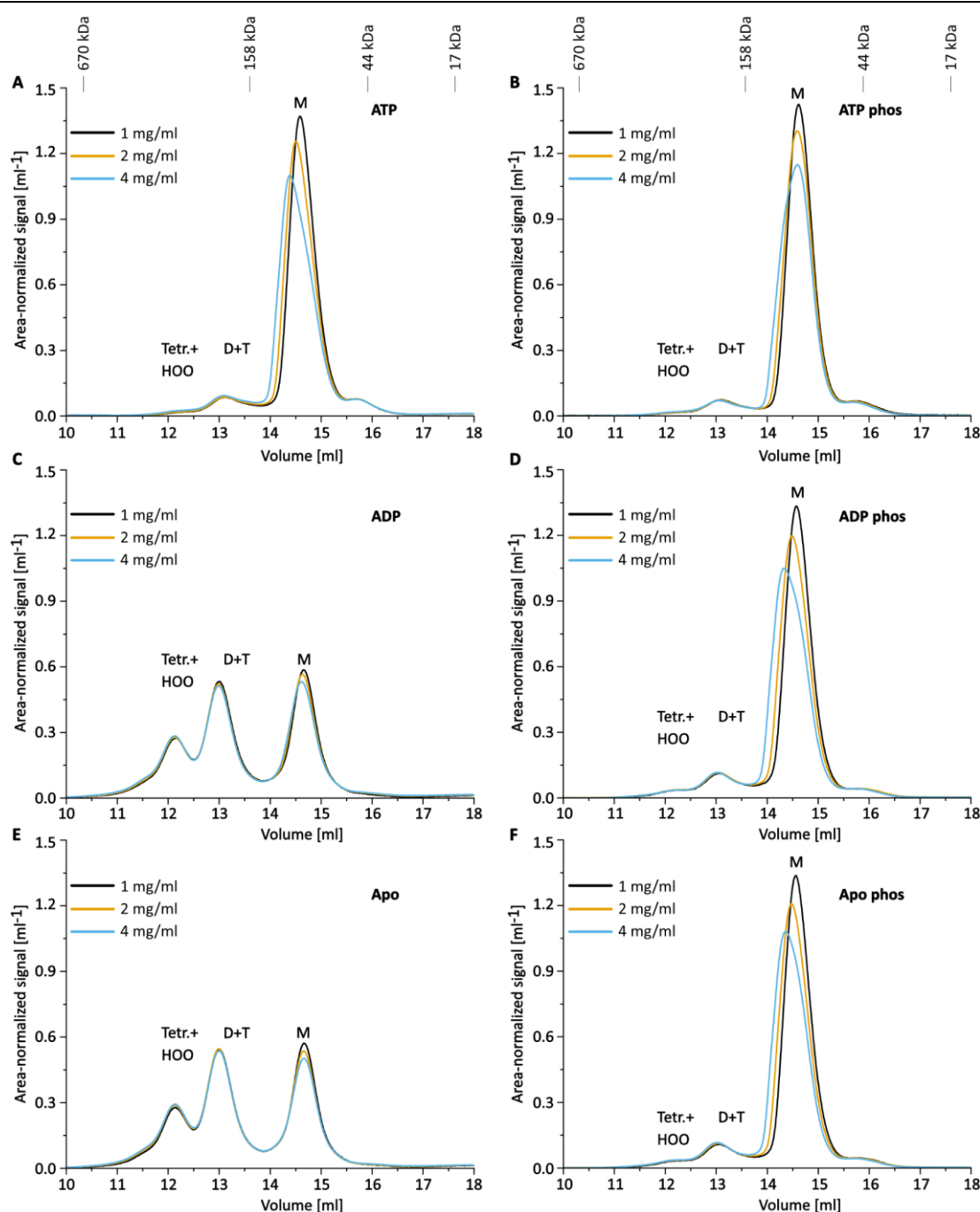


Figure 12: Analytical SEC reveals reduced oligomerization of pHsc70^{T204A} compared with Hsc70^{T204A} across nucleotide states

(A-F) Analytical size-exclusion chromatography (aSEC) of unphosphorylated Hsc70T204A (A, C, E) and phosphorylated pHsc70T204A (B, D, F) at 1, 2, and 4 mg/ml (black, orange, cyan) in ATP (A, B), ADP (C, D), or apo conditions. Chromatograms were baseline-corrected and area-normalized over the displayed elution range (y-axis: area-normalized signal, ml⁻¹). Peak assignments are indicated: M, monomer (including closely eluting larger species); D, dimer; T, trimer; Tetr., tetramer and higher-order oligomers (HOO). Molecular-weight standards are annotated above the chromatograms at the elution volumes corresponding to their respective peak maxima. Measurements were performed in technical duplicates; representative chromatograms are shown here, and the corresponding second replicate is provided in Figure S9. Across conditions, pHsc70^{T204A} shows a markedly reduced contribution of early-eluting oligomeric species compared with Hsc70^{T204A}. While both proteins elute predominantly as monomer in the ATP state (A, B), Hsc70^{T204A} in ADP and apo states displays pronounced additional peaks/shoulders consistent with dimer/trimer and tetramer/higher-order oligomers (C, E). In contrast, these oligomeric features are strongly diminished in pHsc70^{T204A} (D, F), with the signal remaining largely in the peak M.

5.3.5 Limited proteolysis supports domain docking of Hsc70^{T204A} upon T495 phosphorylation

Complementary to SAXS, limited proteolysis is used as an established method for investigating interdomain coupling in Hsp70 proteins: Depending on the protease, characteristic banding patterns are generated that report on conformational accessibility and domain docking/undocking dynamics [6, 92, 93, 96]. In proteinase K digests, cleavage within or near the interdomain linker produces prominent fragments in the expected molecular-weight ranges of the nucleotide-binding domain (NBD; ~43 kDa) and the substrate-binding domain (SBD; ~29 kDa) (Figure 13A). In contrast, a domain-docked state is expected to provide increased protection against linker cleavage, because the linker is tightly engaged by both NBD and SBD (Figure 1).

To assess this experimentally, Hsc70^{T204A} and pHsc70^{T204A} in the apo, ADP, and ATP states were subjected to proteinase K treatment. Upon protease addition, the high-molecular-weight region does not resolve into a single, clean full-length band but instead appears as a mixture of full-length-proximal species and slightly trimmed products (Figure 13A). In particular, a species around ~61 kDa is observed, consistent with partial cleavage affecting the α -helical region and/or trimming within the lid/tail region (Figure 13A, Figure S10A-C). Because individual components in this upper region cannot be reliably separated, quantification was performed using a high-molecular-weight core window (Figure 13A) as a proxy for linker-intact/bi-domain species.

Quantification of this core window reveals that phosphorylation does not strongly alter the high-molecular weight (high-MW) banding pattern overall (Figure S10D). This indicates that phosphorylation does not confer pronounced global protection against proteinase K cleavage, in agreement with the minimal changes in melting temperature upon phosphorylation measured by nanoDSF.

In contrast, analysis of bands near the expected molecular weights for free NBD and free SBD shows a clear phosphorylation-dependent effect (Figure 13B, Figure S10A-C). The summed intensities of intact NBD and SBD fragments are strongly reduced in the phosphorylated protein relative to the unmodified protein in all three nucleotide states. This pattern is consistent with increased protection of the interdomain linker and reduced

domain separation, suggesting that phosphorylation shifts the conformational ensemble toward a linker-protected, i.e., domain-docked, state.

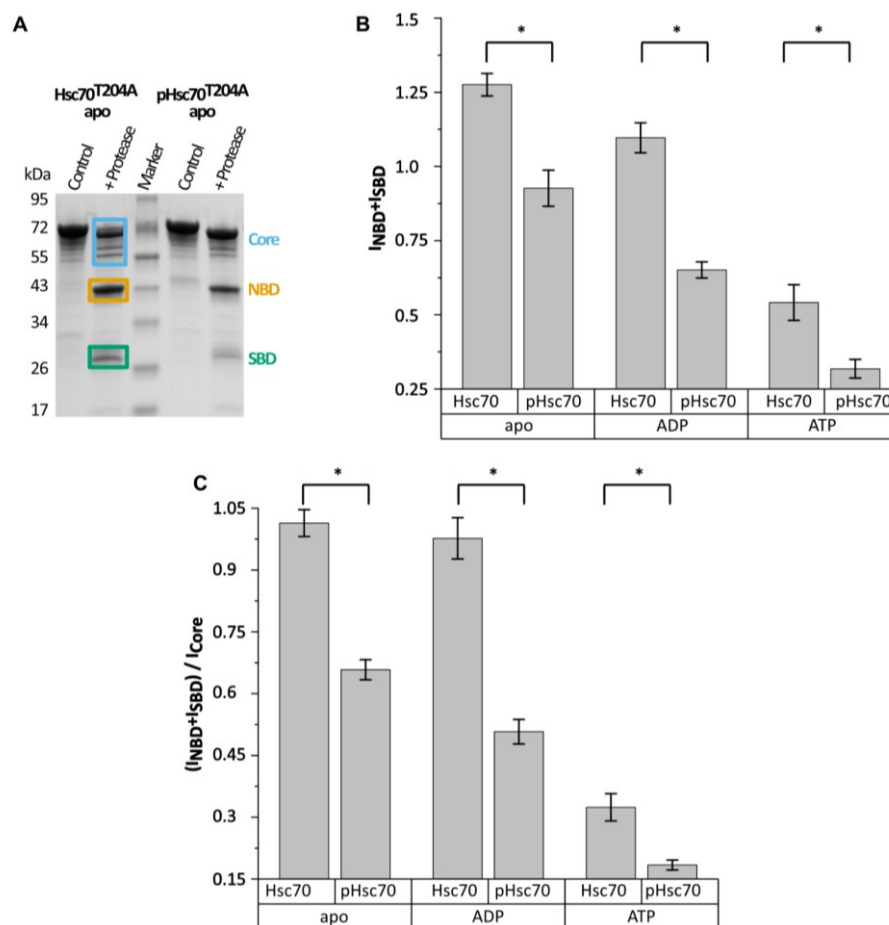


Figure 13: Limited proteolysis supports phosphorylation-dependent domain docking of Hsc70^{T204A} consistent with SAXS

(A) Representative SDS-PAGE gel showing limited proteolysis of Hsc70^{T204A} and monophosphorylated pHsc70^{T204A} in the apo state. Bands corresponding to the protease-resistant core fragment are highlighted in blue. Linker cleavage generates characteristic fragments corresponding to the nucleotide-binding domain (NBD, orange) and substrate-binding domain (SBD, green), which serve as readouts for domain separation following proteolysis. Full, uncropped gels and all technical replicates are provided in Figure S10. (B) Quantification of the combined NBD and SBD fragment intensities (INBD+ISBD), reflecting the extent of linker cleavage. Bars represent mean $\pm$ SD ($n = 3$ technical replicates prepared independently from the same protein preparation). Monophosphorylation significantly reduced linker cleavage compared to Hsc70^{T204A} in all nucleotide states (apo: $p = 0.00017$; ADP: $p = 0.00082$; ATP: $p = 0.0106$; unpaired two-tailed Welch's t-test). (C) Separation ratio ($(INBD + ISBD) / I_{Core}$), representing the relative abundance of linker cleavage products normalized to the protease-resistant core fragment. Monophosphorylation significantly decreased the separation ratio in all nucleotide states (apo: $p = 0.00017$; ADP: $p = 0.00051$; ATP: $p = 0.0106$; Welch's t-test), indicating a phosphorylation-dependent shift toward increased core stability and reduced linker accessibility.

A potential caveat of fragment-based readouts is that reduced fragment formation could, in principle, also reflect differences in overall digestion extent or signal rather than specific suppression of linker cleavage. To address this, NBD and SBD fragment formation was quantified relative to the pool of high-MW, putatively linker-intact (bi-domain)

species by calculating the ratio $(I_{\text{NBD}} + I_{\text{SBD}}) / I_{\text{Core}}$. This ratio reports the extent to which free NBD and SBD fragments accumulate relative to the pool of high-MW, putatively linker-intact/bi-domain species. Phosphorylation markedly reduced this normalized fragment signal in apo, ADP, and ATP conditions (Figure 13C). Thus, even when normalized to the abundance of linker-intact/high-MW species, formation of free NBD and SBD fragments is strongly attenuated by phosphorylation. Together, these data indicate that phosphorylation does not primarily act by globally suppressing proteolysis, but instead specifically reduces the formation of domain-separation products, consistent with increased interdomain coupling and protection of the linker. In this way, limited proteolysis supports the SAXS-derived conclusions ($p(r)$ functions and Kratky analyses) by providing an independent line of evidence (Chapters 5.3.3.2 and Chapters 5.3.3.3).

5.4 Exploration of ATP-analogs for covalent capture of kinase-substrate interaction

Because kinase-target interactions are often transient, structural characterization of such complexes, e.g. by crystallography, remains challenging [132, 133]. To address this, several strategies have been developed to stabilize kinase-target assemblies, including the use of covalent trapping approaches.

Building on the established Hsc70-LegK4 system, this thesis therefore used it as an initial test case to evaluate ATP analogs (TReND-K1-5) designed to stabilize kinase-substrate complexes and to assess whether they can support complex formation at a level suitable for subsequent structural analysis. The ATP analogs tested here carry a polycarbon linker attached to the γ -phosphate that terminates in a chloroacetamide warhead (Figure 3A, C). This electrophile can react with a suitably positioned cysteine thiol in the kinase via nucleophilic substitution, thereby forming a covalent kinase-nucleotide binary adduct (Figure 3D) which can be detected by iMS. If the modified γ -phosphate together with the linker is subsequently transferred to a substrate hydroxyl group, a covalent ternary complex consisting of kinase, nucleotide analog, and substrate would result, which could be detected by SDS-PAGE.

In addition to LegK4-Hsc70, the approach was also evaluated using human protein kinase 2 alpha prime (CK2 α') and its established substrates Huntingtin-associated protein 28 (HAP28) and Eukaryotic translation elongation factor 1 delta (EEF1D), to test whether a

canonical eukaryotic protein kinase might utilize these analogs differently compared to the bacterial effector kinase. Protein kinase CK2 is a highly conserved eukaryotic serine/threonine kinase that typically forms a heterotetrameric holoenzyme composed of two catalytic (CK2 α or CK2 α') and two regulatory β subunits [145]. Importantly, the catalytic subunits are constitutively active and do not require activating phosphorylation or association with the β subunit for activity. Consequently, CK2 α' retains robust catalytic activity when recombinantly expressed in *E. coli*. Owing to its well-characterized substrate spectrum and robust activity, CK2 α' was selected as a representative eukaryotic kinase to evaluate the utilization of the ATP analogs [146]. In the following, point mutants of CK2 α' are denoted as e.g. CK2 α'^{H161C} .

5.4.1 Structural insights into analog binding

To obtain structural insight into the binding mode of the ATP analogs and to assess potential constraints imposed by covalent linkage, crystallographic studies were carried out using CK2 α'^{H161C} in complex with TReND-K1 (Figure S11B). A 2.35 Å crystal structure, solved by Vivian Pogenberg, reveals two molecules in the asymmetric unit (Figure 14A, Table S8). In the literature, both monomeric (e.g., Protein Data Bank (PDB) entries 5CVG, 3NSZ) and dimeric (e.g., PDB entries 5CLP, 8QCG, 3U87) crystal structures of CK2 α and CK2 α' have been reported [147-150]. However, CK2 structure and function are well established, and it is generally accepted that the functional unit is a heterotetramer composed of two catalytic (α/α') and two regulatory (β) subunits, with tetramer formation mediated by the β subunits [151, 152]. Consistent with this, analysis using PDBePISA [153] does not support a stable dimeric assembly. PDBePISA is a web-based tool for the assessment of macromolecular structures that can be used to calculate structural and chemical properties of interfaces and surfaces of macromolecules [153]. The largest observed interface in the CK2 α' crystal structure is relatively small (~ 589 Å²) and displays an unfavorable interaction energy ($\Delta iG = +7.2$ kcal/mol, calculated) together with a high P-value (0.894), indicative of a non-specific contact. Furthermore, the calculated free energy of dissociation for the proposed dimer is negative ($\Delta G_{\text{diss}} = -15.7$ kcal/mol), suggesting that the assembly would be unstable in solution. Taken together, these data indicate that the observed dimer in the asymmetric unit of the crystal is most likely not a biologically relevant assembly.

Importantly, the ligand is bound in the ATP-binding pocket in both chains (Figure 14A). Polder map analysis reveals clear electron density along the ligand, indicating good agreement between the experimental data and the ligand-binding model (Figure 14B, C; shown for chain A). Only weak electron density is observed at the site of the expected location of the covalent linkage between C161 and the ligand (Figure 14C), likely reflecting conformational flexibility of the linker region. This observation is consistent with previously reported structures of cysteine-reactive analogs following chloroacetamide reactions [118, 154]. Measurements of binary adduct formation after 4 h and 22 h show modification levels of $91.5 \pm 5.0\%$ and $94.0 \pm 2.8\%$, respectively (Figure 14D, Chapter 8.3.14). The observed mass shift of the protein signal upon addition of TReND-K1 is 590 Da, corresponding exactly to the expected mass increase upon binary adduct formation (i.e., loss of chloride and formation of a covalent bond), indicating a covalent linkage between ligand and protein.

Superposition of chain A with the reference structures (PDB entries 3U87 and 8QCG), both containing non-hydrolyzable ATP analogs, yields low root-mean-square deviation (RMSD) values ($0.238 \text{ \AA}$ and $0.152 \text{ \AA}$, respectively), indicating a high degree of structural similarity (Figure 14E). Key residues involved in ATP binding and catalysis adopt nearly identical positions across all structures (Figure 14F, G shown for PDB entry 8QCG; Table S4 lists these residues and summarizes their structural and functional relevance), despite the presence of magnesium ions in the reference structures and their absence in the structure presented here. Notably, both reference structures show a well-defined nucleotide analog in the ATP-binding site, supporting a catalytically relevant nucleotide-bound state [149, 150]. Given the conserved arrangement of the key residues involved in ATP binding and catalysis, the structure presented here can be interpreted as adopting a similarly competent nucleotide-binding mode. However, notable deviations are observed in the positioning of the β - and γ -phosphate groups compared to the reference structures (Figure 14F, G shown for PDB entry 8QCG). These shifts likely result from the covalent linkage to Cys161, which imposes geometric constraints on the ligand. Such distortions may impair optimal positioning for catalysis and therefore suggest that, despite competent binding, catalytic turnover could be reduced. Based on these observations, additional analogs with extended linker lengths were synthesized by the Hedberg group (Figure 3A, C).

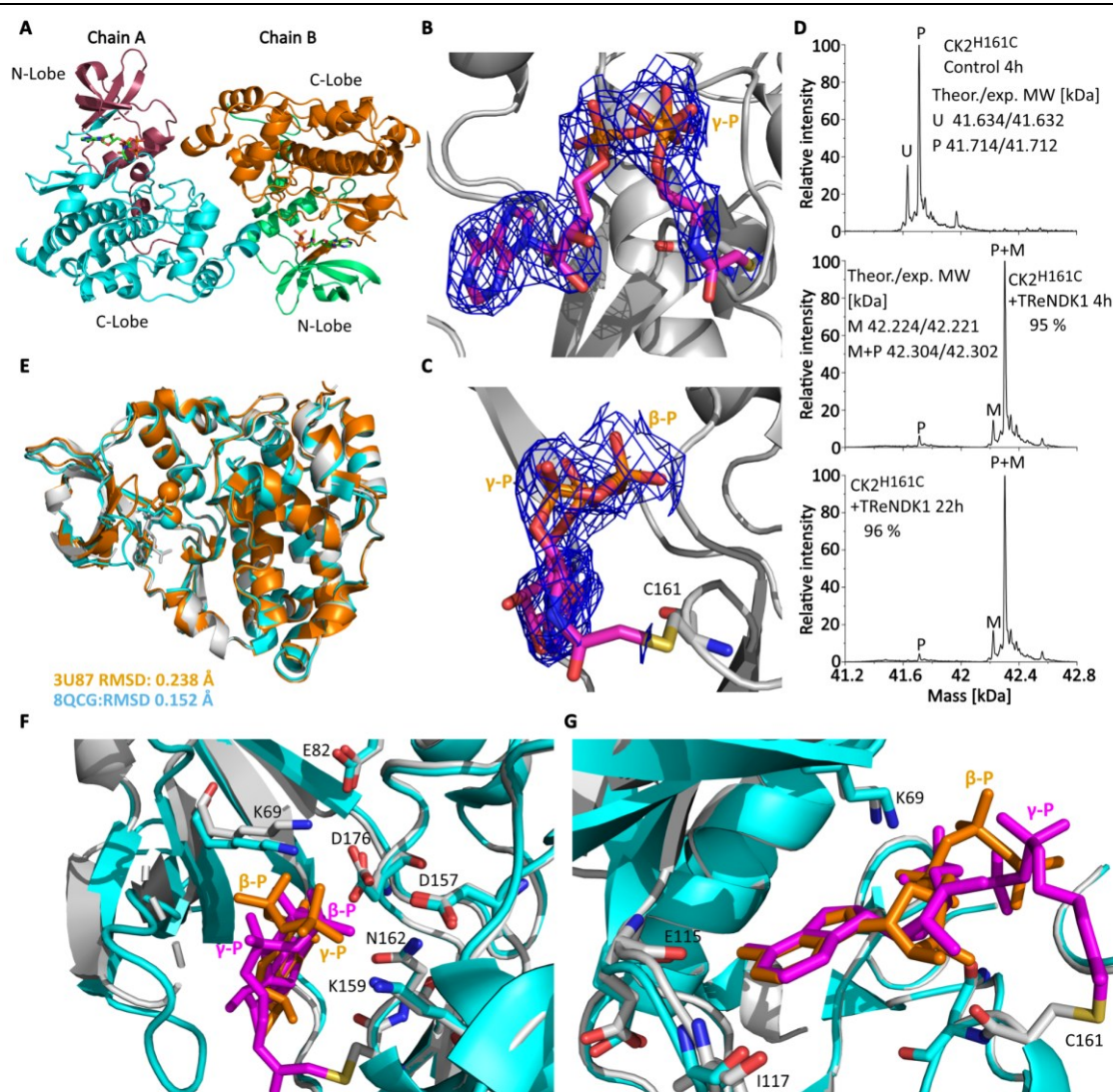


Figure 14: Structural and biochemical characterization of CK2 α' ^{H161C} in complex with the ATP analog TReND-K1

(A) Crystal structure of CK2 α' ^{H161C} in complex with TReND-K1 revealing two protein molecules in the asymmetric unit. The N- and C-lobes of each chain are indicated. Inter-chain contacts are observed but are consistent with a non-biological interface. (B-C) Close-up view of the ATP-binding pocket (chain A) with TReND-K1 shown as sticks and corresponding polder electron density (blue mesh). Clear density is observed for the ligand, while only weak density is present at the proposed covalent linkage site to Cys161, consistent with previous observations for chloroacetamide-based analogs. β -P = β -phosphate, γ -P = γ -phosphate. (D) Intact mass spectrometry demonstrating efficient binary adduct formation between CK2 α' ^{H161C} and TReND-K1 based on independent technical duplicates after 4 h and 22 h incubation, yielding $91.5 \pm 5.0\%$ and $94.0 \pm 2.8\%$ modification, respectively, with a mass shift consistent with covalent bond formation (+590 Da). Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. (E) Structural superposition of CK2 α' ^{H161C} (chain A, this work) with reference structures containing non-hydrolyzable ATP analogs (PDB entries 3U87, 8QCG), showing high structural similarity (low RMSD values). (F,G) Detailed comparison of the ATP-binding site highlighting conserved catalytic residues and deviations in the positioning of the β - and γ -phosphate groups relative to 8QCG (cyan). These shifts likely arise from geometric constraints imposed by covalent linkage to Cys161 and may impair optimal positioning for catalysis. AppNhp is depicted in orange and TReND-K1 in magenta. Heteroatoms are colored as follows: nitrogen (blue), oxygen (red), and sulfur of Cys161 (yellow). The residues shown are described in detail in Table S4. No magnesium ions are observed in the structure presented in this thesis, whereas the magnesium ion in the 8QCG structure has been omitted for clarity. CK2 = CK2 α' .

Together with the synthesis of new ATP analogs varying in linker length, additional cysteine mutations were introduced to probe a broader region of the ATP-binding pocket. Amino acid residues selected for cysteine substitution were chosen along the ATP-binding pocket based on the PDB reference structures 5CKW for LegK4 and 3U87 for CK2 α' (Figure S11). In most cases, residues were selected such that their side chains were oriented toward the γ -phosphate. Distances between the selected residues and the γ -phosphate of bound APPNHP were allowed to vary (8-13 Å, measured from the C α atom of the selected residue to the γ -phosphate), as the different analogs span a range of linker lengths.

5.4.2 Functional evaluation of ATP analogs in kinase systems

Subsequent experiments addressed whether the ATP analogs support efficient binary adduct formation, phosphotransfer, and ternary complex formation in a functional setting. These analyses were first pursued in the established LegK4-Hsc70 system.

Initial experiments showed that truncated wild-type LegK4₅₉₋₄₆₂ forms a binary adduct with the analogs (representative example, Figure 15A). Depending on the analog used, the extent of binary adduct formation was only ~8-11% (Table S6), which is not sufficient for subsequent ternary complex formation at preparative scales. Therefore, based on the crystal structure of truncated LegK4 (5CKW), amino acid residues in the vicinity of bound AppNhp were selected and systematically replaced by cysteines (as described in Chapter 5.4.1) (Figure S11A). From the crystal structure it was also inferred that the endogenous cysteine C145 is the most likely reactive residue through which wild-type LegK4 can bind the analogs. To prevent heterogeneous binary adduct formation, C145 was mutated to serine. It was then tested whether the newly introduced sulfhydryl groups form a covalent linkage with the ATP analogs. While the C145S mutant can no longer form binary adducts with the analogs, the cysteine variants bind the analogs to varying extents (Figure 15A, Figure S13, Figure S14, Figure S15 and Table S6). The extent of modification depends on the specific combination of analog and cysteine variant used. Independent measurements of binary adduct formation after 5 h and overnight indicate that, for most combinations, the level of modification does not change substantially over time. This suggests that the formed binary adducts are stable over the investigated time frame. The

mass shifts of the observed peaks are close to those expected for the covalent adduct formed by nucleophilic substitution (Table S5).

After confirming that the analogs react with cysteins in LegK4₅₉₋₄₆₂, transfer of the γ -phosphate-linked groups from the analogs to Hsc70 at T495 was tested. While LegK4₅₉₋₄₆₂^{C145S} efficiently phosphorylated Hsc70 when ATP was used as the cosubstrate (as indicated by an ~80 Da mass shift in the Hsc70 signal; Figure 15B), no corresponding mass shift was observed upon addition of the analogs (Figure 15B; TReND-K1 and TReND-K4 shown as representative examples). To exclude the possibility that linker transfer failed because Hsc70 in the apo state represents a less favorable substrate, Hsc70 was pre-soaked with ATP prior to dilution and subsequently incubated with LegK4₅₉₋₄₆₂^{C145S} and the nucleotide analogs. However, no corresponding mass shift was detected under these conditions either, with TReND-K1 and TReND-K4 shown as representative examples (Figure S16). Successful transfer of the modified γ -phosphate would be expected to yield an additional mass increase of approximately 200 Da for TReND-K1 and approximately 256 Da for TReND-K4. The absence of such signals indicates that transfer of the analog-derived γ -phosphate moiety to Hsc70 did not occur at detectable levels under these conditions.

To investigate whether the efficiency of phosphotransfer can be increased when the binary adduct of kinase and covalently bound analog is first formed and then incubated with Hsc70, ternary complex formation was attempted using the pre-formed binary adduct. Because ATP and ADP each modulate the conformation of Hsc70 allosterically, complex formation was carried out both in the presence of ATP and in the presence of ADP (Figure 15C, D). As a negative control, LegK4₅₉₋₄₆₂^{C145S} was analyzed alongside the cysteine-substitution variants. As a representative example, the variant LegK4₅₉₋₄₆₂^{C145S D210C} is shown (Figure 15C, D). In neither the ATP nor the ADP condition was a band shift observed. A complex of LegK4₅₉₋₄₆₂ variants with Hsc70 has a theoretical mass of approximately 116 kDa. Since milligram amounts of the ternary complex are required for structural studies, this experiment was not further analyzed using more sensitive methods such as Western blotting. In the absence of a detectable complex at the preparative scale, ternary complex formation was therefore considered unsuccessful for the purpose of structural characterization.

LegK4 exhibits deviations from canonical eukaryotic protein kinases [62]. To test whether eukaryotic protein kinases might use the analogs more efficiently, the approach established for LegK4 was applied to CK2 α' using the known substrates HAP28 and EEf1D.

Since the wild-type kinase did not show detectable binary adduct formation with the analogs (Figure S12A, Figure S17A and

Table S7), several cysteine-substitution variants were generated (Figure S11B). Similar to LegK4₅₉₋₄₆₂, these variants show mutant- and analog-dependent binary adduct formation ranging from low to near-quantitative levels (Figure S12A, Figure S17, Figure S18, Figure S19 and

Table S7). The extent of modification appears to remain largely stable between the 4 h and overnight time points.

When wild-type CK2 α' was used to transfer the γ -phosphate of TReND-K1 or TReND-K2 together with the linker to HAP28 and EEf1D, no signals corresponding to the expected mass shifts were observed (Figure S12B) while the ATP control shows that the kinase is active. For HAP28, phosphorylation was only detected when CK2 α' was incubated with ATP. As observed for LegK4₅₉₋₄₆₂^{C145S}, no detectable transfer of the γ -phosphate together with the attached linker moiety to the substrate was observed under these conditions.

Finally, ternary complex formation was tested for binary adducts formed between the different CK2 α' cysteine mutants and the different analogs, using HAP28 and EEf1D as substrates (Figure S12C, D). CK2 α' ^{S195C} is shown as a representative example. As observed for the LegK4₅₉₋₄₆₂^{C145S} variants, no band shift was detected in the SDS-PAGE analysis.

In summary, ATP analogs designed for complex stabilization were analyzed for their capability of binary adduct and ternary complex formation with two different kinases.

The unmutated enzymes showed no detectable (CK2 α') or only limited (LegK4₅₉₋₄₆₂) binary adduct formation. Introduction of cysteine residues in the vicinity of the ATP-binding pocket enabled efficient binary adduct formation in selected mutants. These binary adducts did not result in ternary complex formation at levels sufficient for structural studies, as judged by SDS-PAGE. Moreover, when variants unable to form binary adducts were used, no phosphotransfer to the substrates could be detected by iMS, indicating that transfer of the γ -modified ATP analogs is highly inefficient under these conditions.

Taken together, these results did not support further use of the analogs for structural complex stabilization, which was their primary intended application.

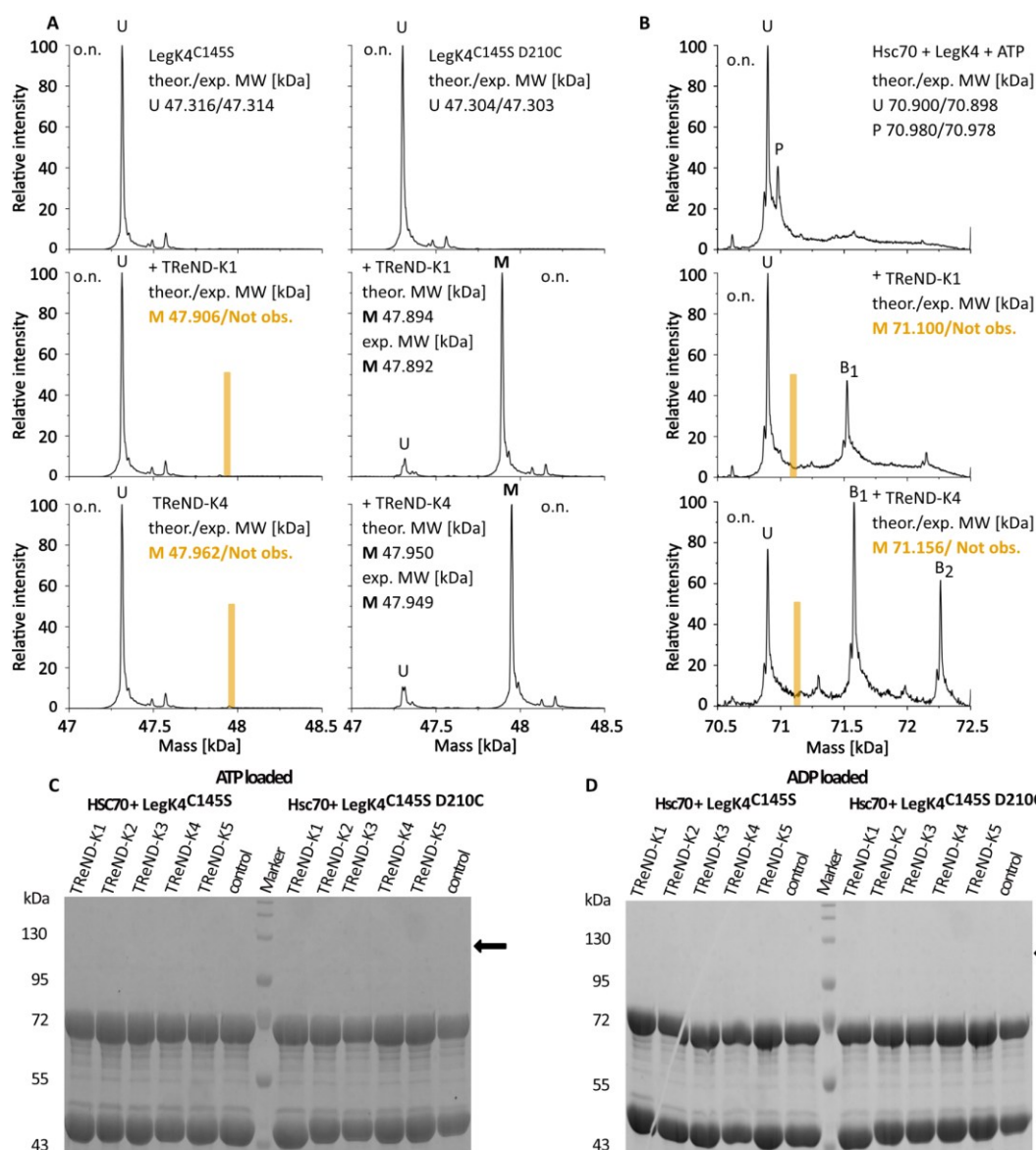


Figure 15: Intact mass spectrometry and SDS-PAGE analysis of potential Hsc70-analog modification, binary adduct formation and potential ternary complex formation

In this figure, LegK4 refers to the truncated construct LegK4⁵⁹⁻⁴⁶².

(A) Intact mass spectrometry (iMS) spectra of LegK4⁵⁹⁻⁴⁶²^{C145S} (left) and LegK4⁵⁹⁻⁴⁶²^{C145S D210C} (right) before and after incubation with TReND-K1 or TReND-K4 overnight (o.n.) at 22 °C. For LegK4⁵⁹⁻⁴⁶²^{C145S}, only the unmodified protein (U) is detected; the theoretical mass of the covalently modified species (M) is indicated by orange bars and not observed (Not obs.). In contrast, LegK4⁵⁹⁻⁴⁶²^{C145S D210C} shows peaks at the expected masses for TReND-K1- and TReND-K4-modified protein, while the unmodified species is largely depleted. Additional mutants and analogs are summarized in Table S6 (B) iMS spectra of 10 μM Hsc70 and 0.5 μM LegK4⁵⁹⁻⁴⁶² after overnight incubation with ATP (top), TReND-K1 (middle) and TReND-K4 (bottom) to assess transfer of the analog linker region. Peaks corresponding to unmodified Hsc70 are labelled U, phosphorylated species are labelled P, analog bound Hsc70 is labelled B1 and B2. The theoretically observable species modified with the linker region of the analogs are indicated with M and the area highlighted in orange. For these species, no peaks are visible. Equivalent negative results were obtained for the other TReND-K: no substrate modification was detected in the presence of the analogs, whereas phosphorylation was observed in the ATP control. Replicate experiments were performed under closely similar, but not fully identical, experimental conditions and gave comparable results. (C,D) SDS-PAGE of ATP-loaded (C) or ADP-

loaded (D), mixtures of Hsc70 with LegK4^{C145S} or LegK4^{C145S D210C} after incubation with TReND-K1-5 or buffer (control). The arrow mark the expected migration position of a covalent Hsc70-LegK4 adduct. Representative single measurements are shown for one mutant; corresponding results for the remaining mutants are summarized in Table S6. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated.

6 Discussion

Post-translational modifications provide a powerful means to regulate Hsp70 chaperones by reshaping their allosteric cycle, interaction networks, and oligomeric equilibria. For Hsc70, phosphorylation at T495 and AMPylation at the homologous site in BiP have been linked to changes in ATPase regulation and conformational state, suggesting that chemical modification at this conserved region can bias Hsp70-family proteins toward distinct functional ensembles [84, 92, 93]. However, despite these functional observations, direct structural characterization of Hsc70 modified at T495, particularly under defined nucleotide conditions and in quantities suitable for biophysical and structural method, has remained limited. A key motivation of this work was therefore to enable a controlled comparison of phosphorylation and AMPylation at the same site by establishing preparative workflows that yield sufficiently modified and temporally stable protein. Accordingly, this thesis first established preparative workflows to generate Hsc70 modified at T495 by phosphorylation and AMPylation. While both modifications could be produced, further optimization of the AMPylation workflow would likely be required to consistently achieve higher modification levels and to fully exploit AMPHsc70 for downstream structural studies. Therefore, subsequent work focused on pHsc70^{T204A}. As isotopic labeling yields were insufficient for methyl-TROSY NMR, nanoDSF, SAXS, analytical SEC, and limited proteolysis were used instead to assess phosphorylation-dependent changes in stability, oligomerization, and domain organization across nucleotide states. Finally, this thesis explored a complementary strategy aimed at stabilizing transient kinase-substrate interactions for downstream structural work. Because kinase-substrate complexes are often weak and short-lived, ATP analogs bearing electrophilic warheads have been developed to covalently trap binary kinase-nucleotide adducts and, upon transfer, ternary kinase-analog-substrate complexes. Here, γ -phosphate-modified ATP analogs synthesized by the Hedberg group were evaluated using the Hsc70-LegK4 system and, additionally, CK2 α' with its substrates, to test whether covalent capture could provide stable complexes suitable for structural analysis.

6.1 Establishment of workflows for site-specific modification of Hsc70 at Thr495

6.1.1 Development of a stable phosphorylation workflow for Hsc70 and role of LegK4-dependent dephosphorylation³

It has long been appreciated that the so-called chaperone code (i.e. post-translational modifications such as phosphorylation) constitutes an additional regulatory layer for Hsp70 chaperones, acting alongside co-chaperone networks, expression control, and proteostasis pathways such as proteolysis [85]. Although functional studies are increasingly clarifying the roles and consequences of phosphorylation in Hsp70 systems [84, 88, 89, 91, 93, 94, 96-99], detailed investigation of phosphorylation-dependent structural effects is often limited by practical challenges in producing phosphorylated protein in a controlled manner. Similar constraints have been reported for other protein systems [155]. A prerequisite is knowledge of the relevant kinase. If the impact of phosphorylation at a defined residue is to be studied, the kinase used must be site-specific. In addition, kinases are known to catalyze reversal of phosphorylation *in vitro* under certain conditions [136, 137]. This necessitates efficient removal of the kinase after the reaction and subsequent monitoring of phosphorylation stability over time. While some studies perform *in vitro* phosphorylation followed directly by biochemical characterization, many workflows include an additional purification step after phosphorylation to ensure a stable phosphorylation state [156, 157]. Depending on the system, kinase depletion can sometimes be achieved by size-exclusion chromatography alone or by heat inactivation of the kinase [158-161]. In other cases, affinity chromatography and/or ion-exchange chromatography (IEX) are required to remove the kinase and to obtain stable, well-defined phosphorylated protein preparations [84, 162, 163]. Using such approaches, a number of mono- and hyperphosphorylated proteins have been purified to date and characterized biochemically and, in some cases, structurally. For Hsp70 chaperones no structural studies based on enzyme-mediated phosphorylation of purified

³ Parts of the following sections are adapted and substantially expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923., in which these findings were originally reported.

Hsp70 proteins had been reported prior to the work presented in this thesis. To investigate the structural consequences of Hsc70 phosphorylation at T495, this thesis built on the discovery that the *Legionella* effector kinase LegK4 phosphorylates Hsc70 at this site [84]. Previously, Hsc70 phosphorylation levels ~53% were achieved, but no information was provided on preparative yield or on the temporal stability of the phosphorylation state [84]. Moreover, subsequent biochemical assays were performed in the presence of ATP [84]. In this work, it became apparent that the Hsc70-LegK4 system belongs to the class of kinase-substrate combinations in which accumulation of phosphorylated substrate is limited by kinase-mediated dephosphorylation, and where simple separation of kinase and substrate by SEC is not sufficient to ensure phosphorylation stability. This was evident from the initial phosphorylation workflow: when LegK4 was used at 700 nM, a combination of IMAC and SEC did not prevent loss of phosphorylation over time. Stable phosphorylation was only achieved when LegK4 was applied at catalytic concentrations (6.72 nM). Under these conditions, the SEC polishing step was optional, whereas the second IMAC step was essential for efficient depletion of the kinase. A follow-up dephosphorylation assay, in which addition of truncated wild-type LegK4, but not its kinase-dead variant, caused pronounced loss of phosphorylation, further confirms that LegK4 is responsible for the observed dephosphorylation. Because the phosphorylation state remained stable after kinase depletion, including under nucleotide-free conditions, the protocol is suitable for structural and biochemical investigations of pHsc70 in ADP-bound and apo states. The phosphorylation level of ~70 % obtained for wild-type Hsc70 is sufficient for many analytical readouts to detect differences between Hsc70 and pHsc70. For applications that benefit from maximal sample homogeneity, such as crystallography, this level may still be limiting. Notably, Hsc70^{T204A} reached higher phosphorylation levels (86 %). There is no direct evidence explaining why phosphorylation levels are higher for Hsc70^{T204A} than for wild-type Hsc70. However, it has been shown that BiP, whose homologous site is AMPylated, is preferentially bound and modified by its effector FicD in the ATP-bound state [118]. Several residues involved in FicD-BiP recognition are conserved or similar between BiP and Hsc70. It is therefore conceivable that LegK4, which can also phosphorylate BiP *in vitro* [84], and FicD share related binding modes and that both enzymes preferentially recognize the ATP-bound conformation of their Hsp70-family substrates. Because the T204A mutation strongly reduces basal ATP hydrolysis

[140] and thereby prolongs the ATP-bound state, this may provide a plausible explanation for the increased phosphorylation levels observed for Hsc70^{T204A}.

With the exception of the ATPase assay, which confirmed that Hsc70 purified using the final protocol remains catalytically competent, most analyses in this thesis were performed using the T204A mutant, for which phosphorylation levels were sufficient for the applied readouts. Nonetheless, further increases in phosphorylation stoichiometry appear feasible. In my view, the most promising approach would be to enrich pHsc70 relative to unmodified Hsc70 by IEX, as has been successfully applied to other phosphorylated protein systems [162, 163].

6.1.2 AMPylation of Hsc70 in comparison to BiP: workflow development and limitations

To date, no published protocol for preparative AMPylation of Hsc70 is available. In contrast, protocols for generating AMPylated BiP have been reported; these often employ the FicD^{E234G} variant and remove the enzyme by GST-tag affinity chromatography [92, 112]. For BiP, these workflows typically yield AMPylation levels close to 100%. Moreover, AMPylated BiP has been obtained in sufficient homogeneity for structural work and has even been crystallized, underscoring how well established preparative BiP AMPylation workflows are [92]. In the Hsc70-adapted protocol developed in this work, a His₁₀-tagged FicD double mutant (E234G/L258D) was used. This variant was chosen because the reduced autoinhibition and impaired dimerization are expected to shift the balance toward net AMPylation by limiting deAMPylation activity [110, 116]. Notably, SEC-purified Hsc70 consistently showed higher AMPylation levels. While this could reflect selection of specific protein populations, a more likely explanation is that SEC purification reduces the nucleotide content of the Hsc70 preparation. Indeed, depending on the purification route, the total nucleotide concentration prior to SEC was approximately 5-10-fold higher than after SEC (Figure S4). The majority of the detected nucleotides consisted of ADP and AMP. Because FicD displays different catalytic behavior depending on the nucleotide state of the substrate, these observations suggest that efficient nucleotide depletion is beneficial for achieving high AMPylation yields.

Publications reporting preparative AMPylation of BiP generally do not describe pronounced deAMPylation during purification when the E234G mutant is used [92, 112],

whereas in this thesis efficient FicD depletion proved critical for maintaining AMPylation levels in Hsc70. It is likely that the loss of AMPylation observed in the initial workflow reflects deAMPylation mediated by FicD. This interpretation is supported by the observation that addition of the FicD mutant used in this study to purified, stably AMPylated Hsc70 results in a clear decrease in AMPylation. Use of a catalytically inactive FicD control mutant would provide additional confirmation; nevertheless, the available data strongly suggest that FicD is responsible for the observed deAMPylation. Accordingly, efficient depletion of FicD in the final AMPylation workflow resulted in stably AMPylated Hsc70. Compared with the BiP *in vitro* assays performed in this work and with high-yield BiP AMPylation protocols reported [92, 112, 118], the AMPylation level achieved here for Hsc70 (approximately 63 %) appears relatively low. However, because insufficient comparative experiments under identical conditions have been performed to date, it cannot yet be concluded whether Hsc70 is intrinsically a poorer FicD substrate than BiP in terms of achievable AMPylation yield. Nevertheless, this thesis provides initial indications in this direction. The reasons for a potentially lower net AMPylation of Hsc70 remain unclear. As discussed above, several residues implicated in FicD binding to BiP are conserved or similar in Hsc70, and the overall structures of Hsc70 and BiP are closely related. Moreover, FicD has been reported to preferentially bind BiP in its ATP-bound state, in which BiP more frequently adopts a domain-docked conformation relative to other nucleotide conditions [118], suggesting that FicD may preferentially recognize the domain-docked state of its substrate. In this context, Hsc70 represents a similar, or even more favorable, target, as it populates the domain-docked conformation to a greater extent than BiP, particularly in the ATP-bound state [6, 10]. Despite these similarities, AMPylation levels of Hsc70 remain lower, indicating that additional factors may influence substrate recognition or catalytic efficiency. Several parameters could, however, still be adjusted to further increase AMPylation yield. The optimization experiments indicated that AMPylation was higher in HEPES-based buffer than in NaH_2PO_4 -based buffer and continued to increase within the 3-6 h incubation window, suggesting that longer incubations (e.g., ~6 h) under HEPES-based conditions may be beneficial. In the current workflow, Hsc70 is purified in NaH_2PO_4 -based conditions during steps in which the protein is concentrated or applied to chromatography columns, in order to maintain protein stability. The subsequent ~5 h dialysis into HEPES-based buffer prior to and during the

AMPylation reaction likely results in residual phosphate being present for a substantial fraction of the incubation period. Extending the incubation time and/or further reducing residual phosphate may therefore improve AMPylation levels. Along the same lines, it may be informative to test lower NaH_2PO_4 concentrations during purification, provided that protein stability is maintained. In addition, it is conceivable that the Hsc70^{T204A} mutant could yield higher AMPylation levels, analogous to the increased phosphorylation levels observed for pHsc70^{T204A} relative to wild-type Hsc70. Finally, only a single FicD concentration was used in the preparative workflow, selected based on the small-scale experiments. Testing moderately lower and substantially lower FicD concentrations may be useful, both to probe whether net AMPylation can be improved and to potentially reduce residual deAMPylation activity during sample preparation for the third IMAC step.

6.2 Phosphorylation enhances domain docking and attenuates oligomerization of Hsc70

To investigate phosphorylation-dependent effects, the ATPase-deficient mutant Hsc70^{T204A} was used for most structural experiments. In the following sections, phosphorylation-induced changes observed for Hsc70^{T204A} are therefore discussed as representative of Hsc70, under the assumption that the T204A substitution does not qualitatively alter the conformational and oligomerization responses examined here. This assumption is supported by previous work showing that the conformational dynamics and self-association of Hsc70 are strongly controlled by nucleotide state: ATP binding favors a predominantly domain-docked conformation with reduced oligomerization, whereas ADP-bound and nucleotide-free (apo) conditions promote more extended, domain-un-docked conformations accompanied by increased oligomer formation [6, 17, 19, 139]. Importantly, Hsc70^{T204A} retains these nucleotide-dependent structural properties and is therefore suitable for probing effects on domain organization and oligomerization [6, 140, 141].

6.2.1 Towards methyl-TROSY NMR of pHsc70: implementation of ILV methyl labeling

Methyl-TROSY NMR is an established approach for studying the allosteric cycle of Hsp70 chaperones [6, 10, 164]. Therefore, one aim of this work was to establish a suitable labeling protocol for the production of isotopically labeled Hsc70 and pHsc70.

Compared to the unlabeled protein, the IMS peaks of the labeled proteins exhibit broader isotopic envelopes. Such broadening is expected for isotopically labeled molecules, because increasing incorporation of ^2H and ^{15}N widens the isotopic distribution around the peak center [165, 166]. Phosphorylation produced a ~77-78 Da mass increase for unlabeled Hsc70^{T204A} expressed in M9 medium, whereas the corresponding mass shifts observed for the labeled protein were ~83 Da, compared with an expected +80 Da. Across this work, technical replicates of the same Hsc70 variant showed deviations of 0.5-4 Da in the reported intact masses, exceeding typical instrument accuracy [121].

A likely contributor to this variability is reduced spectral quality, as lower signal-to-noise ratios can impair peak centroiding and deconvolution accuracy [121]. Consistent with this, the labeled protein exhibited lower signal intensity than the unlabeled variant. In addition, signal broadening due to multiple charge states and isotope incorporation may further reduce mass accuracy.

Overall, the intact-mass results are consistent with successful incorporation of ^{15}N and the intended ^{13}C methyl labeling scheme in a highly deuterated background, while the absolute labeled mass appears to include an additional contribution most consistent with residual deuterium on exchangeable sites.

However, the yield of Hsc70^{T204A} obtained using the ILV labeling strategy applied in this study was only approximately 400 μg per liter of expression culture, while in publications reporting methyl-TROSY NMR measurements on Hsp70 proteins, expression yields are often not reported in a consistent manner [6, 10, 167].

Despite the low yield, protein produced from 2 L of expression culture would in principle be sufficient to meet minimal requirements for an NMR sample (e.g. ~ 50 μM protein in ~ 200 μL) [6]. Two factors are considered the main contributors to the low final yield in this work. Based on the first IMAC step (the earliest stage at which protein concentrations could be quantified consistently across protocols), the amount of unlabeled

Hsc70^{T204A} recovered from M9-grown cells was approximately twofold lower than that obtained using the LB-based protocol. Upon addition of the isotopic labeling components, the recovered amount decreased by a further ~ twofold, resulting in an overall ~ fourfold reduction relative to LB. Second, a SEC step was included to separate HMW species from the target protein fraction. This step introduced additional sample loss and is often omitted in comparable workflows [6, 10, 167]. For future NMR measurements of Hsc70^{T204A} and Hsc70^{T204A}, it will be important, also for cost reasons, to increase the expression yield. This could be achieved, by e.g. supplementing M9 medium with (deuterated) Celtone-based components (e.g., Celtone powder), which are frequently used for this purpose in the literature and would represent a natural next step [168]. In addition, it would be advisable to further adapt the workflow to established protocols and omit the SEC step to reduce sample losses.

6.2.2 Phosphorylation attenuates Hsc70 Oligomerization⁴

Hsc70^{T204A} and pHsc70^{T204A} analyzed in this work predominantly populated oligomeric ensembles, as supported by both SAXS and aSEC. Consistent with this, the R_g and R_{max} values obtained here exceed those reported for monomeric Hsc70^{E543K} in ATP- and ADP-bound conditions, indicating that mixed oligomeric species contribute substantially under the experimental setup [130]. This interpretation agrees with the SEC behavior during purification, which revealed co-existing monomeric, dimeric, trimeric, and higher-order fractions, and is further supported by the aSEC measurements.

Across the concentration range accessible in the SAXS experiments (0.5-7 mg/ml, depending on nucleotide state), the data indicate concentration-dependent shifts in the oligomeric distribution. This is reflected by approximately linear increases in R_g and R_{max} and by rising oligomeric volume fractions derived from OLIGOMER fits. A notable exception was Hsc70^{T204A} under nucleotide-free (apo) conditions, where the oligomeric state showed little concentration dependence. Finally, ATP-bound Hsc70^{T204A} and pHsc70^{T204A} formed smaller assemblies than the corresponding ADP-bound and apo states, consistent with nucleotide-dependent modulation of self-association. This

⁴ Parts of the following sections are adapted and substantially expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923., in which these findings were originally reported.

concentration-dependent oligomerization is not clearly reflected by the aSEC data in the sense of a pronounced shift from monomeric to oligomeric peaks across the three concentrations tested (1, 2, and 4 mg/ml). Instead, peak M, which is expected to be enriched in monomeric species but may also contain contributions from dimers, became broader over the tested concentration range and its peak maximum shifted toward lower elution volumes. This broadening and peak shift were most pronounced in the ATP-bound condition and across all nucleotide states of the phosphorylated protein. Whether these effects primarily arise from increased oligomerization, from conformational changes, or from a combination of both cannot be concluded from the aSEC data alone. Importantly, the rather strong increase in oligomeric fractions inferred from the SAXS-based $p(r)$ analysis and, in particular, from OLIGOMER fitting is not directly observed as a discrete redistribution of peaks by aSEC. Concentration-dependent self-association has been reported for multiple Hsp70 family members, including Hsc70, using both SEC-based approaches and SAXS [18, 129, 169, 170]. In many SEC-based studies, concentration dependence was assessed over broader concentration ranges and with larger step sizes than those used here. This may explain why the concentration-dependent differences observed by aSEC in this thesis are small to moderate. In addition, the dilution inherent to aSEC, particularly compared with static SAXS measurements, may attenuate detectable changes in equilibrium populations, suggesting that higher loading concentrations and/or larger concentration ranges may be required to resolve concentration-dependent oligomerization more clearly by aSEC.

Nucleotide-dependent effects on self-association within the Hsp70 family have been reported to vary between homologs and experimental conditions. For full-length Hsc70, ADP-bound conditions have been described to support a slow equilibrium between monomeric and oligomeric species, which shifts toward smaller assemblies upon ATP binding and upon addition of peptides or unfolded protein substrates *in vitro* [17]. *In vitro*, nucleotide-free Hsp70/Hsc70 often populates mixed oligomeric ensembles, underscoring that apo-state self-association is a common observation [18, 19]. By contrast, a recent comparative analysis across several Hsp70 isoforms (including Hsc70) reported ATP-dependent formation of more defined dimeric species and concluded that dimerization propensity is isoform-specific, with stress-inducible Hsp70 showing the strongest ATP-dependent dimer signal under their conditions [20]. In line with this broader literature,

the SAXS and aSEC data obtained here for Hsc70^{T204A} and pHsc70^{T204A} support a shift toward smaller assemblies in the ATP-bound state, while mixed oligomeric populations persist across the concentration range analyzed. Phosphorylation strongly reduces oligomerization, with the strongest effect observed under ADP-bound and nucleotide-free (apo) conditions, as supported by SAXS and aSEC.

The functional roles of Hsp70 oligomerization in cells (and how oligomerization is integrated with post-translational regulation) remain active areas of research. Distinct oligomeric states are thought to support different cellular functions. For example, dimerization and trimerization of Hsc70 have been implicated in clathrin coat disassembly, whereas monomeric Hsc70 does not efficiently bind clathrin triskelia [171]. In addition, phosphorylation of stress-inducible Hsp70 at T504 has been shown to stabilize an anti-parallel dimer in the context of client transfer to Hsp90 [91]. This is particularly interesting in the context of the present work, as the conserved T504 site lies close to T495 (Figure 2), yet phosphorylation at these two nearby residues appears to have opposite effects on oligomerization.

Notably, the ER-resident homolog BiP can also be phosphorylated by LegK4 *in vitro* at T518, the residue homologous to T495 in Hsc70 [84]. In addition, BiP is AMPylated by the ER Fic enzyme FicD, and AMPylation has been reported to reduce BiP oligomerization [92, 115].

BiP AMPylation reduces high-affinity binding of BiP to client proteins [92]. One hypothesis derived from work on BiP oligomerization proposes that BiP self-association reflects a substrate-like interaction, i.e. BiP can behave as a client for itself [16]. In this model, oligomerization serves as a proxy for substrate engagement, which would provide a plausible explanation for why AMPylation attenuates BiP oligomerization. Whether an analogous mechanism applies to Hsc70 is currently unclear, because substrate binding of pHsc70 has not yet been characterized directly. Notably, a manuscript currently under review reports that a phosphomimetic Hsc70 mutant (T495E) retains substrate binding, which would argue against a simple model in which reduced oligomerization is caused by impaired client interaction [93]. Direct measurements of substrate binding by pHsc70 will therefore be required to clarify the relationship between phosphorylation, client engagement, and oligomerization.

Higher-order oligomeric species have been reported for both Hsc70 and BiP, and these assemblies have been discussed as potential reserve pools that could be shifted towards active monomeric chaperone as demand increases [15, 16]. Early cell-based studies described BiP populations that were differentially modified and assembled, including forms reported as phosphorylated and ribosylated under conditions of low unfolded-protein load; in light of current understanding, the latter modification is likely to correspond to AMPylation [172-174]. More recently, AMPylation of BiP by FicD has been proposed to convert oligomeric BiP into an inactive, predominantly monomeric pool, thereby adding an additional layer of regulation [111, 116]. By analogy, phosphorylation of Hsc70 at T495 may similarly contribute to controlling the equilibrium between oligomeric and monomeric chaperone species (Figure16).

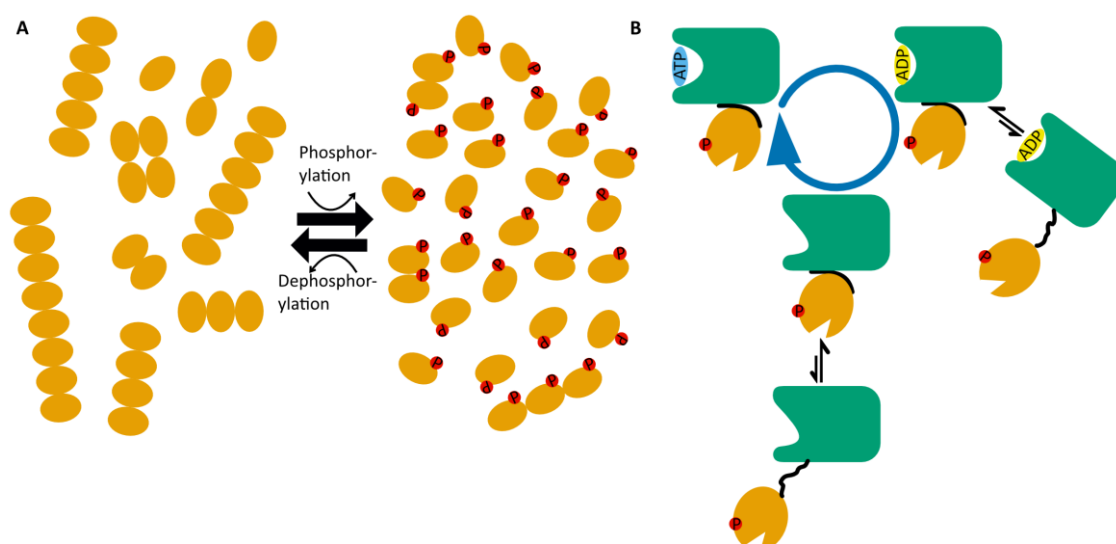


Figure16: Proposed model of the effects of phosphorylation at Thr495

(A) Hsc70 (orange ellipsoids) forms a dynamic mixture of monomers and higher-order assemblies. Phosphorylation (red circles) shifts this equilibrium toward lower-molecular-weight species, consistent with a reduced propensity to oligomerize. Dephosphorylation reverses this effect and restores oligomer formation, suggesting a reversible switch between both states. For clarity, nucleotide-bound states are not explicitly shown. Oligomerization is most pronounced in the ADP-bound and apo states. (B) Simplified schematic of the allosteric cycle. In the ADP-bound and apo states, Hsc70 preferentially populates a domain-undocked conformation in which the interdomain linker and SBD are disengaged from the NBD. In the proposed model, phosphorylation shifts the conformational ensemble toward a domain-docked, ATP-like state even under ADP-bound and apo conditions (indicated by the equilibrium arrows). ATP is shown in blue and ADP in yellow; the NBD in green, the SBD in yellow, and the interdomain linker in black. Phosphorylation is indicated by a red circle. This figure and its caption were adapted from the cited publication [21].

To assess whether and how oligomerization varies with protein concentration and nucleotide state, SAXS datasets of Hsc70^{T204A} and pHsc70^{T204A} were analyzed using, among other tools, OLIGOMER with a basis set comprising monomeric, dimeric, and trimeric

species. Under most conditions, the fitted volume fractions suggested that dimeric and/or trimeric contributions increased as protein concentration rose. In contrast, monomeric fractions were largely absent from OLIGOMER solutions in ADP-bound and nucleotide-free (apo) conditions, with the exception of pHsc70T204A:ADP at 1 mg/ml, for which a minor monomeric component was obtained. At the opposite extremes, OLIGOMER returned a substantial trimer contribution for Hsc70^{T204A}:apo and an almost exclusively dimeric population for Hsc70^{T204A}:ATP at 7 mg/ml. These outcomes do not fully agree with the heterogeneous oligomer distributions apparent from preparative SEC, with the aSEC results presented here, and with published aSEC studies on Hsc70 [17, 19, 170]. Several lines of evidence argue against a strictly quantitative interpretation of the OLIGOMER-derived species fractions. First, many fits resulted in χ^2 values above 1 (Table S2), indicating underfitting of the experimental scattering curves. Second, the basis set was limited to monomer, dimer, and trimer models generated from AlphaFold2-based full-length Hsc70 conformers and RANCH and therefore does not represent higher-order oligomers, which are evident in preparative SEC, aSEC and have been reported previously. Third, the current model set does not explicitly capture all proposed interaction modes. While antiparallel dimer arrangements were represented in the RANCH input structures (Figure S21), other suggested interaction modes were not, including trans-engagement of the interdomain linker by the SBD of another Hsp70 molecule via either the α -helical lid or the canonical substrate-binding groove [16, 18]. Collectively, these constraints can bias the inferred fractions in either direction. In the context of this study, OLIGOMER volume fractions are therefore used primarily as qualitative readouts of oligomerization trends across conditions rather than as accurate estimates of absolute oligomer distributions.

6.2.3 Phosphorylation biases Hsc70 toward domain-docked conformations⁵

The SAXS and limited proteolysis experiments performed in this work are consistent with phosphorylation promoting a more domain-docked organization of Hsc70.

⁵ Parts of the following sections are adapted and substantially expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923., in which these findings were originally reported.

Interpretation of SAXS data is complicated by the presence of oligomeric assemblies, because changes in scattering can arise both from intramolecular rearrangements and from differences in oligomer size or architecture. To contextualize the observations, prior SAXS studies on monomeric Hsc70^{E543K} and on the bacterial Hsp70 homolog DnaK have shown that ADP-bound states adopt more extended, dumbbell-like conformations, typically accompanied by bimodal $p(r)$ distributions that reflect separation between the NBD and SBD [129, 130]. In DnaK, but not explicitly tested for Hsc70^{E543K} in these earlier studies, such nucleotide-dependent rearrangements are also reflected in dimensionless Kratky representations, whereas ATP binding shifts the ensemble toward a more compact state [48] (now understood to correspond to a domain-docked arrangement). In line with this established behavior, Hsc70^{T204A} displayed bimodal $p(r)$ profiles and Kratky features in the ADP-bound and nucleotide-free (apo) states, consistent with a flexible multi-domain arrangement. This indicates that Hsc70^{T204A} undergoes domain rearrangements upon ATP hydrolysis to ADP and subsequent nucleotide release, as reported previously [6]. Strikingly, for pHsc70^{T204A} the second $p(r)$ maximum was strongly reduced in both ADP and apo conditions compared with Hsc70^{T204A}. Concordantly, the phosphorylated samples showed Kratky profiles characterized by a single broadened maximum rather than the bimodal pattern observed for the unmodified protein. Together, these SAXS-derived features are consistent with phosphorylation shifting the conformational ensemble toward more compact, domain-docked arrangements and support phosphorylation-dependent stabilization of domain docking. The limited proteolysis experiments performed with proteinase K support these conclusions. Overall, the digestion patterns observed for Hsc70 were consistent with prior reports that the ATP-bound state is the most protease-resistant, with stability decreasing in ADP-bound and nucleotide-free (apo) conditions, in line with the distinct allosteric landscapes populated across nucleotide states [6, 93]. Importantly, pHsc70 did not appear to be globally protected against protease cleavage, as overall differences between Hsc70^{T204A} and pHsc70^{T204A} were relatively small, suggesting broadly comparable global stability. This interpretation agrees with nanoDSF measurements across the three nucleotide conditions, where melting temperatures differed only modestly between phosphorylated and unmodified protein. Because nucleotide binding strongly increased melting temperatures for both Hsc70 and pHsc70, the nanoDSF data further suggest that nucleotide binding is not markedly

impaired by phosphorylation, consistent with reports on the phosphomimetic mutant Hsc70^{T495E} [93]. Notably, however, phosphorylated samples consistently showed reduced accumulation of the separated NBD and SBD fragments, indicating increased protection of the interdomain linker region and thereby supporting enhanced domain docking of pHsc70. Limited-proteolysis data for the phosphomimetic Hsc70 variant T495E have likewise been interpreted as evidence for a shift toward a more domain-docked arrangement [93]. In a related Hsp70-family context, AMPylation of BiP at T518 has been shown to favor domain docking as well [10, 92]. Taken together, these independent findings are consistent with the results presented here and support the view that post-translational modifications, including phosphorylation and AMPylation, can tune Hsp70 conformational equilibria by biasing the ensemble toward domain-docked states.

Of note, the phosphomimetic mutant Hsc70^{T495E} has been reported to retain substrate binding despite reduced ATPase activity and an apparent bias toward a more domain-docked conformation [93]. This is, at first glance, counterintuitive, because domain docking in Hsp70s is generally associated with reduced substrate affinity. One explanation could be that Hsc70^{T495E} and enzyme-phosphorylated pHsc70 are not fully equivalent and may differ in specific biochemical properties. Direct measurements of pHsc70 substrate binding, for example by isothermal titration calorimetry (ITC), bio-layer interferometry (BLI), or enzyme-linked immunosorbent assay based formats (ELISA), would be required to resolve this point [92, 93]. An alternative possibility is that phosphorylation stabilizes a docked state that is not identical to the canonical ATP-induced docked conformation. Addressing this hypothesis would require methods with sufficient sensitivity to detect relatively subtle intramolecular rearrangements. Approaches such as Förster Resonance Energy Transfer, ion-mobility MS, NMR, or single-molecule force spectroscopy could provide the necessary resolution and have been applied successfully in the Hsp70 field [6, 8, 175-177].

The observation that phosphorylation promotes docking in the present work is also noteworthy because phosphorylation at other sites has been reported to favor undocking of Hsp70-family proteins. For example, an Hsp70 variant phosphorylated at position S66 (generated by genetic code expansion; the native residue at this position is threonine) was reported to exhibit enhanced undocking in the ATP-bound state, together with increased ADP affinity. Functionally, this combination is proposed to reduce substrate

release upon ATP binding and to promote association of Hsp70 with the spindle apparatus during mitosis, thereby supporting spindle assembly and function [96]. Similarly, phosphomimetic substitutions at linker/SBD phosphorylation sites (e.g., S385D/S400D) have been interpreted to promote undocking and stronger substrate binding, with the functional consequence of enhanced chaperone activity [178]. Together, these examples and the data presented in this thesis support the notion that phosphorylation can decouple Hsp70 proteins from their canonical allosteric cycle and, depending on the modified site, either stabilize domain-docked states or shift the ensemble toward undocked conformations.

6.2.4 From structural to functional consequences

Phosphorylation of Hsc70 at T495 was first identified in an infection context [84]. More recent independent work on the yeast Hsc70 homolog Ssa1 indicates that regulation at the homologous site extends beyond infection-related signaling [93, 94]. In mammalian cells, Hsc70 becomes endogenously phosphorylated at T495 in response to DNA damage by DNA-damage response kinases, and this modification has been implicated in regulating cell-cycle progression [93]. A proposed model is that pHsc70 adopts an ATP-like conformational state and engages a client protein involved in cell-cycle control in a manner that promotes G1 retention. In yeast, Ssa1 T492 phosphorylation has been linked to stress signaling as well [94]. Following activation of the mechanosensor Mid2 during heat shock, Pkc1-dependent phosphorylation at this site was reported to support an effective heat shock response by reshaping interactions with clients and co-chaperones, influencing Ssa1-Hsf1 regulation, and modulating cellular translation.

In this context, the structural trends identified in this thesis provide a mechanistic framework for understanding how phosphorylation at this conserved region could reprogram Hsp70 function in cells. Specifically, by reducing oligomerization and biasing the conformational ensemble toward a more domain-docked, ATP-like organization (Figure 16B), which may in turn restrict progression through the canonical allosteric cycle, Hsc70 activity may be reduced or altered. Such a phosphorylation-stabilized ATP-like state would be expected to compromise efficient client capture and release, thereby providing a plausible structural basis for the functional attenuation or rewiring of Hsc70 activity reported in translation, heat shock, and DNA-damage contexts.

6.2.5 TReND-K analogues promote stable binary adduct formation but do not support substrate modification

Kinase-substrate complexes are typically transient and difficult to obtain in quantities suitable for structural analysis [49, 52]. Here, γ -phosphate-modified ATP analogs were therefore evaluated as a covalent-capture strategy: the analogs are designed to form a kinase thiol-linked binary adduct and, upon γ -phosphate transfer, to generate a covalent ternary kinase-analog-substrate complex (Figure 3). The approach was tested using the LegK4-Hsc70 system, which was established in this thesis, and the well-characterized model kinase CK2 α' .

The ATP analogs used here differ in several key structural features from established kinase crosslinking analogs reported in the literature (Figure 3B) [135, 179, 180]. First, the spacer between the γ -phosphate and the electrophile in the TReND-K analogs used here is, in most cases, a predominantly aliphatic polycarbon chain (with one variant deviating from this design), whereas many literature reagents employ PEG-based linkers.

Second, the chemical linkage connecting the linker to the γ -phosphate differs. In many published kinase crosslinking analogs, the linker is attached to the γ -phosphate via a P-N (phosphoramidate) linkage. By contrast, in the analogs used in this work the linker is coupled to the γ -phosphate via a P-O linkage (Figure 3A). Differences in phosphate-linker chemistry can influence stability, the orientation of the substituent within the active site, and compatibility with γ -phosphate transfer, and are therefore potentially relevant for analog performance [181, 182].

In this work, IMS was used to detect formation of the binary adduct. This proximity-induced labeling approach required introduction of cysteine residues at suitable positions. Binary adduct formation was highly efficient, reaching up to ~100% depending on the analog used and the kinase-substrate system tested (Table S9). This interpretation is further supported by the crystal structure of CK2 α' ^{H161C} in complex with TReND-K1, which reveals close agreement with reference structures in terms of overall fold and positioning of catalytic residues [149, 150], while the β - and γ -phosphate groups of the analog adopt altered conformations. The measured efficiencies are sufficient for all subsequent steps toward ternary complex generation. The observation that, in the LegK4₅₉₋₄₆₂ system, only a single endogenous cysteine had to be removed (and that, in the CK2 system,

no endogenous cysteine needed to be removed to prevent covalent coupling in the absence of engineered cysteines) supports the selectivity of the reaction. This selectivity is important because crystallization of a heterogeneous mixture of complexes crosslinked through different kinase cysteines would be challenging. Thus, with respect to binary adduct formation, the analogs performed successfully in this study. For detection of ternary complex formation, SDS-PAGE was deliberately chosen. Compared with iMS and Western blotting, SDS-PAGE is less sensitive; however, successful structural studies require substantial amounts of ternary complex, and SDS-PAGE therefore provides a pragmatic readout for whether complex formation occurs at preparative levels. This approach is established for detecting covalent ternary complexes [118, 154]. The threonine residue phosphorylated by LegK4 in Hsc70 corresponds to the site in BiP that is AMPylated by FicD. Because FicD has been reported to preferentially bind ATP-bound BiP, and because a similar nucleotide-dependent interplay between Hsc70 and LegK4 cannot be excluded, ternary complex formation between the pre-formed binary adduct (LegK4 + TReND) and ATP- or ADP-loaded Hsc70 was tested [118]. Under the conditions examined, no ternary complexes were detected for either CK2 α' or LegK4₅₉₋₄₆₂^{C145S} variants. To probe whether transfer of the γ -phosphate-linked group occurred at all, kinases that do not form detectable binary adducts with the analogs (LegK4₅₉₋₄₆₂^{C145S} and CK2 α') were incubated together with their protein substrates and the analogs. While ATP controls yielded detectable substrate modification, confirming kinase activity, no modification was observed with the tested analogs. This suggests that the analogs are not efficiently accepted as cosubstrates and/or that any transfer occurs below the detection limit of iMS.

As described in the literature, a range of electrophilic warheads coupled via linkers, often attached to the γ -phosphate through a P-N linkage, has been used for kinase-catalyzed labeling and crosslinking. In a comparative study of several such ATP-crosslinker analogs, all compounds showed reduced catalytic efficiency relative to ATP, while remaining catalytically competent substrates [180]. Although warhead identity influenced transfer efficiency, the observed spread (approximately 5-27-fold reduced catalytic efficiencies compared with ATP) is relatively modest when viewed in the context of the overall catalytic power of kinases [180]. Another study reported that γ -phosphoester-modified ATP analogs (P-O-R), as used in this thesis, were not accepted by the tyrosine kinase FAK,

whereas the corresponding γ -phosphoramidate analogs (P-N-R) were enzymatically processed [182]. Importantly, the same trend was observed for UBA1, an E1 ubiquitin-activating enzyme that does not act as a kinase but cleaves the α - β phosphoanhydride bond of ATP during ubiquitin activation. Since both a tyrosine kinase and a mechanistically distinct ATP-consuming enzyme failed to efficiently use phosphoester-linked analogs these findings strongly suggest that poor acceptance of P-O-modified ATP analogs is not limited to a single enzyme class and is therefore likely also relevant for serine/threonine kinases. A possible explanation proposed by the authors is that phosphoramidate analogs retain an NH group that can act as a hydrogen-bond donor, whereas this interaction is lost in phosphoester analogs. This indicates that even a small change from NH to O at the γ -phosphate can strongly affect enzyme recognition and turnover. Together, these results support the idea that the low conversion of P-O-C-modified ATP analogs is likely caused by the chemical nature of the γ -phosphate modification itself rather than by steric bulk alone. In addition, the crystal structure reveals deviations in the positioning of the β - and γ -phosphate groups relative to canonical ATP analogs, likely imposed by the covalent linkage. While this does not explain the absence of phosphotransfer observed with kinases that do not form binary adducts, it suggests that even if transfer were possible, geometric constraints and linker design may further limit efficient phosphotransfer. For the analogs used in this thesis, these observations could imply that the failure to detect γ -phosphate transfer is unlikely to be attributable to the presence of the chloroacetamide electrophile itself. No quantitative information on transfer yields for the analogs used in the literature could be identified [135, 179, 180]. However, given that analog transfer in these studies was typically detected using highly sensitive methods (e.g., Western blotting, peptide enrichment coupled to tandem mass spectrometry), it is reasonable to assume that transfer of the modified γ -phosphate to substrates is often substoichiometric. In contrast, crystallization requires substantial amounts of homogeneous complex. Thus, although literature analogs appear catalytically competent relative to ATP, transfer efficiencies may still be too low to generate high yields of modified substrate protein. The fact that neither the analogs used here nor those reported in the literature have generally been applied for high-yield production supports the notion that reduced transfer efficiency is a broader limitation of γ -phosphate-modified ATP analogs. Consequently, independent of how efficiently the binary adduct is formed, a ternary

complex is unlikely to be produced in amounts sufficient for detection by SDS-PAGE and for subsequent purification toward crystallization. In the context of this work, the limiting step for successful kinase-substrate crosslinking was therefore the inability of the kinases tested (LegK₅₉₋₄₆₂^{C145S} and CK2 α') to efficiently transfer the modified γ -phosphate to their protein substrates (Hsc70, EEF1D, and HAP28). Despite the limitations observed here, the overall concept remains compelling, and a natural next step would be to extend the approach to additional kinase-substrate pairs. Moreover, although γ -phosphoramidate ATP analogs can exhibit reduced chemical stability relative to phosphoester-linked variants, it would be interesting to combine the chloroacetamide electrophile with a P-N linkage. Such a design could potentially unite a relatively selective thiol-reactive warhead with a phosphate-linker connectivity that has been reported to support more efficient transfer, thereby improving the prospects for generating ternary complexes at preparative levels.

7 Outlook

In this work, a phosphorylation protocol for the production of predominantly phosphorylated Hsc70 was successfully established. Biochemical analyses consistently indicate that phosphorylation reduces the oligomerization propensity of Hsc70 and promotes domain docking. In light of previously reported cellular effects of Hsc70 phosphorylation, as well as phosphorylation of the homologous residue in Ssa1, it is proposed that phosphorylation attenuates or reprograms Hsc70 activity by slowing its allosteric cycle and reducing oligomerization. However, the molecular mechanism by which phosphorylation at Thr495 exerts these effects remains unclear, as the resolution of the applied structural methods is insufficient to fully resolve these changes.

To obtain mechanistic insight, high-resolution structural approaches will be required. Crystal structures would represent an important next step; however, as Hsc70 is historically difficult to crystallize, the structurally well-characterized hamster BiP may serve as an alternative model system. In both cases, *in silico* approaches such as molecular dynamics simulations could provide valuable insight into the structural consequences of phosphorylation. Additional mechanistic information could be obtained using methods such as FRET, ion mobility mass spectrometry (IM-MS), NMR, and single-molecule force spectroscopy.

These approaches are also critical to address an apparent discrepancy: phosphomimetic studies suggest that pHsc70 retains substrate binding despite reduced oligomerization and increased domain docking. This observation challenges the current understanding that domain docking is associated with reduced substrate affinity and highlights the need to bridge *in vitro* structural data with *in vivo* functional observations. As a first step, substrate binding of pHsc70 should be directly assessed, for example using peptide substrates or proteins such as Tau in combination with techniques including ITC, bio-layer interferometry (BLI), or ELISA-based assays. If substrate binding is confirmed, possible explanations include the presence of previously uncharacterized conformational states that combine features of docked and undocked conformations, or local structural perturbations induced by phosphorylation that modulate binding properties. High-

resolution techniques such as NMR, FRET, IM-MS, and single-molecule force spectroscopy will be required to resolve such conformational heterogeneity. In particular, NMR studies of the isolated substrate-binding domain, complemented by molecular dynamics simulations, could reveal local structural changes.

Following this, investigation of how phosphorylation affects co-chaperone interactions will be essential to connect molecular mechanisms with cellular function. Another objective of this work was to compare phosphorylation and AMPylation at the same residue. This goal was limited by the relatively low AMPylation levels achieved. Possible routes to further improve AMPylation yields include longer HEPES-based incubations, more complete removal of residual phosphate, optimization of FicD concentration, and testing of Hsc70T204A as an alternative substrate. If AMPylation of Hsc70 cannot be increased sufficiently, an alternative strategy would be to generate and compare AMPylated and phosphorylated BiP instead, provided that the phosphorylation protocol also yields sufficiently modified BiP.

Finally, this work investigated the stabilization of kinase–substrate complexes using ATP analogs developed by the Hedberg group. While binary adduct formation was highly efficient, no transfer of the analog to the substrate or ternary complex formation was detected. Future work could extend this approach to additional kinase–substrate systems and evaluate ATP analogs with a γ -phosphoramidate (P–N) linkage, which may improve transfer efficiency and thereby increase the likelihood of obtaining ternary complexes at preparative levels, provided that sufficient analog stability can be maintained.

8 Materials and Methods

Unless otherwise stated, all chemicals were purchased from the suppliers listed in Table 6.

8.1 Molecular biology methods

8.1.1 Plasmid construction⁶

The pMCSG7 plasmid encoding wild-type Hsc70 was a gift from Prof. Dr. Lila Gierasch (College of Natural Sciences, Biochemistry and Molecular Biology, University of Massachusetts Amherst) (Figure S 20). A T204A mutant was created using NEB Kinase, Ligase and DpnI (KLD) enzyme mix and the respective oligonucleotides (Table S9). Based on both the WT and T204A constructs, additional plasmids encoding residues 1-608 were generated using KLD.

To generate a plasmid encoding full-length LegK4 (LegK4₅₉₋₁₀₁₉, Philadelphia strain annotation) with an N-terminal His₁₀-tag, the psf421 vector (encoding an N-terminal His₆-tag, green fluorescent protein tag (GFP-tag), a streptavidin-binding peptide tag (Strep-tag), and TEV cleavage site) and genomic DNA from *L. pneumophila* subsp. *pneumophila* strain Philadelphia-1 (ATCC 33152) were used in sequence- and ligation-independent cloning (Figure S 20). In a subsequent step, GFP-tag, Strep-tag, and TEV cleavage site were removed and the His₆-tag was extended to a His₁₀-tag by KLD mutagenesis. Analogously, psf421 and the same genomic DNA were used to generate a construct encoding a C-terminally truncated LegK4 variant (LegK4₅₉₋₅₂₀). From this construct, LegK4₅₉₋₄₆₂ carrying an N-terminal His₆-tag, GFP-tag and TEV cleavage site was obtained by deletion of the corresponding coding sequence using KLD. A kinase-dead mutant LegK4 (LegK4₅₉₋₄₆₂^{D253A}) was generated by KLD.

Gene blocks encoding human HAP28 and human EEF1D were codon-optimized for expression in *E. coli* using the Integrated DNA Technologies (IDT) Codon Optimization Tool, purchased from IDT, and cloned into pProEX expression plasmids by sequence- and

⁶ Parts of the following section are reproduced and expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923.

ligation-independent cloning (SLIC) using the oligonucleotides listed in Table S9. The HAP28 construct carries an N-terminal His₆-Tag followed by a TEV cleavage site, whereas the EEF1D construct carries an N-terminal His₆-GFP-Tag followed by a TEV cleavage site. An in-house generated pSF421 vector containing the CSNK2A2 gene was used to produce CK2α' cysteine variants. CSNK2A2 encodes human CK2α'. Site-directed mutagenesis was performed using KLD and the primers listed in Table S9. CK2α' variants with and without a tandem Strep-tag II streptavidin-binding peptide tag (Twin-Strep-tag) were used, if applicable the streptag was deleted using KLD.

In-house pSF421 plasmids containing FicD₁₀₂₋₄₅₈^{E234G L258D} were used for expression. Constructs comprised either an N-terminal His₆-GFP-Tag followed by a TEV cleavage site or a non-cleavable N-terminal His₁₀-Tag.

8.1.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed using the Q5[®] High-Fidelity 2× Master Mix (NEB) or, alternatively, using Q5[®] High-Fidelity DNA Polymerase (NEB) with the corresponding reaction buffer, with or without the Q5[®] High GC Enhancer (NEB) with a T100 thermocycler (Bio-Rad). Reactions contained 0.2 mM dNTPs, 0.5 μM of each primer, and 0.4 ng/μl template DNA. PCR cycling was performed using the following program: initial denaturation at 98 °C for 60 s; 25 cycles of denaturation at 98 °C for 20 s, annealing temperature for 30 s (annealing temperature according to primer) and extension at 72 °C for 25 s per kb; final extension at 72 °C for 5 min; hold at 12 °C.

8.1.3 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (NEB) according to the manufacturer's instructions, with the following modification: instead of incubating the KLD reaction for 5 min at room temperature, samples were incubated for 30 min at 37 °C in a thermomixer (Eppendorf). The reaction was then incubated on ice for 15 min and subsequently added directly to chemically competent *E. coli* cells (Mach1 Mix&Go; Zymo Research) for transformation. Primers were designed using the NEBaseChanger online tool and synthesized by IDT. The annealing temperature used in the PCR cycling program was chosen based on the recommended T_m calculated by the tool for the respective primer pair.

8.1.4 T4-SLIC cloning

For each construct, two 50 µl PCR reactions were set up: one to amplify the insert and one to amplify/linearize the plasmid backbone. PCR products were subsequently treated with in-house DpnI for 20 min at 37 °C in a thermoblock (Thermomixer comfort, Eppendorf) to remove template DNA. Insert and vector fragments were separated by agarose gel electrophoresis, and the corresponding DNA bands were excised and purified using the Monarch® DNA Gel Extraction Kit (NEB). DNA was eluted in 8 µl water and concentrations were determined by NanoDrop (Thermo Fischer Scientific). For SLIC assembly, a vector:insert molar ratio of 1:3 was used. Required DNA amounts were calculated using the NEBioCalculator (ligation module). Reactions were set up according to the protocol in a total volume of 10 µl containing 1 µl 10 x NEBuffer 2.1 (NEB) and 0.2 µl T4 DNA polymerase (NEB; 0.6 U), with vector and insert DNA added accordingly and the remaining volume adjusted with water. T4 DNA polymerase was added last. Immediately upon polymerase addition, the reaction was mixed, a timer was started, and the sample was incubated for exactly 2.5 min at room temperature before being transferred to ice. After incubation on ice for at least 10 min, the complete 10 µl SLIC reaction was added to Mach1 Mix&Go competent cells, and transformants were plated on ampicillin-containing agar plates.

8.1.5 Agarose gel electrophoresis

PCR products intended for SLIC were mixed with 10× loading buffer (50% glycerol, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.2% bromophenol blue, 0.2% xylene cyanol FF) and separated by 1% (w/v) agarose gel electrophoresis in 1× TAE buffer (40 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.6, 20 mM acetic acid, 1 mM EDTA) using a power supply (GENE POWER SUPPLY GPS 200/400). DNA bands stained with G-Stain (Serva) were visualized on a blue-light transilluminator (Biometra TI3; Biometra). Band sizes were estimated using a DNA ladder (2-Log DNA Ladder, 0.1-10.0 kb; NEB).

8.1.6 Transformation for plasmid propagation and plasmid preparation

To propagate plasmids Mach1 Mix&Go cells were used. Aliquots of 50 µl competent cells were thawed on ice and incubated with 100 ng plasmid DNA for 10 min on ice. The cells were plated directly onto agar plates containing Ampicillin and incubated over night at

37 °C. A single colony of Mach1 cells transformed with the target plasmid was grown 8h or overnight at 37 °C and 180 revolutions per minute (rpm) in 4 ml LB medium (Carl Roth) supplemented with Ampicillin. Plasmid DNA was isolated using the PureYield™ Plasmid Miniprep System (Promega) according to the manufacturer's instructions.

8.1.7 Plasmid sequencing

Plasmid sequencing was performed by Microsynth. 10-15 µl plasmid DNA (40-100 ng/µl) was provided together with sequencing primers supplied by the company or specifically designed for the construct and send to the company

8.2 Protein biochemical methods

8.2.1 Transformation for protein expression

For recombinant protein expression, chemically competent BL21(DE3) cells were transformed with 100 ng of the respective LegK4 expression plasmid. Cells were incubated with plasmid DNA on ice, heat-shocked for 30 s at 42 °C, and placed back on ice for 5 min. After heat-shock transformation, 1 ml super optimal broth with catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added and cells were recovered for 60 min at 37 °C and 500-600 rpm. Cells were then pelleted (3000 × g, 5 min), resuspended in water, and plated on LB agar containing ampicillin (125 µg/ml). Plates were incubated overnight at 37 °C. A similar procedure was used for Hsc70, CK2α', HAP28, and EEF1D, except that chemically competent Rosetta™ cells were used and transformants were plated on LB agar containing ampicillin (125 µg/ml) and chloramphenicol (34 µg/ml).

8.2.2 Protein expression⁷

For LegK4 expression, a 40 ml pre-culture (125 µg/ml ampicillin) was cultivated 4-5 h scratching colonies from a pre transformed agar plate and used to inoculate 960 ml of LB medium (125 µg/ml ampicillin). Cells were incubated at 37 °C and 160 rpm (Multitron

⁷ Parts of the following section are reproduced and expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923.

standard shaker, Infors HT and Innova 44 shaker, New Brunswick) until the optical density at 600 nm wavelength (OD₆₀₀) reached 0.6-0.8. Expression was then induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and cultures were further incubated at 18 °C overnight. Cells were centrifuged at 5000 g to remove the medium, washed with phosphate-buffered saline and pelleted by centrifugation. The cells were flash-frozen in liquid nitrogen and stored at -80 °C. The frozen cell pellets were resuspended in ice-cold buffer G (Table 2) and homogenized using a Silent Crusher M (Heidolph Instruments GmbH & Co. KG). A spatula tip of powdered DNase I (AppliChem) was added to the cells, followed by lysis with a Constant Cell Disruption System (Constant Systems Limited, UK) at 1.8 kbar. Subsequently, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. The lysate was cleared by centrifugation at 50,000 g for 30 min. The same procedure was used for Hsc70 with the following modifications: *E. coli* RosettaTM competent cells were transformed with the respective Hsc70 plasmids and incubated on ampicillin-chloramphenicol plates (125 μ g/ml ampicillin, 34 μ g/ml chloramphenicol). The 40 ml pre-culture contained 125 μ g/ml ampicillin and 34 μ g/ml chloramphenicol. Cultures were induced with 0.5 mM IPTG and grown at 23 °C overnight. For lysis, the cells were resuspended in buffer A (Table 2).

The same procedure used for Hsc70 was applied to CK2 α' and its variants. For lysis, the cell pellet was resuspended in buffer O (Table 2). Similarly, HAP28 and EEF1D were expressed and purified using the Hsc70 protocol with the following modifications: cells were induced with 0.3 mM IPTG and incubated overnight at 18 °C. For lysis, the cell pellet was resuspended in buffer K (Table 2).

Table 2: Buffers used for protein purification

Buffer Name	Buffer composition
A	50 mM HEPES, 10 mM imidazole, 500 mM NaCl, pH 7.6, 5 mM MgCl ₂ , 1 mM TCEP
B	80 mM NaH ₂ PO ₄ pH 7.5, 5 mM MgCl ₂ , 100 mM NaCl, 2 mM ATP, 1 mM TCEP
C	50 mM HEPES, 300 mM imidazole, 300 mM NaCl, pH 7.6, 5 mM MgCl ₂ , 1 mM TCEP
D	20 mM HEPES pH 7.6, 5 mM MgCl ₂ , 100 mM NaCl and 5 mM ATP, 1 mM TCEP
E	40 mM NaH ₂ PO ₄ , 5 mM MgCl ₂ , 100 mM NaCl, pH 7.5, 2 mM ATP, 1 mM TCEP
F	40 mM NaH ₂ PO ₄ , 4 mM MgCl ₂ , 100 mM NaCl pH 7.5, 1 mM TCEP
G	50 mM Tris pH 8, 500 mM NaCl, 5 % Glycerol, 20 mM imidazole
H	50 mM Tris pH 8, 500 mM NaCl, 5 % Glycerol, 500 mM imidazole
I	20 mM Tris pH 8, 150 mM NaCl, 5 % Glycerol, 1 mM β-mercaptoethanol
J	20 mM HEPES pH 7.6, 150 mM NaCl, 1 mM TCEP, 1 mM MgCl ₂
K	50 mM NaH ₂ PO ₄ pH 8, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol
L	50 mM NaH ₂ PO ₄ pH 8, 500 mM NaCl, 500 mM imidazole, 1 mM β-mercaptoethanol
M	20 mM NaH ₂ PO ₄ pH 8, 150 mM NaCl, 1 mM β-mercaptoethanol
N	20 mM HEPES pH 7.7, 150 mM NaCl, 1 mM TCEP
O	500 mM NaCl, 20 mM imidazole, 50 mM HEPES pH 7.4, 1 mM β-mercaptoethanol
P	500 mM NaCl, 500 mM imidazole, 50 mM HEPES pH 7.4, 1 mM β-mercaptoethanol
Q	20 mM HEPES, 150 mM NaCl pH 7.4, 1 mM beta mercaptoethanol
R	20 mM HEPES pH 7.4, 1 mM TCEP, 150 mM NaCl
S	50 mM HEPES, pH 7.4, 500 mM NaCl, 1 mM MgCl ₂ , 1 mM β-mercaptoethanol
T	50 mM HEPES, pH 7.4, 500 mM NaCl, 1 mM MgCl ₂ , 1 mM β-mercaptoethanol, 500 mM imidazole
U	20 mM HEPES, pH 7.4, 200 mM NaCl, 1 mM MgCl ₂ , 1 mM β-mercaptoethanol
V	20 mM HEPES, pH 7.4, 150 mM KCl, 1 mM MgCl ₂ , 1 mM TCEP, 10% (v/v) Glycerol

8.2.3 Hsc70 purification- Final phosphorylation protocol⁸

The differences between the initial and final phosphorylation workflows are described in the Results section. Here, purification of Hsc70, pHsc70, and the ATPase-deficient mutant Hsc70^{T204A} is described for the final phosphorylation protocol, which was used to produce the protein preparations analyzed throughout this thesis. After expression and lysis, the clarified lysate was purified using 5 ml EconoFit Nuvia Ni²⁺-nitrilotriacetic acid (NTA) IMAC columns from Bio-Rad with the NGC™ Liquid Chromatography System (Bio-Rad). Buffer A was used to load and wash the sample, followed by an ATP wash with buffer B (Table 2) and elution of the proteins with a gradient of buffers A and C (Table 2). After the first IMAC (1st IMAC), the sample was diluted with 20 mM HEPES, pH 7.5, 5 mM MgCl₂, 100 mM NaCl, and 20 mM ATP until a final concentration of 5 mM ATP was reached. To yield stable phosphorylation, 0.163 nmol His₁₀-tagged LegK4₅₉₋₁₀₁₉ was added per pellet of Hsc70 used. The sample obtained after lysing one pellet of Hsc70 was then dialyzed against 1 L of buffer D (Table 2) using dialysis tubing with a molecular weight cut-off (MWCO) of 12-14 kDa (SERVAPOR). Except for the omission of LegK4₅₉₋₁₀₁₉, the unmodified sample was treated the same way. After 6.5 h of incubation at RT, the samples were dialyzed for 30 min against buffer E (Table 2) (4 L buffer for 2 L expression culture). Homemade Tobacco Etch Virus (TEV) protease was added in a 1:40 mass ratio and the samples were dialyzed against buffer E overnight. After second IMAC (2nd IMAC), the protein was dialyzed four times against 1 L of buffer F (Table 2), concentrated using Amicon 30 kDa MWCO concentrators (Sigma Aldrich), flash-frozen, and stored at -80 °C. In the case of the Hsc70^{T204A} mutant used for SAXS measurements, the last dialysis step was omitted during purification and the protein was subjected to SEC (size-exclusion chromatography) using a Cytiva SEC 26/600 200 prepacked gravity (pg) column with buffer F. After the purification steps 1st IMAC, 2nd IMAC, and SEC, samples were taken for analysis by SDS-PAGE.

In early implementations of the initial purification protocol (Figure 4A), a pET-28a plasmid encoding Hsc70 or Hsc70^{T204A} fused to an N-terminal His₆- small ubiquitin-like

⁸ Parts of the following section are reproduced and expanded from the cited paper 21. Kley, N., M. Hecht-Bucher, and A. Itzen, *Advances on the large-scale bacterial production and stable phosphorylation of Hsc70*. Protein Expression and Purification, 2026. 241, 106923 DOI: 10.1016/j.pep.2026.106923.

modifier (SUMO)-tag was used, and the SUMO-tag was cleaved using homemade ubiquitin-like protease 1 (ULP1) protease. The plasmid encoding Hsc70 was a gift from Prof. Dr. Johannes Buchner (Department of Bioscience, TUM School of Natural Sciences), and the T204A variant was generated by KLD mutagenesis (Table S9). Both pET-28a and pMCSG7 are T7 promoter-based expression vectors and encode N-terminal His₆-tags, but differ in their fusion tag architecture and cleavage sites. Specifically, pET-28a encodes an N-terminal His₆-SUMO fusion, whereas pMCSG7 encodes an N-terminal His₆-tag followed by a TEV protease cleavage site. In addition, the nucleotide sequence of the Hsc70 insert differs between the two constructs, while the resulting amino acid sequence is identical. Expression from the pET-28a construct consistently yielded a significant fraction of truncated protein alongside the full-length protein. In contrast, expression from the pMCSG7 construct resulted in a markedly improved proportion of full-length protein with only a minor truncated fraction. Therefore, the pMCSG7 plasmid was used for all subsequent experiments.

8.2.4 Protein purification of LegK4, HAP28, EEF1D, CK2α' and FicD⁹

LegK4 was purified using the same Ni²⁺-NTA IMAC columns as described for Hsc70. For the 1st IMAC, buffer G (Table 2) was used to wash the protein bound to the column, followed by a gradient of buffers G and H (Table 1). Full-length His₁₀-tagged LegK4 was dialyzed against buffer I (Table 2). For LegK4₅₉₋₄₆₂ and LegK4₅₉₋₄₆₂^{D253A}, a 2nd IMAC was performed after overnight dialysis and TEV cleavage (40:1 mass ratio LegK4:homemade TEV) in buffer I at 4 °C, followed by SEC with a Cytiva 16/600 75 pg column in buffer J (Table 2). All proteins were flash-frozen in liquid nitrogen and stored at -80 °C. Similar to Hsc70, after 1st IMAC, 2nd IMAC and SEC, samples were taken and subjected to SDS-PAGE analysis. Before loading, the samples were boiled for 5 min at 95 °C and were run on homemade 12 % polyacrylamide gels.

In the case of HAP28 and EEF1D, a workflow similar to that used for LegK4 was applied. For the 1st and 2nd IMAC steps, buffers K and L were used, respectively (Table 2). For

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dialysis and subsequent SEC on a Superdex 75 16/600 pg column, buffers M and N were used (Table 2).

Similarly, CK2 α' was purified using an analogous protocol, with buffers O and P for the first and second IMAC steps, respectively, and buffers Q and R for dialysis and SEC (Superdex 75 pg column).

FicD₁₀₂₋₄₅₈^{E234G L258D} with a cleavable His₆-tag was purified using a comparable workflow comprising a first IMAC step, dialysis with simultaneous TEV protease cleavage, a second IMAC step, and subsequent SEC. For the 1st and 2nd IMAC steps, buffers S and T were used, respectively (Table 2). Dialysis with TEV cleavage was performed using buffer U (Table 2), and subsequent SEC was carried out using buffer V (Table 2) with a Superdex 75 16/600 pg column. FicD₁₀₂₋₄₅₈^{E234G L258D} with a non-cleavable His₁₀-tag was purified as described above, except that purification was terminated after the first IMAC step and the protein was subsequently dialyzed into SEC buffer V.

8.2.5 Hsc70 AMPylation protocol

Initial high-yield AMPylation protocol for Hsc70

Hsc70 purification was carried out as described in the section Protein purification up to and including the SEC step (for the dialysis step, ATP was omitted). The SEC-purified sample was adjusted to a volume of 100 ml and supplemented with ATP to a final concentration of 5 mM. FicD₁₀₂₋₄₅₈^{E234G L258D} was then added to a final concentration of 1.15 μ M (corresponding to a FicD/Hsc70 molar ratio of 1:5). The sample was dialyzed for 4.25 h at 22 °C against 1 L of buffer D. After concentration using 30 kDa MWCO Amicon centrifugal filters, the sample was reappplied to a 26/600 200 pg SEC column equilibrated in buffer F (Table 2). The protein was subsequently concentrated and stored as described in the Protein purification section.

Final high-yield and stable AMPylation protocol for Hsc70

The final protocol was adapted from the initial protocol with the following modifications. A third IMAC step was introduced between the first and second SEC steps. Hsc70 was purified as described above, except that after the first SEC step the sample was adjusted to a final volume of 80 ml and supplemented with ATP to a final concentration of 5 mM. His₁₀-tagged FicD₁₀₂₋₄₅₈^{E234G L258D} was used in a 1:5 (FicD/Hsc70) ratio (1.14 μ M FicD) and

the sample was dialyzed for 4.5 h against buffer D. This was followed by brief dialysis against buffer E for 0.5 h, a third IMAC step, and a second SEC after concentration with 30 MWCO Amicon centrifugal filters.

8.2.6 M9 expression of Hsc70 and Hsc70^{T204A} with and without ILV labeling

To enable NMR analysis of Hsc70^{T204A} and pHsc70^{T204A}, ILV labeling was selected to support methyl-TROSY experiments. The labeling procedure followed a protocol shared by Eugenia M. Clerico (College of Natural Sciences, Biochemistry and Molecular Biology, University of Massachusetts Amherst), adapted from published methods [122].

For expression in M9 medium without isotopic supplementation, M9 salts were prepared per liter as follows: 6.5 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl, 120 mg MgSO₄, 11 mg CaCl₂, biotin (10 µg/ml), 1.0 g NH₄Cl, 2 g D-glucose. An antibiotic-supplemented LB preculture (125 µg/ml ampicillin, 34 µg/ml chloramphenicol) was inoculated from freshly transformed Rosetta cells (see above) and grown at 37 °C to OD₆₀₀ 0.4-0.5. For adaptation to minimal medium, an equal volume of M9 medium was added to the same culture, and growth was continued to OD₆₀₀ 0.4-0.5. This stepwise dilution with M9 was repeated once. Cells were then harvested by centrifugation (4000 g) and resuspended in 100 ml M9 medium. After 1 h, an additional 100 ml M9 medium was added, and the culture was incubated at 37 °C overnight.

On the following day, the 200 ml overnight culture was transferred into 755 ml M9 medium (supplemented with ampicillin only) and grown at 37 °C to OD₆₀₀ 0.5. The culture was then shifted to 23 °C, and α-ketobutyric acid (57.14 mg) as well as α-ketoisovaleric acid (117 mg) were added. After 1 h of incubation, expression was induced with IPTG to a final concentration of 0.5 mM, and the culture was grown overnight. Downstream purification was performed as described for LB-grown cultures (see above).

ILV-labeled expression followed the same workflow, except that D₂O-based M9 medium was used and isotope-labeled precursors were supplied. Specifically, the nitrogen source was ¹⁵NH₄Cl (98%), the carbon source was (1,2,3,4,5,6,6-D₇, 98%) D-glucose and the solvent was D₂O (99.9 %). For methyl labeling, α-ketobutyric acid (methyl-¹³C, 99%; pre-incubated in D₂O at pH 10 overnight at 4 °C to exchange labile protons) was added at 70 mg and, α-ketoisovaleric acid (3-methyl- ¹³C,99 %; 3,4,4,4-D₄, 98%; sodium salt) was added at 120 mg.

8.3 Analytic methods

8.3.1 Determination of protein concentration¹⁰

LegK4 concentration was assessed using absorbance at 280 nm (full-length: $\epsilon = 75180 \text{ M}^{-1} \text{ cm}^{-1}$, molecular weight (MW) = 111541 Da; LegK4₅₉₋₄₆₂ $\epsilon = 32890 \text{ M}^{-1} \text{ cm}^{-1}$, MW = 46023 Da). HAP28 ($\epsilon = 4470 \text{ M}^{-1} \text{ cm}^{-1}$, MW = 21034 Da), EEF1D ($\epsilon = 22460 \text{ M}^{-1} \text{ cm}^{-1}$, MW = 31526 Da) and CK2 α' ($\epsilon = 72770 \text{ M}^{-1} \text{ cm}^{-1}$, MW = 44537 Da) concentrations were likewise assessed. Hsc70 concentration before TEV cleavage was routinely assessed using absorbance at 280 nm (Hsc70: $\epsilon = 33350 \text{ M}^{-1} \text{ cm}^{-1}$, MW = 71172 Da). In addition, absorbance measurements were used to determine protein concentration for SAXS experiments. For ATPase and dephosphorylation assays, the protein concentration was assessed using Bio-Rad Protein Assay Dye Reagent Concentrate with F16 Maxisorp loose Nunc-Immuno Modules from Thermo Scientific. Bovine serum albumin from Sigma Aldrich was used to create a standard. A Multiskan FC microplate reader (Thermo Scientific) was used to measure absorbance at 620 nm.

8.3.2 SDS-PAGE analysis

Samples were mixed 1:1 with homemade 2× SDS-PAGE sample buffer prior to loading and incubated for 5 min at 70 °C (for Hsc70) or 95 °C (for all other proteins). The samples were subsequently separated on 12 % homemade polyacrylamide gels using Bio-Rad instrumentation. A prestained protein ladder (Color Prestained Standard Broad Range, 10-250 kDa; New England Biolabs) was used as a molecular weight marker. The compositions of the buffers and solutions used are summarized in Table 3 and Table 4. Gels were stained and destained using SDS-PAGE staining and destaining solutions (Table 3).

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Table 3: Reagents and Solutions for SDS-PAGE Gel Electrophoresis

TEMED = N,N,N',N'-Tetramethylethylenediamine

Solution	Composition
Acrylamide	Acrylamide/Bis solution, 37.5:1
Polymerization Initiator	APS (10% (w/v), 200x); TEMED (2000x)
Sample Buffer (2x)	Tris (0.1 M), pH 6.8; SDS (4% (w/v)); Glycerol (20% (w/v)); β -ME (10% (v/v)); Bromophenol Blue (0.002% (w/v))
Running Buffer (10x)	Tris (0.25 M), pH 6.8; Glycine (2 M); SDS (4% (w/v))
Stacking Gel Buffer (4x)	Tris (0.5 M), pH 6.8; SDS (0.4% (w/v))
Separating Gel Buffer (4x)	Tris (1.5 M), pH 8.8; SDS (0.4% (w/v))
Staining Solution 1	Acetic Acid (12 % (v/v)); Ethanol (44% (v/v)); Coomassie Brilliant Blue R-250 (0.15% (w/v))
Destaining Solution 1	Acetic Acid (12 % (v/v))
Staining Solution 2	Roti Quick (Carl Roth)
Destaining Solution 2	water

Table 4: Composition of a 12 % Polyacrylamide Gel

Ingredient	Stacking Gel	Separating Gel
Stacking gel buffer	0.75 ml	—
Separating gel buffer	—	1.25 ml
Acrylamide	0.50 ml	2.00 ml
APS	24 μ L	20 μ L
TEMED	3 μ L	2.5 μ L
Water	1.75 ml	1.75 ml

8.3.3 Intact mass spectrometry¹¹

For verification and quantification of the modification status, assessment of the synthesized proteins, and determination of the efficiency of binary adduct formation, LC-MS was performed as described previously [183]. Briefly, proteins were diluted to 0.3 mg/ml in ddH₂O (Hsc70) or 0.2 mg/ml (all other proteins), if not otherwise stated, and centrifuged at 21,000 $\times$ g for 5 min at 4 °C. 2 μ l per sample were desalted using a ProSwift™ RP-4H 1 $\times$ 50mm column (Thermo Fisher Scientific) followed by injection into a maXis II electron transfer dissociation ESI LC-MS (Bruker Daltonics) system. The column oven was

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maintained at a constant temperature of 50 °C, while the autosampler was set to 8 °C. Proteins were eluted at a flow rate of 0.3 ml min⁻¹ using an acetonitrile gradient ranging from 5% to 90% over a total run time of 2 min 20 s. Data were analyzed using DataAnalysis (Version 5.1, Bruker Daltonics).

If the modification status of a protein was to be assessed, the percentage of modification was calculated using the formula $I_{\text{modified}}/(I_{\text{modified}}+I_{\text{unmod}})$, with I_{modified} being the intensity of the signal corresponding to the modified protein and I_{unmod} being the intensity of the signal corresponding to the unmodified protein.

8.3.4 Assessment of phosphorylation stability¹²

General stability monitoring during protocol development

40 µl of 30 µM pHsc70/pHsc70^{T204A} were incubated 0 min and over night at RT, unless stated otherwise, in 40 mM NaH₂PO₄, 4 mM MgCl₂, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 100 mM NaCl, pH 7.6 with or without ADP as indicated (ADP concentration stated in the corresponding Results section/figure legends). For iMS analysis, samples were diluted by addition of 50 µl water, and 2 µl were injected into the mass spectrometer immediately after dilution.

Dephosphorylation challenge assay with LegK4 (± kinase; kinase-dead control)

30 µl of 60 µM pHsc70 were incubated with or without 0.4 µM of a truncated version of LegK4, LegK4₅₉₋₄₆₂, or its kinase-dead D253A mutant at 25 °C in 40 mM NaH₂PO₄, 4 mM MgCl₂, 1 mM TCEP, 100 mM NaCl, pH 7.6. At time points 0 min, 20 min, 4 h and 20 h, 4 µl of each sample was treated with APPNHP (Adenosine-5'-[(β,γ)-imido]triphosphate, tetralithium salt) to a final concentration of 9 mM to stop the reaction. After mixing with 50 µl water, 2 µl of each sample were loaded into the mass spectrometer. Three biological replicates were performed.

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8.3.5 HSC70 basal ATPase activity⁶

The Malachite Green Phosphate Assay Kit from Sigma Aldrich was used. 25 µl of 1 µM Hsc70 or pHsc70 were incubated at 30 °C for 1 h in 100 mM Tris at pH 7.4, 100 mM KCl, 6 mM MgCl₂, and 0.01 % Triton X-100 after starting the reaction with 500 µM ATP in a transparent Sarstedt 96-well, F-bottom plate using a Tecan spark instrument from Tecan group. After incubation, the sample was diluted with Malachite Green working reagent and water to a final volume of 100 µl. After incubation at room temperature for 30 minutes, absorption was measured at 620 nm wavelength and the phosphate concentration calculated using the phosphate standard supplied by Sigma Aldrich. Significance was assessed by an independent-samples t-test using Origin software. 8 technical replicates were performed.

8.3.6 Small-scale AMPylation assays for condition screening

Small-scale AMPylation reactions were performed to explore the influence of protein purification strategy, FicD/Hsc70 ratio, buffer composition and incubation time on AMPylation efficiency. As AMPylation enzyme, the catalytically deregulated FicD variant FicD₁₀₂₋₄₅₈^{E234G L258D} was used in all experiments. As substrates, either human BiP or human Hsc70 were employed. BiP was purified by size-exclusion chromatography, whereas Hsc70 was used either after the second IMAC step or after additional SEC purification, as indicated in the Results section.

Unless stated otherwise, reactions contained 10 µM chaperone (BiP or Hsc70) and were incubated with FicD₁₀₂₋₄₅₈^{E234G L258D} at 22 °C for 3-6 h in either a HEPES-based buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.5, 1.5 mM ATP, 1 mM TCEP) or a NaH₂PO₄-based buffer (buffer F + 1.5 mM ATP, Table 2). The FicD/chaperone ratio was systematically varied by adjusting the FicD concentration (e.g. 0.5 µM FicD for a 1:20 FicD/chaperone ratio or 2 µM FicD for a 1:5 ratio), and the exact enzyme concentrations used for each condition are given in the corresponding figure legends and in the Results section. For each condition, one or two independent biological preparations of the respective chaperone were used, and each preparation was analyzed in technical duplicates. After incubation, reactions were processed for iMS as described in the iMS section, and

AMPylation levels were quantified based on the relative intensities of unmodified and AMPylated protein species.

8.3.7 Nucleotide content of selected Hsc70 preparations

Nucleotide content was analyzed by reverse-phase high-performance liquid chromatography using a ProntoSIL C18 RP-column (5 μ m 250x4.6, 120 Å, AQ; Bischoff Chromatography) and a Shimadzu device. Depending on the desired dilution, 0.9-13 μ l of sample were mixed with water to a final volume of 30 μ l, boiled for 5 min at 95 °C, and centrifuged at 21,000 g for 5 min. A 15 μ l aliquot of the supernatant was injected onto the column, which was run with a mobile phase containing 50 mM KH₂PO₄ buffer (pH 6.6), 12 % acetonitrile, and 3 mM tetrabutylammonium bromide at 1 ml/min for 15 min. A homemade ATP standard mix as well as defined ATP concentrations were used for calibration.

8.3.8 Assessment of AMPylation stability

General stability monitoring during protocol development

AMPylation stability was assessed analogously to the general stability monitoring for phosphorylation as described above. Unless stated otherwise, AMPylated Hsc70 samples were incubated under the identical buffer and temperature conditions, and time points were analyzed by iMS as described in the iMS section. Deviations from the general scheme (e.g., temperature, nucleotide supplementation, or enzyme addition) are specified in the corresponding Results section and figure legends.

DeAMPylation challenge assay with FicD

To probe whether residual FicD activity can drive deAMPylation of purified AMPHsc70, an add-back deAMPylation assay was performed using FicD₁₀₂₋₄₅₈^{E234G L258D}.

Reactions contained 60 μ M AMPHsc70 in a total volume of 35 μ l in 20 mM HEPES (pH 7.5), 150 mM KCl, 250 μ M MgCl₂, and 1 mM dithiothreitol. Where indicated, FicD₁₀₂₋₄₅₈^{E234G L258D} was added to a final concentration of 0.4 μ M. Reactions were incubated at 25 °C in a thermocycler.

For the condition without FicD, a 0 min sample was taken. Subsequently, samples were collected at 20 min, 2 h, 4 h, and 20 h for both the –FicD and +FicD conditions. To stop the reaction, 4 μ l of each sample were mixed with 2 μ l EDTA to yield a final EDTA

concentration of 2 mM. The quenched sample (6 μ l) was diluted with 50 μ l water, and 2 μ l were injected for iMS analysis. Independent technical replicates (separate reaction tubes) were performed for each condition and time point. For statistical evaluation, AM-Pylation levels were quantified from iMS data and analyzed in Origin using paired t-tests for matched comparisons ($\pm$ FicD) at the respective time points; $p < 0.05$ was considered statistically significant.

8.3.9 nanoDSF

nanoDSF measurements were performed on a Prometheus Panta instrument (NanoTemper Technologies). Hsc70^{T204A} or pHsc70^{T204A} (4 μ M) were measured in 20 mM HEPES (pH 7.5), 150 mM KCl, 10 mM MgCl₂ and 1 mM TCEP, either in the presence of 1 mM ATP, 1 mM ADP, or under nucleotide-free (apo) conditions. Thermal unfolding was monitored from 20 to 85 °C using a continuous temperature ramp. Data were analyzed using the Prometheus software (NanoTemper Technologies). Because the F_{330}/F_{350} ratio did not yield well-defined unfolding transitions under these conditions, melting temperatures were determined from the intrinsic fluorescence intensity at 330 nm using Inflection Point #1 (maximum of the first derivative).

8.3.10 Thermofluor

Thermofluor (differential scanning fluorimetry, DSF) measurements were performed on a Bio-Rad CFX Connect real-time PCR system using SYPRO Orange as the fluorescent dye. Assays contained 4 μ M Hsc70; buffer components were taken from the RUBIC Buffer Screen (Molecular Dimensions). Samples were heated from 5 to 95 °C at a ramp rate of 1 °C/min while monitoring fluorescence. Melting curves were analyzed using eSPC (an online data analysis platform from EMBL) with the MoltenProt workflow [184]. Raw fluorescence signals were used for normalization, and the temperature range from 6 to 94 °C was included in the analysis. Melting temperatures were determined from the maxima of the first derivative.

8.3.11 SAXS¹³

SAXS measurements of Hsc70^{T204A} and pHsc70^{T204A} in different nucleotide states were performed on PETRA III Beamline P12 on the DESY campus, Hamburg in March 2025 [185]. Purified Hsc70^{T204A} and pHsc70^{T204A} were stored for 4 months at -80 °C after purification including SEC. A Hsc70^{T204A}-fraction eluting at a defined elution window from SEC was collected encompassing species ranging from monomers to higher-order oligomers, while very high-molecular-weight species (i.e., eluting at earlier elution volumes) were excluded. This approach was chosen because Hsc70 undergoes a dynamic, reversible monomer-oligomer equilibrium that would establish itself even after purifying a monomeric protein fraction [17]. The same fractionation strategy was applied to Hsc70^{T204A} and pHsc70^{T204A}, providing a comparable monomeric/oligomeric composition while excluding strongly aggregated or very high-molecular-weight material. The proteins were thawed on ice and subjected to dialysis against 20 mM HEPES pH 7.5, 150 mM KCl, 10 mM MgCl₂, 1 mM TCEP at 4 °C for 4 h in Thermo Scientific Slide-A-Lyzer cassettes 0.5-3 ml, 10 kDa MWCO. The buffer was changed every hour. Prior to measurement, the samples were supplemented with nucleotide to a final concentration of 1 mM, where applicable. Concentrations of 0.5, 1, 2, 3, 4, 5, and 7 mg/ml were measured, and data quality was assessed in two steps: first by automated quality classification at P12 and second by manual inspection of scattering curves and Guinier plots. Measurements classified as insufficient by the beamline pipeline and/or displaying irregularities such as poor signal-to-noise or strong deviations from linear Guinier behavior were not considered further. Based on these criteria, Hsc70^{T204A} measurements in the ATP-state at 1 mg/ml, the ADP-state at 0.5 and 5 mg/ml, and the apo-state at 1 mg/ml were excluded from further analysis. The same applies to pHsc70^{T204A} measurements in the ATP-, ADP-, and apo-states at 0.5 mg/ml. The system setup was as follows: Bragg Angle [°] 10.9433625, wavelength [nm] 0.123979, energy [eV] 10000.4, detector distance [m] 3.0, exposure time [s] 0.09500 per frame (40 sample frames, 2*40 buffer frames), detector

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name pilatus6m, q-range 0.00224730 - 0.7247599 Å⁻¹, using a quartz capillary with continuous flow setup.

Data analysis was performed using the ATSAS package version 4.1.1 from BIOSAXS GmbH. It was used to perform Guinier analysis (AUTORG), to derive the p(r) distance distribution (GNOM), to calculate dimensionless Kratky plots, and to perform OLIGOMER analysis on all samples [186-189]. For the calculation of dimensionless Kratky plots, scattering curves were extrapolated to zero concentration to minimize interparticle effects. Due to the polydisperse nature of the sample, no atomic or rigid-body structural modeling was performed.

For OLIGOMER, AlphaFold2 models of monomeric, dimeric and trimeric Hsc70 were used to build pools using RANCH to reflect a small ensemble of different conformers (Figure S21) [188, 189]. In general, domain-docked and undocked conformers of different orientations were created by different combinations of fixing the NBD, the β-SBD or the α-lid. Different combinations of conformers and oligomeric states were tested for Hsc70^{T204A} and pHsc70^{T204A} in different nucleotide states. The parameters were kept fixed over the whole analysis (minimum/maximum scattering vector q: 0-0.5 Å⁻¹, 1800 points, default settings).

8.3.12 Analytical size-exclusion chromatography

Hsc70^{T204A} and pHsc70^{T204A} were analyzed by analytical size-exclusion chromatography at 1, 2, and 4 mg/ml. Where indicated, nucleotides were added to a final concentration of 1 mM. All samples were incubated for 15 min at room temperature prior to injection. Measurements were performed on a Shimadzu HPLC system using a Cytiva Superdex 200 Increase 10/300 GL column operated at a flow rate of 0.5 ml/min. The running buffer (degassed prior to use) consisted of 20 mM HEPES (pH 7.5), 150 mM KCl, 10 mM MgCl₂, and 1 mM TCEP. A Bio-Rad gel filtration standard was used for column calibration, and vitamin B12 was included as an internal control. Elution was monitored by ultraviolet (UV) absorbance at 280 nm and fluorescence emission at 340 nm; data analysis was performed primarily using the A280 traces, with fluorescence recorded in parallel.

Analytical SEC chromatograms were processed in Origin by applying a user-defined linear two-point baseline to correct detector offsets/drift. The baseline-subtracted traces were then area-normalized by dividing each chromatogram by its total integrated area over a

fixed elution-volume window encompassing all Hsc70 species (9-17 ml), yielding unit area within this range (y-axis reported as ml^{-1}). This normalization corrects for differences in injected protein mass resulting from the tested sample concentrations (at constant injection volume), enabling direct comparison of distribution profiles across concentrations and nucleotide conditions. Technical duplicates were analyzed identically.

8.3.13 Limited proteolysis

Hsc70^{T204A} and pHsc70^{T204A} were used at a final concentration of 40 μM . Proteins were incubated in the absence (apo) or presence of nucleotides (5 mM ADP or ATP) for 60 min at room temperature. Control samples (without protease) were taken prior to protease treatment. Limited proteolysis was initiated by addition of Proteinase K (1 μM ; Thermo Fisher Scientific) and samples were incubated for 15 min at room temperature. Reactions were terminated by addition of freshly prepared PMSF (dissolved in isopropanol) to a final concentration of 200 μM . Samples were mixed with 2 $\times$ SDS loading buffer and immediately loaded onto SDS-PAGE gels, corresponding to 2.84 μg Hsc70 per lane. Technical replicates were prepared independently from the same protein preparation and resolved on the same gel.

Band intensities were quantified from Coomassie-stained SDS-PAGE gels by densitometry using Fiji [190]. The core signal was defined as the combined intensity of the full-length band and all bands migrating above 55 kDa, representing species in which the interdomain linker remained largely intact. In contrast, linker cleavage generated characteristic lower-molecular-weight fragments corresponding to the nucleotide-binding domain (NBD) and substrate-binding domain (SBD), which were assigned based on their apparent molecular weight. Core signal intensities were normalized to the respective untreated control (core signal/control). In addition, the combined intensity of linker cleavage products was calculated as the sum of NBD and SBD fragment intensities (INBD + ISBD), which was normalized either to the control (INBD + ISBD / Icontrol) or to the core signal (INBD + ISBD / Icore). All values represent mean $\pm$ SD from three independently prepared technical replicates. Statistical comparisons between Hsc70^{T204A} and pHsc70^{T204A} within each nucleotide condition were performed using an unpaired two-tailed Welch's t-test in Origin.

8.3.14 Binary adduct formation

Binary adduct formation assays were performed using 50 μM kinase (LegK4₅₉₋₄₆₂^{C145S} and its variants or CK2 α' and its variants) and 100 μM nucleotide analog in buffer containing 20 mM HEPES, 100 mM NaCl, 1 mM MgCl₂, and 1 mM TCEP (pH 7.4). Reactions were incubated for 4 h at 22 °C in case of CK2 α' and 5 h and over night in case of LegK4. Samples were then analyzed by intact mass spectrometry as described in the iMS section. In the case of CK2 α' ^{H161C}, only TReND-K1 was tested, with 250 μM Analogs.

8.3.15 Analog modification assay

Modification reactions were performed by incubating 10 μM Hsc70 with 0.5 μM LegK4₅₉₋₄₆₂^{C145S} or 10 μM EEF1D/HAP28 with 0.5 μM CK2 α' , overnight at 22 °C in 20 mM HEPES, 100 mM NaCl, 1 mM MgCl₂, and 1 mM TCEP (pH 7.4). Reactions contained either 750 μM ATP (control) or the respective nucleotide analog, as indicated. Samples were then analyzed by intact mass spectrometry as described in the iMS section.

8.3.16 Ternary complex formation

Ternary complex formation assays were performed by incubating 30 μM substrate (EEF1D and HAP28 for CK2 α' , or Hsc70 for LegK4₅₉₋₄₆₂^{C145S}) with 30 μM of the corresponding pre-formed kinase-analog binary adduct. In case of Hsc70, the protein was loaded with 800 μM ATP/ADP. For CK2 α' , samples were incubated for 4 h or overnight, whereas LegK4₅₉₋₄₆₂^{C145S} samples were incubated for 5 h or overnight in 20 mM HEPES, 100 mM NaCl, 1 mM MgCl₂, and 1 mM TCEP (pH 7.4). Samples were then analyzed by SDS-PAGE, loading 4 μg of substrate protein per lane.

8.4 Protein crystallization and structure determination by X-ray crystallography

150 μM CK2 α' were mixed with 300 μM TReND-K1 for the following crystallization trials: The following part was written by Dr. Vivian Pogenberg and modified by me.

Sitting drop crystallization trials were carried out at 19°C, by mixing equal volumes (100 nl) of reservoir solution and protein solution. Crystals grew initially in a condition containing 0.1 M Tris pH 8.5 8 % (w/v) and PEG 8000, they were then optimized using a seeding procedure [191]. Crystals were soaked in cryo-solutions containing the

crystallization mother liquor supplemented with 25% [v/v] glycerol, mounted onto a cryo-loop (Hampton Research), and immediately flash-cooled in liquid nitrogen. The synchrotron data was collected at beamline operated by EMBL Hamburg at the PETRA III storage ring (DESY, Hamburg, Germany). We would like to thank the staff for the assistance in using the beamline. Diffraction data were processed using XDS and scaled with Aimless from the CCP4 suite [192].

The complex structure was solved by molecular replacement with Phaser using a search model based on the previously published CK2 structure (PDB: 6hmq). The obtained model solution was then corrected and further built manually with COOT [193] and refined using the PHENIX suite [194]. The quality of the final model was assessed using Molprobity [195].

An OMIT/POLDER map where the ligand TRND-K1 as well as the modified cysteine residue have been omitted was calculated using the PHENIX suite. Data collection and refinement statistics are gathered in Supplementary Table S8.

8.5 Computational and *in silico* analyses

For the analysis of SAXS data, several computational approaches were applied, including Kratky analysis, OLIGOMER fitting, and RANCH modeling. These methods are described in detail in the SAXS Methods section.

8.5.1 Calculation of the theoretical mass of ILV-labeled Hsc70^{T204A}

The intact mass of the labeled proteins was determined by iMS. To assess the incorporated isotopes, the theoretical mass of the labeled protein was calculated using the following assumptions. During MS analysis, all exchangeable deuterium is assumed to back-exchange to hydrogen and therefore appears as ¹H in the measured intact mass [122]. Hydrogens in -NH, -OH, -SH and -COOH groups, as well as those at the N- and C-termini and backbone amide positions were considered exchangeable [122]. The molecular formula of the non-phosphorylated protein is C₃₁₂₀H₅₀₁₂N₈₆₄O₉₉₈S₁₇ [196], comprising 649 amino acids (Table S 10).

Isoleucine contains one terminal methyl group, whereas leucine and valine contain two. For ILV labeling, isoleucine was labeled selectively at the δ 1 methyl (¹³C/¹H, Ile δ 1 only), while leucine and valine were labeled such that only one of the two terminal methyl

carbons carries ^{13}C (the other methyl remaining ^{12}C -deuterated, e.g., $^{12}\text{CD}_3$) [197]. Because the precursors contain 99 % ^{13}C and natural isotope abundance must be considered (approximately 1.07 % ^{13}C), the theoretical total number of ^{13}C atoms is 164.61. The number of ^{15}N atoms is 846.72, based on 98 % ^{15}N in the precursor.

Table 5: Exchangeable sidechain groups in Hsc70^{T204A}

The numbers of exchangeable hydrogens per residue for Arg, His, and Trp were taken from the literature [198-200]. The assignment of exchangeable side-chain groups was based on published sources [122].

Amino Acid	Count in Hsc70 ^{T204A}	Exchangeable side-chain group	Exchangeable H per residue	Contribution (H)
Ser	36	-OH	1	36
Thr	46	-OH	1	46
Tyr	15	-OH	1	15
Asp	46	-COOH	1	46
Glu	49	-COOH	1	49
Asn	35	-CONH ₂	2	70
Gln	26	-CONH ₃	2	52
Lys	54	-NH ₃ ⁺ / -NH ₂	2	108
Arg	28	Guanidinium -NH groups	5	140
His	7	Imidazole -NH	1	7
Trp	2	Indole -NH	1	2
Cys	4	-SH	1	4

To calculate the theoretical mass, the number of exchangeable hydrogens was estimated (Table 5 lists all exchangeable residues and their exchangeable hydrogens). Here, 575 side-chain hydrogens were assumed to be exchangeable. In addition, 626 backbone/terminal hydrogens were assumed to be exchangeable (648 peptide bonds – 25 prolines + 3 terminal hydrogens). Thus, the total number of exchangeable hydrogens is 1201. Since the protein contains 5012 hydrogens in total, the number of non-exchangeable hydrogens is 5012 - 1201 = 3811.

It was further assumed that, among these 3811 non-exchangeable hydrogens, 402 remain as ^1H because they belong to the 134 non-deuterated ^{13}C -methyl groups from Ile, Leu and Val (3 H per methyl group). At a deuteration level of 100 % (defined as the

fraction of ^2H at non-exchangeable hydrogen positions), all remaining 3409 non-exchangeable positions would be ^2H . The theoretical mass $m_{\text{theor.}}$ was calculated using the following isotope counts:

$^{12}\text{C} = 2955.39$, $^{13}\text{C} = 164.61$; $^1\text{H} = 1603$, $^2\text{H} = 3409$; $^{14}\text{N} = 17.28$ and $^{15}\text{N} = 846.72$. The isotope counts, together with the molecular formula, were entered into the Molecular Mass Calculator of the Biological Magnetic Resonance Data Bank (bmr.bio), yielding $m_{\text{theor.}} \approx 75542.2$ Da at 100 % deuteration.

8.5.2 AlphaFold2 prediction of Hsc70 monomers, dimers, and trimers

The predicted structures were used as input for Ranch. For the Hsc70 monomer, the AF-P11142-2-F1-v6 entry from the AlphaFold Protein Structure Database [201], with an average pLDDT of 91.25, was used. AlphaFold2 multimer predictions of dimeric Hsc70 (average pLDDT/pTM = 88.2/0.817) and trimeric Hsc70 (average pLDDT/pTM = 81.4/0.494) were generated by Dr. Michael Hecht-Bucher using ColabFold [202, 203] (Figure S21).

The following settings were used:

ColabFold settings (v1.5.5). General settings: num_queries=1, use_templates=true, initial_guess=null, keep_existing_results=false, host_url=https://api.colabfold.com, user_agent=colabfold/google-colab-main, commit=c3e8ab010a2d2c5f8f18f653817c6bfbf58118e8. Model settings: model_type=alphafold2_multimer_v3, num_models=5, model_order=1,2,3,4,5, num_recycles=6, recycle_early_stop_tolerance=0.0, num_ensemble=1, rank_by=multimer, stop_at_score=100.0. MSA/pairing settings: msa_mode=mmseqs2_uniref_env, max_seq=508, max_extra_seq=2048, pair_mode=unpaired_paired, pairing_strategy=greedy. Relaxation settings: num_relax=0, relax_max_iterations=200, relax_tolerance=2.39, relax_stiffness=10.0, relax_max_outer_iterations=3. Sampling/performance settings: random_seed=0, num_seeds=3, recompile_padding=10, use_dropout=false, use_cluster_profile=true, use_fuse=true, use_bfloat16=true, calc_extra_ptm=false, use_probs_extra=true.

8.5.3 Web based services, software and generative AI

Gene and genome sequences were obtained from UniProt [100] or the National Center for Biotechnology Information (NCBI) [204] national library of medicine. Protein sequences and references were retrieved from UniProt. In this work, PyMOL (Schrödinger, Versions 2.5.4 and 4.6) was used for visualization and superposition of crystal structures. Extinction coefficients and molecular masses were calculated using SnapGene (Dotmatics, Version 4.2.11). OriginPro 2021 (OriginLab Corporation) was used for data analysis as indicated.

The generative AI ChatGPT (2025-2026, OpenAI), including version 5.2, was used throughout the preparation of this thesis, primarily for text optimization. In these cases, I provided the original text, and the AI's suggestions were adopted to improve clarity and flow or to translate sentences originally written in German into English. This was the main use of AI in the present work. In addition, the abstract was translated from English into German using this AI system. The AI was also used to identify and summarize literature sources. These sources were then used by me to summarize the current state of the literature and to place the data generated in this work into the context of existing research. In a small number of cases, individual sentences generated by the AI without prior wording provided by me were adopted. In a similar manner, the AI was also used to summarize analytical methods described in the literature.

All analyses presented in this thesis, unless otherwise indicated in the contributions table, were selected, performed, and interpreted by me. All AI-generated material was critically reviewed and verified by me.

8.6 Materials used and Hazardous substances

Table 6: List of chemicals, reagents and media used in this study and their suppliers

Chemical / substance	Supplier
(1,2,3,4,5,6,6-D7) D-glucose	Sigma Aldrich
15NH ₄ Cl (98%)	Sigma Aldrich
3-methyl-13C α -ketoisovaleric acid (3,4,4,4-D ₄ sodium salt)	Cambridge Isotope Laboratories
Acetonitrile (MS-grade)	Chemsolute
Acrylamide/Bis solution, 37.5:1	SERVA Electrophoresis
ADP	Biosynth
Agar-Agar (Kobe I)	Carl Roth
Ammonium persulfate	SERVA Electrophoresis

Methods

Ampicillin sodium salt	Carl Roth
APPNHP	Jena Bioscience
ATP	Biosynth
Biotin	Biosynth
Bromophenol blue	Merck
CaCl ₂	Carl Roth
Chloramphenicol	MP Biomedicals
Coomassie Brilliant Blue R-250	SERVA Electrophoresis
D ₂ O (99.9%)	TH Geyer
D-glucose	Carl Roth
DTT	Biosynth
EDTA	Merck
Ethanol	Chemsolute
Glycerol	TH Geyer
Glycine	TH Geyer (Biosolute)
HEPES	Biosynth
Imidazole	Carl Roth
IPTG	Biosynth
Isopropanol	Chemsolute
K ₂ HPO ₄	Carl Roth
KCl	Carl Roth
KH ₂ PO ₄	TH Geyer
KOH	Sigma Aldrich
LB Broth (Luria/Miller)	Carl Roth
methyl- ¹³ C α-ketobutyric acid	Cambridge Isotope Laboratories
MgCl ₂	AppliChem
NaCl	TH Geyer
NaH ₂ PO ₄	Sigma Aldrich
NaOH	Carl Roth
NH ₄ Cl	Carl Roth
PMSF	Carl Roth
Roti®-Quick	Carl Roth
SDS	SERVA Electrophoresis
TCEP	Fluorochem
TEMED	TH Geyer
Tetrabutylammonium bromide	Biosynth
Tris-HCl	Sigma Aldrich
Triton X-100	Sigma Aldrich
Water (MS-grade)	Chemsolute
Xylene cyanol FF	Carl Roth
α-ketobutyric acid	Sigma Aldrich
α-ketoisovaleric acid	Sigma Aldrich
β-mercaptoethanol	Merck

Table 7: Hazardous substances and associated hazard classifications

Information sources: GESTIS Substance Database (accessed April 3, 2026); if unavailable, Carl Roth Safety Data Sheets.

Hazardous substances	H-phrases	P-phrases	Pictograms
Acetic acid	226; 314	210; 280; 301+330+331; 303+361+353; 305+351+338; 310	 
Acetonitrile	225; 302+312+332; 319	210; 280 303+361+353 304+340+312 305+351+338	 
Acrylamide	301;312;332; 315;317;319; 340;350;361f; 372	201;280 301+310; 302+352+312; 304+340+312; 305+351+338	 
Ammonium persulfate/ Ammonium peroxodisulfate	272;302;315; 317;319;334; 335	210;280; 301+312; 302+352; 304+340+312; 305+351+338	  
Ammoniumchloride	302;319	264;270;280; 301+312; 305+351+338; 337+313	
Ampicillin	317;334;	261;280; 302+352; 342+311	
Calciumchloride	319	264;280; 305+351+338; 337+313	

Chloramphenicol	318;351;361fd	202;280; 305+351+338; 308+313; 405;501	
Dithiothreitol	302;315;318	264;270;280; 301+312; 302+352; 305+351+338	
Ethanol	225;319	210;233;240; 241;242; 305+351+338	
Ethylenediaminetetraacetic acid	319;332;373	260;264;271; 304+340+312; 305+351+338; 314	
hydrochloric acid	290;314;335	234;261;271; 280; 303+361+353; 305+351+338	
Imidazole	302;314;360D	260;280; 301+312; 303+361+353; 304+340+310; 305+351+338	
Isopropanol	225;319;336	210;233;240; 241;242; 305+351+338	
N,N,N',N'-Tetramethylethylenediamine	225;331;302; 314	210;280 301+312; 303+361+353; 304+340+310; 305+351+338	
Phenylmethylsulfonyl fluoride	301;314	260;280; 303+361+353; 304+340+310; 305+351+338; 363	
Potassium hydroxide	290;302;314	234;260;280; 301+312; 303+361+353; 305+351+338	
Proteinase K	315;317;319; 334;335	260;280; 342+311	

Sodium dodecyl sulfate	228; 302+332; 315; 318;335;412	210;273;280 301+312; 305+351+338;	  
Sodium hydroxide	290;314;	234;260;280; 303+361+353; 304+340+310; 305+351+338	
Tetrabutylammonium bromide	302;315;319; 361fd;412	202;273; 301+312; 302+352; 305+351+338; 308+313	 
Tris(2-carboxyethyl)phosphine hydrochloride	302;351;360F; 411	201;264;270; 273;280; 308;313	  
Triton X-100	302;318;411	270;273;280; 305+351+338; 310	  
β -mercaptoethanol	301+331; 310;315;317; 318;361f;373; 410	273;280; 301+310; 302+352+310; 304+340+311; 305+351+338	   

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10 Appendix

10.1 Supplementary Figures

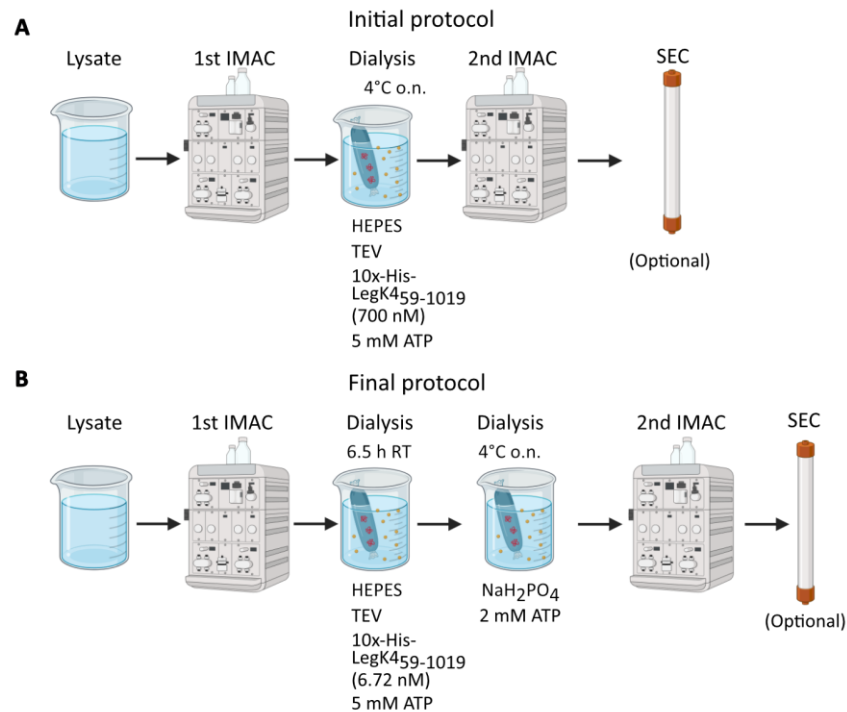


Figure S1: Initial and final workflows for preparative phosphorylation of Hsc70 by LegK4

(A) Initial protocol. Following expression and lysis, His₆-tagged Hsc70 is purified by a first IMAC step and incubated with His₁₀-tagged LegK4₅₉₋₁₀₁₉ (700 nM) during overnight dialysis at 4 °C in HEPES-based buffer in the presence of TEV protease. LegK4 is removed by a second IMAC step, followed by optional SEC polishing. (B) Final protocol. The workflow is identical up to the first IMAC step, but phosphorylation is carried out with catalytic amounts of His₁₀-tagged LegK4₅₉₋₁₀₁₉ (6.72 nM) during a two-step dialysis (6.5 h at room temperature in HEPES-based buffer, followed by overnight dialysis at 4 °C in NaH₂PO₄-based buffer), again in the presence of TEV protease. The kinase is depleted by a second IMAC step, followed by optional SEC polishing. The figure was created using BioRender. Created in BioRender. Kley, N. (2026) <https://BioRender.com/dpq64qd>.

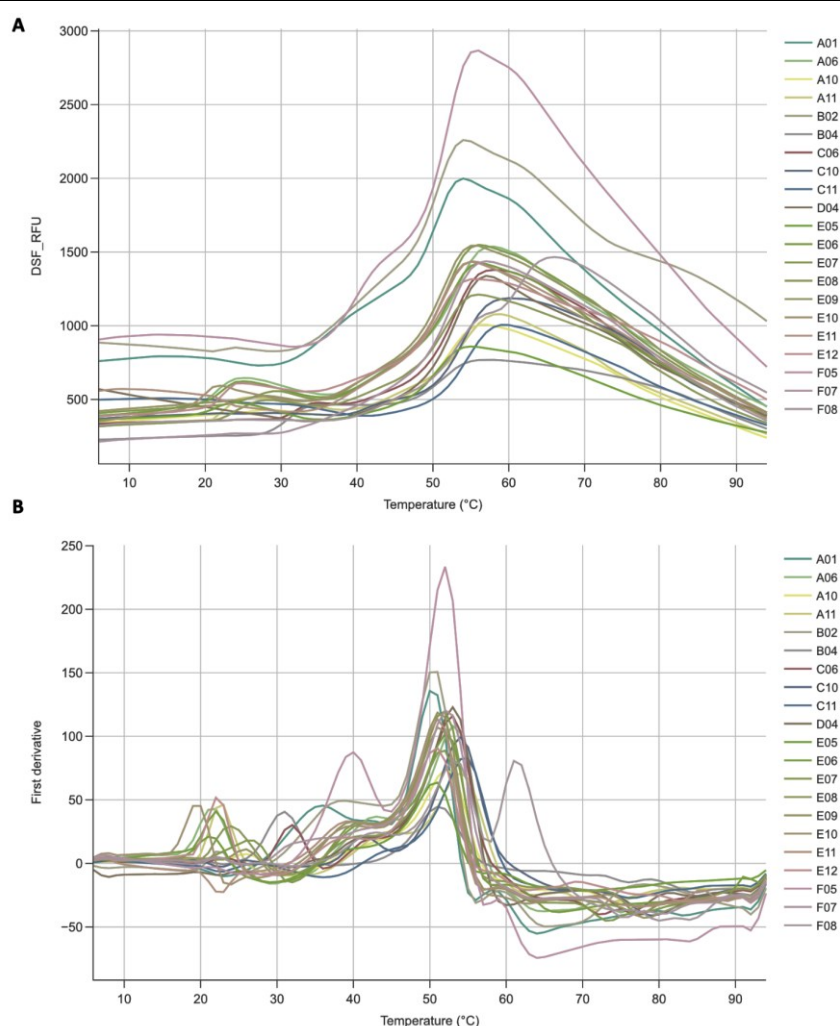


Figure S2: Differential scanning fluorimetry analysis of Hsc70

Fluorescence signals (DSF_RFU) recorded as a function of temperature (top) and their first derivatives (bottom). Shown are the 20 conditions with the highest melting temperatures, together with the water control arranged in alphabetical order. All curves represent single measurements. Melting temperatures were extracted from the maxima of the derivative curves. Data analysis and figure generation were performed using MoltenProt.

A01 (Water/Control), A06 (0.1 M potassium phosphate, pH 6.0), A10 (0.1 M sodium phosphate monobasic, pH 7.0), A11 (0.1 M potassium phosphate monobasic, pH 7.0), B02 (0.1 M ammonium acetate, pH 7.3), B04 (0.1 M sodium phosphate monobasic, pH 7.5), C06 (0.25 M sodium chloride, 0.1 M potassium phosphate monobasic, pH 6.0), C10 (0.25 M sodium chloride, 0.1 M sodium phosphate monobasic, pH 7.0), C11 (0.25 M sodium chloride, 0.1 M potassium phosphate monobasic, pH 7.0), D04 (0.25 M sodium chloride, 0.1 M sodium phosphate monobasic, pH 7.5), E05 (0.1 M SPG, pH 6.0), E06 (0.1 M SPG, pH 6.5), E07 (0.1 M SPG, pH 7.0), E08 (0.1 M SPG, pH 7.5), E09 (0.1 M SPG, pH 8.0), E10 (0.1 M SPG, pH 8.5), E11 (0.1 M SPG, pH 9.0), E12 (0.1 M SPG, pH 10.0), F05 (0.02 M sodium phosphate, pH 7.5), F07 (0.125 M sodium phosphate, pH 7.5), F08 (0.25 M sodium phosphate, pH 7.5).

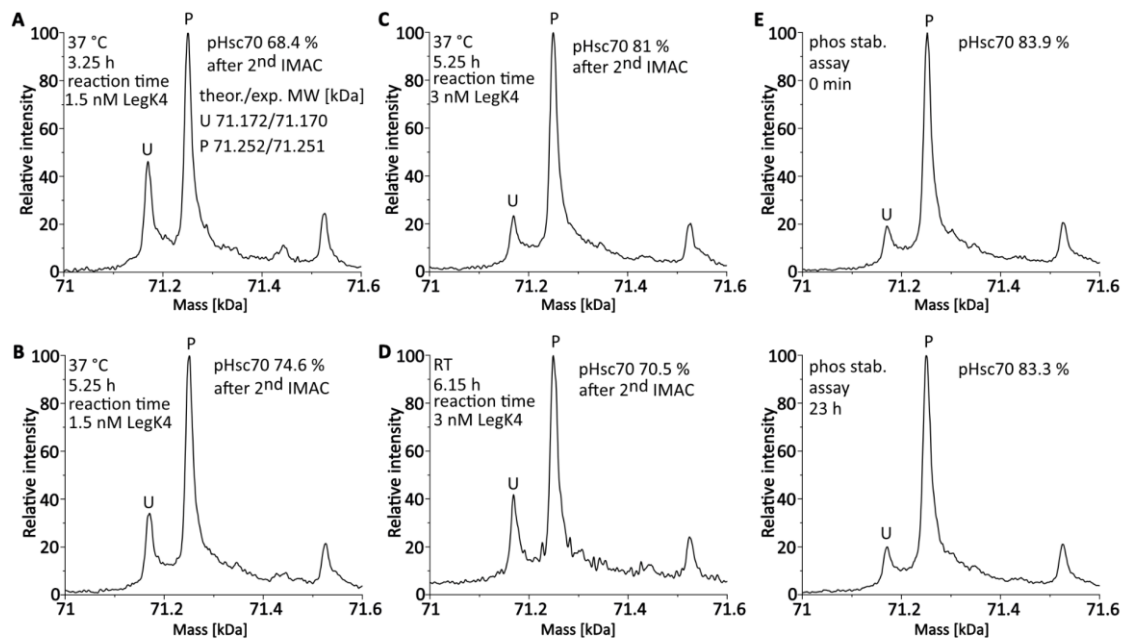


Figure S3: Optimization of the preparative Hsc70 phosphorylation protocol by varying LegK4 concentration, incubation time, and temperature

(A-D) Intact mass spectrometry (iMS) spectra from individual optimization experiments acquired under the indicated reaction conditions (temperature, reaction time, and LegK4 concentration), measured directly after the 2nd immobilized metal affinity chromatography (IMAC) step as single point and time measurements. Hsc70 was used as substrate in all cases. Phosphorylation levels were quantified from the relative intensities of the unmodified (U) and phosphorylated (P) species and are indicated in each panel together with the theoretical/experimental molecular weights. (E) Phosphorylation stability assay of pHsc70 from panel (C) after subsequent SEC. Shown are iMS spectra directly after purification (0 min, top) and after 23 h incubation in the presence of ADP (bottom), indicating stable phosphorylation over time. Each spectrum corresponds to a single time measurement. All measurements represent single experiments without replicates.

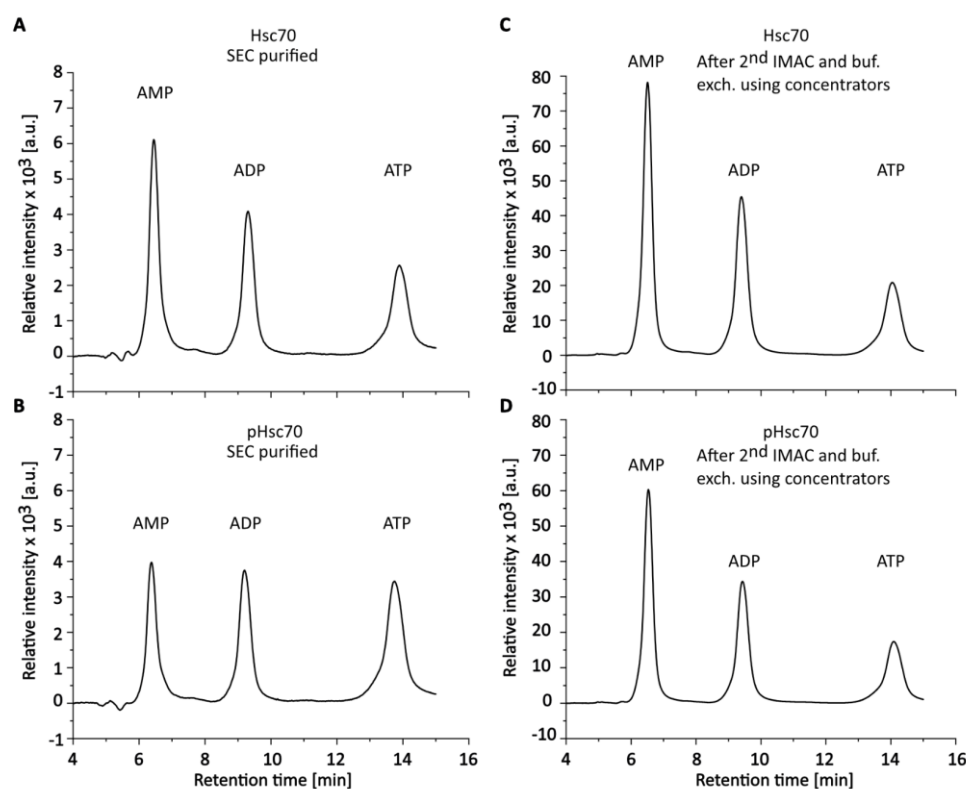


Figure S4: HPLC measurement of the nucleotide content of Hsc70 samples after selected purification protocols

(A-B) Nucleotide content of Hsc70 (A) and pHsc70 (B) after purification including SEC, analyzed by reverse-phase HPLC on a C18 column using tetrabutylammonium bromide. After accounting for dilution factors, the nucleotide content is 18 μM for Hsc70 and 20 μM for pHsc70. **(C-D)** Nucleotide content of Hsc70 (C) and pHsc70 (D) after purification up to the 2nd IMAC step and buffer exchange using Amicon centrifugal filters, analyzed by reverse-phase HPLC on a C18 column with tetrabutylammonium bromide. After accounting for dilution factors, the nucleotide content is 132 μM for Hsc70 and 124 μM for pHsc70. A representative measurement from one purification is shown; comparable results were obtained across multiple independent purifications.

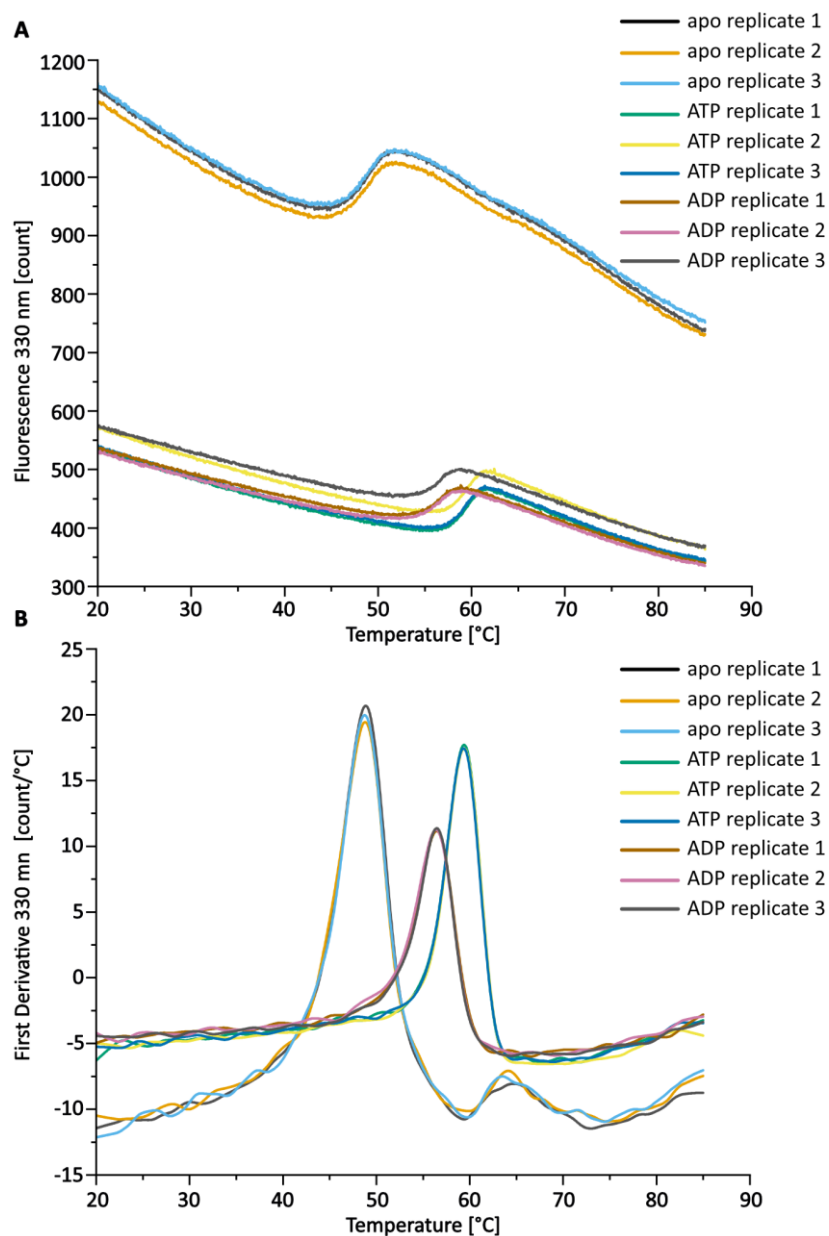


Figure S5: NanoDSF-based thermal unfolding analysis indicates no major differences between Hsc70^{T204A} and pHsc70^{T204A}

(A) Intrinsic protein fluorescence at 330 nm plotted as a function of temperature. **(B)** First derivative of the fluorescence signal with respect to temperature (dF/dT, a.u./°C). Each condition was measured in technical triplicates, and individual replicates are shown.

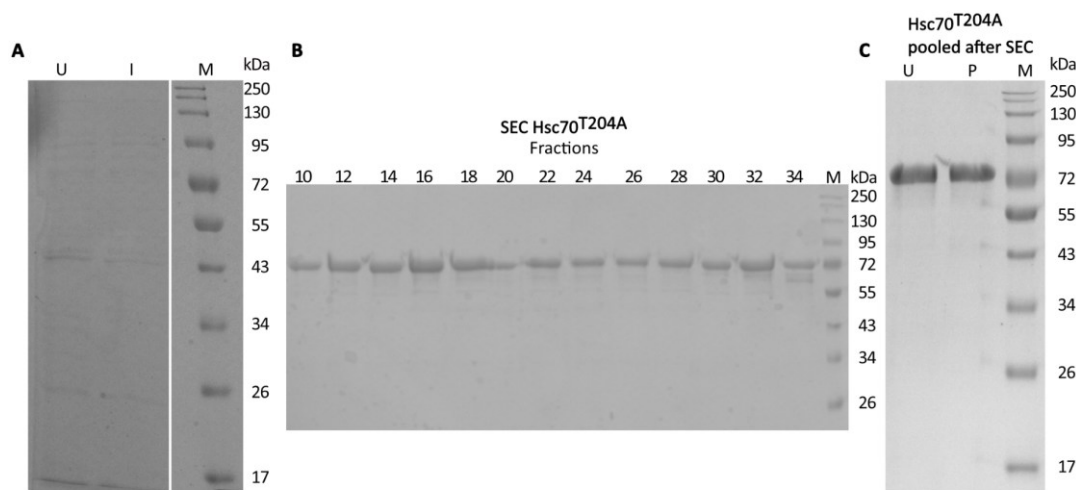


Figure S6: Supplementary SDS-PAGE analysis of Hsc70^{T204A} expression for isotopic labeling experiments and purification by the standard LB-based workflow with final phosphorylation protocol

(A) SDS-PAGE analysis of Hsc70^{T204A} expression in M9 medium without isotopic supplementation. Lanes labeled U and I correspond to uninduced and induced samples, respectively. Only a weak overall signal is visible, and no pronounced difference between uninduced and induced samples is apparent. (B) Representative SDS-PAGE analysis of fractions obtained after size-exclusion chromatography (SEC) of unmodified Hsc70^{T204A} purified from LB-grown cells using the final phosphorylation protocol. Consistent with the SEC elution profile shown in Figure 4F, Hsc70^{T204A} elutes across multiple fractions, reflecting the presence of oligomeric species. (C) SDS-PAGE analysis of pooled SEC fractions containing unmodified (U) and phosphorylated (P) Hsc70^{T204A} prepared from LB-grown cells using the final phosphorylation protocol.

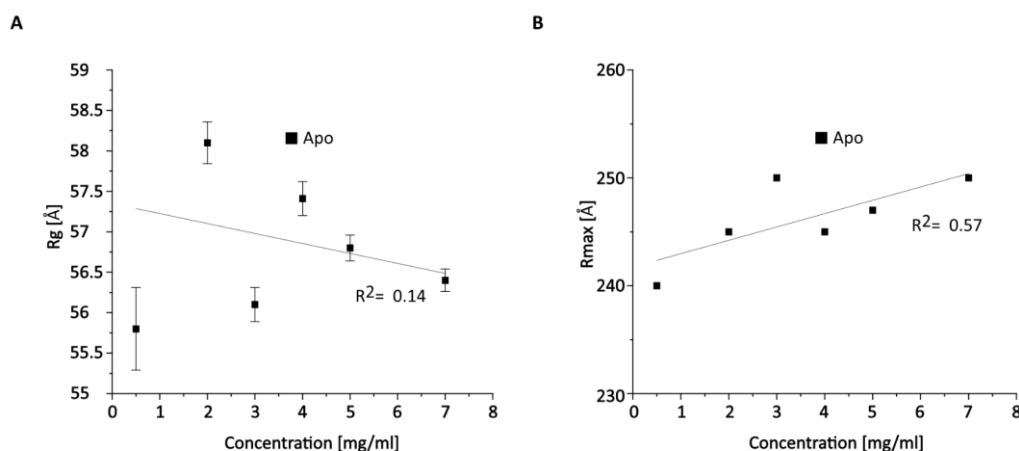


Figure S7: Rg and Rmax of Hsc70^{T204A}:apo as a function of concentration

(A-B) Rg (A) and Rmax (B) of Hsc70^{T204A}:apo plotted against concentration. These panels reproduce the data shown in Figure 9C and Figure S8C, respectively, but with rescaled y-axes so that deviations from linearity are more clearly visible. This figure and its caption were adapted from the cited publication [21].

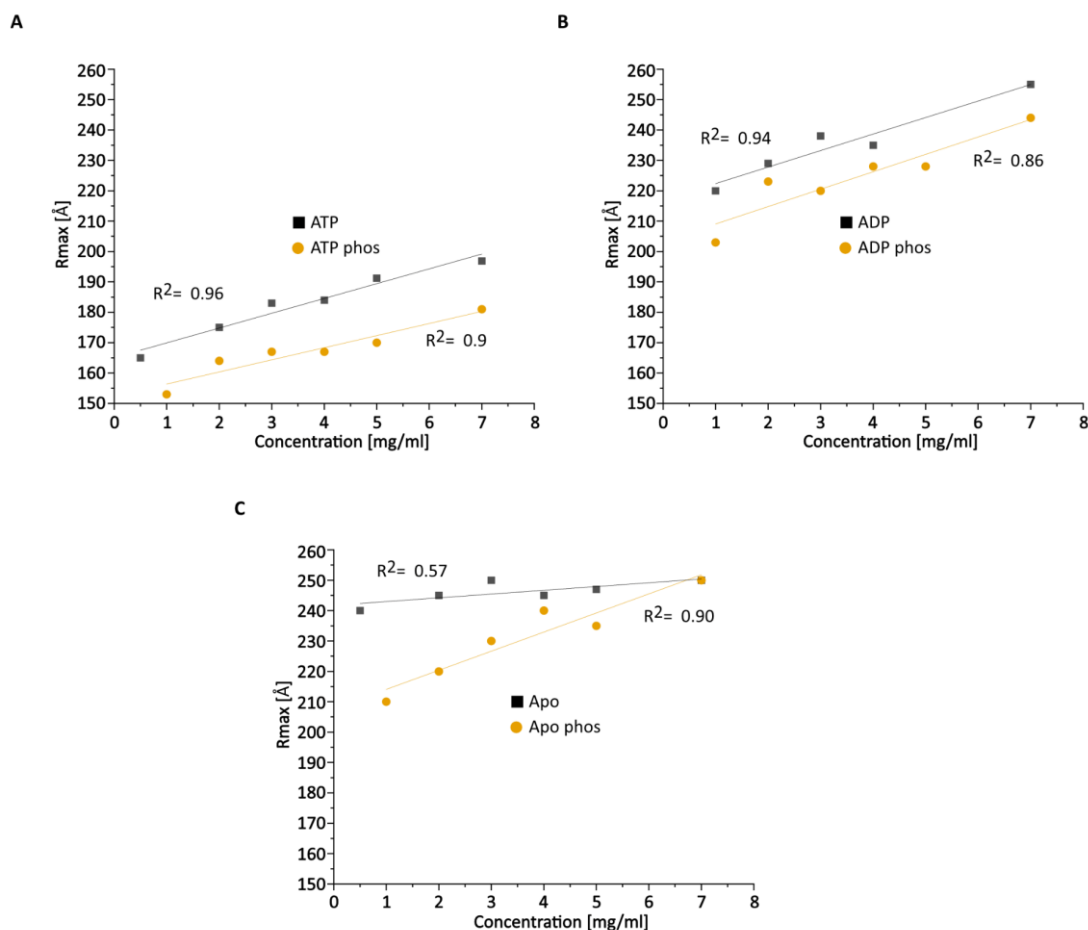


Figure S8: Rmax increases as a function of concentration, indicating oligomerization

(A-C) Rmax values obtained from the p(r) distance distribution functions are plotted against concentration for Hsc70^{T204A}/pHsc70^{T204A}:ATP (A), Hsc70^{T204A}/pHsc70^{T204A}:ADP (B), and Hsc70^{T204A}/pHsc70^{T204A}:apo (C). In all cases except for Hsc70^{T204A}:apo, Rmax increases approximately linearly with concentration, indicating oligomerization in these nucleotide states. For Hsc70^{T204A}:apo, deviations from linearity are more clearly visible in the rescaled representation shown in Figure S7B. Across all conditions, pHsc70^{T204A} tends to display smaller Rmax values than Hsc70^{T204A}. This figure and its caption were adapted from the cited publication [21].

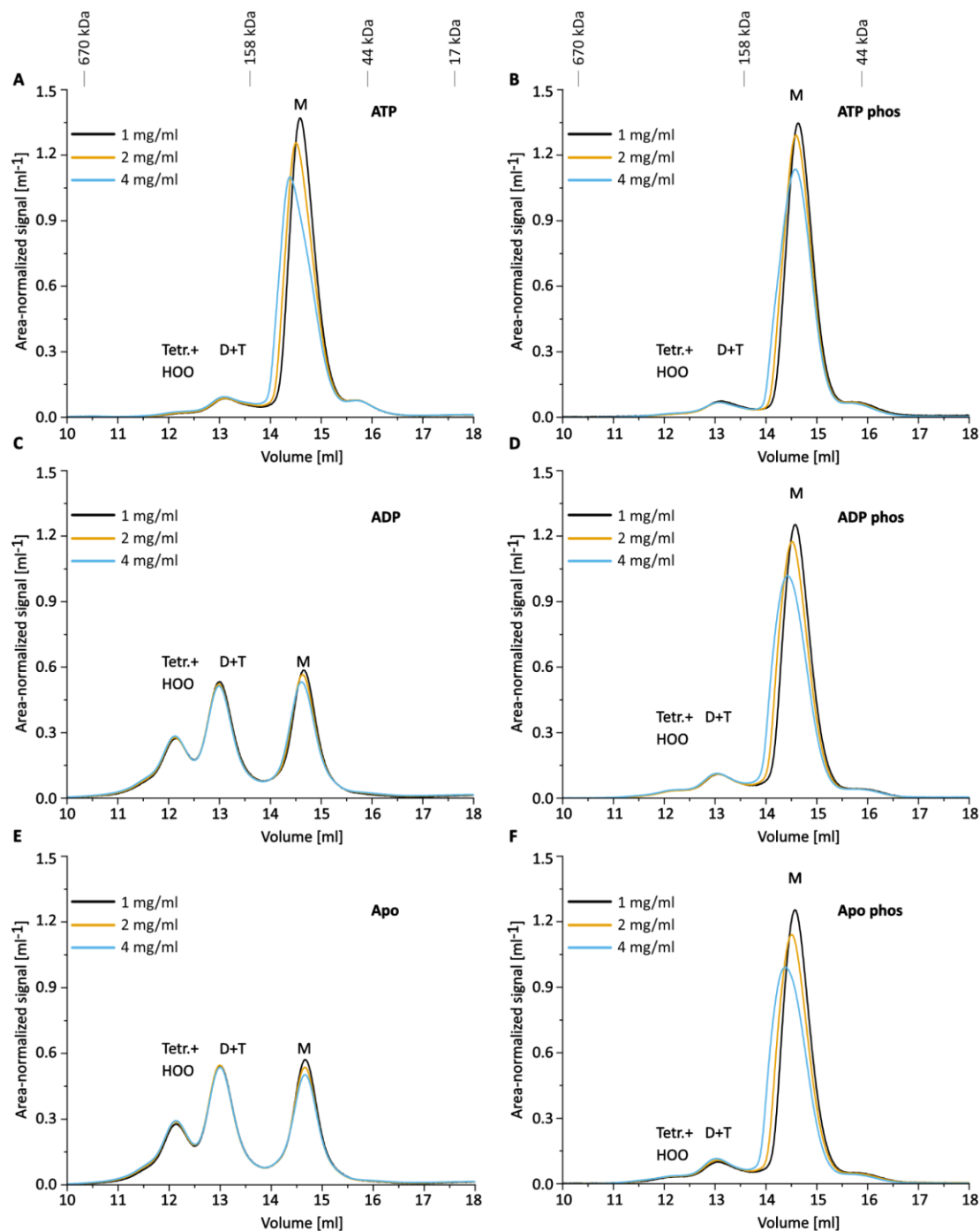


Figure S9: Technical replicate of the Hsc70^{T204A} and pHsc70^{T204A} aSEC measurement

Analytical SEC chromatograms corresponding to the second technical replicate of Figure 9, showing unphosphorylated Hsc70^{T204A} and phosphorylated Hsc70^{T204A} (pHsc70^{T204A}) under ATP, ADP, and apo conditions at 1, 2, and 4 mg/ml (color coding and peak annotations as in Figure 12). The replicate reproduces the trends shown in Figure 12; for experimental details and interpretation, see the Figure 12 caption.

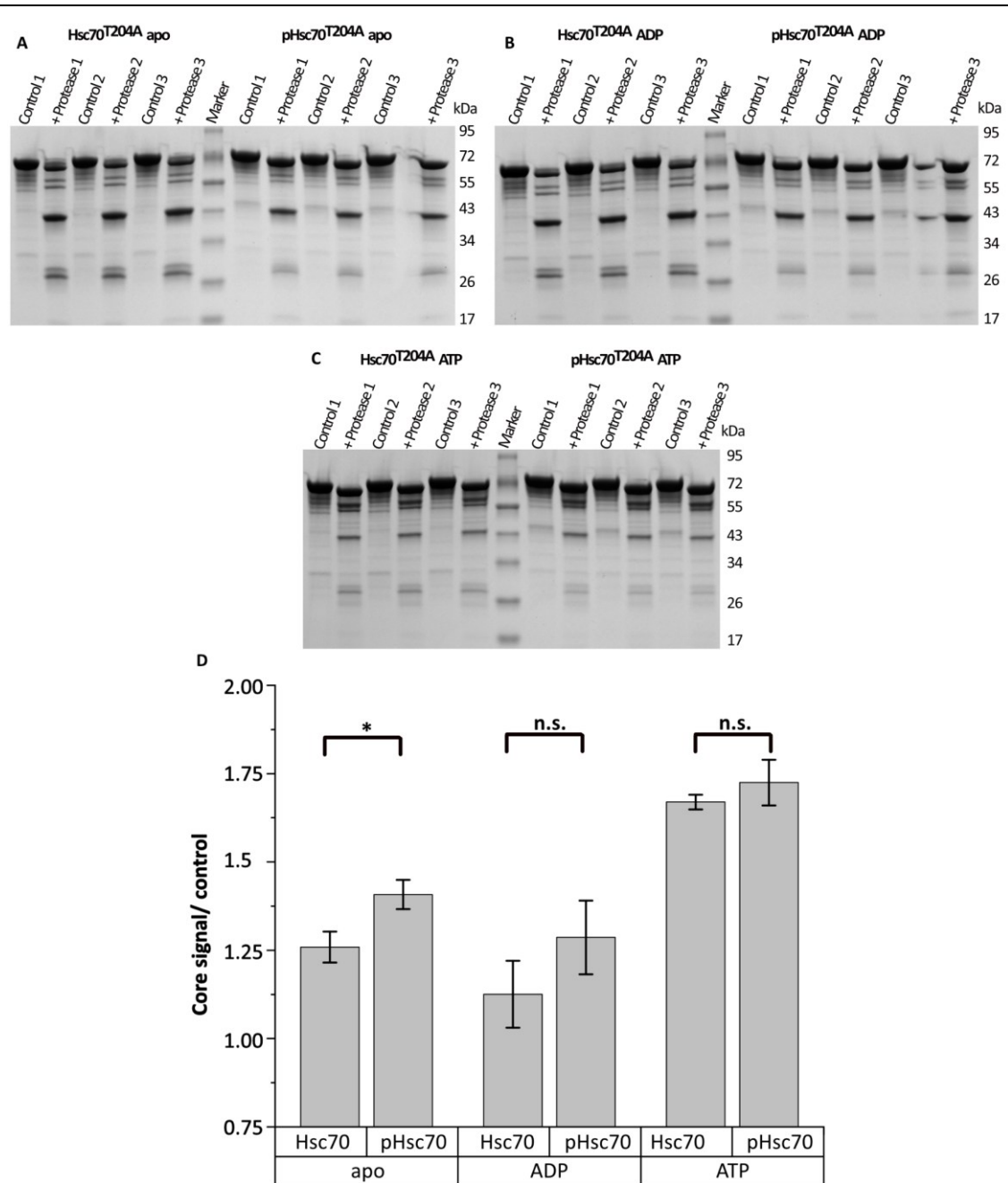


Figure S10: Protease susceptibility and core signal quantification of Hsc70^{T204A} and monophosphorylated pHsc70^{T204A} in different nucleotide states

(A-C) Representative SDS-PAGE gels showing limited proteolysis of Hsc70^{T204A} and monophosphorylated pHsc70^{T204A} in the apo (A), ADP-bound (B), and ATP-bound (C) states. For each condition, three technical replicates were prepared independently and resolved on the same gel. Control samples (Control) and protease-treated samples (+Protease) are shown for each replicate. Molecular weight markers are indicated in kDa. (D) Quantification of the core band signal normalized to the respective control for Hsc70^{T204A} and monophosphorylated pHsc70^{T204A} in the apo, ADP-bound, and ATP-bound states. Bars represent mean $\pm$ SD ($n = 3$ technical replicates prepared independently from the same protein preparation). Statistical significance between Hsc70^{T204A} and pHsc70^{T204A} within each nucleotide condition was assessed using an unpaired two-tailed Welch's t-test. A statistically significant difference was observed in the apo state ($p = 0.0131$). However, the magnitude of the effect is moderate. No statistically significant differences (n.s.) were detected in the ADP-bound ($p = 0.120$) or ATP-bound ($p = 0.258$) states.

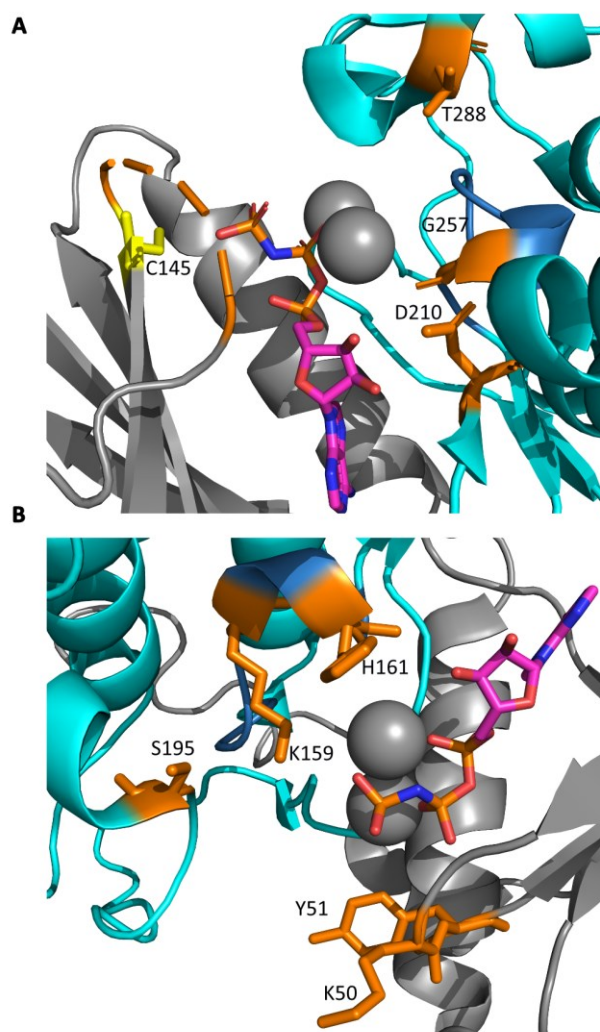


Figure S11: Mutated residues selected for cysteine substitution for binary adduct formation

(A) Close-up view of a LegK4 crystal structure in complex with the non-hydrolyzable ATP analog adenosine 5'-(β,γ -imido)triphosphate (APPNHP) (PDB: 5CKW). (B) Close-up view of a CK2 α' crystal structure bound to APPNHP (PDB: 3U87). In both structures, the N-terminus to the N-lobe is colored gray, and the C-lobe to the C-terminus is colored cyan. Residues mutated in this study are highlighted in orange. For orientation, the catalytic loop is shown in blue, except at positions corresponding to residues mutated in this study. Magnesium ions are shown as spheres. In the LegK4 structure, Cys145 is shown in yellow, as this endogenous cysteine was mutated to serine. Residues K142 and S143 belong to the glycine-rich loop, which is unresolved in the structure and indicated in orange. In APPNHP, carbon atoms are shown in magenta, nitrogen in blue, oxygen in red, and phosphorus in orange. For both CK2 α' and LegK4, residues selected for cysteine substitution were chosen based on their proximity to the γ -phosphate of APPNHP.

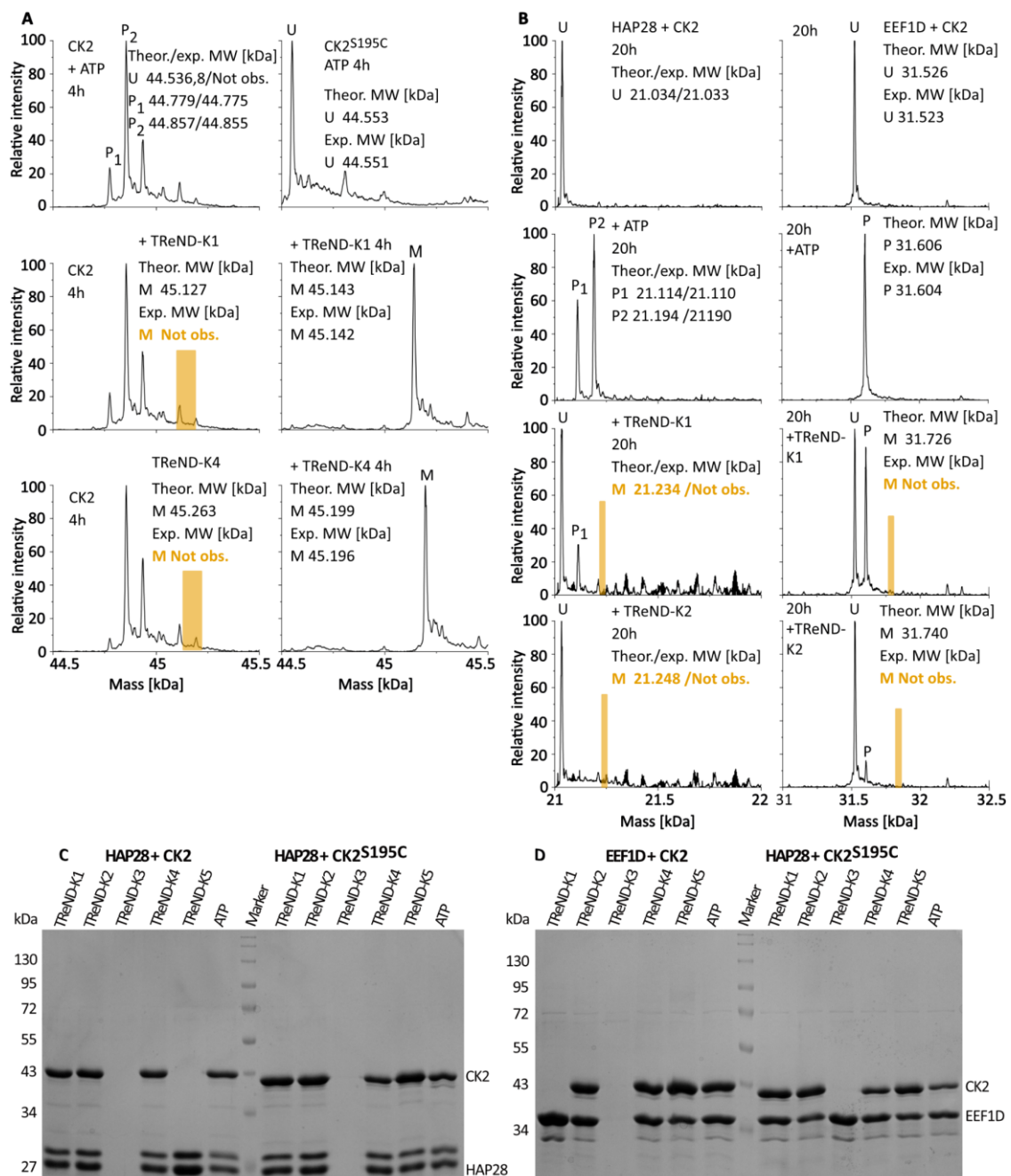


Figure S12: Intact mass spectrometry and SDS-PAGE analysis of potential CK2 α' -substrate-analog modification, binary adduct formation and potential ternary complex formation

(A) iMS spectra of CK2 α' (left) and CK2 α' ^{S195C} (right) before and after 4 h incubation with TReND-K1 or TReND-K4. For CK2 α' , only the unmodified protein (U) is detected; the expected masses of the covalent adducts (M) are marked by orange bars, but no peaks are observed. In contrast, CK2 α' ^{S195C} shows peaks at the expected masses for TReND-K1- and TReND-K4-modified protein, with depletion of the unmodified species. (B) iMS spectra of 10 μ M HAP28/EEF1D and 0.5 μ M CK2 α' after overnight incubation with ATP (top), TReND-K1 (middle) and TReND-K2 (bottom) to assess transfer of the analog linker region. Peaks corresponding to unmodified substrates are labelled U, phosphorylated species are labelled P. The theoretically observable species modified with the linker region of the analogs are indicated with M and the area highlighted in orange. For these species, no peaks are visible. The spectra shown represent technical replicates measured from the same sample. (C-D) SDS-PAGE of mixtures of HAP28 (C) or EEF1D (D) with CK2 α' and CK2 α' ^{S195C} after incubation with TReND-K1-5 or buffer (control). The arrow mark the expected migration position of a covalent HAP28-CK2 or EEF1D-CK2 ternary complex. Representative single measurements are shown for one mutant; corresponding results for the remaining mutants are summarized in Table S6. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. CK2 = CK2 α' .

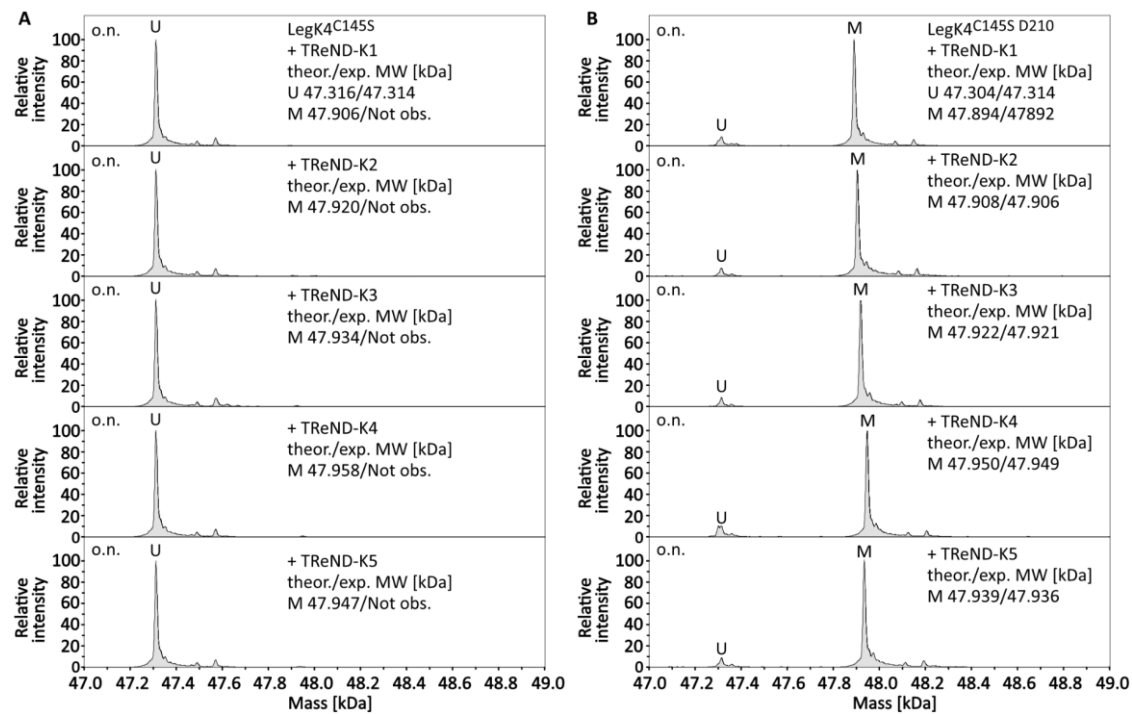


Figure S13: iMS spectra of LegK4⁵⁹⁻⁴⁶²^{C145S} and LegK4⁵⁹⁻⁴⁶²^{C145S D210C} after overnight incubation with TReND-K analogs show cysteine-position-dependent binary adduct formation

Intact mass spectrometry (iMS) data of LegK4⁵⁹⁻⁴⁶²^{C145S} (**A**) and LegK4⁵⁹⁻⁴⁶²^{C145S D210C} (**B**) after overnight (o.n.) incubation with TReND-K analogs 1-5, showing binary adduct formation. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. All measurements represent single determinations. The spectra for TReND-K1 and TReND-K4 are reproduced from Figure 15 for completeness and direct comparison.

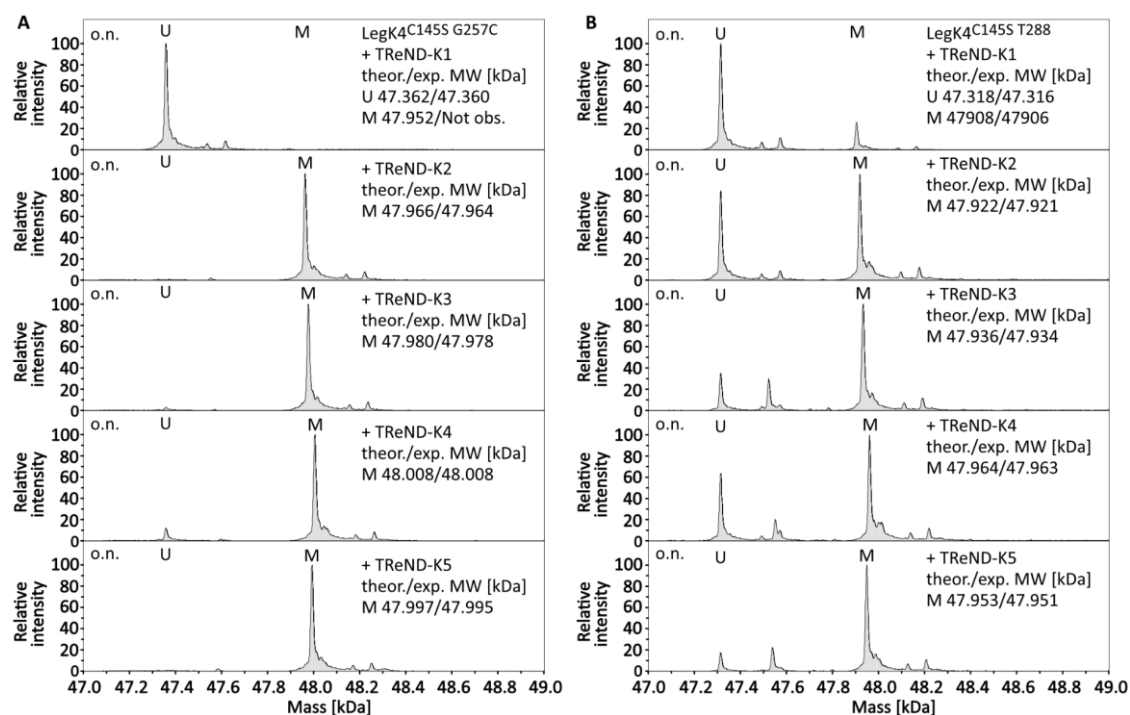


Figure S14: iMS spectra of LegK4₅₉₋₄₆₂^{C145S G257C} and LegK4₅₉₋₄₆₂^{C145S T288C} after overnight incubation with TReND-K analogs show cysteine-position-dependent binary adduct formation

Intact mass spectrometry (iMS) data of LegK4₅₉₋₄₆₂^{C145S G257C} (**A**) and LegK4₅₉₋₄₆₂^{C145S T288C} (**B**) after overnight (o.n.) incubation with TReND-K analogs 1-5, showing binary adduct formation. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. All measurements represent single determinations.

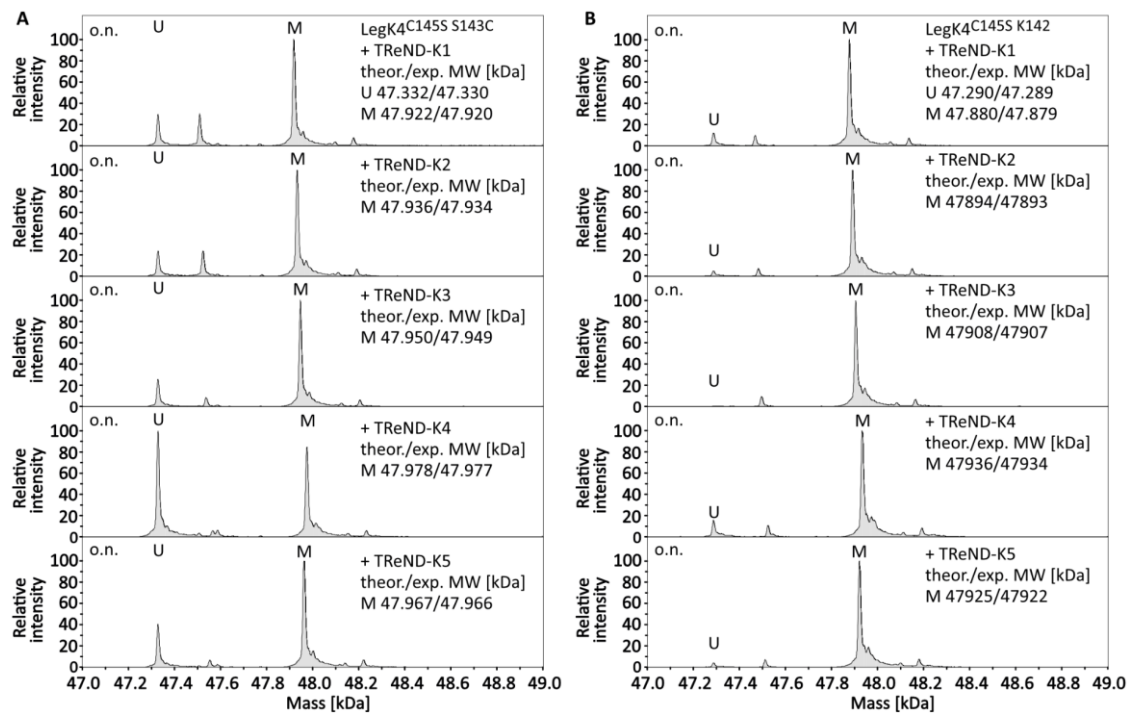


Figure S15: iMS spectra of LegK4₅₉₋₄₆₂^{C145S S143C} and LegK4₅₉₋₄₆₂^{C145S K142C} after overnight incubation with TReND-K analogs show cysteine-position-dependent binary adduct formation

Intact mass spectrometry (iMS) data of LegK4₅₉₋₄₆₂^{C145S S143C} (**A**) and LegK4₅₉₋₄₆₂^{C145S K142C} (**B**) after overnight (o.n.) incubation with TReND-K analogs 1-5, showing binary adduct formation. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. All measurements represent single determinations.

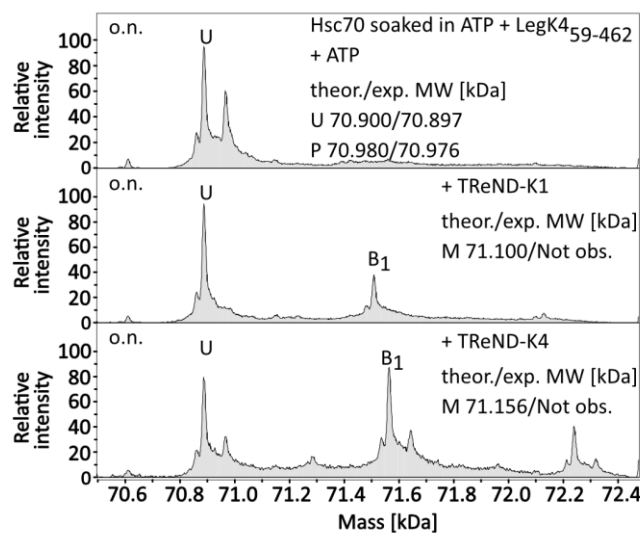


Figure S16: iMS analysis of ATP-pre-soaked Hsc70 reveals no detectable transfer of TReND-K linker moieties

iMS spectra of 10 μ M Hsc70, derived from a sample pre-soaked with 250 μ M ATP at 100 μ M protein concentration prior to dilution, and 0.5 μ M LegK4₅₉₋₄₆₂ after overnight incubation with ATP (top), TReND-K1 (middle), and TReND-K4 (bottom), to assess possible transfer of the analog linker region. Peaks corresponding to unmodified Hsc70 are labelled U, phosphorylated species are labelled P, analog bound Hsc70 is labelled B1 and B2. The theoretically observable species modified with the linker region of the analogs are indicated with M. For these species, no peaks are visible. Equivalent negative results were obtained for the other TReND-K: no substrate modification was detected in the presence of the analogs, whereas phosphorylation was observed in the ATP control.

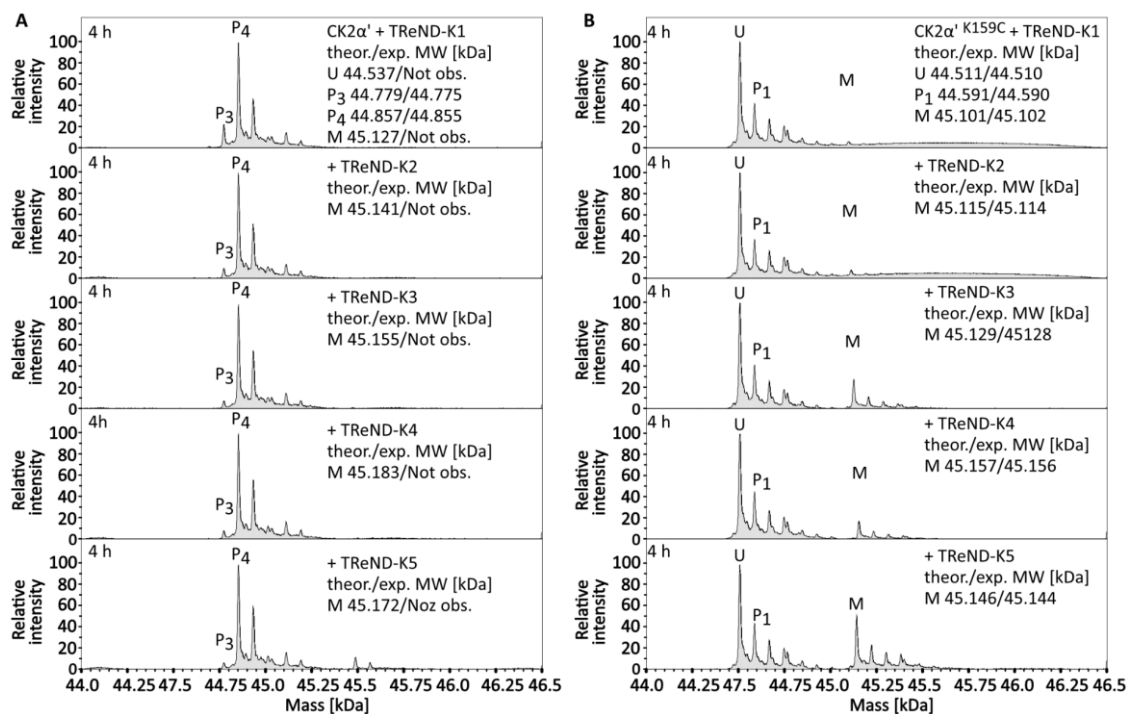


Figure S17: iMS spectra of CK2α' and CK2α'^{K159C} after 4 h incubation with TReND-K analogs show cysteine-position-dependent binary adduct formation

Intact mass spectrometry (iMS) data of CK2α' (**A**) and CK2α'^{K159C} (**B**) after 4 h incubation with TReND-K analogs 1-5. CK2α' serves as a negative control and shows no binary adduct formation, whereas CK2α'^{K159C} exhibits analog-dependent binary adduct formation. No unmodified peak is detected for CK2α'; instead, the protein appears to be hyperphosphorylated. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. All measurements represent single determinations. U = unmodified; P_x = species carrying x phosphate groups; M = modified (binary adduct).

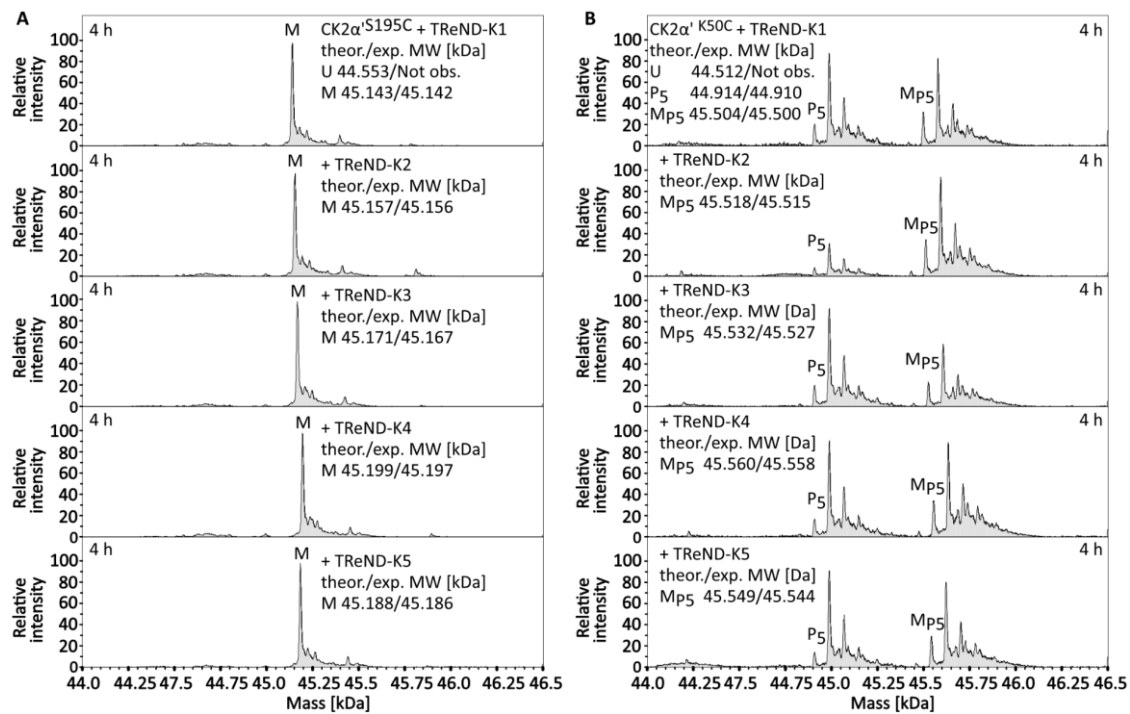


Figure S18: iMS spectra of CK2 α ^{S195C} and CK2 α ^{K50C} after 4 h incubation with TReND-K analogs show cysteine-position-dependent binary adduct formation

Intact mass spectrometry (iMS) data of CK2 α ^{S195C} (**A**) and CK2 α ^{K50C} (**B**) after 4 h incubation with TReND-K analogs 1-5. CK2 α ^{K50C} appears to be hyperphosphorylated. Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. All measurements represent single determinations. U = unmodified; P_x = species carrying x phosphate groups; M = modified (binary adduct); Mp_x = modified species carrying x phosphate groups.

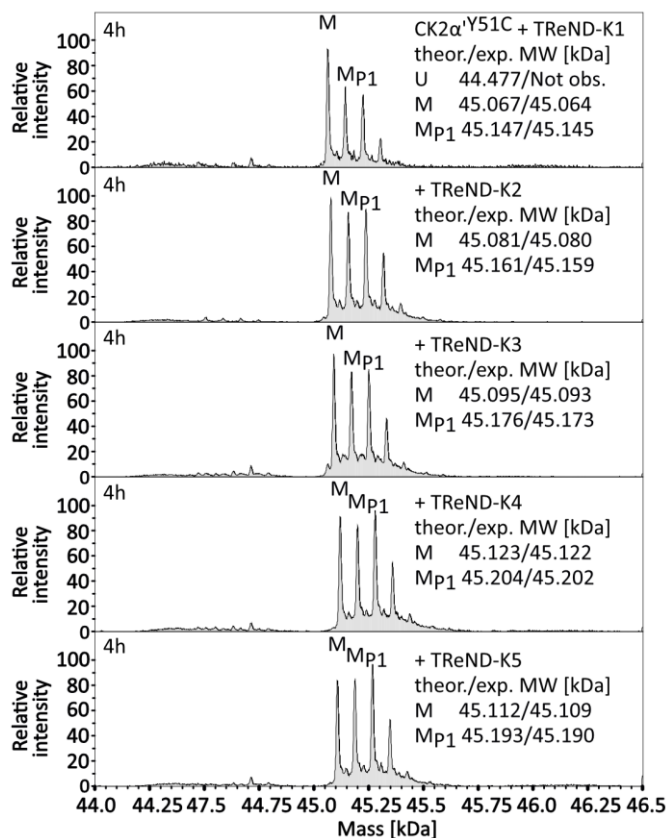


Figure S19: iMS spectra of CK2 α^{Y51C} after 4 h incubation with TReND-K analogs show cysteine-position-dependent binary adduct formation

Theoretical (theor.) and experimental (exp.) molecular weights (MW) are indicated. All measurements represent single determinations. U = unmodified; P_x = species carrying x phosphate groups; M = modified (binary adduct); M_{Px} = modified species carrying x phosphate groups.

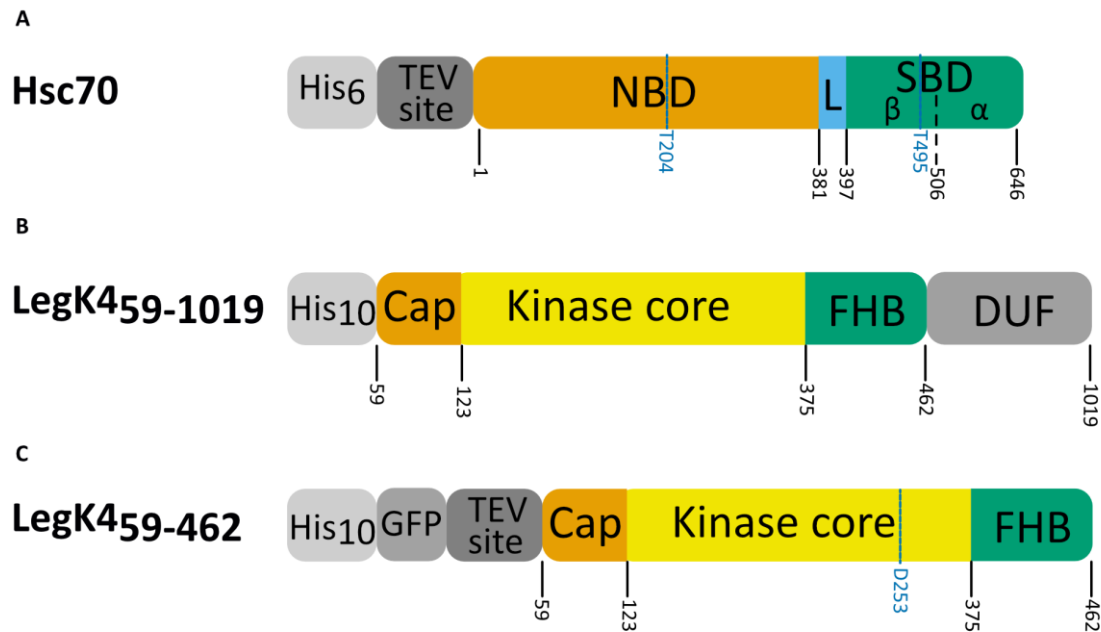


Figure S 20: Schematic overview of Hsc70 and LegK4 constructs

(A) Schematic of the Hsc70 expression construct showing domain architecture and key sites. The N-terminal nucleotide-binding domain (NBD), the interdomain linker (L), and the C-terminal substrate-binding domain (SBD) are indicated. The SBD comprises a β -sandwich subdomain (β -SBD) and an α -helical lid subdomain (α -SBD), with residue 506 marking the transition between the β -sandwich and the α -helical lid. An N-terminal His₆-tag (labeled His6) followed by a TEV protease cleavage site is shown. Positions of amino acids T204 (mutated to alanine for SAXS experiments) and the phosphorylation site T495 are highlighted in blue. Amino-acid numbers 1-646 refer to the canonical Hsc70 sequence. (B) Domain organization of full-length LegK4 (Philadelphia strain annotation). The construct comprises an N-terminal His₁₀-tag followed by a cap domain (Cap), the central kinase core, a four-helix bundle (FHB), and a C-terminal domain of unknown function (DUF). (C) Domain organization of the truncated LegK4₅₉₋₄₆₂ construct. The construct comprises an N-terminal His₆-tag followed by a GFP-tag and a TEV protease cleavage site and contains the Cap domain, the kinase core, and the four-helix bundle (FHB), but lacks the C-terminal DUF. The catalytic aspartate D253 (D253A mutant) is indicated. (GFP: green fluorescent protein). This figure and its caption were reproduced from the cited publication [21].

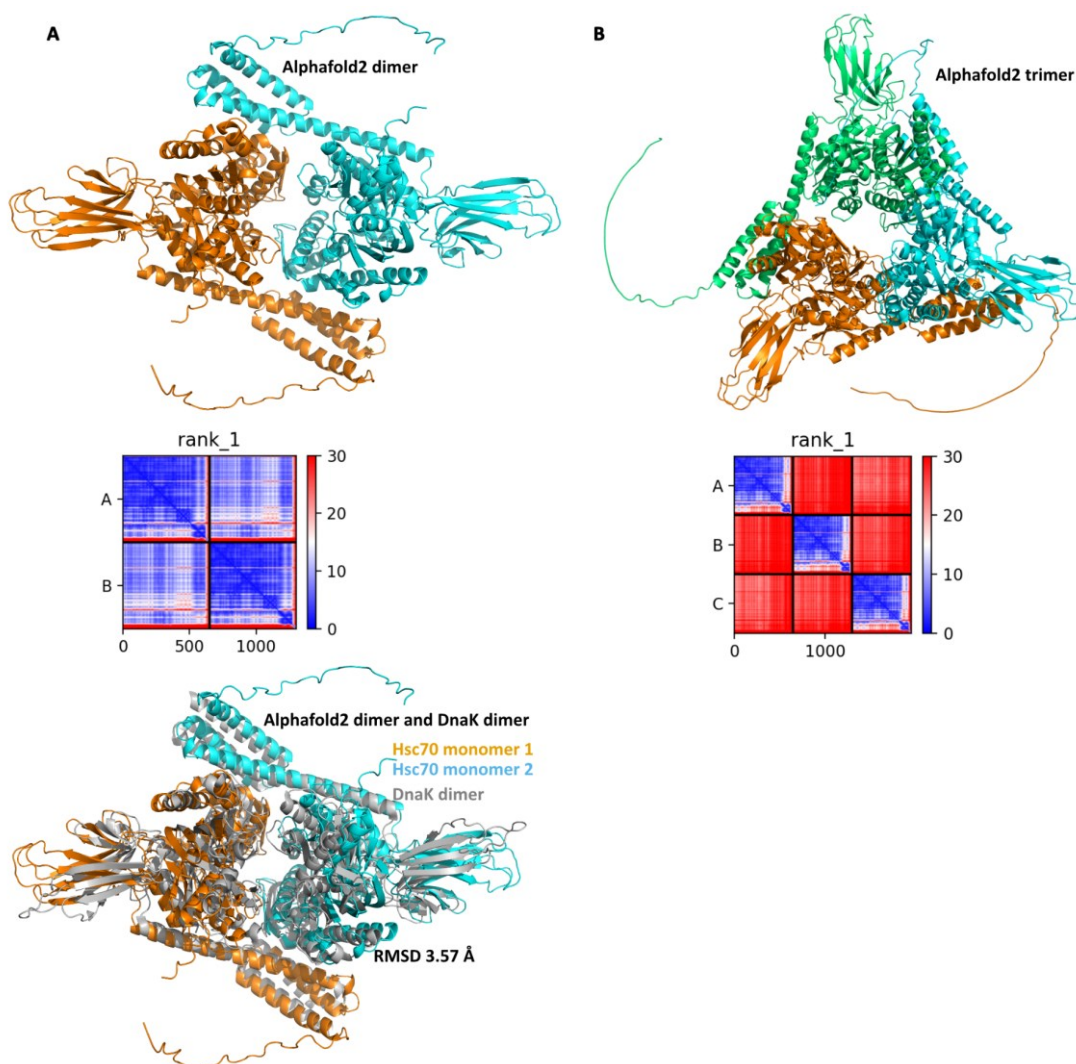


Figure S21: AlphaFold2 models of Hsc70 used as RANCH/OLIGOMER input for SAXS analysis

(A) Top: AlphaFold2 rank 1 model of dimeric Hsc70 used as input for RANCH to generate conformational ensembles for OLIGOMER analysis, with the individual monomers highlighted in orange and cyan. Middle: interface predicted template modeling (iPTM) score of the rank 1 model. According to the iPTM metric, prediction confidence is highest for intramolecular contacts within each monomer but remains moderate for intermolecular contacts across the dimer interface. Bottom: Superposition of the Hsc70 dimer (colored as above) with an antiparallel DnaK dimer (PDB 4JNE, shown in gray). Despite a root-mean-square deviation (RMSD) of 3.57 Å, the overall orientation and the domains involved in the interaction are similar. This similarity to the antiparallel DnaK dimer therefore suggests that the AlphaFold2 model of Hsc70 adopts an antiparallel binding mode. (B) AlphaFold2 rank 1 model of trimeric Hsc70 used as input for RANCH to generate conformational ensembles for OLIGOMER analysis, with the monomers highlighted in orange, cyan, and green. As in the dimeric model, the nucleotide-binding domain and the α -helical substrate-binding domain contribute to the intermolecular interfaces, while the β -substrate-binding domain is oriented away from the interface. Bottom: iPTM score of the rank 1 model. While the intramolecular domain organization within each monomer is predicted with high confidence, the iPTM score for intermolecular contacts is low, indicating poor confidence in the predicted trimeric assembly. Nevertheless, the model was used as a starting point for RANCH to generate alternative conformations, since an initial spatial arrangement of the monomers was required, even though the low-confidence trimer prediction represents a limitation of this approach.

10.2 Supplementary Tables

Table S1: Thermofluor (DSF) buffer screen highlights phosphate-based conditions among the highest-T_m hits for Hsc70

Shown is an excerpt of the RUBIC Buffer Screen results for Hsc70, comprising the 20 highest melting temperatures (T_m), listed from top to bottom, together with the control. All values shown represent single measurements and were analyzed using MoltenProt. Several of the best-performing conditions contain phosphate buffers, indicating that phosphate-based buffer systems confer increased thermal stability under the tested conditions. Based on this trend, sodium phosphate buffers were preferentially used in the final purification/phosphorylation workflow. SPG = succinic acid-sodium dihydrogen phosphate-glycine buffer system.

Rank	Condition	T _m (°C)
1	0.25 M Sodium chloride, 0.1 M Potassium phosphate monobasic pH 7	55
2	0.1 M Potassium phosphate monobasic pH 7	54
3	0.25 M Sodium chloride, 0.1 M Sodium phosphate monobasic pH 7	54
4	0.1 M Potassium phosphate pH 6	53
5	0.25 M Sodium chloride, 0.1 M Potassium phosphate monobasic pH 6	53
6	0.25 M Sodium chloride, 0.1 M Sodium phosphate monobasic pH 7.5	53
7	0.25 M Sodium phosphate pH 7.5	53
8	0.1 M Sodium phosphate monobasic pH 7.0	52
9	0.1 M SPG pH 6.5	52
10	0.1 M SPG pH 7	52
11	0.1 M SPG pH 7.5	52
12	0.02 M sodium phosphate pH 7.5	52
13	0.125 M sodium phosphate pH 7.5	52
14	0.1 M Ammonium acetate pH 7.3	51
15	0.1 M Sodium phosphate monobasic pH 7.5	51
16	0.1 M SPG pH 6.0	51
17	0.1 M SPG pH 8.0	51
18	0.1 M SPG pH 8.5	51
19	0.1 M SPG pH 9.0	51
20	0.1 M SPG pH 10	51
23	Water/Control	50

Table S2: χ^2 values of OLIGOMER fits

This Table was reproduced from the cited publication [21]

Sample	Concentration (mg/ml)						
	0.5	1	2	3	4	5	7
χ^2 Hsc70 ^{T204A} :ATP	1.02		1.74	1.56	2.36	3.56	4.46
χ^2 pHsc70 ^{T204A} :ATP		1.2	2.98	3.19	5.16	3.44	9.48
χ^2 Hsc70 ^{T204A} :ADP		1.13	1.73	5.88	2.16		20.05
χ^2 pHsc70 ^{T204A} :ADP		1.12	1.86	3.2	6.38	16.49	38.35
χ^2 Hsc70 ^{T204A} :Apo	1.07		1.64	1.96	3.09	4.68	7.68
χ^2 pHsc70 ^{T204A} :Apo		1.14	1.97	3.77	7.48	15.61	40.17

Table S3: Apparent molecular masses of aSEC peaks assigned to monomeric and oligomeric Hsc70 species

Apparent molecular masses (kDa) derived from analytical size-exclusion chromatography (aSEC) for the peaks assigned to monomeric (M), dimeric/trimeric (D+T), and tetrameric/higher-order oligomeric (Tetr. + HOO) Hsc70 species under different nucleotide conditions at 1 mg/ml protein concentration. For samples in which a concentration-dependent shift of the peak M was observed, apparent molecular masses determined at 4 mg/ml are additionally shown. Apparent molecular masses were calculated based on a Bio-Rad gel filtration standard. Values represent mean $\pm$ standard deviation from technical duplicates. N.s. indicates that no corresponding peak was detected or that no reliable peak maximum could be assigned because the signal intensity was too low. For reference, the theoretical molecular masses of monomeric, dimeric, and trimeric Hsc70 are 71, 142, and 213 kDa, respectively.

Sample	Measured mass (kDa)		
	M	D+T	Tetr. + HOO
U ATP 1 mg/ml	84.81 $\pm$ 0.06	174.92 $\pm$ 0.06	N.s.
U ADP 1 mg/ml	81.61 $\pm$ 0.01	189.36 $\pm$ 0.01	289.42 $\pm$ 2.04
U apo 1 mg/ml	81.61 $\pm$ 0.01	188.15 $\pm$ 0.01	289.59 $\pm$ 0.03
P ATP 1 mg/ml	83.06 $\pm$ 0.25	180.05 $\pm$ 1.86	N.s.
P ADP 1 mg/ml	85.20 $\pm$ 0.11	182.92 $\pm$ 0.18	N.s.
P apo 1 mg/ml	85.53 $\pm$ 0.08	179.31 $\pm$ 1.87	N.s.
U ATP 4 mg/ml	93.47 $\pm$ 0.00	179.14 $\pm$ 1.83	N.s.
P ADP 4 mg/ml	94.09 $\pm$ 3.04	184.99 $\pm$ 0.05	N.s.
P apo 4 mg/ml	94.38 $\pm$ 0.42	185.23 $\pm$ 0.26	N.s.

Table S4: Key residues involved in nucleotide binding and catalysis in CK2 α' [205]

Lys69	Interacts with and stabilizes the α - and β -phosphate groups of the nucleotide
Glu82	Forms a stabilizing interaction with Lys69 and is indicative of the active kinase conformation (α C-helix in-position)
Glu115 and Ile117	Located in the hinge region, mediating backbone hydrogen bonds to the adenine moiety of the nucleotide
Asp157	Catalytic aspartate of the HRD motif, involved in phosphotransfer
Lys159	Contributes to stabilization of the γ -phosphate group
Asn163	Involved in coordination of the catalytic magnesium
Asp176	Participates in Mg^{2+} coordination and is part of the conserved DWG motif, characteristic of the active conformation in CK2 α'
Trp177	Replaces the phenylalanine of the canonical DFG motif (DWG in CK2 α') and plays an important role in stabilizing the active kinase conformation

Table S5: Mass increases upon binary adduct formation between TReND-K analogs and cysteine residues of the target kinase

Analog	Mass difference after binary adduct formation [Da]
TReND-K1	590
TReND-K2	604
TReND-K3	618
TReND-K4	646
TReND-K5	635

Table S6: Binary adduct formation and temporal stability of LegK4 variants with TReND-K analogs

Percent modification (% mod.) determined by intact mass spectrometry after incubation of the indicated LegK4₅₉₋₄₆₂ variants with the respective TReND-K analogs. Binary adduct formation was assessed after 5 h and after overnight incubation at 22 °C. Values are given as 5 h / overnight. The two time points derive from two independent experiments, with each time point measured once per experiment. Values of 0 indicate that the analog was tested but no modification was detected under the conditions used; NT = not tested. WT = wild type. Spectra from overnight samples are shown (Figure S13, Figure S14 and Figure S15). These are representative of both the 5 h and overnight measurements.

5 h/overnight					
Protein	TReND-K1 (% mod.)	TReND-K2 (% mod.)	TReND-K3 (% mod.)	TReND-K4 (% mod.)	TReND-K5 (% mod.)
WT	10.87	8.1	NT	NT	NT
LegK4 C145S	0/0	0/0	0/0	0/0	0/0
LegK4 C145S D210C	93/92	93/93	NT/92	88/90	93/92
LegK4 C145S G257C	100/0	100	100/97	100/89	100/100
LegK4 C145S T288C	10/20	29/54	55/74	57/61	67/85
LegK4 C145S S143C	75/75	64/81	57/79	35/46	54/71
LegK4 C145S K142C	85/89	91/95	100/91	92/86	98/96

Table S7: Binary adduct formation of CK2 α ' variants with TReND-K analogs

Percent modification (% mod.) determined by intact mass spectrometry (iMS) after incubation of the indicated CK2 α ' variants with the respective TReND-K analogs. Binary adduct formation was assessed after 4 h and after overnight incubation at 22 °C. Values are given as 4 h/overnight. The 4 h and overnight values were obtained from the same reaction mixture, with one measurement per time point. The corresponding iMS spectra after 4 h incubation are shown in Figure S17, Figure S18, and Figure S19. Values of 0 indicate that the analog was tested but no modification was detected under the conditions used; NT = not tested. All measurements represent single determinations. WT = wild type.

Protein	TReND-K1 (% mod.)	TReND-K2 (% mod.)	TReND-K3 (% mod.)	TReND-K4 (% mod.)	TReND-K5 (% mod.)
WT	0/0	0/0	0/0	0/0	10/10
CK2 α ' K50C	49/100	75/100	39/90	49.5/94	46.8/94
CK2 α ' Y51C	100/100	96/100	91/100	93/100	93/100
CK2 α ' K159C	9/12.3	0/20	22/42.5	15/34	34/68.8
CK2 α ' S195C	100/100	100/100	100/100	100/100	100/100

Table S8: CK2 α '^{H161C}-TReND-K1 crystal data collection and crystal refinement parameters

a = Values in parentheses correspond to the highest-resolution shell. $R_{\text{merge}} = \sum hkl \sum i |I_i(hkl) - \langle I(hkl) \rangle| / \sum hkl \sum i I_i(hkl)$, where $I_i(hkl)$ is the i^{th} observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations of reflection hkl . Crystallography was performed by Dr. Vivian Pogenberg.

Data collection	
Parameter	Value
Wavelength (Å)	0.976256
Resolution range (Å)	47.63-2.35 (2.44-2.35) ^a
Space group	P1
Unit cell a (Å)	46.7
Unit cell b (Å)	62.2
Unit cell c (Å)	75.0
Alpha (°)	81.0
Beta (°)	75.1
Gamma (°)	71.9
Total reflections	114,692 (11,934)
Unique reflections	29,547 (3,061)
Multiplicity	3.9 (3.9)
Completeness (%)	91.9 (91.9)
Mean I/sigma(I)	4.8 (1.0) ^a
R_{merge}	0.111 (0.744) ^a
$CC_{1/2}$	0.998 (0.683) ^a
Refinement and model statistics	
Parameter	Value
R_{work}	0.2090
R_{free}	0.2276
Non-hydrogen atoms	5590
Macromolecules	5344
Ligands	72
Solvent	172
RMSD bonds (Å)	0.002
RMSD angles (°)	0.521

Ramachandran favored (%)	96.17
Ramachandran allowed (%)	3.51
Ramachandran outliers (%)	0.32
Rotamer outliers (%)	0.17
Average B-factor ($\text{\AA}^2$)	50.8
Macromolecules B-factor	50.7
Ligands B-factor	59.7
Solvent B-factor	46.1

Table S9: Oligonucleotides used for the synthesis of plasmids in this study

Fw: forward primer; Rv: reverse primer.

Oligonucleotide primers		Sequence 5'-3'
LegK4 to pSF421 F	Fw	CCACCCGCGAGTTCGAAAAGGGAGGCGGCATGAAATT-GCTTCGGTTTCATGAATTAAAATC
LegK4 to pSF421 R	Rv	ACAGGAGTCCAAGCTCAGCTAATTAAGCTTAATATGG-CAAAATGATGACGTTGCG
LegK4 59-1019 10x-Histag F	Fw	CATCATGGAGGCGGCATGAAATTG
LegK4_59-1019 10x-Histag R	Rv	ATGATGATGGTGATGATGGTGATGC
LegK4 59-520 to pSF421 F	Fw	AGCCACCCGCGAGTTCGAAAAGGGAGGCGG-CATTCTGTTTATTACCACCGAGGCATC
LegK4 59-520 to pSF421 R	Rv	CAGGAGTCCAAGCTCAGCTAATTAA-GCTTAATCTGAAACTTGTTTAATCGCTGCC
LegK4 59-462 F	Fw	TAAGCTTAATTAGCTGAGC
LegK4 59-462 R	Rv	ATTAGCAAGCGATTTAATC
LegK4 59-462 Tag deletion F	Fw	GGAGGCGGCATGAAATTG
LegK4 59-462 Tag deletion R	Rv	GCCCTGAAAATAAAGATTCTCATC
LegK4 59-462 D253A F	Fw	AATTCACCGCGCGCTTAAACCTG
LegK4 59-462 D253A R	Rv	AAGCCTAATTCATGGAATTG
LegK4 K142C F	Fw	AGGAGGTGGCTGTTCTTATTCTGAG
LegK4 K142C R	Rv	GAAATCTTGTTTTTCAGTGG
LegK4 S143C F	Fw	AGGTGGCAAATGTTATTCTGAGC
LegK4 S143C R	Rv	CCTGAAATCTTGTTTTTCAGTG
LegK4 C145S F	Fw	CAAATCTTATTCTGAGCAATTTAAAGTTTATG
LegK4 C145S R	Rv	CCACCTCCTGAAATCTTG
LegK4 G257C F	Fw	TCTTAAACCTTGTAATATTATGCTGGATGCG
LegK4 G257C R	Rv	TCGCGGTGAATTAAGCCT
LegK4 D210C F	Fw	AAAAGGGATTTGTTTATCCCGGTATAAAAAC
LegK4 D210C R	Rv	ATAAAAGTTGTGAGAATATAATGC
LegK4 T288C F	Fw	ATCATGGGGATgtGCAAGCTATTTAGC
LegK4 T288C R	Rv	ACCGGTTTAGGTTCTTTATC
Hsc70 T204A F	Fw	AAGTCAAAAATGAGCACATTCC
Hsc70 T204A R	Rv	GGGAGGTGGCGCGTTTGATGTGT
CK2 α' S195C F	Fw	TCGTGTAGCCTGCAGGTACTTCAAG

CK2 α' S195C R	Rv	ACATTGTACTCCTGAGCAG
CK2 α' K50C F	Fw	TGGTCGGGGAtgcTATAGTGAAGTATTTG
CK2 α' K50C R	Rv	AGTTTTTCGAACCAGTTGG
CK2 α' Y51C F	Fw	TCGGGGAAAAAtgcAGTGAAGTATTTG
CK2 α' Y51C R	Rv	CCAAGTTTTTCGAACCAGTTG
HAP28 fragment to pProEX F	Fw	AACCTGTATTTTCAGGGCGCCATGG- GATCCATGCCCAAGGGAGGTCGTAAAG
HAP28 fragment to pProEX R	Rv	GCTTGGTACCGCATGCCTCGAGTTACTATTATTAA- GCTAAGCGATTGCATGCGTTTTTC
EEF1D fragment to pProEX F	Fw	AACCTGTATTTTCAGGGCGCCATGG- GATCCATGGCGACTAACTTCCTG
EEF1D fragment to pProEX R	Rv	AAGCTTGGTACCGCATGCCTCGAGTTACTA- AATCTTATTAAATGCGGCAATG
HSC70_1-608_fw	Fw	TAACATTGGAAGTGGATAAC
HSC70_1-608_rv	Rv	CTGGTACAGCTTGGTG
delTwinStrep_CKalpha'_fw	Fw	GGTGCAATGCCCGGCCCG
delTwinStrep_CKalpha'_rv	Rv	CATATGGCCCTGAAAATAAAGATTCTCATCAGCCTTG

Table S 10: Amino Acid composition of Hsc70^{T204A}

Amino acid composition of Hsc70T204A, determined using SnapGene software, including residual amino acids originating from a TEV cleavage site

Amino Acid	Count
Ala	52
Arg	28
Asn	35
Asp	46
Cys	4
Gln	26
Glu	49
Gly	53
His	7
Ile	44
Leu	44
Lys	54
Met	13
Phe	24
Pro	25
Ser	36
Thr	46
Trp	2
Tyr	15
Val	46

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Statement of Individual Contribution and External Contributions

This work would not have been possible without both advisory support and direct contributions from others. The table below lists the contributions of individuals and research groups that were analyzed and/or presented in this thesis.

Table S11: List of individual contribution and external contributions in this thesis

The column “Chapters/Figures/Tables” indicates those chapters, figures, and tables for which contributions from others were important, for example through scientific advice, provision of chemicals, or support during data acquisition.

Author	Type of Contribution	Chapters/Figures/Tables containing data
Nicholas Kley	With the exception of CK2 crystallization and structure determination, TReND-K analogue synthesis, and AlphaFold2 structure prediction, all data presented in the Results section were generated and analyzed by Nicholas Kley. Unless otherwise indicated, all proteins used were produced by Nicholas Kley, with the exception of CK2 α' H161C and common in-house reagents such as TEV. Scientific advice provided by others during Thermoshift, nanoDSF, and SAXS measurements and data analysis was also important. For details, see the list of collaborators and colleagues below.	All figures and tables in this thesis were prepared by Nicholas Kley, with the exception of Table S8. The chapters, figures and tables based on paper [21] were originally prepared in their published form together with the co-authors Prof. Dr. Aymelt Itzen and Dr. Michael Hecht-Bucher.
Collaborators/groups involved	Type of Contribution	Chapters/Figures/Tables containing data
Maruthi Konda and Christian Pett (Hedberg Group, Umeå University, Sweden)	Synthesis and analytical characterization (e.g., by NMR) of TReND-K1 to TReND-K5	Figure 3, Figure 14, Figure 15, Figure S12, Figure S13, Figure S14, Figure S15, Figure S16, Figure S17, Figure S18, Figure S19, Table S5, Table S6, Table S7, Table S8
Dr. Aleksi Sutenin	Scientific advice on the SAXS measurement and data analysis performed by Nicholas Kley	Chapter 5.3.3

Dr. Daniel Franke	Scientific advice on the SAXS data analysis performed by Nicholas Kley	Chapter 5.3.3
Colleagues involved	Type of Contribution	Figures/Tables containing data
Dr. Joel Fauser	Co-design of the CK2 α ' cysteine mutants H161C and Y51C together with Nicholas Kley; purification of CK2 α ^{H161C} ; coordination of CK2 α ^{H161C} crystallization together with Nicholas Kley. Scientific advice and guidance during the early stages of the project.	Figure 14 shows the crystal structure of CK2 α ^{H161C} synthesized (and co-designed) by Dr. Joel Fauser
Dr. Vivian Pogenberg	Crystallization of CK2 α ^{H161C} , collection of diffraction data, and structure determination. Scientific advice on thermoshift and nano-DSF measurement and analysis	Figure 14 shows the crystal structure of CK2 α ^{H161C} solved by Dr. Vivian Pogenberg. Table S8 contains information provided by Dr. Vivian Pogenberg. The crystallography Methods section was written predominantly by Dr. Vivian Pogenberg and subsequently adapted by Nicholas Kley. Figure 7, Figure S2, Table S1
Dr. Michael Hecht-Bucher	Generation of AlphaFold2 predictions for Hsc70 dimers and trimers; scientific advice on the SAXS measurement and data analysis performed by Nicholas Kley	Figure S21 shows AlphaFold2 structures and fits provided by Dr. Michael Hecht-Bucher; the corresponding data summarized in Section 8.5.2 were also provided by him. Figure 9, Figure 10, Figure 11, Figure S7, Figure S8, Table S2

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Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben. Sofern im Zuge der Erstellung der vorliegenden Dissertationsschrift generative Künstliche Intelligenz (gKI) basierte elektronische Hilfsmittel verwendet wurden, versichere ich, dass meine eigene Leistung im Vordergrund stand und dass eine vollständige Dokumentation aller verwendeten Hilfsmittel gemäß der Guten wissenschaftlichen Praxis vorliegt. Ich trage die Verantwortung für eventuell durch die gKI generierte fehlerhafte oder verzerrte Inhalte, fehlerhafte Referenzen, Verstöße gegen das Datenschutz- und Urheberrecht oder Plagiate.

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