The characterization of the spermidine synthase from *Plasmodium falciparum* (Welch 1897) and *Caenorhabditis elegans* (Maupas, 1900)

Dissertation

Submitted in partial fulfilment of the requirements for a doctoral degree

from the Department of Biology, Faculty of Mathematics, Informatics and

Life Sciences at the University of Hamburg

by

Nashya Haider

(from Dhaka, Bangladesh)

Hamburg 2005

Genehmigt vom Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Herrn Professor Dr. R. D. WALTER
Weitere Gutachterin der Dissertation:
Frau Priv.-Doz. Dr. S. MÜLLER
Tag der Disputation: 16. Dezember 2005

Hamburg, den 02. Dezember 2005

Professor Dr. Arno Frühwald
Dekan

Abbreviations

AdoDATO	S-adenosyl-1,8-diamino-3-thiooctane
AdoMet	S-adenosylmethionine
AdoMetDC	S-adenosylmethionine decarboxylase
AHT	Anhydrotetracycline
Amp	Ampicillin
APA	3-Aminooxy-1-Aminopropane
APE	5-amino-1-pentene hydrochloride
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	Base pair
BLAST	Basic local alignment search tool
BPB	Bromophenol blue
BSA	Bovine serum albumin
cDNA	Complementary DNA
Da	Dalton
dATP	2'-deoxyadenosine 5'-triphosphate
dcAdoMet	Decarboxylated S-adenosylmethionine
dNTP	Deoxyribonucleotide triphosphate
DAPI	4'-6-Diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
DFMO	Difluoromethylornithine
ddH_2O	Double Distilled Water
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBSS	Earle’s balanced salt solution

ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
eIF-5A	Eukaryotic translation initiation factor
EGF	Epidermal growth factor
EtBr	Ethidium bromide
g	Gram
h	Hour
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
IC_{50}	The IC_{50} (or EC_{50} - effective concentration 50%) is the concentration required for 50% inhibition.
IPTG	Isopropyl-ß-D-thiogalactopyranoside
K_i	Inhibitor constant
K_m	Michaelis-Menten constant
kb	Kilobase
kDa	Kilo Dalton
l	Litre
LB	Luria-Bertani medium
M	Molar
MDL 73811	5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine
MEA	2-Mercaptoethylamine
MW	Molecular weight
MGBG	Methylglyoxal bis(guanylhydrazone)
min	Minute
mg	Milligramm
ml	Millilitre

mM	Millimolar
mRNA	Messenger RNA
MTA	5'-Methylthioadenosine
mV	Millivolt
μg	Microgram
μl	Microlitre
μM	Micromolar
N	Normal
N^1-AcSpm	N^1-acetylspermine
N^1-AcSpd	N^1-acetylspermidine
Ni-NTA	Nickel-nitrilotriacetic acid
nCi	Nanocurie
ng	Nanogram
nm	Nanometer
nmol	Nanomol
OD	Optical Density
ODC	Ornithine-Decarboxylase
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAO	Polyamine oxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Picomol
PMSF	Phenylmethylsulfonylfluoride
PP5	protein phosphatase 5 antibodies
Put	Putrescine
PV	Parasitophorous vacuole
rpm	Rotations per Minute

RBC	Red Blood Cell
RNA	Ribonucleic acid
RT	Room Temperature
s	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
Spd	Spermidine
Spm	Spermine
SpmOx	Spermine oxidase
SP	Sulfadoxine/pyrimethamine
SSAT	Spermidine/Spermine-N^1-Acetyltransferase
SSC	Standard Saline-Citrate
4MCHA	Trans-4-methylcyclohexylamine
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-ß	Transforming growth factor beta
TRBC	Trophozoite infected Red Blood Cells
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit
5' UTR	5' Untranslated Region
v	Volume
v/v	volume per volume
v/w	volume per weight
V	Volt
W	Watt
WHO	World Health Organisation

X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Table of Contents

Chapter 1	Review of the literature	1
1.1	The Polyamines	1
1.1.1	Properties and function of the polyamines	1
1.1.2	Polyamine metabolism	3
1.1.3	Regulation of ODC and AdoMetDC	5
1.1.4	Spermidine synthase	7
1.1.5	Regulation of spermidine synthase expression	9
1.1.6	Spermine synthase	10
1.2	Inhibitors of spermidine synthase	11
1.2.1	Polyamine biosynthetic enzymes as drug targets in parasitic infections	12
1.3	Malaria Overview	14
1.4	*P. falciparum* life cycle	17
1.4.1	*Anopheles* stages	17
1.4.2	Human stages	18
1.5	Pathobiology of malaria	20
1.6	Antimalarial drugs and malaria parasite drug resistance	21
1.7	Polyamine metabolism in *P. falciparum*	23
1.8	Nematodes	25
1.8.1	*Caenorhabditis elegans* as a model organism for eukaryotic organisms	25
1.8.2	Polyamines and the nematodes	26
1.9	Objectives and aims for the current study	27

Chapter 2 Materials and methods 29

2.1 Chemicals and bioreagents 29

2.2 Preparation of standard solutions 32

2.3 Vectors and cells 33

2.4 Culture of *P. falciparum* 34

2.4.1 Percoll-alanine separation of *P. falciparum* in culture 34

2.4.2 *In vitro* assay in *P. falciparum* culture 35

2.5 Preparation of DNA and RNA 35

2.5.1 Preparation of genomic DNA from *P. falciparum* 35

2.5.2 Southern blot analysis 36

2.5.3 Preparation of RNA from *P. falciparum* 36

2.5.4 Northern blot analysis 37

2.5.5 DNA preparation from plasmid DNA 38

2.5.5.1 Mini-preparation of plasmid DNA 38

2.5.5.2 Midi-preparation of plasmid DNA 38

2.6 Polymerase Chain Reaction (PCR) 38

2.7 Restriction enzyme digestion of DNA 39

2.8 Agarose gel electrophoresis of DNA 39

2.9 Purification of DNA by the GeneClean technique 39

2.10 Ligation of DNA 40

2.11 Transformation 40

2.11.1 Competent bacteria production 40

2.11.2 Transformation of competent cells 40

2.12 DNA sequencing and analyses 41

2.12.1 Phylogenetic analysis 41

2.13 Expression constructs for spermidine synthase 42

2.13.1	Cloning and expression of recombinant *P. falciparum* spermidine synthase	42
2.13.2	Cloning and expression of recombinant *C. elegans* spermidine synthase	43
2.13.3	Cloning and expression of recombinant *B. malayi* spermidine synthase	44
2.13.4	Co-expression of *P. falciparum* ODC/AdoMetDC with *P. falciparum* spermidine synthase	44
2.13.5	Purification of recombinant *P. falciparum* spermidine synthase	46
2.13.6	Purification of recombinant *C. elegans* spermidine synthase	46
2.13.7	Purification of the recombinant *B. malayi* spermidine synthase	47
2.14	Site-directed mutagenesis	47
2.14.1	Deletion mutagenesis	48
2.15	Bradford assay for protein determination	49
2.16	Protein gel electrophoresis	49
2.16.1	SDS-PAGE	49
2.16.2	Native gel electrophoresis	50
2.16.3	Protein staining in gels	50
2.17	Production of Antiserum	51
2.18	Western blotting	51
2.18.1	Western blot analyses of *P. falciparum* spermidine synthase	51

2.18.2 Western blot analyses of the co-expressed *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase 52
2.19 Immunofluorescence 53
2.20 Aminopropyltransferase enzyme assays 53
2.21 Product analyses by thin layer chromatography (TLC) 54
2.22 Product analyses using high performance liquid chromatograhy (HPLC) 54

Chapter 3 Results 56
3.1 Genomic organisation 56
3.2 Stage specific expression of spermidine synthase in *P. falciparum* 57
3.3 Amino acid sequence analysis 57
3.4 Recombinant expression and biochemical characterization 60
3.5 Western blot analyses 62
3.6 Localization of the *P. falciparum* spermidine synthase 63
3.7 Kinetic analyses 64
3.8 The effect of synthetic spermidine synthase inhibitors on enzyme activity and cultured *P. falciparum* 67
3.9 *P. falciparum* spermidine synthase mutations 70
3.10 Co-expression of *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase 71
3.11 Analysis of the deduced amino acid sequences of *C. elegans* and *B. malayi* spermidine synthase 72
3.12 Expression and characterization of the recombinant *C. elegans* and *B. malayi* spermidine synthase 75

3.13 Kinetic analyses of the *C. elegans* spermidine synthase 77
3.14 *C. elegans* spermidine synthase deletion mutant 79

Chapter 4 Discussion 80
4.1 Properties of spermidine synthases 81
4.1.1 The active site of the *P. falciparum* and *C. elegans* spermidine synthases 83
4.1.2 Localization of the *P. falciparum* and *C. elegans* spermidine synthase 87
4.2 Uniqueness of polyamine metabolism in *P. falciparum* 89
4.2.1 Protein-Protein Interactions 91
4.3 *P. falciparum* spermidine synthase: a potential drug target 93

Chapter 5 Summary 97
References 100
Appendix 121

Chapter 1 Review of the literature

1.1 The Polyamines

1.1.1 Properties and function of the polyamines

The naturally occurring polyamines, putrescine, spermidine and spermine are found in almost all organisms (Figure 1.1). In mammalian cells, the polyamines are present in millimolar concentrations (Pegg and McCann, 1982). They are organic polycations since the primary and secondary amino groups are protonated at physiological pH. Therefore, putrescine is divalent, spermidine trivalent and spermine tetravalent (Wallace, 1998).

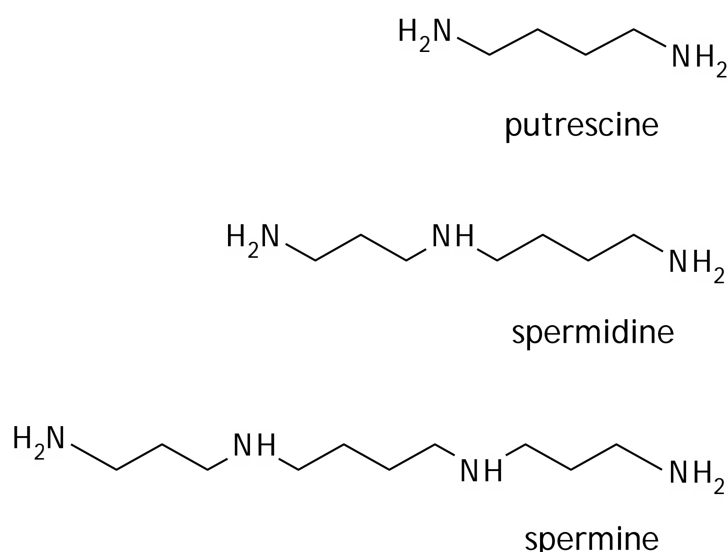


Figure 1.1 The polyamines

Numerous studies have illustrated that the multi-cationic polyamines react directly with the multi-anionic polyphosphorylated DNA molecules in cell free systems. The charge distribution in the spermine molecule makes it able to bind strongly to two phosphate groups in each strand of the DNA double helix. Spermidine and spermine causes DNA to condense, to aggregate, to induce B-to-Z and B-to-A transitions, and to bend or contort the normal alpha helical structure. Polyamines have been shown to stabilize the DNA helix and also

other double-helical structures such as stems and loops in ribosomal RNAs, messenger RNAs and transfer RNAs. These interactions are considered to be the basis for their stimulatory effects on DNA, RNA and protein synthesis. Some studies support the theory that polyamine binding to chromatin DNA may cause an increased or decreased availability of genomic sites for DNA and RNA synthetases, and hence alter DNA and RNA synthesis. Furthermore, polyamine depleted chromatin was much more susceptible to DNAse digestion (Cohen, 1998).

The polyamines are essential for normal cell growth of prokaryotes and eukaryotes (Pegg *et al.*, 1995). When cells are depleted of polyamines, for example after exposure to specific biosynthesis inhibitors, they cease to multiply. Studies with polyamine inhibitors and null mutants have shown that polyamines play an important role in cell growth and differentiation. The effects of polyamine starvation include a decrease in the elongation rates of protein and nucleic acid synthesis, and impairment in the fidelity of translation (Cohen, 1998). A mutant strain of *Saccharomyces cerevisiae* that cannot make polyamines because of a deletion-insertion mutation in the gene for S-adenosylmethionine decarboxylase (AdoMetDC), (SPE2) has revealed that spermidine or spermine is absolutely essential for growth, and that polyamine deficient cells develop marked morphological changes (Cohn *et al.*, 1978). A spermidine synthase knockout strain of *Leishmania donovani* was created by double targeted gene replacement (Roberts, 2001). The data established that spermidine synthase is an essential enzyme in *L. donovani* promastigotes. A null mutant of the *Dictyostelium discoideum* spermidine synthase gene was unable to grow in the absence of exogenous spermidine. Development of spermidine synthase null cells grown in the absence of spermidine produced fruiting bodies that had abnormally short stalks (Guo *et al.*, 1999). In addition, polyamines seem to be involved in the protection of the cell and cellular components from oxidative damage in yeast. However, in prokaryotic cells spermidine was not essential for growth, since null mutants of *Escherichia coli* that cannot synthesize spermidine because of deletions in the gene encoding AdoMetDC, were still able to grow at nearly normal rate in purified media deficient in polyamines (Xie *et al.*, 1993).

Eukaryotic translation initiation factor (eIF-5A) is a highly conserved and essential protein that contains the unique amino acid hypusine. One of the few specific roles that have been found for spermidine in macromolecular synthesis is the synthesis of hypusine, a post-translationally modified lysyl residue found in eukaryotic translation initiation factor 5A (Molitor *et al.*, 2004). A depletion of spermidine and thereby hypusine in eIF-5A was shown to be associated with cytostasis induced by AdoMetDC inhibition (Byers *et al.* 1992). Furthermore spermidine seems to have a critical role in controlling inwardly rectifying potassium channels and the normal electrical activity of cells (Lopatin *et al.*, 2000).

1.1.2 Polyamine metabolism

In mammals polyamine metabolism is well characterized and the cellular polyamines are regulated by a complex circuitry of synthesis, degradation as well as cellular uptake and efflux (Figure 1.2) (Tabor and Tabor, 1984; Pegg, 1986; Jänne *et al.*, 1991; Heby *et al.*, 2003). Putrescine is synthesized by the decarboxylation of ornithine by the rigorously regulated enzyme ornithine decarboxylase (ODC). S-Adenosylmethionine decarboxylase (AdoMetDC) generates decarboxylated S-adenosylmethionine (dcAdoMet), which serves as the aminopropyl group donor for spermidine and spermine synthesis. The latter reactions are catalysed by spermidine synthase and spermine synthase, respectively. Spermidine and spermine can be converted back to their precursors via acetylation and oxidation reactions. The acetylation and catabolism of polyamines in eukaryotes is controlled by the inducible enzyme spermidine/spermine N^1-acetyltransferase (SSAT) (Casero and Pegg, 1993). The acetylated products, N^1-acetylspermidine and N^1-acetylspermine, are then substrates for the constitutive enzyme, the FAD-dependent polyamine oxidase (PAO) (Hölttä, 1977), which completes the conversion of spermidine to putrescine and spermine to spermidine. Acetylation is also a physiological way to convert excess polyamines to a physiologically inert form which can be readily excreted. Both putrescine and N^1-acetylspermidine are excreted from cells (Hyvonen, 1989). Intracellular oxidation of polyamines can occur via a second recently characterized FAD-dependent PAO in the cell, spermine oxidase (Vujcic *et al.*,

2002). Extracellular oxidation of polyamines catalysed by a series of copper-dependent amine oxidases was found to be involved in the terminal catabolism of polyamines (Seiler e*t al*., 1981). Several studies have shown that the oxidation products generated during polyamine catabolism are responsible for cytotoxicity in mammalian cells (Parchment, 1993).

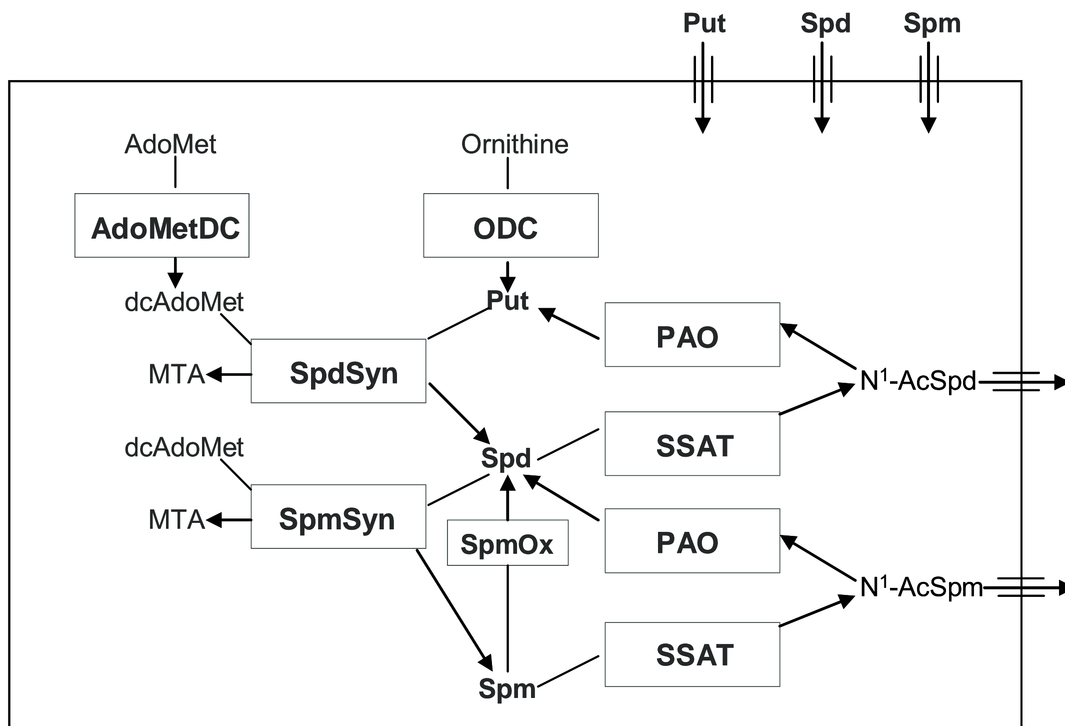


Figure 1.2 Polyamine metabolism in mammalian cells
AdoMet: S-adenosylmethionine; AdoMetDC: S-adenosylmethionine decarboxylase; dcAdoMet: decarboxylated S-adenosylmethionine; ODC: ornithine decarboxylase; SpdSyn: spermidine synthase; SpmSyn: spermine synthase; PAO: polyamine oxidase; SSAT: spermidine/spermine N^1-acetyltransferase; Put: putrescine; Spd: spermidine; Spm: spermine; MTA: 5'-methylthioadenosine; SpmOx: spermine oxidase; N^1-AcSpm: N^1-acetylspermine; N^1-AcSpd: N^1-acetylspermidine.

The cellular requirements for polyamines are met through either their endogenous biosynthesis or their transport into the cell from the environment. Exogenous polyamines are provided both by release from other cells and via external polyamine sources. Polyamines are present in most foods and are produced and excreted by intestinal microorganisms. Especially tissues with a high demand of polyamines, such as tumours or normal but rapidly proliferating cells have been shown to enhance their polyamine transport (Seiler and Dezeure, 1990). Since putrescine and spermine concentrations in blood are low compared with spermidine concentrations, it is predominantly the uptake of spermidine from the circulation into tissues that is physiologically important. Polyamine transport systems have

been characterised from *E. coli*, yeast and *Leishmania* (Pistocchi *et al.*, 1993; Igarashi and Kashiwagi, 1999; Hasne and Ullman, 2005). However, in mammalian cells the properties and regulation of the polyamine uptake system are still poorly understood and its structure is as yet completely unknown. The uptake system resembles in many respects the uptake systems of amino acids. It has been shown to be energy and temperature dependent, and saturable, suggesting a carrier-mediated transport (Seiler and Dezeure, 1990). Polyamine depletion was shown to induce enhanced uptake of the polyamines (Alhonen-Hongisto *et al.*, 1980). Enhanced polyamine transport rates were observed during cell transformation and in cells stimulated by serum, growth factors and hormones (Seiler and Dezeure, 1990). Moreover putrescine and spermidine transport was strongly enhanced by hypoosmotic stress. Stringent feedback control mechanisms were shown to regulate the polyamine transport system (Mitchell *et al.*, 1994). The free intracellular spermidine concentration was postulated to play the most important role as an uptake regulator. Recently it was shown, that antizyme, besides regulating ODC degradation, also negatively regulates polyamine transport (Mitchell *et al.*, 1994).

1.1.3 Regulation of ODC and AdoMetDC

Intracellular polyamine concentrations vary throughout the cell cycle. The polyamine pools are tightly regulated by mechanisms that control biosynthesis, degradation, and uptake (Morgan, 1999). In the synthesis pathway ODC and AdoMetDC represent the rate limiting enzymes.

ODC activity increases rapidly and dramatically when growth is stimulated as in regenerating tissues, hormonally stimulated tissues, and mitogenically activated cells in culture (Pegg, 1988). The expression of ODC activity is regulated at multiple levels including the rate of transcription (Katz and Kahana, 1987), the translational efficiency of mRNA and enzyme degradation. The ODC gene has been found to be transcriptionally regulated by growth factors (Katz and Kahana, 1987) and tumour promoters (Abrahamsen and Morris, 1990). Recently it was shown that ODC transcription is increased by c-Myc (Bello-Fernandez *et al.*,

1993) and by TGF-ß in response to H-ras (H-ras is downstream of TGF-β) (Hurta *et al.*, 1993). Furthermore, in many neoplastic cells ODC mRNA levels were constitutively elevated. The regulation of ODC by polyamines seems to be at the translational level. All mammalian ODC mRNAs have been reported to contain a long, untranslated leader sequence (5' UTR) of about 300 nucleotides, and an even longer 3' untranslated region (Hickok *et al.*, 1987). The GC-rich sequence in the 5'-UTR of ODC mRNA was found to be involved in the stimulation of ODC synthesis by low spermidine concentrations and in the inhibition of ODC synthesis at high spermidine concentrations. Moreover, the translation of ODC mRNA was suppressed by its 5' UTR, which is able to form a very stable secondary structure (Grens and Scheffler, 1990).

The ODC protein has a half-life of the order about 30 min while the half life of ODC mRNA is rather long, varying from 2 to 5 h, depending on the cell type (Abrahamsen and Morris, 1990). Thus, the rapid regulation of ODC enzyme depends on its rapid turnover rate. An ODC inhibitory protein, antizyme, was found to be induced by polyamines and accelerate ODC degradation (Heller *et al.*, 1976). Later antizyme was shown to inhibit ODC activity by forming an ODC antizyme complex and by rendering the enzyme susceptible to proteolysis (Murakami *et al.*, 1994). The C-terminal region of the ODC protein was shown to be associated with the constitutive and polyamine-induced turnover of the enzyme (Ghoda *et al.*, 1990).

Variations in AdoMetDC activity seem to have a very important role in the regulation of cellular polyamine levels. This is partly due to the very short half-life of the enzyme and partly because AdoMetDC expression is regulated at multiple levels; transcriptional, translational as well as post-translational (Pegg *et al.*, 1988).

AdoMetDC activity has been found to increase in response to a variety of stimuli that increase cell growth. Several studies have shown that agents causing a decrease in the polyamines lead to an increased level of AdoMetDC activity (Alhonen-Hongisto, 1980) or protein (White *et al.*, 1990). The processing of the proenzyme and catalytic activity of the final enzyme were stimulated by putrescine (Pegg and Williams-Ashman, 1969). However,

spermidine and spermine were shown to inhibit the proenzyme synthesis (Pajunen *et al.*, 1988). Spermine affected primarily the translation of the AdoMetDC mRNA, whereas spermidine affected the content of this mRNA (Shantz *et al.*, 1992). The results suggested that there is a spermidine responsive element in the first 72 nucleotides from the 5'-end of AdoMetDC mRNA (Shantz *et al.*, 1992). In addition, the GC-rich 5'-end of 5'-UTR was involved in inhibition of translation at high spermine concentration and in stimulation at low concentrations (Suzuki *et al.*, 1993). Unlike ODC, the secondary structure of AdoMetDC mRNA did not seem to be the limiting factor in its translation, since the overexpression of translation initiation factor eIF-4E does not increase AdoMetDC synthesis (Shantz and Pegg, 1994).

1.1.4 Spermidine synthase

Spermidine synthase is an aminopropyltransferase that catalyzes the transfer of an aminopropyl moiety of dcAdoMet on putrescine leading to the production of spermidine and MTA. Spermidine synthase has been purified in homogeneous form from *E. coli* (Bowman *et al.*, 1973), bovine brain (Raina *et al.*, 1984), rat prostrate, rat liver (Samejima and Yamanoha, 1982) and pig liver (Yamanoha *et al.*, 1984). The enzyme has also been cloned and characterized from bacterial (Bowman *et al.*, 1973), plant (Hashimoto *et al.*, 1998) and mammalian sources (Wahlfors *et al.*, 1990). The active enzyme is usually a dimer consisting of two identical subunits and the activity of this synthase is regulated by the availability of its substrates, putrescine and dcAdoMet (Pegg *et al.*, 1995). The reaction products, spermidine and MTA inhibit the enzyme (Pegg *et al.*, 1995).

Cadaverine can also act as a substrate for the mammalian enzyme but the reaction proceeds at only one twentieth of the rate with putrescine. Spermidine synthase from bovine brain can also utilize 1,6-diaminohexane as the aminopropyl receptor but only at 1% of the rate with putrescine (Raina *et al.*, 1984). A model for the active site of aminopropyltransferase has been proposed. This seems to have a relatively large hydrophobic cavity adjacent to a negatively charged site, to which a protonated amino group of putrescine binds, with another

amino group of putrescine being situated in the hydrophobic cavity as a free form to be aminopropylated by decarboxylated AdoMet (Shirahata *et al.*, 1991).

Recently, the crystal structure of the spermidine synthase from *Thermatoga maritima* was reported (Korolev *et al.* 2002). The structure has been solved to 1.5 A resolution in the presence and absence of AdoDATO (S-adenosyl-1,8-diamino-3-thiooctane), a compound containing both substrate and product moieties (Figure 1.3).

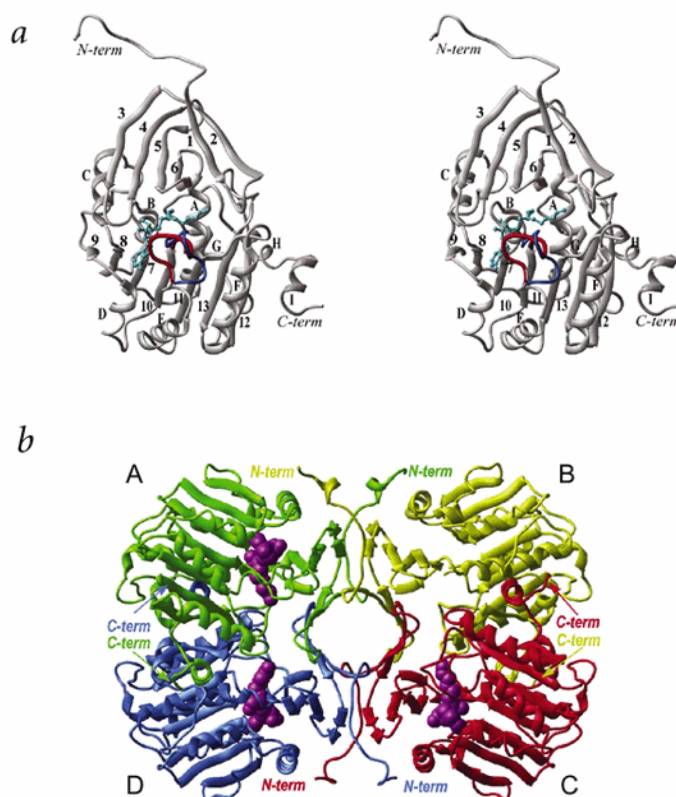


Figure 1.3 Overall structure of *T. maritima* spermidine synthase monomer and architecture of the tetramer.
a. Stereo view of a ribbon diagram representation of *T. maritima* spermidine synthase subunit D. The α-helixes are lettered, and β-strands are numbered. AdoDATO is shown in ball-and-stick representation in cyan. b. Architecture of the *T. maritima* spermidine synthase tetramer. Ribbon diagrams of subunits A, B, C and D are shown in green, yellow, red and blue, respectively. AdoDATO is shown in representation in magenta (Korolev *et al.*, 2002).

This first structure of an aminopropyltransferase reveals deep cavities for binding substrate and cofactor, and a loop that envelops the active site. The AdoDATO binding site is lined with residues conserved in spermidine synthase enzymes from bacteria to humans, suggesting a universal catalytic mechanism. Other conserved residues act sterically to

provide a structural basis for polyamine specificity. The enzyme is tetrameric unlike all other characterized spermidine synthases. Each monomer consists of a C-terminal domain with a Rossmann-like fold and an N-terminal beta-stranded domain. The tetramer is assembled using a novel barrel-type oligomerization motif.

1.1.5 Regulation of spermidine synthase expression

In contrast to ODC and AdoMetDC, spermidine synthase is a stable enzyme with a molecular half-life of over 12 hours. Elucidation of the relationship between the enzyme activity and protein in normal tissues showed that difference in spermidine synthase activity among rat tissues depends solely on the difference in the amount of the enzyme.

In most instances the induction of ODC with concurrent accumulation of putrescine greatly enhances the activity of pre-existing AdoMetDC, which is the rate-controlling enzyme in the synthesis of spermidine. Therefore the induction of spermidine synthesis is sometimes concomitant with the induction of the decarboxylases. In addition, increased spermidine synthase activity has been reported in regenerating tissues (Hannonen *et al.*, 1972), in hormonally stimulated tissues (Oka *et al.*, 1977), and in mitogenically activated cells in culture (Korpela *et al.*, 1981). It has also been shown that spermidine synthase activity is elevated in some malignant human tumours and cultured malignant cells (Kajander *et al.*, 1988). Large increases in cellular levels of polyamines spermidine and spermine have been found in lymphocytes induced to transform by concanavalin A. The increased polyamine levels in stimulated lymphocytes were required for DNA synthesis (Fillingame *et al.*, 1975). Later it was shown that spermidine synthase activity is increased in stimulated lymphocytes in parallel to the elevation in spermidine content (Korpela *et al.*, 1981). This induction of spermidine synthase in phytohemagglutinin-induced stimulation seemed not to be influenced by polyamines.

After partial hepatectomy, rat liver spermidine synthase activity was shown to increase for up to four days (Hannonen *et al.*, 1972). Nishikawa and co-workers showed that spermidine synthase gene expression is low in normal rat liver, but its expression is rapidly induced after

partial hepatectomy with a mRNA peak already after 6 hours. In cultured rat hepatocytes, the spermidine synthase expression was suppressed by the absence of epidermal growth factor (EGF) and by the presence of transforming growth factor beta (TGF-ß). Thus, it was suggested that both growth factors are involved in the regulation of spermidine synthase gene expression. Recently, it was shown that increased spermidine or spermine level is essential for hepatocyte growth factor- induced DNA synthesis in cultured rat hepatocytes (Higaki *et al.*, 1994). Hepatocyte growth factor is a potent mitogen for mature hepatocytes and seems to act as a trigger for liver regeneration.

Various hormones have been shown to stimulate spermidine synthase expression. In mouse mammary epithelial cells spermidine synthase activity was found to increase 2-3 hours after the induction of culture with insulin, cortisol and prolactin (Oka *et al.*, 1977). The addition of actinomycin D or cycloheximide inhibited completely the hormonal stimulation, suggesting that the increase in enzyme activity may require both new RNA and protein synthesis. Spermidine synthase activity was also enhanced in hormone dependent growth stimulation of rat uterus and male accessory sexual glands (Käpyaho *et al.*, 1980).

1.1.6 Spermine synthase

Spermine synthase is the second aminopropyltranferase which has been found only in some eukaryotes. Analogous to the spermidine synthase reaction spermine synthase catalyzes the formation of spermine from spermidine and dcAdoMet (Seiler, 2003). Sequence comparisons between human spermine synthase and spermidine synthases from bacterial and mammalian sources revealed only a modest similarity that was most pronounced in a relatively short peptide domain apparently involved in the binding of decarboxylated S-adenosylmethionine, the common substrate for both enzymes (Korhonen *et al.*, 1995). The apparent lack of an overall similarity may indicate that spermine synthase, the enzyme found only in eukaryotes, and spermidine synthase with more universal distribution, although functionally closely related, have branched into separate groups (Korhonen *et al.*, 1995). The enzyme, like the spermidine synthase, consists of two subunits of equal size. Spermine

synthase showed strict specificity to spermidine as the propylamine acceptor (Pajula *et al.*, 1979). It is, like spermidine synthase, expressed and regulated mainly by the availability of dcAdoMet. The activity of the enzyme was inhibited by both reaction products, spermine and MTA (Raina *et al.*, 1982; Yamanaka *et al.*, 1987). Putrescine was a competitive inhibitor at high concentrations (Pajula and Raina, 1979).

1.2 Inhibitors of spermidine synthase

Inhibitors of the spermidine synthase may be substrate analogues or transition state analogues. Among the putrescine analogues cyclohexylamine and *trans*-4-methyl-1-cyclohexylamine have been studied the most extensively (Shirahata *et al.*, 1988; Pegg *et al.*, 1995; Kobayashi *et al.*, 2005). Tight binding inhibitors of methyl- and aminopropyltransferases have systematically been synthesized (Coward, 1989). In some of the structural analogues of dcAdoMet, for instance in 5´-deoxyadenosylspermidine, an amino group substitutes the thiol group. It is considered as one of the most potent inhibitors of spermidine synthase. A transition state analogue of the reaction product of spermidine synthase is AdoDATO (Coward and Pegg, 1987). Among the spermidine synthase inhibitors AdoDATO became popular because of its high selectivity and potency. Nanomolar concentrations inactivate the enzyme due to tight binding of the compound within the active site (Seiler, 2003). 1-Aminooxy-3-aminopropane was shown to be a potent competitive inhibitor of homogenous mouse kidney ornithine decarboxylase, a potent irreversible inhibitor of homogeneous liver adenosylmethionine decarboxylase and a potent competitive inhibitor of homogeneous bovine brain spermidine synthase. It did not inhibit homogeneous bovine brain spermine synthase and it did not serve as a substrate for spermidine synthase (Khomutov *et al.*, 1985). Spermidine synthase activity from *Trypanosoma brucei brucei* was markedly inhibited in vitro by dicyclohexylamine (Bitonti *et al.*, 1984). It was also found that the spermidine synthase activity in bacteria was also specifically inhibited by the addition of dicyclohexylamine (Mattila *et al.*, 1984). Spermidine synthase from rat ventral prostate was

inhibited by 2-mercaptoethylamine (MEA). Inhibition of spermidine synthase by MEA was competitive with respect to one of the substrates putrescine. (Hibasami *et al.*, 1988).

1.2.1 Polyamine biosynthetic enzymes as drug targets in parasitic infections

The polyamines and their biosynthetic enzymes are present in high concentrations in proliferating cells, including tumour cells and parasitic organisms, leading to the suggestion that the inhibition of polyamine biosynthesis is an approach for chemotherapy and antiparasitic drugs (Heby *et al.*, 2003). The antiproliferative effects of polyamine depletion have been one strategy in cancer treatment and chemoprevention, and in infectious diseases caused by viruses, bacteria, fungi and parasitic protozoa (Jänne *et al.*, 1991).

Since the polyamine biosynthetic enzymes of the parasites may exhibit features that are different from those of the human host and also because some enzymes that use polyamines as substrates are unique to the parasites, it is thought that the exploitation of this pathway could lead to the design of new antiparasitic drugs that will selectively kill the parasites (Müller *et al.*, 2001).

Due to their roles as rate-limiting enzymes in polyamine biosynthesis, ODC and AdoMetDc serves as obvious targets for chemical intervention aimed at depletion of the polyamine pool. Inhibition of ODC leads to decreased putrescine and spermidine levels (Pegg, 1989) whereas inhibition of AdoMetDC leads to large increases in putrescine and a decline in spermidine and spermine due to the absence of dcAdoMet (Pegg, 1989). A major breakthrough in ODC inhibition was the synthesis of the substrate analogue, DL-α-difluoromethylornithine (DFMO), which is enzymatically decarboxylated and generates an irreversible alkylation of the enzyme at or near the active site to cause a rapid loss of enzyme activity (Cohen, 1998). The only physiologically meaningful inhibitor of AdoMetDC was synthesized as an antileukemic agent, methylglyoxal bis(guanylhydrazone) (MGBG) (Seiler, 2003).

The antiprotozoal activity of ODC inhibitors, have been very promising. DFMO exerts a dramatic and rapid therapeutic effect in African sleeping sickness caused by *Trypanosoma brucei gambiense*. DFMO was approved as the first drug for the treatment of this disease (Müller *et al.*, 2001). Reasons for the sensitivity of these parasites to DFMO include an increased half-life of the parasitic ODC (> 6 h) compared to the human ODC enzyme (<1 h), implying that newly synthesised, active ODC rapidly replaces DFMO-inactivated ODC in the human host but not in the parasite. The reduced production of putrescine and spermidine in DFMO treated *T. brucei* cannot be compensated by uptake of polyamines from the blood of the host because they have an inefficient polyamine-transport system (Pegg, 1989). Because of the DFMO-treated depletion of intracellular spermidine, the parasites cannot synthesize trypanothione, a product of spermidine and glutathione that is unique to trypanosomes and *Leishmania.* This product is necessary for many protective and regulatory functions, and its depletion in DFMO-treated *T. brucei* leads to parasite death (Heby *et al.*, 2003).
Irreversible inhibition of AdoMetDC, using 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine (MDL 73811), a structural analogue of dcAdoMet, was very effective against *T. b. brucei* infections in mice and rats (Byers *et al.*, 1991). MDL 73811 was more potent than DFMO, and a combination of the two drugs were synergistic, even curing mice infected with clinical isolates of *T. b. rhodesiense* (Bacchi *et al.*, 1992).
T. cruzi causes Chagas' disease in South America. This parasite lacks ODC and therefore cannot synthesise putrescine *de novo* (Persson *et al.*, 1998). The parasite relies upon putrescine uptake and AdoMetDC for spermidine synthesis (Müller *et al.*, 2001). DFMO is completely inactive against all stages of the parasite and the expression of a foreign ODC gene in *T. cruzi* overcomes the requirement of exogenous polyamines for growth (Carrillo *et al.*, 1999). Because of the efficient polyamine transporter in *T. cruzi* the inhibitors of AdoMetDC and spermidine synthase are most probably ineffective against these parasites as they can derive a rich source of polyamines from the host cells. A good target would be the blockage of the parasitic transporter in order to achieve a polyamine deficient state in the *T. cruzi* cells (Heby *et al.*, 2003).

L. donovani and *L. infantum* are causative agents of visceral leishmaniasis. DFMO is also cytotoxic to *L. donovani* and *L. infantum* promastigotes (Heby *et al.*, 2003). To investigate the importance of ODC, AdoMetDC and spermidine synthase in *L. donovani*, the corresponding null mutants of these genes were created in promastigotes. The ODC-mutant was unable to grow in polyamine deficient medium (Jiang *et al.*, 1999). This auxotrophy was however overcome by the addition of putrescine or spermidine. Putrescine restored the intracellular pools of both putrescine and spermidine, but spermidine was not back converted to putrescine. This indicates that spermidine alone is sufficient for *L. donovani* growth and that the organism lacks a polyamine interconversion pathway (Jiang *et al.*, 1999). The AdoMetDC and spermidine synthase knock-out mutants were also unable to grow without polyamines. Auxotrophy was overcome by spermidine but not by the addition of putrescine. The stability of *L. donovani* AdoMetDC (> 24 h) suggests that irreversible inhibitors of AdoMetDC may also be able to eradicate leishmanial infections (Roberts *et al.*, 2002). Biochemical and genetic elucidation of the polyamine metabolic pathway in parasites indicates marked differences with the mammalian host pathway and therefore represents an important avenue for the development of antiprotozoal drugs.

1.3 Malaria Overview

Malaria is a parasitic infection of global importance and is one of the most prevalent human infections worldwide with over 40% of the world's population living in malaria-endemic areas (Figure 1.4) (Suh *et al.*, 2004). The World Health Organisation (2001) estimates, that there are 300-500 million clinical episodes of malaria worldwide each year and approximately 0.7-2.7 million annual deaths (Bozdech, *et al.*, 2003). Ninety percent of deaths occur in sub-Saharan Africa, the majority involving children less than 5 years of age. In addition, pregnant women and non-immune people, for example, travellers and foreign workers are at highest risk of severe disease (Suh *et al.*, 2004). All age groups may be at risk of severe disease during malaria epidemics, which occur either when changes in the physical environment (caused by climatic variation, agricultural projects or mining, for example) increase the

capacity of mosquitoes to transmit the disease or when population displacements (natural disasters, war) expose populations to infection (Breman *et al.*, 2004). These estimates designate malaria among the top three communicable diseases.

The full impact of the relationship between poverty and malaria has only recently been appreciated. The impoverished communities that are burdened by malaria lack the means to adequately prevent, control or treat the disease. Therefore, in many endemic countries, malaria is responsible for economic stagnation and lowering the annual economic growth (Sachs and Malaney, 2002).

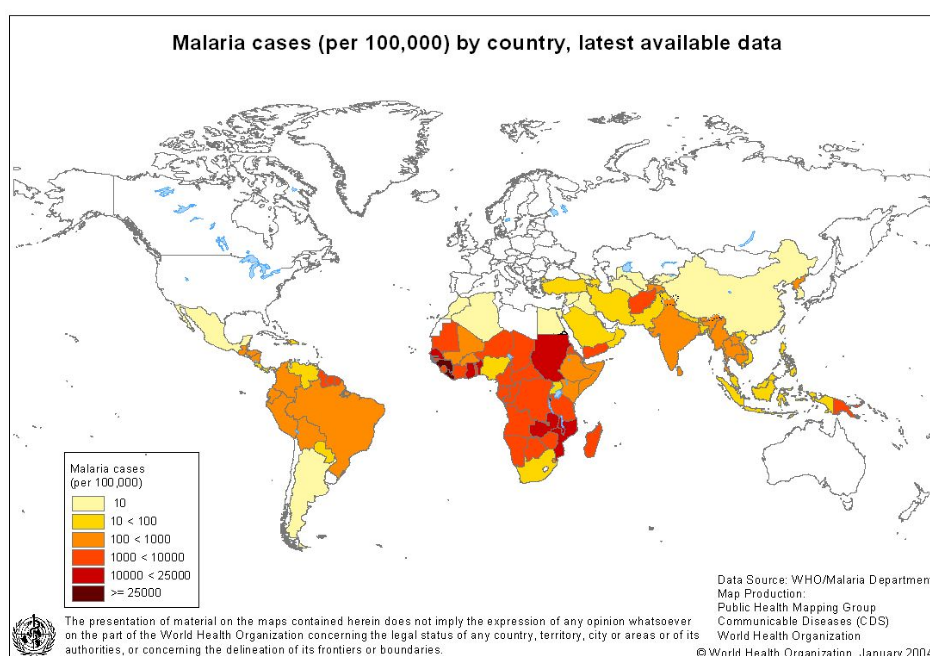


Figure 1.4 Global malaria cases

The malaria parasite *P. falciparum* is presented here classified according to Lucius and Loos-Frank (1997):

Kingdom	Protista
Phylum	Apicomplexa
Class	Hematozoa
Order	Haemosporida
Family	Plasmodiidae

The genus *Plasmodium* consists of nearly 200 species that parasitize reptiles, birds and mammals (Ayala *et al*., 1998). Human malaria is caused by four species of the parasitic protozoan genus *Plasmodium*: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale*. Of these four species, an infection with *P. falciparum* parasites has the greatest medical impact and is almost solely responsible for malaria fatalities and is therefore the focus of the work in this study. The parasite is transmitted to a human host from the bite of an infected female mosquito of the genus *Anopheles*, including the three species *A. gambiae*, *A. arabiensis* and *A. funestus*, of which *A. gambiae* is the most important (Cox, 1993). Warm, moist climates where the infrastructure is undeveloped are ideal conditions for the anthrophilic *Anopheles* vector to thrive (Breman, 2001).

While efforts to curtail malaria with combinations of vector control, education and drugs have proven successful, a global solution has not been accomplished. There are few antimalarial chemotherapeutics available that serve as both prophylaxis and treatment (Bozdech *et al*., 2003). Compounding this scarcity of drugs is a worldwide increase in *P. falciparum* strains resistant to the available antimalarial drugs (Ridley, 2002). Antimalarial drug resistance is now acknowledged to be one of the greatest threats against the reduction of malaria. The situation is aggravated, with the geographic spread of resistance widening to previously unaffected areas (Yeung *et al*., 2004). Chloroquine-resistant *P. falciparum* now predominates in Southeast Asia, South America, and increasingly in Africa. Resistance to sulfadoxine-pyrimethamine is widespread in Asia and South America and is spreading in Africa (Roper *et al*., 2003; Takechi *et al*., 2001). The effects of resistance on morbidity and mortality are usually underestimated (White, 2004). Hence, the search for effective, safe and affordable drugs for *P. falciparum* malaria is one of the most important health priorities worldwide (Winstanley, 2000). Despite earlier promising results in the 1960's with prototype vaccines, there is no effective vaccine against malaria available today. Experimental vaccination with irradiated sporozoites can protect animals and humans against the disease, demonstrating the feasibility of developing an effective malaria vaccine. However, developing a universally

effective, long lasting vaccine against this parasitic disease has been a difficult task (Tsuji *et al.*, 2001).

1.4 *P. falciparum* life cycle

The malaria parasite *P. falciparum* passes its life cycle in two different hosts, in the female *Anopheles* mosquito and in humans (Figure 1.5). Sexual replication and an asexual sporogony occur in the mosquito, two further asexual replications take place in the human host (Wahlgren and Perlmann, 1999).

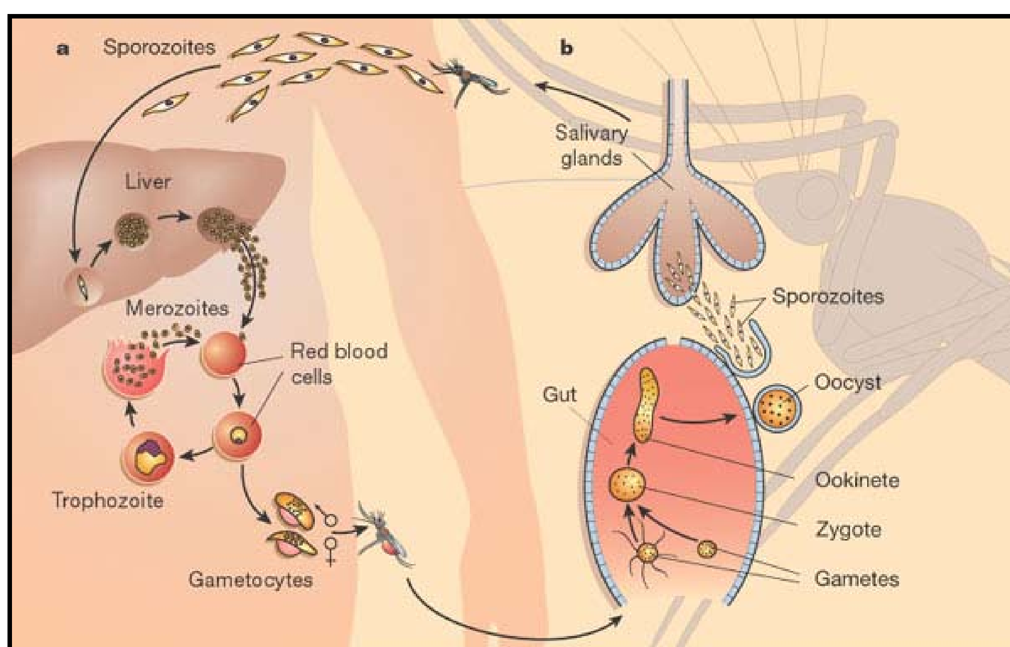


Figure 1.5 The life cycle of the parasite *P. falciparum* (http://www.sanger.ac.uk)

1.4.1 *Anopheles* stages

Female *Anopheles* mosquitoes that take a blood meal from an infected human ingest humoral and cellular components of the blood stream plus male and female *P. falciparum* gametocytes, respectively. The haploid gametocytes undergo transformation to microgametes and macrogametes, respectively, due to stimulations that arise from the ingestion by the mosquito, as for example, a drop in temperature, an increase in pH and xanthurenic acid (Sinden & Smalley, 1976). Fertilization of the female gamete by the

microgamete takes place in the mosquito midgut to generate a diploid zygote that over the next 24 h undergoes morphological changes to form a motile ookinete (Kumar et al., 1985). Meiosis is initiated immediately after zygote formation (Sinden & Hartley, 1985). The ookinete then passes through the peritrophic membrane, which encapsulates the blood meal, before invading the midgut epithelium (Meis & Ponnudurai, 1987). The midgut epithelium lining is traversed from the luminal to the haemocoel side, where the ookinete lies below the outer limiting membrane of the stomach wall and develops into the oocyst stage (Sinden, 1978). The oocyst reorganises internally into multiple haploid nucleated masses, which divide repeatedly to form thousands of sporozoites (Sinden & Hartley, 1985). The sporozoites from mature oocysts migrate to the salivary glands of the mosquito via the hemolymph system and await injection into the human host when the mosquito takes its next blood meal (Pimenta *et al.*, 1994).

1.4.2 Human stages

A new human infection of *P. falciparum* starts with the injection of between 25 and 100 sporozoites from the female *Anopheles* mosquito bite (Vanderberg, 1997). Sporozoites are transported by the bloodstream to the liver microcirculation and enter hepatocytes a few minutes after injection (Shin *et al.*, 1982). Sporozoites are first arrested in the liver sinusoid by binding to extracellular matrix proteoglycans on the surface of Kupffer cells, which they use to traverse the sinusoidal cell barrier (Pradel *et al.*, 2002). The sporozoites appear to pass through the cytosol of several cells before invading a hepatocyte and then develop inside the parasite-generated parasitophorous vacuole (PV) (Mota *et al.*, 2001). The detailed mechanism of hepatocyte entry is not known but is presumed to involve complex molecular interactions between sporozoite and hepatocyte molecules (Cerami *et al.*, 1992). Upon entry into the hepatocyte, the sporozoite metamorphoses into a trophozoite. The mature trophozoite undergoes asexual reproduction (schizogony) to generate up to 30,000-40,000 merozoites. 5 to 6 days after the penetration of the hepatocyte by the sporozoite, the merozoites are released into the blood stream.

After release the merozoites have a short life expectancy and must quickly invade an erythrocyte to survive (Johnson *et al.*, 1980). During this invasion process the merozoite attaches to specific receptors on the RBC surface. It re-orientates its apical end towards the RBC and forms a tight junction with the erythrocyte. The merozoite then moves into an invagination in the RBC and continues to develop in a membrane-bound parasitophorous vacuole (Wahlgren and Perlmann, 1999).

In the erythrocyte the merozoite transforms from the ring stage into trophozoites, and it is during this stage that the infected RBC acquires the ability to bind to vascular endothelium via parasite-derived knob structures on the erythrocyte surface. The parasite utilises haemoglobin in the RBC cytoplasm as a food source and completely alters the internal and external structure of the erythrocyte for its own growth and replication. At around 30 h the trophozoite stage starts dividing asexually within the host RBC. The parasite is now identified as a schizont, which contains multiple developing merozoites that are generated by successive divisions. After a total of 48 h from erythrocyte invasion, up to 32 new merozoites are released from the infected RBC into the blood stream. They go on to find new erythrocytes to invade, thereby perpetuating the infection in the human host (Lucius and Loos-Frank, 1997).

The *P. falciparum* life cycle is complete when some asexual blood-stage parasites differentiate into sexual forms by a process known as gametocytogenesis. There is evidence to support the hypothesis that an individual merozoite is committed to gametocytogenesis in the preceding asexual generation (Inselberg, 1983). Host environmental factors are implicated in the initiation of gametocytogenesis and basically relate to parasite density and the host immune response in addition to an underlying default rate of differentiation (Dyer & Day, 2000). Once a gametocyte is formed it no longer participates in the asexual blood stage, but relies on a mosquito for ingestion in a blood meal to further participate in the sexual reproduction stages.

1.5 Pathobiology of malaria

The morbidity and mortality that are consequences of the disease malaria arise solely from the direct effects of the asexual blood-stage. The intensity of the disease is due to both parasite and host factors which combine to produce an outcome that ranges from increased resistance against a parasite infection to death. Parasite factors include the transmission rates, multiplication rates, cytoadherence ability, the set of antigens expressed by the parasite, drug resistance and clonal load (Marsh & Snow, 1997). Host factors include congenital disposition, gravidity, age, period of prior exposure and constancy of exposure. The severity of symptoms range from non-specific febrile illness to severe anaemia and cerebral malaria, which can result in coma and death.

The pathological aspects of the disease derive from the metabolic products of parasite growth, the destruction of the host RBC and the modification of the RBC membrane that enables an infected erythrocyte to adhere to the vascular endothelium, and the impact of the host immune response (Miller *et al.*, 2002). A principle pathophysiological feature of malaria is metabolic acidosis, predominantly lactic acidosis (Marsh *et al.*, 1996). Acidosis is also strongly correlated with reduced deformability of infected RBC (Miller *et al.*, 1971), which in turn impedes blood microcirculation and oxygen delivery. Another metabolic aspect of malaria pathology is hypoglycaemia, which results from an increased demand for glucose from the parasites and as a consequence of febrile illness (Davis *et al.*, 1993). The rapid expansion of infected RBC mass and the destruction of both infected and uninfected erythrocytes can combine to cause anaemia (Achidi *et al.*, 1996). Anaemia in turn can add to the impact of cerebral malaria because of reduced oxygen availability. Lethargy and a diminished capacity to withstand immunological stress can result from anaemia and these effects are amplified by malnutrition (Newton *et al.*, 1997).

The ability of *P. falciparum* infected RBC to adhere to the epithelium of small blood vessels in vital organs is considered to be a contributing factor to the development of cerebral malaria (White and Ho, 1992). This adhering ability is thought to have evolved so infected erythrocytes could avoid circulating through the spleen, which destroys infected or damaged

RBC (Silverman *et al.*, 1987). Infected RBC that bind to the vascular epithelium impede blood flow and this affect can be exacerbated by the rossetting of parasitized cells and the reduced elasticity of uninfected RBC (Roberts *et al.*, 2000). The resulting obstruction is suspected of decreasing oxygen supply to the brain and contributes to an inflammatory response.

1.6 Antimalarial drugs and malaria parasite drug resistance

There are a limited number of drugs which can be used to treat or prevent malaria. Chloroquine is a 4-aminoquinoline derivative of quinine first synthesized in 1934 and since then been the most widely used antimalarial drug (Winstanley, 2000). Unfortunately, chloroquine resistance is now extensive in Southeast Asia, South America and Africa. Another quinine-related compound in common use is mefloquine. Mefloquine is effective against non-severe *P. falciparum* infections which are thought to be chloroquine and sulfadoxine/pyrimethamine (SP) resistant. However, resistance against mefloquine is frequent in some areas of Southeast Asia and has been reported in Africa and South America (Bloland, 2001).

The antifolate combination drugs are various combinations of dihydrofolate-reductase inhibitors (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and sulfa drugs (dapsone, sulfalene, sulfamethoxazole and sulfadoxine). In combination, they produce a synergistic effect on the parasite and can be effective to some extent even in the presence of resistance to the individual components. Typical combinations include, for example, sulfadoxine/pyrimethamine (SP), sulfalene/pyrimethamine and chlorproguanil/dapsone (Lap-Dap). Unfortunately, SP resistance occurs frequently in Southeast Asia and South America, and is more prevalent in Africa as that drug is increasingly being relied upon as a replacement for chloroquine (Bloland, 2001).

Artemisinin compounds, which are sesquiterpine lactone compounds (artesunate, artemether, arteether) have been derived from the plant *Artemisia annua*. These compounds

have the advantage of greater antimalarial potency, but with the disadvantage that they are more expensive (White, 1999).

Halofantrine is a phenanthrene-methanol compound with activity against the erythrocytic stages of the malaria parasite. Its use has been especially recommended in areas with multiple drug resistant *P. falciparum.* Studies have indicated, however, that the drug can produce potentially fatal cardiac conduction abnormalities, limiting its usefulness (Nosten *et al.*, 1993). New drugs on the market are Lumefantrine, a fluoromethanol compound, being produced as a fixed combination tablet with artemether (van Vugt *et al.*, 1999). These drugs are effective against non-severe *P. falciparum* infection but are rather expensive.

Malarone^{TM}, a fixed dose combination of atovaquone (a hydroxynapthoquinone) and proguanil is used for the treatment of multidrug resistant *P. falciparum* but is also very costly.

Antimalarial drug development is constrained by the same factors as any drug development programme in that new agents must demonstrate efficacy, be safe and have additional properties important for the specific disease. In the malaria case, the major need is for widespread treatment of malaria in developing countries where resistance to currently available drugs is spreading. Considering resource limitations in this setting, it is generally agreed that new antimalarials should be dosed orally and be effective with single-daily dosing, and that curative regimens should be short. The critical consideration in antimalarial drug development is economic. Financial constraints are relevant in two key regards. First, to be widely useful, antimalarial drugs must be very inexpensive so that they are routinely available to populations in need in developing countries. Secondly, since malaria markets are primarily in poor countries, marketing opportunities have generally been considered to be limited. Thus, drug discovery directed toward malaria is particularly reliant upon shortcuts that may obviate excess cost.

1.7 Polyamine metabolism in *P. falciparum*

The life cycle of *Plasmodium* can be interrupted at several stages by treatment with ODC inhibitors. When administered to the mosquito vector, DFMO inhibits the sporogonous cycle of *Plasmodium berghei*, and mice bitten by the mosquitoes do not contract malaria (Gillet *et al.*, 1983). DFMO also blocks exoerythrocytic schizogony and limits erythrocytic schizogony of *P. berghei* in mice (Bitonti *et al.*, 1987). Several ODC inhibitors block erythrocytic schizogony of *P. falciparum in vitro*. In human *P. falciparum*-infected erythrocytes, DFMO inhibits growth and maturation of the intracellular parasite at the trophozoite stage and decreases the levels of putrescine and spermidine but not spermine (Assaraf *et al.*, 1984; Assaraf *et al.*, 1987). Inhibition of polyamine synthesis inhibited synthesis of selected proteins, caused a slight inhibition of RNA synthesis and blocked DNA synthesis. This led to the proposition that the polyamines are required for the synthesis of malaria parasite proteins involved in DNA synthesis (Assaraf *et al.*, 1987). Prolonged treatment of the parasites with DFMO induced massive accumulation of pigment followed by death (Assaraf *et al.*, 1987). Unfortunately, DFMO inhibition of ODC seems to be circumvented by an exogenous supply of polyamines (Assaraf *et al.*, 1987). The intracellular polyamine pool is simply maintained by decreased polyamine excretion and increased polyamine uptake. Furthermore, DFMO might be transported poorly into the intraerythrocytic *P. falciparum* (Müller *et al.*, 2001). *In vitro* growth inhibition is also observed when AdoMetDC is inhibited with MGBG (Rathauer and Walter, 1987). Irreversible inhibition of AdoMetDC with the compound MDL 73811, inhibited growth of chloroquine-sensitive and -resistant strains of *P. falciparum* equally (Wright *et al.*, 1991).

When the polyamine biosynthetic enzymes from *P. falciparum* were studied at the molecular level, it was demonstrated that the ODC and AdoMetDC enzymes were components of a bifunctional protein (Müller *et al.*, 2001). The N-terminal region of the newly synthesized protein contains an AdoMetDC proenzyme, which cleaves itself to produce an active enzyme with a catalytically essential pyruvoyl residue at the new N-terminus. The AdoMet domain is connected to the C-terminal ODC domain through a hinge domain. The mature enzyme is a

heterotetrameric complex, containing two sets of the cleavage products. The significance of the bifunctional nature of this protein remains obscure, but the unique organisation of AdoMetDC and ODC may offer the possibility of therapeutic intervention, particularly because of regulatory differences between the enzymes of the parasite and the human host cells (Müller *et al.*, 2001). The polyamine enzymes exhibit features that differ significantly between the parasites and the human host. The ODC/AdoMetDC protein differs from its mammalian counterparts in its response to putrescine. The *P. falciparum* ODC is more strongly feedback regulated by its product putrescine than the mammalian enzyme (Wrenger *et al.*, 2001). The *Plasmodium* AdoMetDC is not stimulated by putrescine which is the case of the mammalian enzyme. Moreover, the *Plasmodium* ODC/AdoMetDC has a half-life of more than 2 h which is in contrast to the extremely short half-life of the mammalian monofunctional counterparts (Wrenger *et al.*, 2001). The generation of mutations in either the *Plasmodium* ODC or AdoMetDC domains revealed that the exchange of amino acids essential for the activity of one domain had no effect on the enzyme activity of the other domain. These results suggest that no domain-domain interactions occur between the two enzymes of the bifunctional protein and that both enzymatic activities can operate independently (Krause *et al.*, 2000; Wrenger *et al.*, 2001). Furthermore, it was shown that the hinge region of the bifunctional ODC/AdoMetDC is important for the catalytic activity of the *Plasmodium* ODC domain as it is necessary for the correct folding of the ODC domain (Krause *et al.*, 2000). The importance of the parasite-specific inserts for activity and protein–protein interactions of the bifunctional protein was investigated by deletion of these inserts in the bifunctional protein (Birkholtz *et al.*, 2004). These mutations diminished the corresponding enzyme activity and in some instances also decreased the activity of the neighbouring, non-mutated domain. In contrast to previous results, it was suggested that intermolecular interactions between AdoMetDC and ODC appear to be vital for optimal ODC activity. It was also shown that co-incubation of the monofunctional *Plasmodium* AdoMetDC and ODC domains produced an active hybrid complex of 330 kDa. The unique hinge region was proposed to be important for bifunctional complex formation (Birkholtz *et al.*, 2004)

rather than for catalytic activity of the ODC domain (Birkholtz *et al.*, 2004). It is conceivable that exploitation of such differences between the human and the *P. falciparum* polyamine biosynthesis enzymes can lead to the design of new inhibitors that will selectively kill the parasites while exerting minimal effects on the parasite-infected host.

1.8 Nematodes

1.8.1 *Caenorhabditis elegans* as a model organism for eukaryotic organisms

The phylum Nematoda consists of over 30,000 species although more than 500,000 different species are suspected to exist. Nematodes are one of the most ecologically diverse animal groups on earth and can be found in terrestrial and marine habitats, as free-living and parasitic pathogens of humans, animals and plants. Different species of nematodes may eat algae, bacteria, fungi, yeasts, diatoms or several kinds of small animals in soil or sediments. There are even large nematodes that eat smaller nematodes. Many serious tropical diseases of man and many diseases of domestic animals are caused by nematodes. While most nematodes in soil are actually beneficial, farmers are most concerned with nematodes that are pathogens of the roots, stems, leaves or seeds of plants. Only about 10% of all nematode species are plant parasites, however. Most are free-living species that feed on other organisms in marine sediments (50%) or nematodes in freshwater sediments or soil (25%). The remaining 15% are parasites of animals or man (http://plpnemweb.ucdavis.edu/nemaplex/General/general.htm).

Caenorhabditis elegans is a small (1.2 mm), free-living, bacteriovorous nematode. The classification of the free-living nematode *C. elegans* according to Blaxter *et al.* (1998) is the following:

Phylum Nematoda

Class Secernentia

Order Rhabditida

Superfamily Rhabditoidea

Family Rhabditidae

It is a representative of a large phylum with many parasitic members, for example *Brugia malayi* and *Ascaris lumbricoides*. The two sexual forms of *C. elegans* are the self-fertilizing hermaphrodites and the males. During embryogenesis about 550 cells are generated. Postembryonic cell divisions approximately double the cell number, so that the hermaphrodite adult has 959 somatic nuclei while the adult male has 1031 somatic nuclei (Bürglin *et al*., 1998). The haploid genome consists of six chromosomes with a total of 8 x 10^7 nucleotides (Wood, 1988). The full genome of the *C. elegans* organism has been sequenced in the *C. elegans* genome project, and the organism consists of approximately 20,000 genes (http://www.sanger.ac.uk/Projects/C_elegans/Genomic_Sequence.html).

C. elegans offers many features that make it a model system. Its life-cycle is very rapid; at 25°C it takes 2-3 days from zygote to zygote. *C. elegans* can easily be maintained on agar plates that have been inoculated with *E. coli* as a food source. Since this relatively simple organism is accessible for molecular, genetic and biochemical experiments it is an ideal model organism for “higher” organisms. Genes cloned from parasitic nematodes have usually an orthologue in *C. elegans* and therefore the *C. elegans* counterpart could provide useful insights about the parasitic genes and proteins (Bürglin *et al*., 1998).

1.8.2 Polyamines and the nematodes

The polyamine biosynthetic enzymes that have been characterised in *C. elegans* are the ODC and AdoMetDC (Macrae *et al*., 1995; Da’dara *et al*., 1998; Ndjonka *et al*., 2003). A gene of a potential antizyme has also been found in *C. elegans* (Ivanov *et al*., 2000). The spermine synthase is absent in *C. elegans* and therefore the worms cannot produce spermine. Polyamines are essential for the development of *C. elegans*. It has been shown that ODC-null-mutants can grow in a polyamine containing medium but produce less offspring (Macrae *et al*., 1995). ODC-null-mutants have two different fates, depending upon the developmental stage at which polyamines are removed. When the polyamines are

removed at the L1 larval stage, they develop into adult hermaphrodites that produce very few or no eggs. In contrast, if mutant larvae at the later L4 stage of development are transferred to polyamine-deficient medium, they develop and lay eggs normally. However, 90% of the eggs yielded embryos that did not develop. These effects can be reversed by the addition of putrescine and spermidine into the growth medium. This shows that the worms are capable of taking up polyamines from the medium (Macrae *et al.*, 1998). Furthermore, it has been shown that wild-type *C. elegans* can grow in axenic medium that is free from polyamines (Macrae *et al.*, 1998; Szewczyk *et al.*, 2003). This proves that the uptake of polyamines from the medium is not essential for wild-type *C. elegans* and that they are able to biosynthesize the polyamines.

The regulatory enzymes ODC has been found in the parasitic nematode *Haemonchus contortus* (Klein *et al.*, 1997) but the presence of an ODC is questionable in the filarial nematodes *Onchocerca volvulus* and *B. malayi*. However, a putative ODC-antizyme gene has been identified from *O. volvulus* (Ivanov et al., 2000). Therefore it is suggested that the parasitic nematodes can derive putrescine, spermidine and spermine from the medium and can catabolise the higher polyamines to putrescine via an interconversion pathway (Wittich *et al.*, 1987; Walter, 1988; Müller *et al.*, 1991).

1.9 Objectives and aims for the current study

The essential function of the polyamines for cell proliferation and differentiation make the enzymes of the polyamine metabolic pathway an important chemotherapeutic target not only for cancer but also for parasitic infections. In particular, ODC inhibitors have been successful for the therapy of West African sleeping sickness (Bacchi *et al.*, 1987). There are only a few chemotherapeutics available for the treatment of malaria. This is compounded by the increasing prevalence of multi-resistant *P. falciparum* worldwide that has become a serious public health threat to the global control of malaria. Therefore, there is an urgent and pressing need for new drugs attacking novel targets in the metabolism of the malaria parasite.

The biochemical characterization of the polyamine metabolic pathway of *P. falciparum* is essential to illustrate differences between the parasite and its human host. The polyamines are suggested to be more critical for rapidly growing and replicating *P. falciparum* than for normal human cells, and it is conceivable that exploitation of this pathway could lead to the detection of antiparasitic inhibitors that will have a more adverse effect on the parasites than the human host. The ODC and AdoMetDC from *P. falciparum* have been characterized. However, the third enzyme of the polyamine biosynthetic pathway, spermidine synthase, has not been investigated so far. The spermidine synthase from *P. falciparum* was suggested to be a drug target since the *P. falciparum* polyamine biosynthesis enzymes may exhibit features that are significantly different from those of the human host and also because the enzyme may have unique substrate preferences in comparison to the human enzyme.

The spermidine synthase enzyme from *P. falciparum* should be cloned, recombinantly expressed in *E. coli* and biochemically characterized. For further analyses mutations should be made of the recombinant protein to identify crucial amino acid residues which may be important for enzyme activity. Inhibitors should be tested on the molecular and whole parasite level, using the recombinant *P. falciparum* spermidine synthase and cultured *P. falciparum*, respectively.

The free-living nematode *C. elegans* has been found to be a good model system for investigating genes in eukaryotic organisms. The spermidine synthase from *C. elegans* should be cloned, recombinantly expressed in *E. coli* and biochemically characterized. Inhibition studies should be conducted and deletion mutations prepared with the *C. elegans* enzyme to investigate which amino acid residues are essential for enzyme activity. In collaboration with Dufe *et al.* at the University of Lund, Sweden, the recombinant *C. elegans* spermidine synthase protein should be used for crystallization and subsequent structural analyses. Furthermore, in comparative studies it was planned to clone, recombinantly express and characterize the spermidine synthase from the parasitic nematode *B. malayi*.

Chapter 2 Materials and methods

2.1 Chemicals and bioreagents

Amersham-Biosciences (Freiburg):

[α-^{32}P] dATP (3000 Ci / mmol), [α-^{35}S] dATP (1000 Ci / mmol), S-adenosyl-L-[carboxy$^{14}CO_2$] methionine (56 mCi / mmol), [1, 4-^{14}C]-putrescine dihydrochloride (107 mCi / mmol, [^{14}C]-spermidine trihydrochloride (112 mCi / mmol), Sequenase Version 2.0 DNA sequencing kit, ECL Plus western blotting detection reagents.

Becton Dickinson (Cockeysville):

BBL granulated agar, BBL bacto yeast extract, BBL bacto-tryptone.

Biomol (Hamburg):

Bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal), 1,4 dithiothreitol (DTT), glycerol, imidazole, lysozyme, phenol.

Bio-Rad (München):

Goat Anti-Mouse IgG (H + L)-HRP conjugate, Precision Plus Protein Standards.

Canberra-Packard (Frankfurt)

UltimaGold™ liquid scintillation cocktail.

Curasan (Kleinostheim):

Gencin (gentamicin).

Fluka AG (Neu-Ulm):

Putrescine, spermidine, spermine.

Gerbu (Gaiberg):

Ethylenediamine tetraacetic acid (EDTA), glycine, sodium chloride,
N’, N’, N’, N’-Tetramethylethylendiamine (TEMED).

Gibco BRL, Life Technologies (Eggenstein):

Agarose gel 1-kb ladder, albumax, RPMI 1640 medium, low melting point agarose.

IBA (Göttingen):

Anhydrotetracycline (AHT), desthiobiotin, Strep-Tactin sepharose, *Strep*-tag® II specific monoclonal antibody.

Invitrogen (Karlsruhe):

PCR Supermix, Taq DNA Polymerase, TOPO TA Cloning Kit, TRIzol™.

Jackson ImmunoResearch Europe Ltd. (Cambridgeshire):

Alkaline phosphatase-conjugated anti-rabbit IgG (H+L).

Macherey-Nagel GmbH (Düren):

Nucleobond AX DNA purification kit.

MBI (St. Leon-Rot):

High Fidelity PCR enzyme mix, dNTP set.

Merck (Darmstadt):

Acetic acid (glacial), Brij 35, chloroform, Coomassie-Brilliant Blue R-250, ethanol, formaldehyde, *D*(+)-glucose-monohydrate, hydrochloric acid, ß-mercaptoethanol, methanol, ortho-phosphoric acid, potassium dihydrogen phosphate, silica gel 60 TLC plates, sodium dihydrogen phosphate, sodium hydroxide, Triton X-100, Tween 20.

Molecular Probes Inc. (Eugene):

Cy3-labelled goat anti-rabbit IgG antibodies, 4',6-diamidino-2-phenylindole (DAPI).

New England Biolab (Frankfurt am Main):

Restriction enzymes, T4-DNA-ligase.

Novagen (Darmstadt):

His-Tag® Monoclonal Antibody.

Omnilab (Göttingen):

Sodium dodecylsulfate (SDS), urea.

Qiagen (Hilden):

Ni-nitrilotriacetic acid resin (Ni-NTA), QIAquick PCR purification kit, QIAGEN Plasmid Mini Kit.

Packard (Jügesheim):

Pico Prias vials.

Pharmacia (Freiburg):

HiLoad 16 / 60 Superdex 75 Column, Superdex S-200 column.

Promega (Mannheim):

Pfu DNA polymerase.

Roche (Mannheim):

DNase, DNA polymerase I (Klenow-Fragment), Random Primed DNA labelling kit, nylon membranes positively charged, RNase.

Roth (Karlsruhe):

Ampicillin, Rotiphorese® NF-Acrylamide / Bis-LSG 40% (29:1), isopropyl-β-D-thiogalactopyranoside (IPTG), Roti-Mark protein standard, tris-(hydroxymethyl)-aminomethane.

Serva (Heidelberg):

N-(2-Hxdroxyethyl) piperazin-N^1-2-ethanesulfonic acid (HEPES).

Sigma (München):

Ammonium persulphate (APS), S-adenosylmethionine, 5-bromo-4-chloro-3-indolylphosphate (BCIP), cyclohexylamine, dicyclohexylamine, ethidium bromide, gel filtration molecular weight marker kit, hypoxanthine, Kodak BioMax MR film**,** mineral oil, MTA, nitrobluetetrazoliumchloride (NBT), phenylmethylsulfonylfluoride (PMSF), sodium bicarbonate.

Schleicher & Schuell (Dassel):

Nitrocellulose transfer membrane Protran R.

Stratagene (La Jolla):

C. elegans λ-Zap cDNA library.

Whatman (Göttingen):

Chromatogragphy paper 1.

Vectors, inhibitors and antibodies from other laboratories

B. malayi cDNA library in λ Screen-1-vector was a kind gift from Dr. Peter Fischer (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany).

Trans-4-methylcyclohexylamine (4MCHA), 5-amino-1-pentene hydrochloride (APE) and (*S, R*)-dcAdoMet were generous gifts from Keijiro Samejima (Josai University, Saitama, Japan). 1-Aminooxy-3-aminopropane (APA) was kindly provided by Alex R. Khomutov (Russian Academy of Sciences, Moscow, Russia).

Plasmodium falciparum protein phosphatase 5 antibodies (anti-*Pf*PP5) were generously given by Dr. Mo Klinkert (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany).

2.2 Preparation of standard solutions

Earles Balanced Salt Solution (EBSS):

6.8 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4.7H_2O$, 0.158 g $NaH_2PO_4.2H_2O$, 0.264 g $CaCl_2.2H_2O$, 2.2 g $NaHCO_3$, 1.0 g *D*-glucose. The $CaCl_2.2H_2O$ was dissolved in 400 ml H_2O, mixed with the others substances and finally filled to 1 litre with H_2O. The solution was sterile filtered before use.

LB-medium:

1% Bacto tryptone, 0.5% Bacto yeast extract and 1% NaCl.

LB-agar:

LB-medium + 1.5% agar.

Agarose gel electrophoresis:

5 X TBE Buffer	450 mM Tris / 450 mM borate, 10 mM EDTA, pH 8.0.
5 X TAE Buffer	200 mM Tris, 1.5 M sodium acetate, 5 mM EDTA, pH 8.0.
6 X Loading Buffer	0.25% bromophenol blue, 0.25% xylene-cyanol, 40% glycerol.

Stock solutions for hybridization:

Southern hybridization solution	0.1% SDS, 5 X SSC, 5 X Denhardt's solution, 50 μg/ ml denatured herring sperm DNA, 1% NaPPi.
Northern hybridization solution	7% SDS, 0.5 M NaH_2PO_4, pH 7.2, 2% dextran sulphate.
20 X SSC	3 M NaCl and 0.3 M trisodium citrate dihydrate, pH 7.0.
100 X Denhardt's solution	2% Ficoll, 2% polyvinyl pyrrolidone and 2% BSA.
NaPPi	5% sodium pyrophosphate solution.

Stock solutions for DNA preparation:

STET-buffer	8% sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% Triton X-100.
NEW WASH	50% ethanol, 10 mM Tris-HCl, pH 7.5, 0.05 mM EDTA.

Stock solutions for recombinant protein purification and dialysis:

Lysis buffer	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl, 10 mM imidazole.
Wash buffer	50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl and 20 mM imidazole.
Elution buffer	50 mM NaH_2PO_4 pH 8.0; 300 mM NaCl and 1 M imidazole.
Buffer A	50 mM KH_2PO_4, pH 7.4, containing 1 mM EDTA, 0.1 mM DTT and 0.1 mM PMSF.

2.3 Vectors and cells

Plasmid vectors

pTrcHisB (Invitrogen), pASK-IBA3 (Institut für Bioanalytik, Göttingen), pACYC184 (New England Biolabs).

***E. coli* strain genotypes**

BL21 (DE3): F´ *dcm omp hsd*S($^{r}B^{-m}B^{-}$) gal, λ(DE3), (Stratagene).

BLR (DE3): F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) Δ (*srl-recA*)*306*::Tn*10* (Tet^R), (Novagen).

DH5α: Φ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_k-,m_k+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169, (Hanahan, 1985).

EWH331: Hfr, Δ(*spe*A *spe*B) Δ (*spe*C *glc*) Δ*spe*D *thr*-1 *pro*A2 *thi*-1 *lac*Y1 *gal*K2 *mtl*-1 *xyl*-5 *ara*-14 *str*-25 *spc sup*E44, λ^-, (Hafner *et al.*, 1979).

2.4 Culture of *P. falciparum*

P. falciparum 3D7 parasites were maintained in continuous culture according to Trager and Jensen (1976). Parasites were grown in human erythrocytes (blood group A+) in RPMI 1640 medium, supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, 40 μg ml^{-1} gentamicin, and 0.5% albumax at 4% hematocrit in 150-cm^2 flasks at 37°C in an atmosphere of 90% N_2, 5% O_2, and 5% CO_2. The percentage of infected erythrocytes and the development of the parasites were determined by light microscopy of Giemsa-stained thin blood smears.

For saponin lysis *P. falciparum* infected erythrocytes at a hematocrit of 25% were incubated for 10 min in ice-cold EBSS containing 0.15% (w/v) saponin before the addition of four volumes of ice-cold EBSS. The reaction mixture was centrifuged at 1500 X *g* for 5 min at 4°C, and the resulting pellet was washed twice in ice-cold EBSS.

2.4.1 Percoll-alanine separation of *P. falciparum* in culture

The *P. falciparum* culture was grown until approximately 15% parasitemia. The cells were then placed in 50 ml Falcon tubes and centrifuged at 2000 X *g* for 10 min. The *P. falciparum* pellet was washed once with EBSS without alanine. The resulting pellet was then mixed with EBSS containing 3% alanine. The different stages of the *P. falciparum* 3D7 culture were separated over a discontinuous Percoll-alanine gradient. The following solutions were used for the separation:

1) 90% hypertonic Percoll solution (3% alanine in 9 volumes Percoll + 1 volume 10 X RPMI 1640-medium).

2) 3% alanine in EBSS.

These two standard solutions were then used to make 80%, 70% and 60% dilutions of the original Percoll solution. The hypertonic Percoll solutions were then heated to 37°C.

The Percoll gradient was made in the following manner:

4 ml of 90% Percoll solution was placed into a 50 ml Falcon tube, followed by 3 ml of the 80%, 2 ml of the 70% and 2 ml of the 60% Percoll solutions. These Percoll layers were overlayed with *P. falciparum* infected red blood cells. The separation was conducted by centrifugation in a Heraeus (Megafuge 1.0 R) centrifuge for 30 min at 4000 X *g*. The separated stages were then placed into different tubes and washed twice with EBSS without alanine. For subsequent RNA isolation, the pellets were mixed in ½ volume of TRIzol™ per pellet volume and frozen at -20°C.

2.4.2 *In vitro* assay in *P. falciparum* culture

Spermidine synthase inhibitors were tested for their plasmodicidal activity using a [^{3}H]-hypoxanthine incorporation assay (Desjardins *et al.*, 1979). Parasitized erythrocytes (1.5% haematocrit at 1% parasitemia) were distributed in 96-well plates (Filtermat A 1450-421, Wallac). APA, APE, cyclohexylamine, dicyclohexylamine, 2-mercaptoethylamine and 4MCHA were used in concentrations ranging from 0.01 – 1.00 mM. After incubation for 24 h 100 nCi of [^{3}H]-hypoxanthine (Amersham Biosciences) was added to each well. Cultures were incubated for a further 24 h, before the contents of each well were collected on standard filter microplates and washed using a cell harvester (Inotech). Parasite growth was assessed by measuring the radioactivity incorporated by the parasites using a multi-detector liquid scintillation and luminescence counter (Wallac). The 50% inhibitory concentrations (IC_{50}) were calculated from inhibition curves using GraphPad Prism 1.02 (GraphPad Software).

2.5 Preparation of DNA and RNA

2.5.1 Preparation of genomic DNA from *P. falciparum*

Saponin isolated *P. falciparum* pellet was homogenised in 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% SDS and incubated overnight at 37°C with 100 µg/ ml Proteinase K. The following day, the mixture was placed on ice for 10 min before saturated NaCl solution (1/4 of the end

volume) was added. This mixture was vortexed and placed on ice for a further 5 min. Following centrifugation at 2000 X *g* for 15 min at 4°C, the supernatant was incubated with 20 µg/ ml RNase for 15 min at 37°C. The DNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) before precipitation using 100% ethanol. The pellet was washed with 70% ethanol and then dissolved in double distilled water.

2.5.2 Southern blot analysis

10 µg aliquots of genomic DNA were digested with *Nde*I, *Hind*III and *Bam*HI, respectively, before being resolved electrophoretically on a 0.8% agarose gel. After rinsing the gel in ddH_20, the DNA was denatured in 0.5 M NaOH, 1.5 M NaCl and transferred onto a nylon membrane as described by Southern's method (Sambrook *et al.* 1989). Following an overnight capillary transfer of the DNA in 10 X SSC onto a positively charged nylon membrane, the membrane was washed in 6 X SSC and dried at 80°C for 1 h (Sambrook *et al.* 1989). Subsequently, the membrane was pre-hybridized at 65°C in hybridization solution for 3 h. The membrane was hybridised overnight at 65°C with a ^{32}P radiolabelled probe corresponding to the coding region of *P. falciparum* spermidine synthase (Random Primed DNA Labeling Kit, Roche). The membrane was washed twice for 15 min in 2 X SSC, 0.1% SDS at room temperature (RT) and twice for 1 h in 1 X SSC, 0.1% SDS at 65°C. Signals were revealed by autoradiography using a Kodak Biomax-MR film.

2.5.3 Preparation of RNA from *P. falciparum*

The parasitized red blood cells were lysed in ½ volume of TRIzol™ per pellet volume as described by the manufacturer's instructions. The homogenized sample was incubated for 5 min at 37°C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of TRIzol™ was added to the tube and then the sample was mixed vigorously for 15 sec before incubation at 30°C for 2-3 min. This was followed by a centrifugation at 10,000 X *g* for 15 min at 4°C. Afterwards, the aqueous phase containing the

RNA was transferred to a fresh tube. The RNA was precipitated by mixing with ½ volume of isopropyl alcohol per 1 volume of TRIzol™ reagent. The samples were incubated at RT for 10 min and centrifuged at 12,000 X *g* for 10 min at 4°C. The supernatant was removed carefully and the pellet was washed once with 75% ethanol, adding 1 volume of 75% ethanol per volume of TRIzol™. The sample was mixed by vortexing and then centrifuged at 7,500 X *g* for 5 min at 4°C. The supernatant was removed and the RNA pellet was allowed to dry at RT for 5-10 min. The RNA was then redissolved in 20 µl of 100% formamide and stored at -70°C until used.

2.5.4 Northern blot analysis

Northern blotting was conducted according to the procedures of Kyes *et al*. (2000). Total RNA was extracted from asynchronous as well as from three stages of a synchronous *P. falciparum* 3D7 culture (young trophozoites 12 $\pm$ 4 h post invasion, old trophozoites 26 $\pm$ 4 h post invasion and schizonts 40 $\pm$ 4 h post invasion) using TRIzol™ reagent as described in section 2.5.3. 30 µg of RNA was separated on an agarose formaldehyde gel and transferred to a positively charged nylon membrane (Sambrook *et al.* 1989). The membrane was hybridized with a ^{32}P radiolabelled probe of the coding region of *P. falciparum* spermidine synthase in Northern hybridization solution at 45°C overnight. The membrane was then washed in 6 X SSC, 0.1% SDS at 45°C for 10 min followed by one wash in 2 X SSC, for 10 min at 45°C. Signals were visualized by exposure to a Kodak BIOMAX-BR film overnight. As a loading control the stage specific blot was hybridized with a *P. falciparum* rRNA probe.

2.5.5 DNA preparation from plasmid DNA

2.5.5.1 Mini-preparation of plasmid DNA

Small scale plasmid DNA preparations were made from 2 ml overnight *E. coli* cultures using the QIAGEN-Plasmid mini-kit as described by the manufacturer. Typically, about 15 µg of plasmid DNA was obtained.

Alternatively, an aliquot of 2 ml overnight *E. coli* culture was centrifuged at 13,000 X *g* and the bacterial pellet then resuspended in 250 µl STET-buffer. 100 µg of lysozyme was added to this suspension. This was followed by an incubation step on ice for 10 min and subsequent boiling for 1 min. The tube was then placed on ice for 30 sec, followed by centrifugation at 13,000 X *g* for 15 min. The supernatant was placed in a new tube and mixed with 750 µl of 6 M sodium iodide solution and 7.5 µl of glass milk (silicon dioxide) suspension. To bind the DNA, the suspension was shaken at 37°C for 5 min. The pellet was washed once with 400 µl NEW WASH and dried at 56°C for 5 min. The glass milk pellet was resuspended with 80 µl distilled water and incubated for 5 min at 56°C. The DNA was eluted by centrifugation.

2.5.5.2 Midi-preparation of plasmid DNA

Large-scale purification of plasmid DNA was performed using the Nucleobond AX DNA purification kit from Macherey-Nagel as described by the manufacturer. Typically, about 400 µg of high quality supercoiled plasmid DNA could be obtained from a 100 ml overnight *E. coli* culture.

2.6 Polymerase Chain Reaction (PCR)

PCRs were performed in 50 µl reaction volumes containing 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg / ml Nuclease-free BSA, 200 µM deoxyribonucleotides (dNTPs), 10 pmol of each oligonucleotide, 100 ng DNA-template and 1.0-2.5 U of polymerase. Samples were covered with mineral oil and 35 PCR cycles

were performed, consisting of an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing of the oligonucleotide at the appropriate temperature for 0.5-1.5 min, followed by a polymerase dependent extension at 68-72°C for a specific time depending on the expected fragment size. A final 10 min extension step was conducted at 68°C.

2.7 Restriction enzyme digestion of DNA

PCR products, vectors and DNA plasmid constructs were digested with the appropriate restriction endonucleases in the suitable buffer system according to the manufacturer's instructions.

2.8 Agarose gel electrophoresis of DNA

For analytical purposes, and depending on the size of the DNA fragments to be resolved, samples were mixed with loading buffer and separated on 0.8-1.5% agarose gels containing 0.5 µg/ ml ethidium bromide in TBE buffer. The 1 kb ladder (Gibco BRL) was used as the standard marker and the resolved DNA was visualized over UV light.
Low melting point agarose in TAE buffer was used to resolve DNA fragments for preparative purposes. The DNA bands of interest were excised from the gel and purified using the Gene-Clean technique.

2.9 Purification of DNA by the GeneClean technique

The gel slices excised from TAE agarose gels were mixed with sodium iodide (1:3 w/v) and incubated at 56°C until the agarose was completely dissolved. Glass milk (10 µl) was added and the mixture was shaken at RT for 10 min. The matrix was pelleted at 14,000 X *g* for 30 sec and washed twice with NEW WASH buffer. The glass milk was dried at 56°C and the bound DNA was eluted by resuspending the pellet in distilled water at 56°C for 5 min.

2.10 Ligation of DNA

DNA fragments were cloned into plasmid vectors as described by Sambrook *et al.* (1989). The vectors were digested either by single or double digestion with the appropriate restriction enzymes. The vector DNA and the DNA fragment were then mixed in a molar ratio of 1:3 and ligated in a 15 µl ligation volume overnight at 14°C, using 1 U of T4 DNA-ligase.

2.11 Transformation

2.11.1 Competent bacteria production

Bacteria were made competent for transformation by the method of Hanahan (1985). A bacterial colony was inoculated in 10 ml LB-medium and left to grow overnight at 37°C. The culture was diluted 1:100 in 100 ml LB-medium and allowed to grow until OD_{600} = 0.5. The cells were chilled on ice for 15 min. The following steps were performed at 4°C. After centrifugation for 10 min at 4000 X *g* the resulting bacterial pellet was resuspended and incubated in ice cold 80 mM $CaCl_2$ for 30 min. The cells were pelleted again and then resuspended in 10 ml ice-cold 100 mM $CaCl_2$ and 20% glycerol solution, aliquoted and stored at -70°C.

2.11.2 Transformation of competent cells

The transformation of the bacterial cells was performed according to the method of Cohen *et al.* (1972). The competent cells were slowly allowed to thaw on ice, and were then incubated for 30 min with plasmid DNA or 5 µl of ligation product. The cells were heat shocked at 42°C for 1 min and subsequently cooled on ice for 2 min. 700 µl of LB medium was added and the mixture was agitated at 37°C for 1 h. The cells were then plated on LB agar plates containing the appropriate selection antibiotic for the vector used and incubated overnight at 37°C.

2.12 DNA sequencing and analyses

DNA sequencing was performed according to the dideoxy chain termination method of Sanger *et al.* (1977). The Sequenase Version 2.0 DNA sequencing kit was used, with vector or gene-specific oligonucleotides. The double-stranded DNA was denatured for 10 min with 0.2 M NaOH at 37°C. The DNA was finally precipitated with 3 M ammonium acetate (pH 5.2) and 100% ethanol, washed with 70% ethanol, dried at 56°C and eventually resuspended with distilled water in order to prepare it for the sequence reaction. The sequence reaction was conducted according to the manufacturer's instructions. Prepared samples were heat-denatured at 95°C for 2 min and loaded on a 8% polyacrylamide gel. Following electrophoresis at 80 W for 2-4 h the gel was dried at 80°C under vacuum and autoradiographed overnight. Homology comparisons were performed using the BCM Search Launcher programme (http://searchlauncher.bcm.tmc.edu/), the National Center for Biotechnology and Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and PlasmoDB databases (http://www.plasmodb.org/).

2.12.1 Phylogenetic analysis

The sequences of spermidine and spermine synthases from various organisms were selected by PubMed (NCBI). The *P. falciparum* spermidine synthase sequence was also included into the dataset. Amino acid sequence alignment was made using the ClustalX program [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 2.1. Cladograms were constructed by a minimum evolution tree analysis using the neighbour-joining algorithm. The degree of confidence for the internal linage was determined by the bootstrap method (Kumar *et al.*, 2001).

2.13 Expression constructs for spermidine synthase

2.13.1 Cloning and expression of recombinant *P. falciparum* spermidine synthase

The gene encoding *P. falciparum* spermidine synthase (accession no. AJ271622) was identified by BLAST search in PlasmoDB (http://www.plasmodb.org/) using the protein sequence of human spermidine synthase (accession no. AAH33106) as query.

The coding region of *P. falciparum* spermidine synthase was amplified from *P. falciparum* cDNA using the sense oligonucleotide SPDS-S1: 5'-GCGCGCGGATCCCATG GATAAATTAATATCAAACAAT-3' containing a *Bam*HI restriction site and the antisense oligonucleotide SPDS-AS1: 5'-GCGCAAGCTTTTATATGTTTTCTATTTCTTTTAAAAG-3' containing a *Hind*III restriction site. The PCR product was made by denaturing for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, 2.5 min at 68°C with an extension time of 10 min at 68°C. The obtained PCR fragment was cloned into pCRII™ (Invitrogen) resulting in the construct pCRII:*Pf*SPDS1. The nucleotide sequence for the construct was determined according to the method in section 2.12.

The complete open reading frame of *P. falciparum* spermidine synthase (construct pCRII:*Pf*SPDS1) was cloned into pTrcHisB vector leading to the expression construct pTrcHisB:*Pf*SPDS. Alternatively, a *P. falciparum* spermidine synthase fragment lacking the first 87 bp was amplified by PCR with the oligonucleotides SPDS-S2 5'-GCGCGGATCC GAATAAATTTCATCTTTCCCAG-3' and SPDS-AS1 using pCRII:*Pf*SPDS1 as template. The fragment was cloned into pTrcHisB vector leading to the expression construct pTrcHisB:*Pf*SPDS2. The sequences of the spermidine synthase insert of both expression constructs were verified by sequencing. Positive clones were transformed into *E. coli* BLR (DE3) cells.

A fresh overnight culture initiated from one bacterial colony of the *E. coli* expression cells containing the plasmid pTrcHisB:*Pf*SPDS2 was diluted 1:100 in Luria-Bertani medium supplemented with 100 µg ml^{-1} ampicillin and allowed to grow at 37°C to OD_{600} = 0.5.

Expression of the recombinant protein was initiated with 1 mM IPTG and incubation continued for a further 4 h at 37°C. The cells were then harvested by centrifugation at 10,000 X *g* for 30 min at 4°C, resuspended in lysis buffer containing 0.1 mM PMSF and sonicated. Insoluble material was removed by high speed centrifugation at 100,000 X *g* for 1 h at 4°C using a TFT 55.38 rotor and Centricon T-1065 from Kontron. The supernatant was applied to nickel-chelating affinity matrix for subsequent protein purification.

2.13.2 Cloning and expression of recombinant *C. elegans* spermidine synthase

A putative *C. elegans* spermidine synthase is annotated in the *C. elegans* genome on chromosome II (gene Y46G5A.19, EMBL accession no. AL110485). The corresponding open reading frame was amplified by PCR using *C. elegans* cDNA or a *C. elegans* λ-Zap cDNA library (Stratagene) as template and gene specific oligonucleotides based on the identified genomic sequence CeSPDSExS: 5‘-GGATCCCATGAACAAGCTGCACAAGGGA-3‘ and CeSPDSExAS: 5‘-AAGCTTCTACTCCAAAGCATT TTTGAC-3‘ (introduced restriction sites for *Bam*HI and *Hin*dIII are underlined). PCR was performed as follows: 95°C for 2 min, 50°C for 1 min, and 68°C for 2 min, for 30 cycles using Elongase amplification system (Invitrogen). The PCR product was subcloned for sequence analysis into pCRII™ vector using TA cloning (Invitrogen). The open reading frame of *C. elegans* spermidine synthase was cloned into pTrcHisB vector (Invitrogen) to produce a His-tag fusion protein. The recombinant expression plasmid pTrcHisB:*Ce*SPDS was sequenced to ensure that the insert was in the correct reading frame. Subsequently, the *E. coli* strain BLR (DE3) was transformed with pTrcHisB:*Ce*SPDS. The expression of the construct and handling thereafter was conducted as above in section 2.13.1.

2.13.3 Cloning and expression of recombinant *B. malayi* spermidine synthase

Using the protein sequence of *C. elegans* spermidine synthase as query (gene Y46G5A.19, EMBL accession no. AL110485), the gene encoding the *B. malayi* spermidine synthase was identified by BLAST search in the Nematode database (http://www.nematodes.org/ncbi_blast.html).

The coding region of *B. malayi* spermidine synthase was amplified with High Fidelity PCR enzyme mix from *B. malayi* cDNA library in λ Screen-1-vector using the sense oligonucleotide 5'-CGGGATCCGATGAATGTCTTTCGAGATGG-3' containing a *Bam*HI restriction site and the antisense oligonucleotide 5'-CCGCTCGAGTTACACA CATAAAGCTTCTTTTATG-3' containing a *Xho*I restriction site. The PCR product was made by denaturing for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, 1.5 min at 72°C with a final extension time of 10 min at 72°C. The resulting fragment was cloned into pCRII vector (Invitrogen). Following a digestion with *Bam*HI and *Xho*I the ORF of *B. malayi* spermidine synthase was subcloned into the expression vector pTrcHisB (Invitrogen) and transformed into DH5α *E. coli* cells.

The sequence of the *B. malayi* spermidine synthase insert in pTrcHisB vector was verified using manual sequencing as stated in the Sanger method. The construct was subsequently transformed into competent BLR (DE3) cells.

The expression of the construct and handling thereafter was conducted as in section 2.13.1. A modification to the above method was that after induction with 1 mM IPTG, the recombinant protein expression was incubated for 12 h at a temperature of 25°C.

2.13.4 Co-expression of *P. falciparum* ODC/AdoMetDC with *P. falciparum* spermidine synthase

The role of the bifunctional organisation of the *P. falciparum* ODC/AdoMetDC is still unclear. One explanation may be that this organisation allows the efficient transfer of putrescine and

dcAdoMet, the products of ODC and AdoMetDC respectively, to spermidine synthase. The reaction is suggested to take place via an enzyme-complex that is built between the two participating proteins. Hence it is suggested that substrate channelling may take place between *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase.
The intermolecular protein-protein interactions between *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase were analysed. In collaboration with Birkholtz, the separately expressed and isolated recombinant *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase were co-incubated at RT for 10 min. The protein mixture was subjected to FPLC and the *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase activities determined as described in Birkholtz (2002).
A second strategy was followed for the coexpression of the two proteins in *E. coli*. In this approach the *P. falciparum* spermidine synthase including the expression cassette was extracted from the construct pTrcHisB:*Pf*SPDS2 and cloned into the pACYC184 vector. Co-expression was achieved by co-transformation of two different expression plasmids. One of them pASK-IBA3 contains an ampicillin resistance marker and a Strep-tag. The plasmid pACYC184 contains a chloramphenicol resistance marker and a His-tag.
The oligonucleotides used for the PCR were the following:-

pTrcHisB-*EcoR* I-Sense: GCGC<u>GAATTC</u>CGCGAGGCAGCAGATCAATTCG

pTrcHisB-*Sac* II-Antisense: GAGA<u>CCGCGG</u>AACCCCCCATGGTTTATTCCTC

This construct pACYC184:*Pf*SPDS2 was co-transformed and co-expressed in EWH331 *E. coli* cells along with the expression plasmid *P. falciparum* ODC/AdoMetDC in the pASK-IBA3 vector (Müller *et al.*, 2000). The bacterial culture was grown at 37°C until OD_{600} = 0.5. The expression of *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase was initiated with 200 ng/ ml anhydrotetracycline and 1 mM IPTG, respectively. The cells were grown an additional 12 h at 25°C before being harvested. The expression culture was centrifuged for 30 min at 4,000 X *g* at 4°C and the cell pellet resuspended in PBS solution, pH 7.0. This mixture was sonicated on ice and then centrifuged at 100,000 X *g* for 1 h. The resulting supernatant was analysed using a native PAGE (see section 2.16.2).

2.13.5 Purification of recombinant *P. falciparum* spermidine synthase

His-tagged recombinant protein was purified batch-wise by nickel-chelating affinity chromatography (Qiagen) according to the manufacturer's recommendations. All solutions were used at the concentrations stated and all steps were done at 4°C. 500 µl of His-Bind resin (Qiagen) was packed at 400 X *g* for 4 min. The overlying solution was discarded and protein supernatant samples were applied and incubated for 4 h at 4°C.
Non-specifically bound contaminating proteins were washed from the resin twice with 20 ml of wash buffer. The bound *P. falciparum* spermidine synthase protein was eluted with 2 ml of elution buffer. For further purification and in order to determine the molecular weight of *P. falciparum* spermidine synthase, the eluate was applied to fast protein liquid chromatography on a calibrated Superdex S-75 gel sizing column (Hiload 16/60, Pharmacia Biotech), previously equilibrated with 50 mM potassium phosphate buffer pH 7.0, 1 mM DTT, 1.25 mM Na_2EDTA, 0.02 % Brij 35. The Superdex column was calibrated with the molecular weight marker kit, with a range of 1350-158,000 to determine the molecular size of the active enzyme.

2.13.6 Purification of recombinant *C. elegans* spermidine synthase

Recombinant *C. elegans* spermidine synthase was purified from the supernatant as stated above in section 2.13.5. In modification to that section, the protein was dialysed against 1000 volumes of buffer A. To determine the molecular weight of the *C. elegans* spermidine synthase, the eluate of the chelating chromatography was subjected to fast protein liquid chromatography on a calibrated Superdex S-200 column (2.6 cm x 60 cm) equilibrated with buffer A at a flow rate of 2 ml min^{-1}. The Superdex column was calibrated with the following standard proteins: (1) thyroglobulin (670,000 Da) (2) alcohol dehydrogenase (150,000 Da) (3) bovine serum albumin (67,000 Da) (4) carbonic anhydrase (29,000 Da)

(5) cytochrome C (12,400 Da).

2.13.7 Purification of the recombinant *B. malayi* spermidine synthase

The recombinant *B. malayi* spermidine synthase was purified according to the method in section 2.13.6.

2.14 Site-directed mutagenesis

The replacement of amino acids from the *P. falciparum* spermidine synthase was conducted by PCR mutagenesis (Sambrook *et al.*, 1989). Complementary oligonucleotides were designed to mutate the sites of interest.

Primers	Primer Sequence
Asp-Asn196-Sense	TATGATGTTATTATCGTA**AAT**AGTTCAGATCCAATAGGA
Asp-Asn196-Antisense	TCCTATTGGATCTGAACT**ATT**TACGATAATAACATCATA
Ser-Ala197-Sense	GATGTTATTATCGTAGAT**GCT**TCAGATCCAATAGGACCA
Ser-Ala197-Antisense	TGGTCCTATTGGATCTGA**AGC**ATCTACGATAATAACATC
Tyr-Ala102-Sense	GAAAAAGATGAATTTGCT**GCT**CATGAAATGATGACACAT
Tyr-Ala102-Antisense	ATGTGTCATCATTTCATG**AGC**AGCAAATTCATCTTTTTC

Table 2.1 List of primers used to mutate *P. falciparum* spermidine synthase. Mutated triplets are shown in bold.

The wild-type expression construct pTrcHisB:*Pf*SPDS2 was used as the template. 50 µl reaction mixture was made which contained 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 1 % Triton X-100, 1 mg/ ml nuclease-free BSA, 200 µM deoxyribonucleotides 5´-dNTP's, 10 pmol from each oligonucleotide, 100 ng DNA template, 1-2.5 U of *Pfu*-polymerase. The DNA was first denatured for 2 min at 95°C. 18 PCR cycles then followed, each cycle consisting of a denaturation step at 95°C for 1 min, one

oligonucleotide hybridization step at 48°C for 1 min and an extension step at 68°C for 10 min. After the PCR, the product was mixed with 10-15 U of *Dpn*I-enzyme and placed at 37°C for 1 h. This step made sure that the methylated template DNA was digested. The PCR product was then transformed into DH5α cells. The construct was sequenced to check whether the mutagenesis was successful. Positive clones were transformed into *E. coli* BLR (DE3) cells. The expression of the mutants and handling thereafter was conducted as in sections 2.13.1 and 2.13.5.

2.14.1 Deletion mutagenesis

PCR mutagenesis was conducted to create a deletion mutant from the wild-type construct pTrcHisB:*Ce*SPDS. 1µl of pTrcHisB:*Ce*SPDS DNA was used as the template for the PCR using the sense oligonucleotide *Ce*-Spd-Mut-S:
5’-AAGGGATGGTTCACCGAGTTCTCGCCGGCGTGGCCGGGACAGGCGTTCTCGTTG-3’
and the antisense oligonucleotide *Ce*-Spd-Mut-AS:
5’-CAACGAGAACGCCTGTCCCGGCCACGCCGGCGAGAACTCGGTGAACCATCCCTT-3’
The technique used overlapping oligonucleotides which were designed in inverted tail-to-tail directions to amplify the cloning vector together with the target sequence. A deletion was generated by amplification with these primers because they have a corresponding gap between their 5' ends. After the PCR with these primers, amplified linear DNA was selfligated, and used to transform appropriate competent cells.
The PCR reaction was catalysed using *Pfu* polymerase enzyme by denaturing for 1 minute at 94°C, followed by 18 cycles of 1 minute at 94°C, 1 minute at 55°C, with an extension time of 10 minutes at 68°C. The PCR product was digested with *Dpn*I enzyme and then transformed into DH5α cells. The sequence of the deletion mutant *C. elegans* spermidine synthase insert in pTrcHisB vector was verified by sequencing and the construct was then transformed into competent BLR (DE3) cells. The expression of the deletion mutant and handling thereafter was conducted as in sections 2.13.1 and 2.13.6.

2.15 Bradford assay for protein determination

The concentration of soluble proteins were determined as described by Bradford (1976), using BSA as the standard.

2.16 Protein gel electrophoresis

2.16.1 SDS-PAGE

The homogeneity of the enzyme preparations were analysed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Stock solutions

Monomer stock solution: 29.1 acrylamide, 0.9% bis-acrylamide

Electrode buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS (w/v).

4 X Sample buffer: 200 mM Tris-HCl, pH 6.8, 400 mM DTE, 8% SDS (w/v), 40% glycerine, 0.1% bromophenol blue (w/v).

4% Stacking gel:

1 ml Tris-HCl buffer, pH 6.7, 540 µl bis-acrylamide, 5 µl TEMED, 0.1% SDS, 25 µl 10% APS, 2.44 ml H_2O.

10% Separating gel:

1.5 ml Gel buffer, 2 ml bis-acrylamide, 5 µl TEMED, 0.1% SDS, 25 µl 10% APS,
2.5 ml H_2O.

For analytical purposes, protein samples were resolved by discontinuous polyacrylamide gel electrophoresis under denaturing conditions, according to the method of Laemmli (1970). Samples were mixed with sample buffer and denatured for 5 min at 100°C. Following centrifugation at 10,000 X *g* for 1 min, samples were loaded onto a 4% stacking, 10% separating gel. Protein molecular weight markers (Roth) were used as standards. Electrophoresis was carried out in SDS running buffer at 25 mA for 2 h. The gel was then recovered and proteins were visualized by Coomassie Brilliant Blue staining.

2.16.2 Native gel electrophoresis

7 X Native gel upper (stacking) buffer:	5.7 g Tris base, pH to 6.7 with H_3PO_4, ad 100 ml H_2O.
4 X Native gel lower (separating) buffer:	18.2 g Tris Base, pH to 8.9 with HCl, ad100 ml H_2O.
50 X Running buffer:	7.5 g Tris base, 36 g Glycine, H_2O to 250 ml.
3 X Sample buffer:	3 ml glycerol, 0.6 ml 50 X Running Buffer, 6.4 ml H_2O, bromophenol blue.

Native gel recipe:

Separating layer (lower; 7.5%):	6 ml H_2O, 3 ml lower buffer, 3 ml Acrylamide, Bis (30/0.8), 7 µl TEMED, 84 µl 10% APS.
Stacking layer (upper; 4.3%):	5 ml H_2O, 1 ml upper buffer, 1 ml Acrylamide, Bis (30/0.8), 8 µl TEMED, 40 µl 10% APS.

The bacterial sample from the co-expression of *P. falciparum* ODC/AdoMetDC with *P. falciparum* spermidine synthase (see section 2.13.4) was mixed with 5 µl native sample buffer and then loaded onto the native gel. The gel was run at 15 V for 12 h at 4°C. The protocol was modified from Clos *et al.* (1990). (For subsequent Western blotting of the native gel see section 2.18.2).

2.16.3 Protein staining in gels

Gels were stained for 1 h in Coomassie stain solution (0.1% Coomassie Brilliant Blue R-250 (w/v in ethanol), 10% glacial acetic acid). Afterwards, the gel was placed in destaining solution (20% ethanol, 10% glacial acetic acid) until the background cleared and the protein bands became visible.

2.17 Production of Antiserum

The production of *P. falciparum* spermidine synthase specific antibodies were made by immunising rabbits with recombinant spermidine synthase protein (EurogenTec, Belgium). Antibodies were stored at -20 ^{o}C until required.

2.18 Western blotting

2.18.1 Western blot analyses of *P. falciparum* spermidine synthase

85 ng of recombinant *P. falciparum* spermidine synthase and 10 µg from a 15,000 X *g* supernatant of isolated *P. falciparum* cells obtained by saponin lysis were applied to SDS-PAGE. Protein was blotted onto a nitrocellulose membrane following SDS-PAGE, by the method of Towbin *et al.* (1984), using the electrophoresis transfer unit 2117-250 NOVABLOT (Pharmacia, Freiburg). After blocking overnight with BSA blocking buffer (3% BSA in PBS) at 4°C, the membrane was incubated for 2 h in primary antibody, rabbit anti-*P. falciparum* spermidine synthase serum, 1:500 diluted in 1% BSA in PBS. Subsequently, the membrane was washed three times for 5 min in 0.05% Tween 20 in PBS and incubated for 1 h in horseradish peroxidase conjugated anti-rabbit IgG (Amersham Biosciences) diluted 1:10,000 in 1% BSA in PBS. The protein was visualised with freshly prepared ECL detection mixture following the manufacturer's protocol (Amersham Biosciences).

Stage specific expression of *P. falciparum* spermidine synthase during the asexual life cycle of the parasite was analysed by using aliquots of synchronized parasites that were harvested at three time points post invasion according to 2.4.1. After saponin lysis, parasites were washed 3 times with PBS and subsequently resuspended in Laemmli Buffer. Parasite proteins were separated on SDS-PAGE gels (10% acrylamide) and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with skim milk blocking buffer (5% (w/v) skim milk in PBS) for 1 h, and either incubated with rabbit anti-*P. falciparum* spermidine synthase serum or anti-*P. falciparum* protein phosphatase 5 (PP5)

serum diluted 1:1000 in skim milk blocking buffer for 1 h. The blot was consequently washed 3 times for 10 min with 0.05% Tween20-PBS. The membranes were incubated with horseradish peroxidase sheep anti-rabbit IgG antibodies (1:5000, Molecular Probes) for 1 h and developed by chemiluminescence using ECL (Amersham International). *P. falciparum* PP5 was used as loading control, since it has been reported to be equally expressed throughout the erythrocytic cycle of *P. falciparum* (Lindenthal and Klinkert, 2002).

2.18.2 Western blot analyses of the co-expressed *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase

The gel from section 2.16.2 was incubated for 4 h in a 0.1% SDS running buffer and then transferred onto a nitrocellulose membrane (see section 2.18.1). This blot was subsequently hybridized with monoclonal anti-Strep-Tag antibody which would detect the Strep-tag of the bifunctional *P. falciparum* ODC/AdoMetDC. An anti-mouse-HRP-antibody was used as the second antibody. The bands were detected by chemiluminescence, according to the manufacturer's instructions using the ECL Plus western blotting detection reagents.

The blot was stripped using 10 ml 0.2 M glycine, HCl (pH 2.8) by incubation in this buffer for 5 min, followed by neutralisation with the addition of 10 ml of 1 M NaOH. The blot was rinsed once with 0.05% Tween20-PBS and finally washed with PBS. The membrane was subsequently blocked overnight at 4°C using BSA blocking buffer (3% BSA in PBS) and then hybridized with a His-Tag® monoclonal antibody which was intended to detect the 6 X His-Tag of the *P. falciparum* spermidine synthase. An anti-mouse-HRP-antibody was used as the second antibody. The bands were detected by chemiluminescence, according to the manufacturer's instructions using the ECL Plus western blotting detection reagents.

2.19 Immunofluorescence

Immunofluorescence assays (IFAs) of synchronised infected erythrocytes were performed on air-dried thin blood smears after fixation in 100% methanol at -20°C for 5 min. The slides were dried and stored at -70°C (Tonkin *et al.*, 2004). Slides were incubated at RT for 1 h with rabbit anti-*P. falciparum* spermidine synthase serum (1:2000), washed 3 times for 10 min with 0.05% Tween20-PBS and then incubated for 1 h with Cy3-labelled goat anti-rabbit IgG antibodies (1:1000) and DAPI DNA stain (1:2000). Dual-colour fluorescence images were captured using a Zeiss Axioskop 2 microscope and a digital camera (PCO sensicam).

2.20 Aminopropyltransferase enzyme assays

Aminopropyltransferase activity was assayed by measuring the formation of [^{14}C] labelled reaction products from [1,4-^{14}C]-putrescine or [^{14}C]-spermidine following Zappia *et al.* (1980). The standard reaction mixture in a final volume of 100 µl contained 50 mM potassium phosphate buffer pH 7.0, 1 mM DTT, 1.25 mM Na_2EDTA, 200 µM of (*S,R*)-dcAdoMet, and 0.2 µg to 1 µg of recombinant *P. falciparum* spermidine synthase, *C. elegans* spermidine synthase and *B. malayi* spermidine synthase protein respectively, along with 200 µM putrescine or spermidine. Dejima *et al.* (2003) have previously demonstrated that the biologically inactive *R*-isomer of dcAdoMet that is present in equal amounts in the preparation did not significantly affect spermidine synthase reaction. Assays were incubated at 37°C for 30 min and then terminated by placing the reaction tubes at 100°C for 5 min. Reaction products were identified by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) analyses as described below. For the determination of K_m values, concentrations of putrescine varied from 25 – 1000 µM and of (*S*)-dcAdoMet from 10 – 300 µM.

Inhibition tests were performed in standard assays supplemented with varying concentrations of the inhibitors APA (5 – 500 µM), APE (1 – 1000 µM), cyclohexylamine (1 – 1000 µM), dicyclohexylamine (10 – 1000 µM), 2-mercaptoethylamine (10 –1000 µM), 4MCHA (0.5 – 50

µM) and MTA (5 – 1000 µM). For the determination of the K_i values 4MCHA was used in the range of 0.5 – 2 µM. Kinetic parameters were calculated by Lineweawer-Burk plots using the program GraphPad Prism 1.02 (GraphPad Software, San Diego, CA).

2.21 Product analyses by thin layer chromatography (TLC)

For TLC analyses, 10 µl of the assays together with 2 µl of the respective polyamine standards (50 mM) were separated on silica gel 60 (Merck) using the solvent system: ethyleneglycol-monomethylether / propionic acid / NaCl (saturated) (140:30:30, v/v/v) (Abo-Dalo, 2004). Reaction products were visualised either by ninhydrine staining at 60°C or by autoradiography. Spots were cut out and radioactivity was measured by means of a liquid scintillation analyzer (United Technologies Packard 2000CA Tri-CARB®) using 4 ml Packard UltimaGold™ liquid scintillation cocktail.
In spermidine synthase assays the amount of spermidine formed from labelled putrescine was calculated from the [^{14}C] spermidine / [^{14}C] putrescine ratio on the thin-layer plate. This was possible since in the assays spermidine was the only labelled metabolite produced from putrescine (Wiest and Pegg, 1998).

2.22 Product analyses using high performance liquid chromatograhy (HPLC)

For HPLC analyses the samples from section 2.20 were dansylated. 100 µl samples were placed into a glass tube and the volume filled up to 1 ml with distilled water. The pH was neutralized by adding $NaHCO_3$ until it was saturated in the mixture. Then 1 ml dansylchloride (2 mg/ ml acetone) was added after which the sample was incubated overnight in the dark at RT. This is a method used to add a dansyl group to the free amino groups at the terminal ends of the polyamines. The dansylated amino groups, isolated after hydrolysis of the polyamines, are fluorescent and may be detected in nanomolar quantities by HPLC. The dansylated polyamine sample was extracted with 1 ml toluene and dried in a vacuum

centrifuge SpeedVac SVC 100 (Savant). The dried sample was resuspended in 100 µl methanol/ acetic acid (95/5, v/v) and stored at -20°C until used for HPLC analysis.

50 µl aliquots of the dansylated samples were applied to a HPLC apparatus fitted with the Spherisorb ODS II column (5 µm, 250 x 3 mm, Machery-Nagel). Separation was achieved at a flow rate of 0.6 ml / min by using the following mobile phase and gradient programme:

Phase A: 10 mM Na_2HPO_4, 0.2% triethylamine, pH 7.17 (The pH was adjusted using concentrated acetic acid), 30% acetonitrile.

Phase B: 100 % acetonitrile.

The programme was the following:

0	20	36	42	54	68	min
0	38	50	82	87	0	% B

Dansylated derivatives were detected with a fluorescence spectrophotometer (excitation 365 nm, emission 425 nm, SFM 25, Kontron) and radioactivity measured by a flow-through radiodetector (LB 506, Berthold). To find out the retention times of the polyamines, the dansylated standards putrescine, spermidine and spermine were applied to the HPLC column (Seiler and Knodgen, 1978).

Chapter 3 Results

3.1 Genomic organisation

The nucleotide sequence of *P. falciparum* spermidine synthase was determined by BLAST search using known spermidine synthase amino acid sequences as query. The gene that consists of four exons and three introns is situated on chromosome 11 between two hypothetical proteins PF11_0300 (1245 bp upstream of the translational start site) and PF11_0302 (2575 bp downstream of the stop codon) (PlasmoDB; http://plasmodb.org/). Southern blot analysis using *P. falciparum* genomic DNA digested with the endonucleases *Nde*I, *Hin*dIII and *Bam*HI suggests that *P. falciparum* contains only one copy of the spermidine synthase gene (Figure 3.1). Executing additional BLAST searches using known spermine synthase amino acid sequences as query gave no suggestion for a second aminopropyltransferase in the genome of *P. falciparum*.

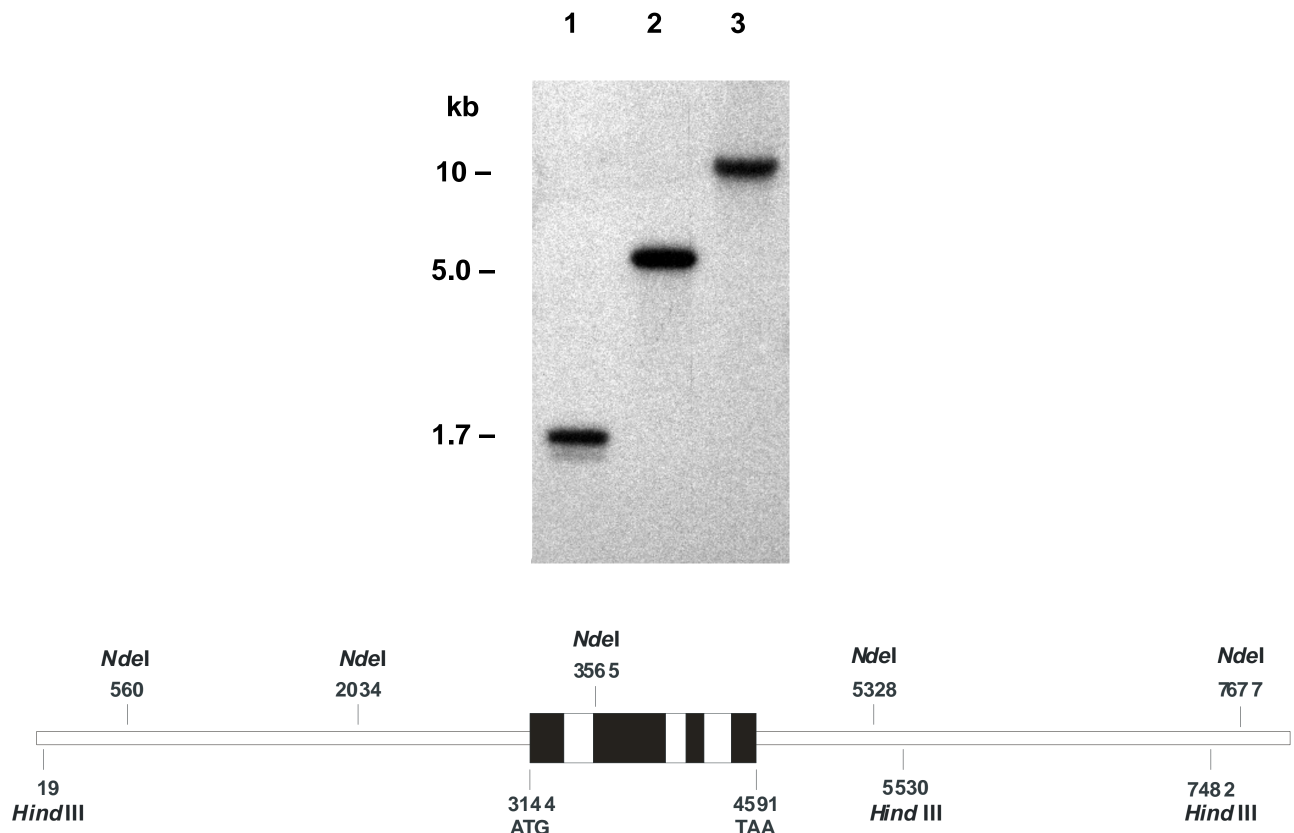


Figure 3.1 Genomic structure and Southern blot analyses of the *P. falciparum* spermidine synthase gene. Genomic DNA of *P. falciparum* 3D7 was digested with (1) *Nde* I, (2) *Hind* III, (3) *Bam*HI. Following Southern transfer, the membrane was hybridised with a radiolabelled probe of the complete ORF of *P. falciparum* spermidine synthase. The appearing bands are in accordance with the genomic sequence shown below.

3.2 Stage specific expression of spermidine synthase in *P. falciparum*

Equal amounts of total RNA from three stages of highly synchronised *P. falciparum* 3D7 culture were analysed by Northern blot hybridization using the *P. falciparum* spermidine synthase open reading frame as probe. A transcript of approximately 1.6 kb was identified (Figure 3.2), exhibiting nearly one and a half times the size of the corresponding open reading frame. *P. falciparum* spermidine synthase is stage specifically expressed with the amount of transcript peaking at the old trophozoite stage (Figure 3.2).

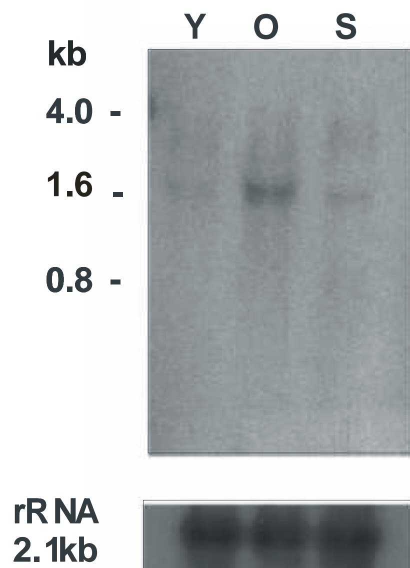


Figure 3.2 Northern blot analyses.
Total RNA from three different developmental stages of *P. falciparum* (Y, young trophozoites; O, old trophozoites; S, schizonts) were investigated using a radiolabelled probe that encompasses the ORF of *P. falciparum* spermidine synthase. The maximal amount of transcript is seen at the old trophozoite stage. As a loading control the blot was reprobed with radiolabelled *P. falciparum* rRNA.

3.3 Amino acid sequence analysis

The 966 bp open reading frame (ORF) of *P. falciparum* spermidine synthase was amplified by PCR from *P. falciparum* cDNA. The nucleotide sequence encodes a deduced polypeptide of 321 amino acids that exhibits a moderately high degree of identity to the amino acid sequences of human (37.4%), *Arabidopsis thaliana* (45.5%) and *Leishmania donovani* (36.8%) spermidine synthases (Figure 3.3 A). The spermidine synthase from *P. falciparum* possesses an N-terminal sequence extension that is not present in the spermidine synthases

```
Pf     1 --------------MDKLISNNKLKLSVVLLGGLCSLAYYHLKNKFHLSQFCFSKKWFSE
At     1 MEGDVGIGLVCQNTMDGKASNGNGLEKTVPS--CCLKAMACVPEDDAKCHSTVVSGWFSE
Hs     1 -------------------------------------MEPGPDGPAASGPAAIREGWFRE
Ld     1 ----------------------------------------------MPGPGLLPDGWFRE
Tm     1 ---------------------------------------MRTLKELERELQPRQHLWYFE

Pf    47 FS---------------IMWPGQAFSVEIKKILYETKSKYQNVLVFES---TTYGKVLVL
At    59 PHPRSGKKGGKAVYFNNPMWPGEAHSLKVEKVLFKDKSDFQEVLVFES---ATYGKVLVL
Hs    24 ----------------TCSLWPGQALSLQVEQLLHHRRSRYQDILVFR---SKTYGNVLVL
Ld    15 ----------------ESTMWPGQAQGLKVEKVLYDQPTEFQHLTVFESDPSGPWGTVMTL
Tm    22 YYTG----------------NNVGLFMKMNRVIYSGQSDIQRIDIFEN---PDLGVVFAL

Pf    89 DGVIQLTEKDEFAYHEMMTHIPMTVSKEPKNVLVVGGGDGGIIRELCKYKS-----VENI
At   116 DGIVQLTEKDECAYQEMIAHLPLCSISSPKNVLVVGGGDGGVLREISRHSS-----VEVI
Hs    66 DGVIQCTERDEFSYQEMIANLPLCSHPNPRKVLIIGGGDGGVLREVVKHPS-----VESV
Ld    60 DGAIQLTDYDEFVYHEMLANLSLTCHHKPERVLIIGGGDGGVVREVLRHKSEKDGIVQSV
Tm    63 DGITMTTEKDEFMYHEMLAHVPMFLHPNPKKVLIIGGGDGGTLREVLKHDS-----VEKA

Pf   144 DICEIDETVIEVSKIYFKNISCGYEDKRVNVFIEDASKFLENVTN-TYDVIIVDSSDP-I
At   171 DICEIDKMVIDVSKKFFPELAVGFDDPRVQLHIGDAAEFLRKSPEGKYDAIIVDSSDP-V
Hs   121 VQCEIDEDVIQVSKKFLPGMAIGYSSSKLTLHVGDGFEFMKQNQD-AFDVIITDSSDP-M
Ld   120 ELVDIDGAVIQQSKKHFPQIACGFANPCVTATVGDGAAFVKRAPDSVYDVIIIDTTDP-K
Tm   118 ILCEVDGLVIEAARKYLKQTSCGFDDPRAEIVIANGAEYVRKFKN-EFDVIIIDSTDPTA

Pf   202 GPAETLFNQNFYEKIYNALKPNGYCVAQCESLWIHVGTIKNMIGYAKK--LFKKVEYANI
At   230 GPALALVEKPFFETLARALKPGGVLCNMAESMWLHTHLIEDMISICRQT--FKSVHYAWS
Hs   179 GPAESLFKESYYQLMKTALKEDGVLCCQGECQWLHLDLIKEMRQFCQS--LFPVVAYAYC
Ld   179 GPASELFGADFYTNVLRILRPGGVLCNQGESVWLHRPLIEMMMGFLKKDIGFATVNYAMI
Tm   177 GQGGHLFTEEFYQACYDALKEDGVFSAETEDPFYDIGWFKLAYRRISK--VFPITRVYLG

Pf   260 SIPTYPCGCIGILCCSKT--DTGLTKPNKKLESKEFAD-----LKYYNYENHSAAFKLPA
At   288 SVPTYPSGVIGFVLCSTEGPAVDFKNPINPIEKLDGAMTHKRELKFYNSDMHRAAFALPT
Hs   237 TIPTYPSGQIGFMLCSKN-PSTNFQEPVQPLTQQQVAQM---QLKYYNSDVHRAAFVLPE
Ld   239 YIPTYPCGSIGTLVCAKS-ADTDVTVPMRPVESLGFAD----QLKYYSSDMHKAAFVLPR
Tm   235 FMTTYPSGMWSYTFASKG---IDPIKDFDPEKVRKFNK----ELKYYNEEVHVASFALPN

Pf   313 FLLKEIENI---
At   348 FLRREVASLLAS
Hs   293 FARKALNDVS--
Ld   294 FAAHLNE-----
Tm   288 FVKKELGLM---
```

Figure 3.3 A. Amino acid sequence analyses of *P. falciparum* spermidine synthase.
(A) The multiple alignment shows the comparison of the deduced amino acid sequence of *P. falciparum* spermidine synthase with the respective sequences from *Arabidopsis thaliana* (accession no. NP_200124), *Homo sapiens* (accession no. A32610), *Leishmania donovani* (accession no. AAG24612) and *Thermotoga maritima* (accession no. TM0654). Amino acid residues that are identical in at least three sequences are highlighted in dark boxes, similar residues in grey boxes. Gaps (-) are introduced to increase similarity.

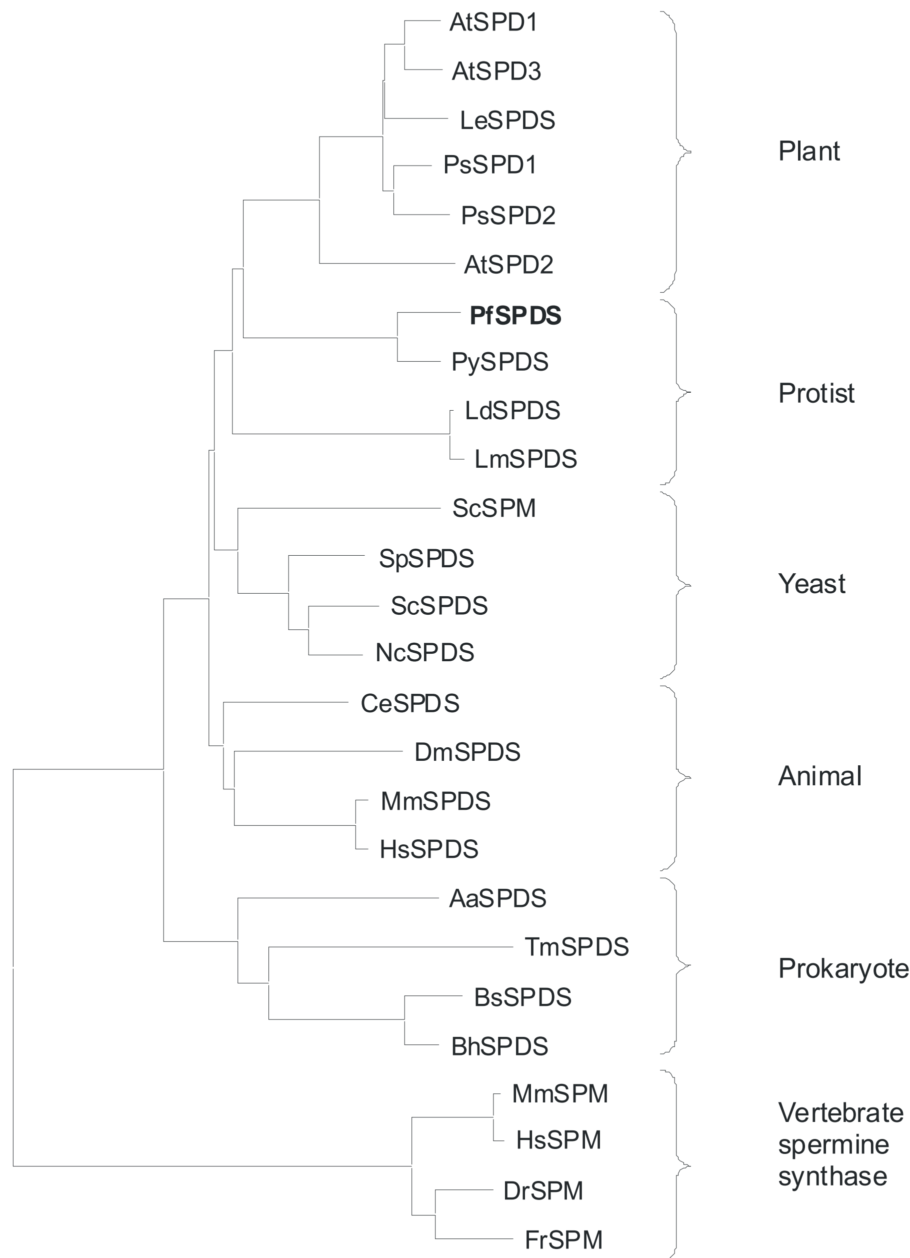


Figure 3.3 B. Protein sequences of spermidine and spermine synthases were aligned using ClustalX. Subsequently, the phylogenetic tree was generated using the neighbour-joining method.

The accession numbers for the proteins used are as follows: *Pf*SPDS (*Plasmodium falciparum*; CAB71155), AtSPD1 (*Arabidopsis thaliana*; CAB61614), AtSPD2 (*Arabidopsis thaliana*; AY040013), AtSPD3 (*Arabidopsis thaliana*; CAB61615), ScSPD (*Saccharomyces cerevisiae*; U27519), ScSPM (*Saccharomyces cerevisiae*; AF067970), MmSPDS (*Mus musculus*; NM_009272), MmSPM (*Mus musculus*; NM_009214), HsSPDS (*Homo sapiens*; M64231), HsSPM (*Homo sapiens*; NM_004595), DrSPM (*Danio rerio*; AJ009633), FrSPM (*Fugu rubripes*; AJ009865), PsSPD1 (*Pisum sativum*; AF043108), PsSPD2 (*Pisum sativum*; AF043109), LeSPDS (*Lycopersicon esculentum*; AJ006414), TmSPDS (*Thermotoga maritima*; TM0654), BsSPDS (*Bacillus subtilis*; CAB15777), BhSPDS (*Bacillus halodurans*; NP_244678), AaSPDS (*Aquifex aeolicus*; AE000672), CeSPDS (*Caenorhabditis elegans*; AJ306734), NcSPDS (*Neurospora crassa*; AB001598), LdSPDS (*Leishmania donovani*; AF298195), DmSPDS (*Drosophila melanogaster*; NP_731384), PySPDS (*Plasmodium yoelii yoelii*; EAA16925), LmSPDS (*Leishmania major*; CAC44919) and SpSPDS (*Schizosaccharomyces pombe*; NP_596015).

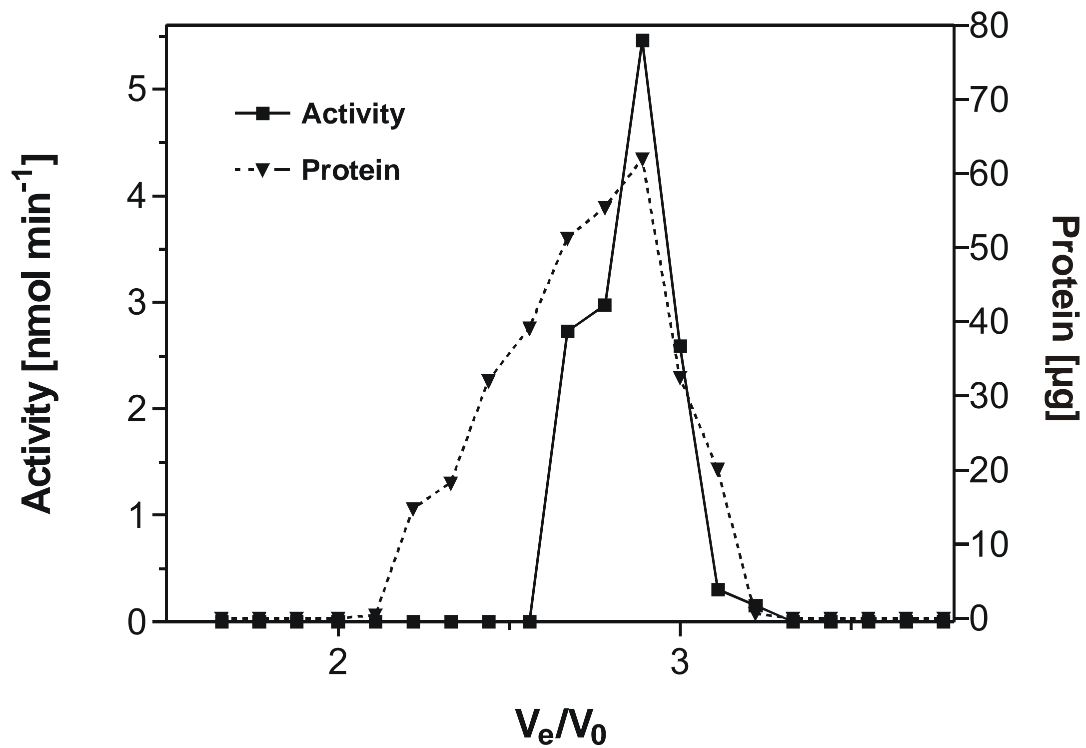
from other organisms apart from certain plant sequences (see *A. thaliana* spermidine synthase, Figure 3.3 A). In this regard it is noteworthy that the *P. falciparum* spermidine synthase is predicted to be more closely related to its plant than to its animal orthologues (Figure 3.3 B). The spermine synthases were introduced into the phylogenetic tree analysis because they possess several conserved amino acid residues that are involved in the binding of dcAdoMet and the acceptor amine. The result from the phylogenetic tree analysis illustrates that the *P. falciparum* spermidine synthase belongs to the spermidine synthase cluster and is not ordered to the spermine synthases.

3.4 Recombinant expression and biochemical characterization

Recombinant expression of the full length *P. falciparum* spermidine synthase protein including the N-terminal extension in *E. coli* was not successful. However, when the first 29 amino acids were omitted, spermidine synthase expressed abundantly. The recombinant protein was purified by metal affinity chromatography and gel filtration on a calibrated Superdex S-75 column with a yield of approximately 0.5 mg l^{-1} expression culture.

In gel filtration, spermidine synthase activity eluted at a fraction that corresponds to a molecular mass of approximately 70 kDa, indicating a protein with a dimeric structure (Figure 3.4 A and B).

A



B

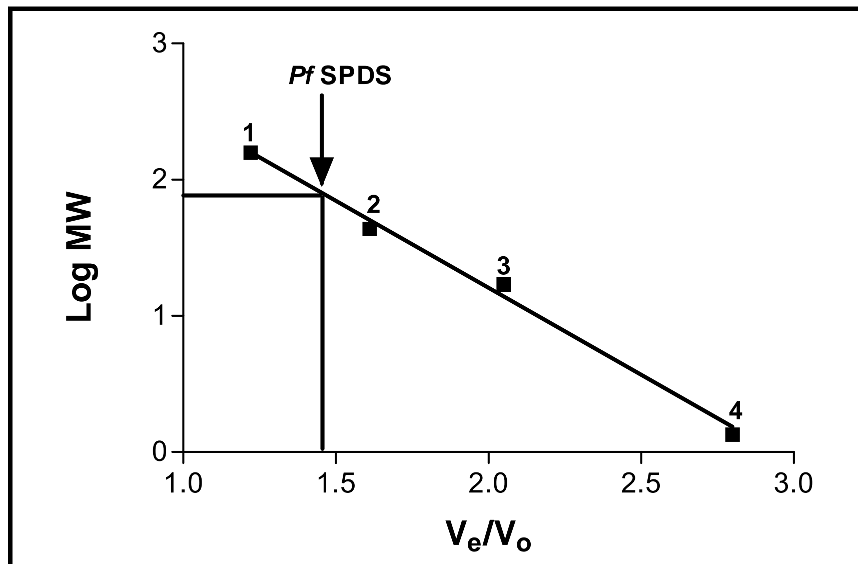


Figure 3.4 Elution profile for the *P. falciparum* spermidine synthase.
A. The determination of the molecular weight (MW) of the active recombinant *P. falciparum* spermidine synthase (*Pf* SPDS) enzyme by gel filtration on a Hiload 16/60 Superdex S-75 column. B. The following protein standards were used to calibrate the column: (1) Gamma globulin (158,000) (2) Ovalbumin (44,000) (3) Myoglobin (17,000) (4) Vitamin B-12 (1,350). The arrow shows the determined molecular weight of the *P. falciparum* spermidine synthase at approximately 70,000.

In accordance with the molecular mass deduced from the cDNA sequence, SDS-PAGE analysis of the truncated recombinant spermidine synthase protein that carries the N-terminal His_6-tag of 3.6 kDa shows a homogeneous band at 37 kDa (Figure 3.5).

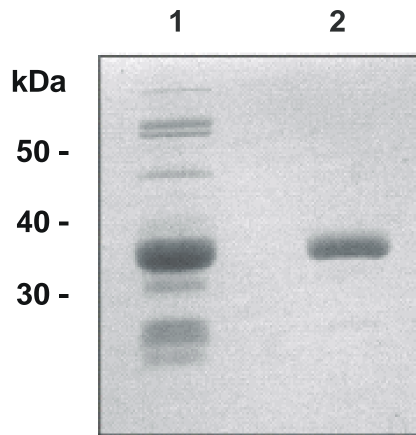


Figure 3.5 SDS-PAGE of the *P. falciparum* spermidine synthase.
The figure shows the recombinant truncated His-tagged *P. falciparum* spermidine synthase protein after nickel-chelating affinity chromatography (lane 1) and gel filtration (lane 2). The size of the protein standard is shown on the left.

3.5 Western blot analyses

Rabbit polyclonal antibodies were prepared against the recombinant *P. falciparum* spermidine synthase. As shown in Figure 3.6A the antiserum recognised the recombinant truncated *P. falciparum* spermidine synthase fusion protein that carries the N-terminal His_6-tag (33.4 + 3.6 kDa). Moreover, when extracts of *P. falciparum* cells were subjected to Western blot analysis, a band corresponding to the molecular size of the deduced full length spermidine synthase polypeptide including the N-terminal extension (36.6 kDa) was specifically detected. In accordance with the stage specific Northern blot analyses (Figure 3.2), the spermidine synthase protein also peaks at the old trophozoite stage (Figure 3.6B).

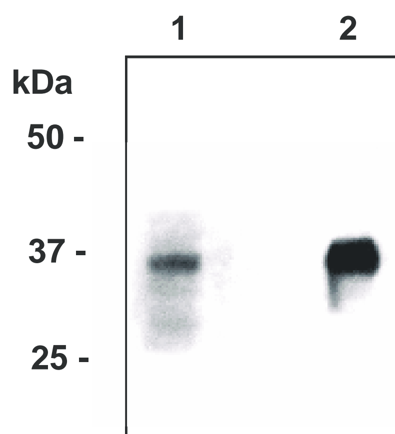


Figure 3.6 Western blot analyses of *P. falciparum* spermidine synthase.
A) Extract of *P. falciparum* 3D7 cells (lane 1) and the recombinant truncated *P. falciparum* spermidine synthase that carries the His_6-tag of 3.6 kDa (lane 2) were subjected to Western blot analysis. The blot was probed with a polyclonal rabbit antiserum against the recombinant *P. falciparum* spermidine synthase

B) Stage specific Western Blot analyses of spermidine synthase expression in *P. falciparum*. Immunodetection of *P. falciparum* spermidine synthase was achieved by using a *P. falciparum* spermidine synthase specific polyclonal antibody. Maximal expression of *P. falciparum* spermidine synthase occurs in old trophozoite stages. The identical blot was incubated with anti-*P. falciparum* PP5 antibody to ensure equal loading of parasite proteins for each time point.

3.6 Localization of the *P. falciparum* spermidine synthase

IFA analyses with trophozoite-infected erythrocytes indicate that *P. falciparum* spermidine synthase protein is localized throughout the parasite, suggesting a cytoplasmic localization (Figure 3.7).

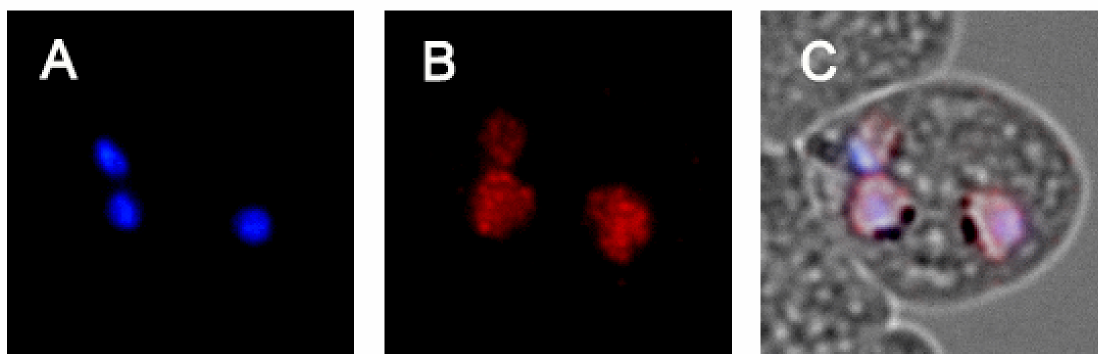


Figure 3.7 Localization of *P. falciparum* spermidine synthase.
Methanol fixated smears of synchronized 3D7 parasites (trophozoites) were incubated with anti-*P. falciparum* spermidine synthase serum followed by the DNA specific stain DAPI (blue) and Cy3-labelled anti-rabbit antibody (red). Within a triple infected erythrocyte (A) the *P. falciparum* spermidine synthase protein localized throughout the parasite, indicating cytoplasmatic localisation (B). The merged picture (C) shows that the distribution of the *P. falciparum* spermidine synthase protein is restricted to the parasite.

3.7 Kinetic analyses

The radiolabelled substrate putrescine and product spermidine of the spermidine synthase assay were separated by thin layer chromatography (Figure 3.8). The retention factor for putrescine and spermidine were 0.66 and 0.44 respectively. The recombinant *P. falciparum* spermidine synthase catalyses the formation of spermidine from putrescine and dcAdoMet with a specific activity of 820 ± 95 nmol min^{-1} mg^{-1} and a k_{cat} of 0.48 ± 0.05 s^{-1} (n = 3).

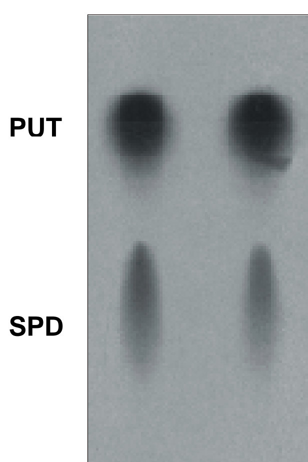


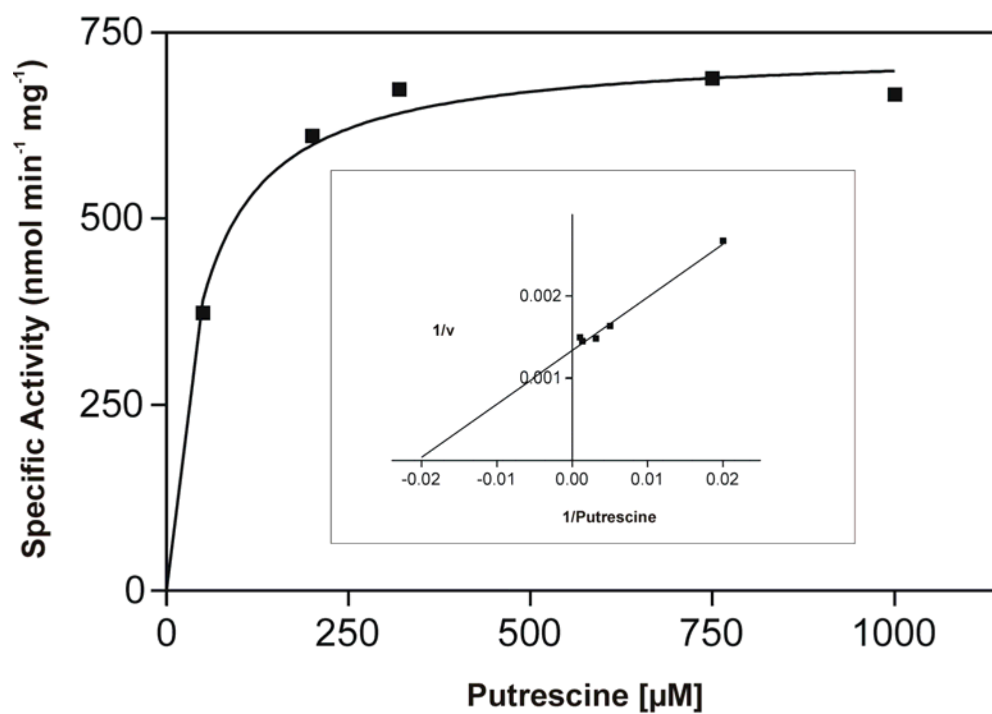
Figure 3.8 TLC of the *P. falciparum* spermidine synthase assay.

The autoradiograph shows the separation of the substrate [^{14}C] putrescine and the reaction product [^{14}C] spermidine after thin layer chromatography. The retention factor or R_f for putrescine and spermidine were 0.656 and 0.44 respectively.

The K_m values for the substrates putrescine and dcAdoMet were determined to be 52.0 ± 0.6 μM (n= 3) (Figure 3.9A) and 35.3 ± 4.1 μM (n= 3) (Figure 3.9B), respectively. DcAdoMet concentrations up to 150 μM did not inhibit enzyme activity. MTA, the second product of the spermidine synthase reaction, acts as a feedback inhibitor. The IC_{50} value for the *P. falciparum* spermidine synthase was determined to be 159 ± 27 μM (n = 3).

Furthermore, when using [^{14}C]-spermidine in the reaction assay, HPLC analyses revealed that *P. falciparum* spermidine synthase has also spermidine aminopropyltransferase activity (Figure 3.10). The specific activity in a standard assay was 75.3 ± 8.6 nmol min^{-1} mg^{-1} (n=4), which is approximately 15% of the reaction rate with putrescine as an acceptor.

A)



B)

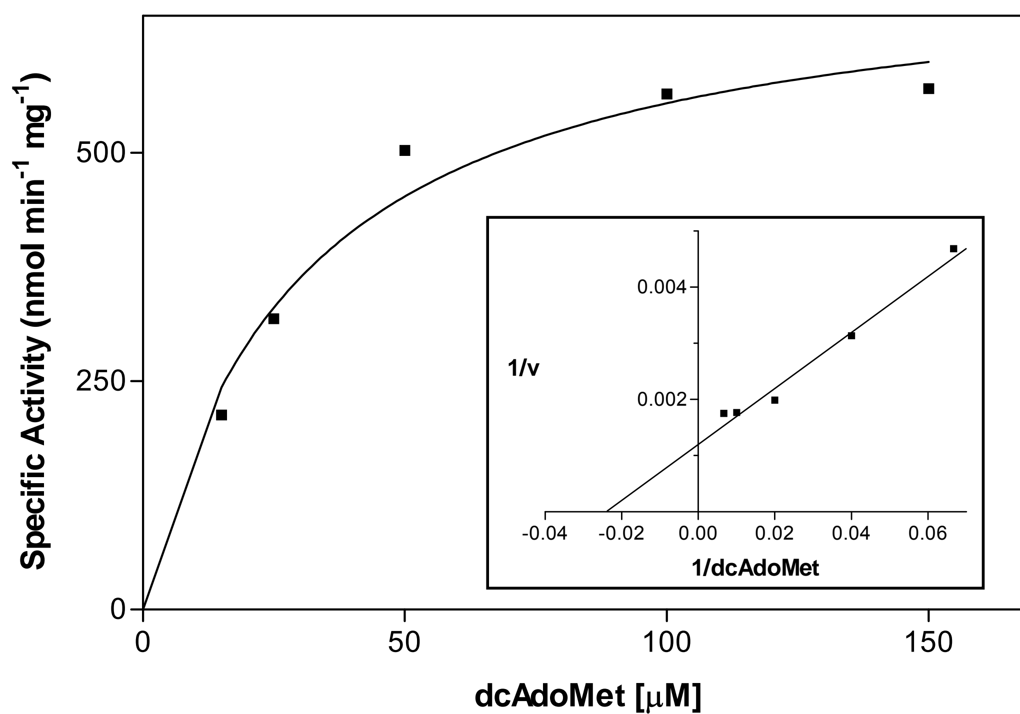


Figure 3.9 Kinetic analyses of *P. falciparum* spermidine synthase.
(A) The Michaelis-Menten and the corresponding Lineweaver-Burk plot for the determination of the K_m value for putrescine and (B) dcAdoMet.

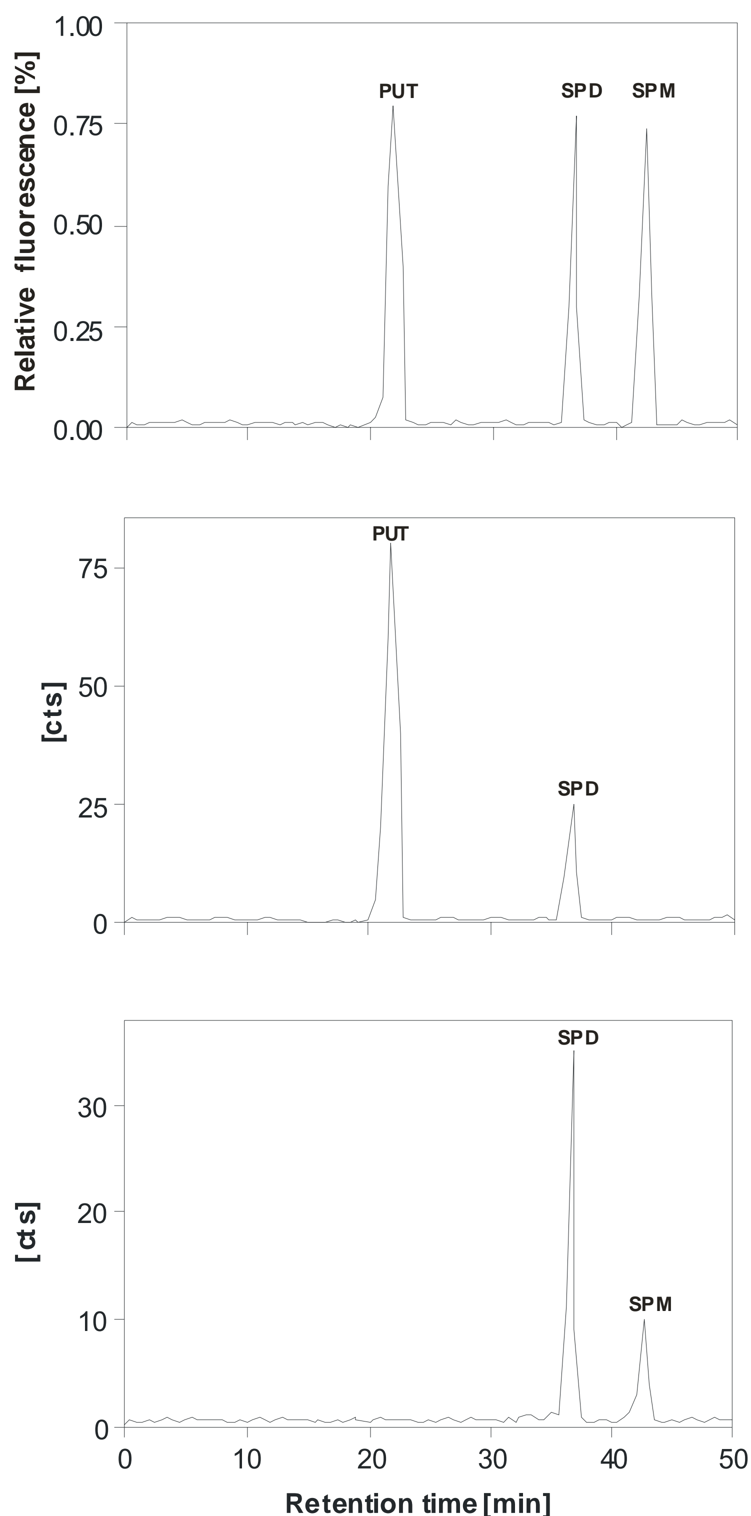


Figure 3.10 HPLC analyses of the reaction products of *P. falciparum* spermidine synthase. (A) Chromatogram of the dansylated polyamine standards putrescine (PUT), spermidine (SPD) and spermine (SPM). (B) Putrescine was incubated with 200 ng recombinant *P. falciparum* spermidine synthase at 37 °C for 30 min producing spermidine. (C) Spermidine was incubated with 1 µg recombinant *P. falciparum* spermidine synthase at 37 °C for 30 min producing spermine. The axes of ordinates in (B) and (C) indicate the radioactivity given as counts per second [cts].

3.8 The effect of synthetic spermidine synthase inhibitors on enzyme activity and cultured *P. falciparum*

The recombinant *P. falciparum* spermidine synthase was tested with the synthetic inhibitors 4MCHA, APE, cyclohexylamine, dicyclohexylamine, APA and 2-mercaptoethylamine (Table 3.1). 4MCHA exhibits the best IC_{50} value with 1.4 µM ± 0.1. The corresponding K_i value for the competitive inhibitor was determined to be 0.18 µM ± 0.02 (n = 3) (Figure 3.11). APE and cyclohexylamine inhibited enzyme activity by 50% at concentrations of 6.5 µM and 19.7 µM, respectively, whereas APA, 2-mercaptoethylamine and dicyclohexylamine were only poor inhibitors of recombinant *P. falciparum* spermidine synthase (Table 3.1).

The inhibitors of *P. falciparum* spermidine synthase were consequently tested for their antimalarial activity in culture (Table 3.1). The efficacy of APA on the growth of *P. falciparum* with an IC_{50} of 1.0 µM has been shown previously to be attributable to ODC inhibition, since APA inhibits *P. falciparum* ODC enzyme activity in the low nanomolar range and hence, more potently than the spermidine synthase (Das Gupta *et al.*, 2005). 4MCHA was most effective with an IC_{50} value of 34 µM, followed by APE with 83.3 µM (Table 3.1). Supplementation of the medium with 500 µM putrescine or 500 µM spermidine did not significantly rescue the growth of the parasites (Figure 3.12).

Inhibitor	Enzyme Inhibition IC_{50} [µM]	Growth inhibition IC_{50} [µM]
4MCHA	1.4 ± 0.1	34.2 ± 4.0
APE	6.5 ± 2.1	83.3 ± 3.3
Cyclohexylamine	19.7 ± 3.1	198 ± 47
APA	84 ± 21	1.0 ± 0.3
MTA	159 ± 27	N.D.
2-Mercaptoethylamine	76 ± 10	254 ± 42
Dicyclohexylamine	> 1000	342 ± 57

Table 3.1 The effect of various inhibitors on *P. falciparum* spermidine synthase enzyme activity and the growth of malaria culture. Results are the means ± SD of at least three independent determinations.

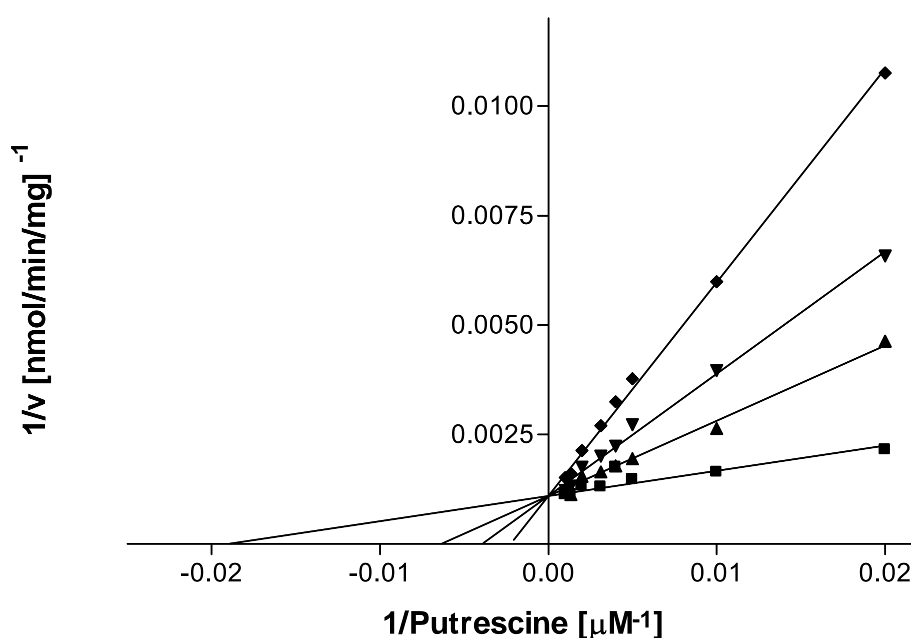


Figure 3.11 K_i analyses of *P. falciparum* spermidine synthase with the inhibitor 4MCHA. Lineweaver-Burk plot of inhibition of *P. falciparum* spermidine synthase by 4MCHA at concentrations of 0 (■), 0.5 (▲), 1.0 (▼) and 2.0 µM (◆).

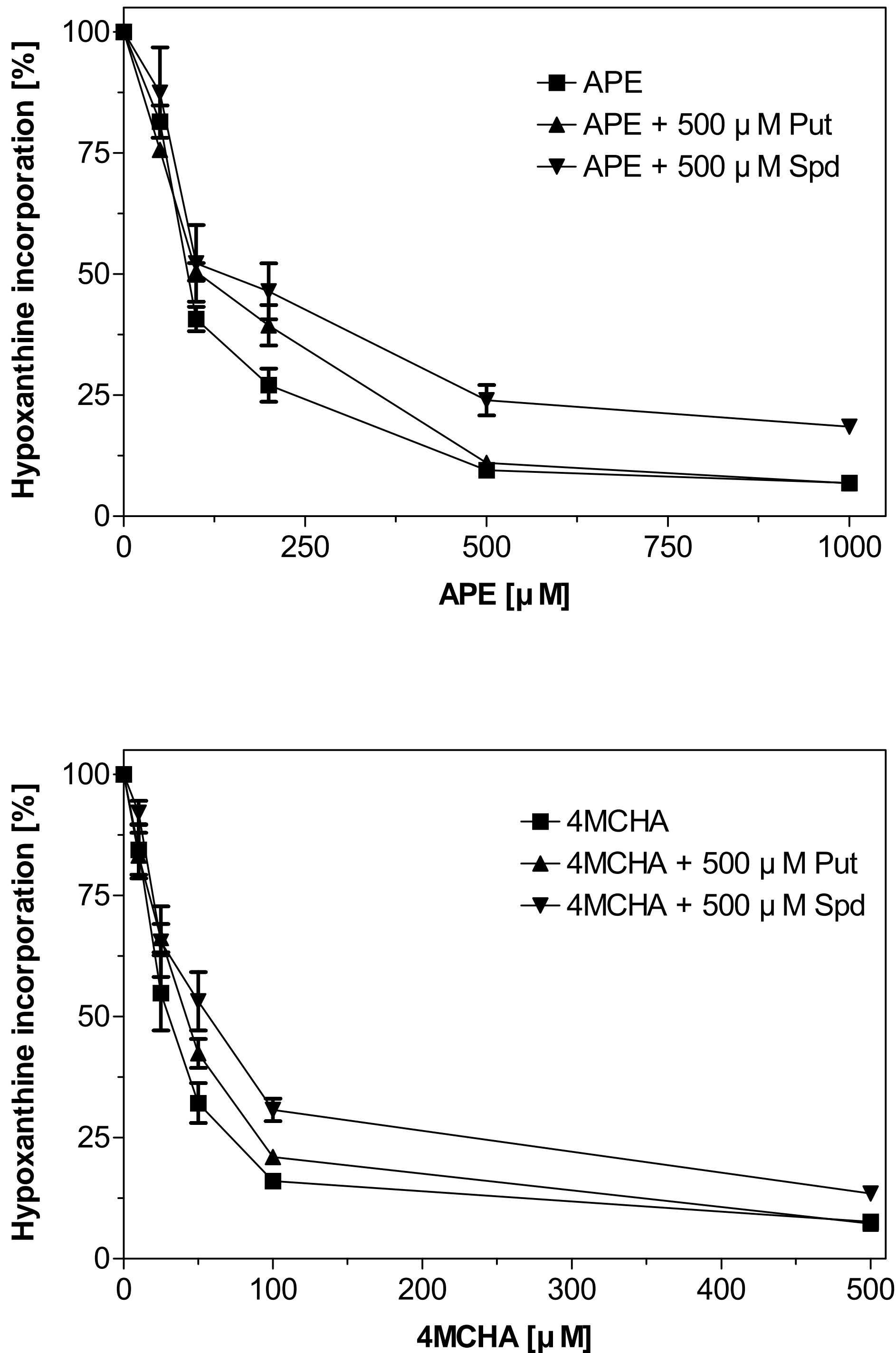


Figure 3.12 The effect of exogenous polyamines on growth inhibition by APE and 4MCHA.
P. falciparum culture medium containing increasing concentrations of APE (upper panel) or 4MCHA (lower panel) was supplemented with 500 µM putrescine or 500 µM spermidine as indicated. Control cells were grown without exogenous polyamines. Proliferation was determined after 48 h incubation by measuring [^{3}H]-hypoxanthine incorporation.

3.8 *P. falciparum* spermidine synthase mutations

In most spermidine synthases, the amino acid residues Y^{102}, D^{196} and S^{197} are highly conserved. According to the *Thermotoga maritima* crystal model it has been suggested that these particular amino acids are important for substrate binding and protonation. Therefore the respective amino acid residues were mutated in the *Plasmodium* spermidine synthase to investigate whether they affected the activity of the enzyme.

A

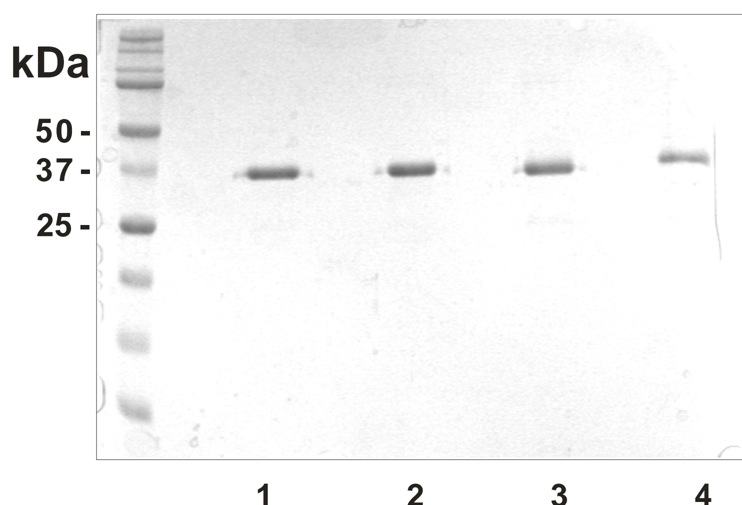


Figure 3.13 A. SDS-PAGE of purified recombinant wild-type *P. falciparum* spermidine synthase (lane 1), and the corresponding mutants S^{197}A (lane 2), D^{196}N (lane 3) and Y^{102}A (lane 4).

B

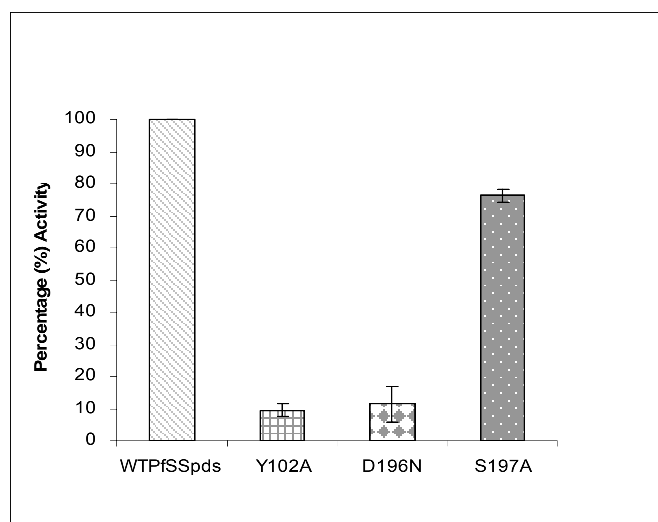


Figure 3.13 B. Activity of the wild-type *P. falciparum* spermidine synthase enzyme and the corresponding mutants. The percentage activity of the mutants is shown in comparison to the wild-type activity.

As can be observed from the bar graph (Figure 3.13), the $Y^{102}A$ and $D^{196}N$ mutations have a great impact on the enzyme. Compared to the wild-type enzyme they have a percentage rest activity of 9 ± 2 % and 11 ± 2 %, respectively. The $S^{197}A$ mutant exhibited only a slight reduction of the specific activity and had a rest activity of 76 ± 6 %. This suggests that this residue may not be so critical for substrate binding.

3.10 Co-expression of *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase

It is hypothesised that the bifunctional nature of *P. falciparum* ODC/AdoMetDC is to allow substrate channelling to occur, whereby the spermidine synthase, is required as a second enzyme that uses the decarboxylated products of *P. falciparum* ODC/AdoMetDC, namely, putrescine and dcAdoMet, as substrates to produce spermidine. It is therefore suggested that the spermidine synthase docks onto the bifunctional protein and forms a multicomplex with it in order to acquire the two substrates for the subsequent spermidine synthase reaction. Thus, the intermolecular protein-protein interactions between bifunctional *P. falciparum* ODC/AdoMetDC and spermidine synthase were analysed. As shown in Birkholtz (2002), *P. falciparum* ODC/AdoMetDC protein and activity was observed at approximately 330 kDa, the size of the wild-type bifunctional protein. Spermidine synthase protein and activity did not co-elute with the decarboxylase activities but eluted solely at the expected size of approximately 70 kDa for the active dimeric form of spermidine synthase. None of the protein activities were found in fractions corresponding to a complex between *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase of approximately 400 kDa. It therefore seems that no interactions occur between *P. falciparum* ODC/AdoMetDC and spermidine synthase under the *in vitro* conditions used or that the interactions are transitory and not stable enough to survive size exclusion chromatography (Birkholtz, 2002).

The second strategy that was used for the determination of protein-protein interactions was the bacterial co-expression of *P. falciparum* ODC/AdoMetDC and *P. falciparum* spermidine synthase. The pACYC184:*Pf*SPDS2 construct was co-transformed and co-expressed along

with the expression plasmid *P. falciparum* ODC/AdoMetDC in *E. coli* EWH331 cells. After expression, the *E. coli* cells were sonificated. Insoluble material was removed by high speed centrifugation at 100,000g for 1.05 h at 4 ^{o}C, and the supernatant was subsequently applied to a native PAGE and Western blot analyses (see section 2.16.2). The Western blot analysis showed a band for the Strep-tagged *P. falciparum* ODC/AdoMetDC of 330 kDa (Figure 3.14, lane 1) after it was probed with monoclonal Anti-Strep antibody. The blot was stripped and reprobed with Anti-His-Tag antibody. A band with a size of approximately 70 kDa was visualised, indicating the His_6-tagged spermidine synthase dimer (Figure 3.14, lane 2). Since the signal of the spermidine synthase is separate from the *P. falciparum* ODC/AdoMetDC band, it shows that there is no protein-protein interaction between the two enzymes under the conditions used in these experiments.

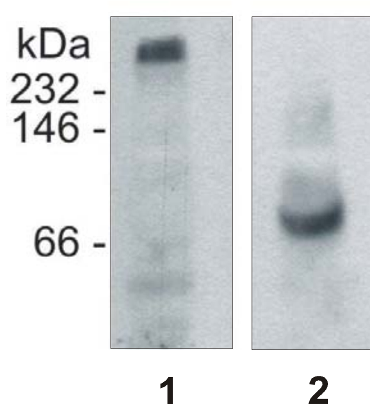


Figure 3.14 Western blot analyses of the co-expression of *P. falciparum* ODC/AdoMetDC and spermidine synthase.

Lane 1 shows a band for the Strep-tagged *Pf*ODC/AdoMetDC of approximately 330 kDa, after probing with the monoclonal Strep-antibody. Probing with an Anti-His-Tag antibody revealed a band with a size of approximately 70 kDa indicating the His_6-tagged spermidine synthase dimer (Lane 2). The marker used was the high molecular weight (HMW Native marker Kit (66,000-669,000) from Amersham Biosciences).

3.11 Analysis of the deduced amino acid sequences of *C. elegans* and *B. malayi* spermidine synthase

A putative 945 bp spermidine synthase PCR fragment was amplified from *C. elegans* cDNA using gene specific oligonucleotides based on the EMBL genomic nucleotide sequence accession number AL110485. The cDNA sequence contains an open reading frame that shows high similarity to amino acid sequences of known spermidine synthases (Figure 3.15 A). However, the obtained nucleotide sequence differs from the cDNA sequence published in

the Sanger Centre gene bank [accession number AL110485] in that an additional exon of 156 bp was proposed within intron IV.

Furthermore a putative spermidine synthase was amplified from the *B. malayi* cDNA using gene specific primers based on the *B. malayi* genomic nucleotide sequence. The 945 bp cDNA sequence codes an open reading frame which shows similarity to amino acids of other established spermidine synthases (Figure 3.15 A). *B. malayi* spermidine synthase cDNA encodes a polypeptide of 315 amino acids with a calculated molecular mass of 35.6 kDa. The *C. elegans* spermidine synthase cDNA encodes a polypeptide of 314 amino acids with a deduced molecular mass of 35.0 kDa. Pairwise sequence alignment using BCM search launcher (http://searchlauncher.bcm.tmc.edu/) revealed that the amino acid sequence of *C. elegans* spermidine synthase is very similar to the counterpart of the human-parasitic nematode *B. malayi* (55%) and also to other known spermidine synthases with sequence identities of 57% to the human (Wahlfors et al., 1990), 48% to the *Nicotiana sylvestris* (Hashimoto et al., 1998), 41% to the *E. coli* (Tabor *et al.*, 1986) and 43% to the *T. maritima* (Korolev *et al.*, 2002) proteins (Figure 3.15 A).

The alignment in Figure 3.15 A revealed that the *C. elegans* and the *B. malayi* spermidine synthase contains an insertion of 27 amino acids close to its N-terminus. Similar insertions are present in all putative nematode but not in other spermidine synthases from prokaryotic or eukaryotic origin (Figure 3.15 A, 3.15 B), thus, probably representing a nematode-specific characteristic. The amino acid sequences of these insertions reflect the phylogenetic relationship of the nematodes according to the taxonomic classification by Blaxter *et al.* (1998). *C. briggsae*, *Ancylostoma caninum, Ascaris suum* and *Haemonchus contortus* are members of the same clades as *C. elegans* and the identity of their spermidine synthase insertions, with respect to the *C. elegans* sequence, is 74% to 62%. The value for *Meloidogyne hapla* is 30% and *B. malayi* with a value of 11% (3 identical amino acids in 27) exhibits the greatest phylogenetic distance to *C. elegans*.

A

```
CeSPDS    1 --MNKLHKGWFTEFSPDDLEKMNGASDEEPTKVLKSDGQEMGGAWPGQAFSLQVKKVLFH
BmSPDS    1 --MNVFRDGWFTELPPSLKNDDP-EKTKLVANKDDDLDSFDNHIWSGQAFSLKVDKVLAH
HsSPDS   11 SGPAAIREGWFRETCS--------------------------LWPGQALSLQVEQLLHH
NsSPDS   16 PYISSVLPGWFSEISP--------------------------LWPGEAHSLKVEKILFQ
EcSPDS    1 ---MAEKKQW-HE-T----------------------------LHDQFGQYFAVDNVLYH
TmSPDS    9 RELQPRQHLWYFEYY---------------------------TGNNVGLFMKMNRVIYS

CeSPDS   59 EKSKYQDVLVFESTTYGNVLVLDGIVQATERDEFSYQEMLAHLPMFAHPDPKRVLIIGGG
BmSPDS   58 ERSKYQDILIFKSSTHGNVLVLDGVIQCTEHDEFAYQEMVTHLPLCSHPNPRKVLVIGGG
HsSPDS   44 RRSRYQDILVFRSKTYGNVLVLDGVIQCTERDEFSYQEMIANLPLCSHPNPRKVLIIGGG
NsSPDS   49 GKSDYQNVMVFQSSTYGKVLVLDGVIQLTERDECAYQEMIAHLPLCSIPNPKKVLVIGGG
EcSPDS   28 EKTDHQDLIIFENAAFGRVMALDGVVQTTERDEFIYHEMMTHVPLLAHGHAKHVLIIGGG
TmSPDS   41 GQSDIQRIDIFENPDLGVVFALDGITMTTEKDEFMYHEMLAHVPMFLHPNPKKVLIIGGG

CeSPDS  119 DGGILREVLKHESVEKVTMCEIDEMVIDVAKKFLPGMSCG-FSHPKLDLFCGDGFEFLKN
BmSPDS  118 DGAVLREVLKHECVESVTICEIDETVINLSKKFLPHMSLA-FSSSKLKLAVQDGFDFLKE
HsSPDS  104 DGGVLREVVKHPSVESVVQCEIDEDVIQVSKKFLPGMAIG-YSSSKVTLHVGDGFEFMKQ
NsSPDS  109 DGGVLREVSRHSSVEQIDICEIDKMVVEVSKQFFPDVAVG-YEDPRVNLHIGDGVAFLKN
EcSPDS   88 DGAMLREVTRHKNVESITMVEIDAGVVSFCRQYLPNHNAGSYDDPRFKLVIDDGVNFVNQ
TmSPDS  101 DGGTLREVLKHDSVEKAILCEVDGLVIEAARKYLKQTSCG-FDDPRAEIVIANGAEYVRK

CeSPDS  178 HKN-EFDVIITDSSDP-VGPAESLFGQSYYELLRDALKEDGILSSQGESVWLHLPLIAHL
BmSPDS  177 HKG-EFDVIITDSSDP-IGPAKKLFSKTYYDLIKEALTEKGVLSSQGECPWLDMKLIKNV
HsSPDS  163 NQD-AFDVIITDSSDP-MGPAESLFKESYYQLMKTALKEDGVLCCQGECEWLHLDLIKEM
NsSPDS  168 VAAGTYDAVIVDSSDP-IGPAQELFEKPFFESIARALRPGGVVSTQAESIWLHMHIIEEI
EcSPDS  148 TSQ-TFDVIISDCTDP-IGPGESLFTSAFYEGCKRCLNPGGIFVAQNGVCFLQQEEAIDS
TmSPDS  160 FKN-EFDVIIIDSTDPTAGQGGHLFTEEFYQACYDALKEDGVFSAETEDPFYDIGWFKLA

CeSPDS  236 VAFNRKIFP-AVTYAQSIVSTYPSGSMGYLICAKN----ANRDVTTPARTLTAEQIKALN
BmSPDS  235 IKHVSTLYP-RVAYAVGFVPTYPSGQMGYLLCSKD----EKHDLTIPQKMLSESEVRRMN
HsSPDS  221 RQFCQSLFP-VVAYAYCTIPTYPSGQIGFMLCSKN----PSTNFQEPVQPLTQQQVAQMQ
NsSPDS  227 VANCRQIFKGSVNYAWTTVPTYPSGMIGFMLCSTEGPAVDFKNPINPIDDDASHNKTLGP
EcSPDS  206 HRKLSHYFS-DVGFYQAAIPTYYGGIMTFAWATDN----DALRHLSTEIIQARFLASGLK
TmSPDS  219 YRRISKVFP-ITRVYLGFMTTYPSGMWSYTFASKG---IDPIKDFDPEKVRKFN----KE

CeSPDS  291 LRFYNSEVHKAAFVLPQFVKNALE----
BmSPDS  290 LKYYNSDIHRSAFILPQFIKEALCV---
HsSPDS  276 LKYYNSDVHRAAFVLPEFARKALNDVS-
NsSPDS  287 MKFYNSELHKASFCLPSFAKRVIESKGK
EcSPDS  261 CRYYNPAVHTAAFALPQYLQDALASQPS
TmSPDS  271 LKYYNEEVHVASFALPNFVKKELGLM--
```

B

```
CeSPDS    4 LHKGWFTEFS  PDDLEKMNG-  -ASDEEPTKV  LKSDGQEMGG  AWPGQAFSLQVK
CbSPDS    4 LHKGWFTEFS  PDDLEKMSNP  DAPEDSSSKL  LKSDGQEMGG  AWPGQAFSLQVK
AcSPDS    4 LHKGWFTEFS  PDDLEKMKQ-  --EGSGDSKQ  LKSDGIAMGG  AWPGQAFSLKVK
HcSPDS      SAKGWFTEFS  PDDLERMKQ-  --GEASDSKQ  LKSDGVVMGG  AWPGQAFSLKVK
AsSPDS    4 LQKGWFTEFS  PDDLQKLQQ-  --TESGDEKQ  MKSDGVSMGG  AWSGQAFSLQVN
MhSPDS    4 LHKGWFTEFS  PDDADRISG-  ---SEDSGKM  MHLDGQEISG  AWTGQAFSLDIE
BmSPDS    4 FRDGWFTELP  PSLKNDDPE-  -KTKLVANKD  DDLDSFDN-H  IWSGQAFSLKVD
HsSPDS   16 IREGWFRETC  S---------  ----------  ----------  LWPGQALSLQVE
```

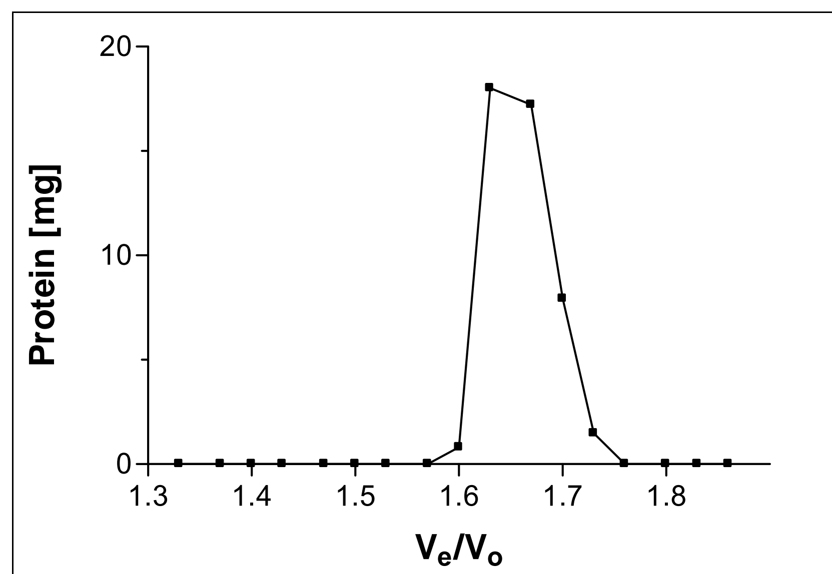
Figure 3.15 Alignment of spermidine synthase amino acid sequences
(A) The amino acid sequences of *C. elegans* spermidine synthase are compared with the *B. malayi*, the human [EMBL accession no. AAA36633], the *N. sylvestris* [EMBL accession no. BAA24535], the *E. coli* [EMBL accession no. P09158] and *T. maritima* spermidine synthase. Amino acid residues that are invariant in at least five additional sequences are shaded in black, similar amino acids in grey. The

N-terminus of the human, the *N. sylvestris* and the *T. maritima* sequence is omitted as indicated. Gaps (-) are introduced to provide maximum similarity.
(B) The nematode-specific insertion of *C. elegans* spermidine synthase is aligned with the respective regions of putative spermidine synthases from the nematodes *C. briggsae* [www.genome.wustl.edu/gsc/Projects/C.briggsae/], *A. caninum* [EMBL accession no. AW626946], *H. contortus* [EMBL accession no. BF060177], *A. suum* [EMBL accession no. BI783107] *M. hapla* [EMBL accession no. BM883048] and *B. malayi*. The N-terminus of the *H. contortus* sequence is unknown. The respective region of the human spermidine synthase is aligned to indicate the insertion. Identical amino acid residues are shaded in black, similar in grey. Gaps (-) are introduced to provide maximum similarity.

3.12 Expression and characterization of the recombinant *C. elegans* and *B. malayi* spermidine synthase

C. elegans spermidine synthase was recombinantly expressed as His-tagged fusion protein in *E. coli*. One litre of bacterial culture yielded about 10 mg purified protein. Performing gel filtration on a calibrated Superdex S-200 column resulted in a single peak corresponding to a molecular weight of 78,000, indicating a dimeric structure of the enzymatically active nematode spermidine synthase (Figure 3.16).

A



B

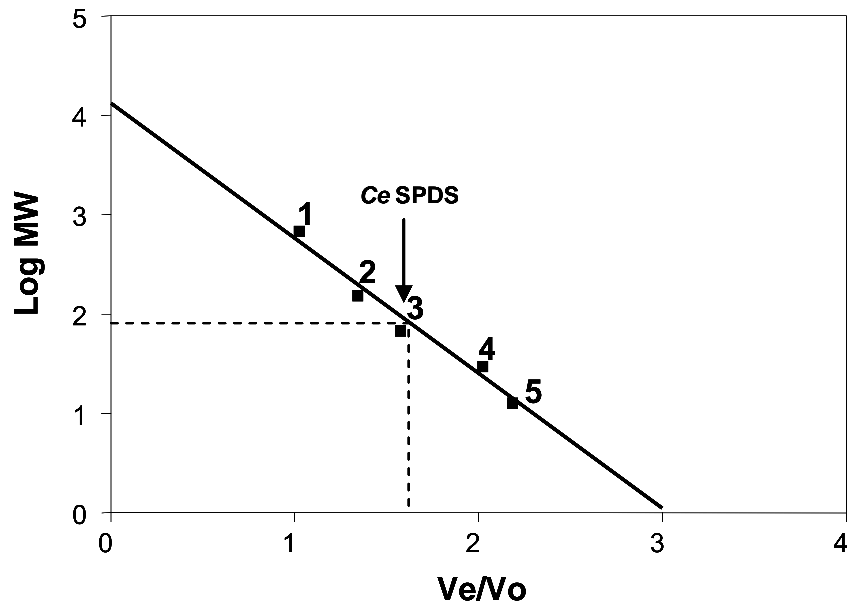


Figure 3.16 A. The determination of the molecular weight (MW) of the active recombinant *C. elegans* spermidine synthase (*Ce* SPDS) enzyme by gel filtration on a Hiload Superdex S-200 column.
B. The following standard proteins were used to calibrate the column: (1) thyroglobulin (670,000) (2) alcohol dehydrogenase (150,000) (3) bovine serum albumin (67,000) (4) carbonic anhydrase (29,000) (5) cytochrome C (12,400). The arrow shows the determined molecular weight of the *C. elegans* spermidine synthase at approximately 78,000.

SDS/PAGE analysis revealed a single band with a molecular mass corresponding to 38.6 kDa including the His-tag of 3.6 kDa (Figure 3.17). This is in good accordance with the predicted molecular mass of 35.0 kDa based on the deduced amino acid sequence of the cDNA sequence.

The *B. malayi* spermidine synthase was cloned and expressed and has a similar size to the *C. elegans* spermidine synthase. The *B. malayi* protein was however expressed in an inactive form (Figure 3.18).

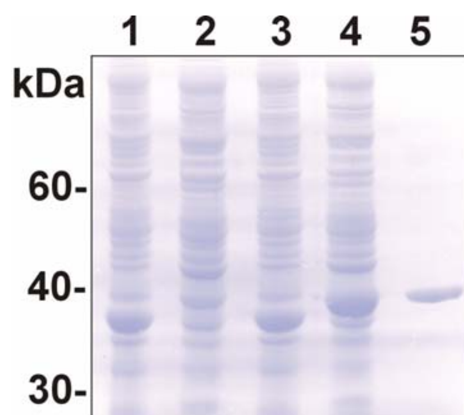


Figure 3.17 SDS-PAGE analysis of the recombinant *C. elegans* spermidine synthase
Lanes 1-5 represent protein extracts from *E. coli* BL21 (DE3) induced with 1 mM IPTG (see Materials and Methods). Coomassie blue-stained SDS-PAGE (10% polyacrylamide separation gel) of the 100,000 xg pellet (lane 1) and supernatant (lane 2) of lysed cells containing pTrcHisB without insert, of the 100,000 xg pellet (lane 3) and supernatant (lane 4) of lysed cells containing pTrcHisB:*Ce*SPDS and of recombinant His-tagged *C. elegans* spermidine synthase purified by Ni-NTA-chelating chromatography (lane 5). The size of the protein standard is shown in kDa on the left.

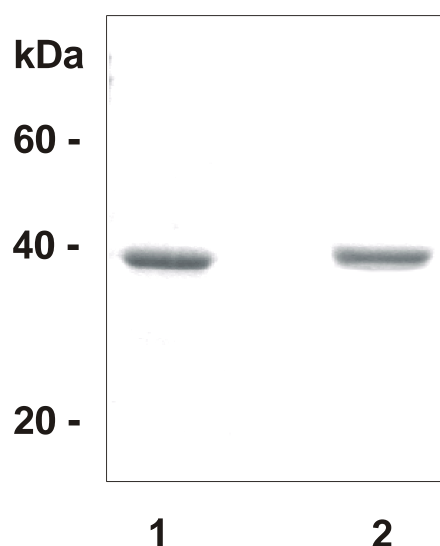
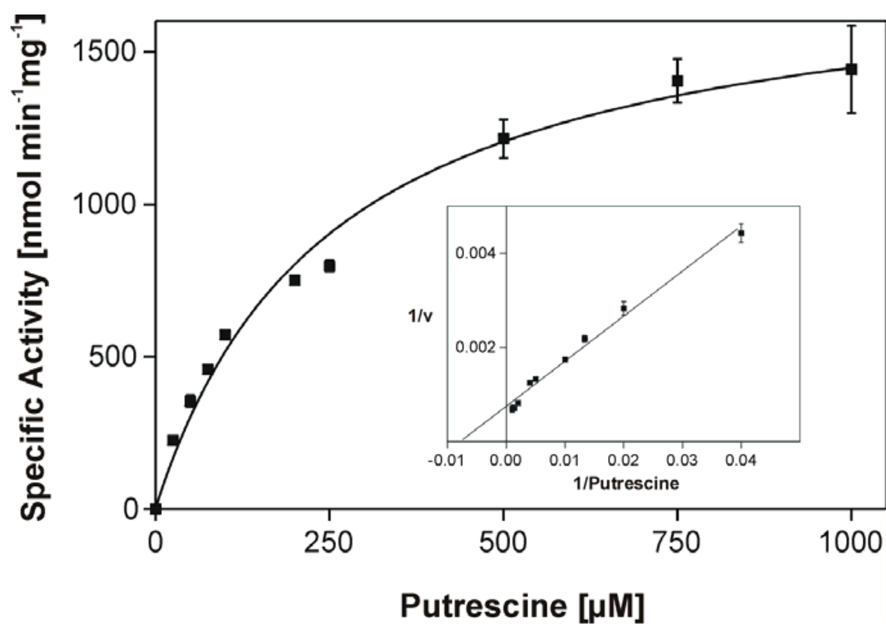


Figure 3.18 SDS-PAGE analyses of the recombinant *C. elegans* spermidine synthase and *B. malayi* spermidine synthase. Lane 1 *C. elegans* spermidine synthase and Lane 2 *B. malayi* spermidine synthase. Both enzymes were purified by Ni-NTA-chelating chromatography and subsequent gel filtration on a Hiload Superdex S-200 column.

3.13 Kinetic analyses of the *C. elegans* spermidine synthase

The recombinant *C. elegans* spermidine synthase enzyme has a specific activity of 1.8 µmol min^{-1} mg^{-1} protein resulting in a k_{cat} of 69.2 min^{-1}. The K_m value for the substrate putrescine was calculated to be 158 ± 27 µM (n = 5) (Figure 3.19 A). The spermidine synthase is characterised by a high specificity towards putrescine. Hence, spermidine could not replace putrescine as propylamine acceptor. The K_m value for the second substrate dcAdoMet was determined to be 111 ± 5 µM (Figure 3.19 B). The enzyme reaction of *C. elegans* spermidine synthase is inhibited by the second product MTA with an IC_{50} of 430 µM (n = 2).

A



B

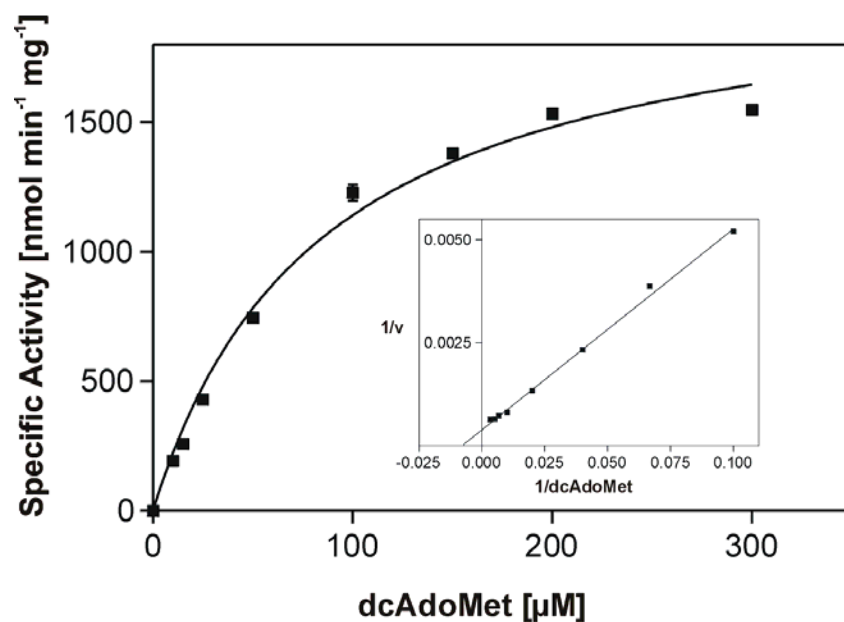


Figure 3.19 The K_m values of the *C. elegans* spermidine synthase enzyme for the substrates putrescine and dcAdoMet.

C. elegans spermidine synthase activity was affected by the synthetic inhibitors 4-MCHA, cyclohexylamine and dicyclohexylamine that are known competitive inhibitors with respect to putrescine. Cyclohexylamine and 4-MCHA with IC_{50} values of 2.4 ± 0.7 µM (n = 4) and 7.2

μM (n = 2) respectively were more potent inhibitors than dicyclohexylamine with an IC_{50} value of 154 μM (n = 2).

3.14 *C. elegans* spermidine synthase deletion mutant

The deletion mutant for *C. elegans* spermidine synthase that lacks the nematode specific sequence close to the N-terminal of the protein was expressed in *E. coli*. According to the deduced size, the deletion mutant is approximately 9 kDa smaller than the wild-type *C. elegans* spermidine synthase and runs at approximately 30 kDa in the SDS-PAGE (Figure 3.20). The specific activity of the *C. elegans* deletion mutant was determined under standard assay conditions. It was not different from the wild type recombinantly expressed *C. elegans* spermidine synthase protein. The specific activity was also 1.8 μmol min^{-1} mg^{-1} (n = 2).

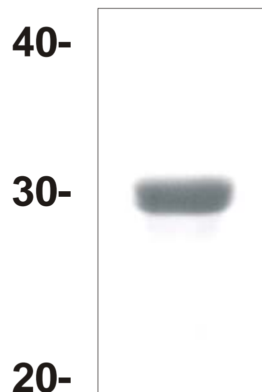


Figure 3.20 SDS-PAGE of the *C. elegans* spermidine synthase deletion mutant.
The *C. elegans* spermidine synthase deletion mutant is running at approximately 30 kDa.

Chapter 4 DISCUSSION

Most eukaryotes contain three polyamines putrescine, spermidine and spermine. They are essential cell components and are involved in a multitude of functions that are integral to macromolecular syntheses, cell proliferation and differentiation. Interference with the usual functions of polyamines has been one strategy in the search for effective antiparasitic drugs, which was encouraged by several promising early findings on polyamine inhibition in tumour cells and in *T. brucei gambiense* (Pegg, 1986; Müller *et al.*, 2001).

Malaria remains one of the most devastating tropical infectious diseases affecting humankind (Greenwood and Mutabingwa, 2002). Resistance of *P. falciparum*, the most virulent parasite infecting humans, to current anti-malarials is widespread, while control programmes are hindered by insecticide resistance, and social and environmental factors (Winstanley *et al.*, 2002). In order to intervene with a disease such as malaria, a comprehensive approach must be pursued. The parasite-specific characteristics of enzymes and their metabolic pathways can be biochemically investigated and exploited for rational drug development. The polyamine metabolism of *P. falciparum* has been proposed to be a promising drug target (Müller *et al.*, 2001). In the *P. falciparum* polyamine synthesis pathway a unique bifunctional enzyme, ODC/AdoMetDC has been elucidated (Müller *et al.*, 2000; Wrenger *et al.*, 2001; Birkholtz *et al.*, 2004).

The free-living nematode *C. elegans* is generally proposed to be a good model for eukaryotic organisms since it is accessible for molecular, genetic and biochemical experiments (Bürglin *et al.*, 1998). Polyamines have been shown to be essential for the development of this metazoa and the ODC and AdoMetDC have been characterized (Da'dara *et al.*, 1998; Ndjonka *et al.*, 2003; Macrae *et al.*, 1995, 1998).

In contrast to ODC and AdoMetDC, the third enzyme of the polyamine biosynthetic pathway, spermidine synthase, has not been investigated in most organisms including *P. falciparum* and *C. elegans*. Therefore, in the present research spermidine synthase has been cloned, recombinantly expressed and characterized from *P. falciparum* and *C. elegans*.

4.1 Properties of spermidine synthases

The molecular masses of *P. falciparum* and *C. elegans* spermidine synthases predicted from the cDNA sequences are in a range of 35 kDa which is similar to the size of spermidine synthases from other organisms. Gel filtration studies suggest that both spermidine synthases have a dimeric structure. The X-ray crystallographic structure of the *C. elegans* enzyme confirms a homodimer (Dufe *et al.*, in press). Each subunit consists of two domains, a six-stranded β-sheet builds up the structural framework of the N-terminal domain (residue 3-92) and a Rossman-fold like unit builds up the C-terminal domain (residue 93-314) (Dufe *et al.*, in press). The structure of the spermidine synthases in *Homo sapiens*, *E. coli*, and *N. sylvestris* are also dimeric (Kajander *et al.*, 1989; Tabor and Tabor, 1985; Hashimoto *et al.*, 1998) and the single subunits have been reported to be 35 kDa, 36.5 kDa and 38.7 kDa respectively. Contrary to that the soybean spermidine synthase was found to have a monomeric structure with a molecular mass of 74 kDa (Yoon *et al.*, 2000) and the *Sulfolobus solfataricus* enzyme has a molecular mass of 110 kDa and is a trimer composed of three identical subunits (Cacciapuoti *et al.*, 1986). Furthermore, resolution of the first crystal structure of an aminopropyl transferase has revealed that *T. maritima* spermidine synthase is a tetramer of 136.4 kDa (Korolev *et al.*, 2002). It was suggested that tetramerization of spermidine synthase monomers possibly underlies their thermostability, indicating that these enzymes may need some type of quaternary organization to increase protein stability. The fact that *P. falciparum* and *C. elegans* spermidine synthase are dimeric does not distinguish these proteins from the human counterpart. The spermidine synthase of both *P. falciparum* and *C. elegans* were recombinantly expressed and biochemically characterized to find out peculiarities of the enzymes which are different from the human enzyme.

P. falciparum spermidine synthase catalyses the formation of spermidine with a K_m for putrescine of 52 µM. The corresponding K_m value for the *C. elegans* enzyme is 158 µM. These values are in a similar range as those for the spermidine synthases from plants such as soy bean 32 µM and *Senecio vulgaris* 21 µM (Yoon *et al.*, 2000; Graser *et al.*, 2000), mammals such as human 80 µM and rat 15 µM (Pegg *et al.*, 1995; Pegg *et al.*, 1981;

Kajander *et al.*, 1989) and bacteria, for example, *T. maritima* 20 µM and *E. coli* 90 µM (Korolev *et al.*, 2002; Tabor and Tabor, 1983) whereas the K_m value for putrescine from *T. brucei brucei* is 200 µM (Bitonti *et al.*, 1984).

The K_m for dcAdoMet (35 µM) for the *P. falciparum* spermidine synthase is higher than the values reported for mammalian (7-25 µM) (Pegg, 1969; Kajander, 1989), *E. coli* (2 µM) (Bowman, 1973) and plant enzymes 0.4-4 µM (Graser *et al.*, 2000; Yoon *et al.*, 2000). Compared to that, the *C. elegans* spermidine synthase K_m of 110 µM for dcAdoMet is 3-fold higher than the *P. falciparum* enzyme and about 15-fold to 100-fold higher than the above mentioned enzymes.

Substrate inhibition by dcAdoMet at concentrations higher than 20 µM has been shown in some studies on mammalian enzymes (Coward *et al.*, 1977; Hibasami *et al.*, 1980). Such an effect was not found with the *P. falciparum* and the *C. elegans* enzyme up to dcAdoMet concentrations of 150 µM and 300 µM, respectively. The *P. falciparum* spermidine synthase is inhibited by the reaction product MTA with an IC_{50} of 160 µM which is in accordance with the data on the *E. coli* enzyme (Bowman, 1973). The *C. elegans* spermidine synthase is inhibited by the second product MTA with an IC_{50} of 430 µM. Therefore, a physiological function of feedback inhibition by MTA seems to be unlikely in *C. elegans*. In contrast to that, the mammalian spermidine synthases are more sensitive to MTA inhibition. A concentration of about 30 µM MTA is needed for 50% inhibition of enzyme activity (Hibasami *et al.*, 1980).

In conclusion, the spermidine synthases from *P. falciparum* and *C. elegans* resemble their mammalian counterparts in their organisation. However, the K_m for the substrate dcAdoMet and the inhibition by MTA are very different compared to the human spermidine synthase.

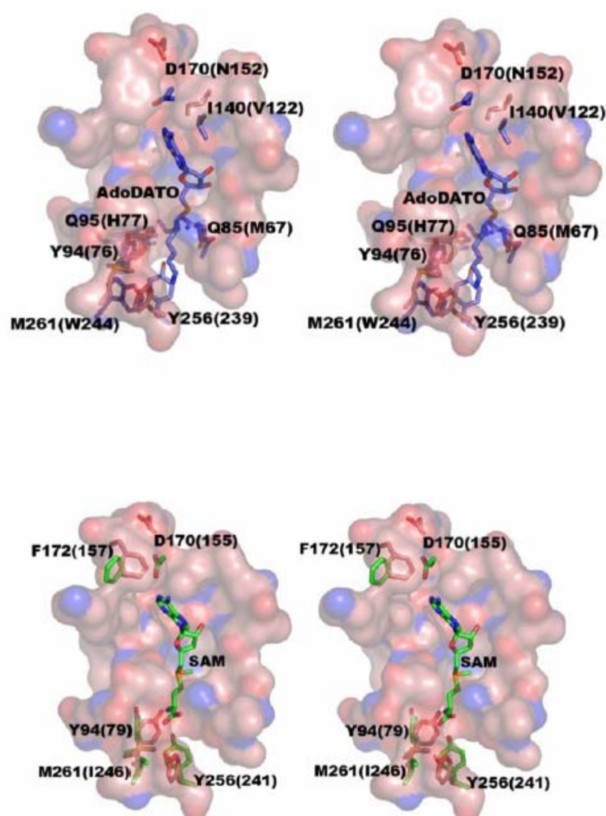
4.1.1 The active site of the *P. falciparum* and *C. elegans* spermidine synthases

Based on the crystal structures of the *C. elegans* and *T. maritima* spermidine synthase several amino acids residues that are highly conserved among spermidine synthases have been shown to build up the active site of the protein (Dufe *et al.*, in press; Korolev *et al.*, 2002) (Figure 4.1 A and B). Of the 20 amino acid residues that form the active site of the *C. elegans* spermidine synthase 16 of these residues are conserved in *T. maritima*, whereas four are exchanged Q^{85} to M^{67}, Q^{95} to H^{77}, P^{195} to Q^{178} and M^{261} to W^{245}. 17 of these 20 amino acid residues are present in the *P. falciparum* protein, whereas in comparison to the *C. elegans* protein the following differences are found in the *P. falciparum* spermidine synthase: Q^{95} by H^{103}, G^{171} by A^{179}, M^{261} by I^{269}.

The putrescine binding site of spermidine synthase is a hydrophobic cavity lying adjacent to two negatively charged sites responsible for anchoring the amino groups of putrescine. According to the *C. elegans* and *T. maritima* crystal structures three amino residues of spermidine synthase, Y^{94} (Y^{76}), D^{188} (D^{170}) and S^{189} (S^{171}) (the residues for the *T. maritima* sequence are in brackets), have been suggested to be crucial for catalysis (Dufe *et al.*, in press; Korolev *et al.*, 2002). In particular, D^{188} (D^{170}) are proposed to be responsible for deprotonating the attacking amino group of putrescine, whereas Y^{94} (Y^{76}) and S^{189} (S^{171}) are thought to be involved in substrate binding and proper orientation of the diamine (Dufe *et al.*, in press; Korolev *et al.*, 2002).

Y^{102}, D^{196} and S^{197} are the respective amino acid residues in the *P. falciparum* enzyme. To investigate whether these amino acid residues are crucial for enzyme activity, they were

A



B

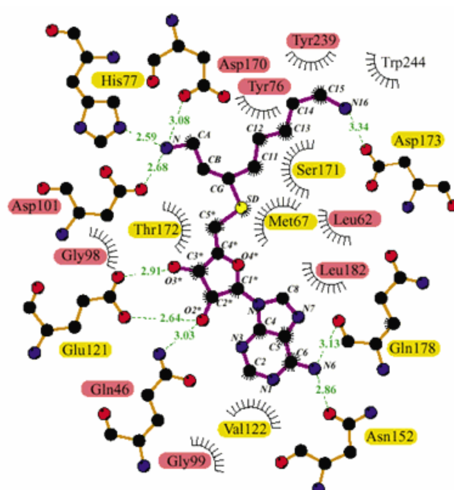


Figure 4.1 Crystal structure models of the *C. elegans* and *T. maritima* spermidine synthases.
A. Interaction of AdoDATO and S-adenosylmethionine (SAM) with *C. elegans* spermidine synthase (Dufe *et al.*, in press).
B. Schematic LIGPLOT[29] diagram of the interactions between AdoDATO (bonds shown in violet) and *T. maritima* spermidine synthase (Korolev *et al.*, 2002).

exchanged by site-directed mutagenesis in the *P. falciparum* spermidine synthase resulting in the mutant proteins $Y^{102}A$, $D^{196}N$ and $S^{197}A$. In accordance with the proposed crucial role, mutation of the Y^{102} to A and D^{196} to N led to inactive enzymes. In contrast to that, substitution of the S^{197} to A has only a slight effect on enzyme activity and hence may not be so critical for substrate binding or may be compensated by other amino acid residues which are present in the active site cavity.

The use of specific inhibitors is another approach in the study of the substrate-binding site (Goda *et al.* 2004). Using a number of monoamine and diamine compounds, a model has been proposed for the putrescine binding site of pig spermidine synthase. In accordance with the *T. maritima* crystal structure, the pig enzyme features a relatively large hydrophobic cavity adjacent to a negatively charged site (Goda *et al.* 2004). Based on the inhibitory activities of the 4MCHA analogs, it is reasonable to define the compounds that enter this groove and inhibit spermidine synthase activity as possessing as primary amine group, a flexible structure, and a distance of about 0.6 nm between the N-atom and terminus C-atom. Probably, one of the amino groups of putrescine is protonated and binds to this charged site, and the other amino group is not protonated and binds to the hydrophobic cavity, to be aminopropylated by dcAdoMet. The substrate binding site of the rat model shows good agreement with that of the crystal structure of the *T. maritima* enzyme.

A conspicuous feature of *T. maritima* spermidine synthase is a loop that consists of the amino acid residues 171-180, a conserved region that is also present in the *P. falciparum* sequence at position 197-205. This loop is proposed to be a dynamic structure functioning as a “gatekeeper” to or from the active site (Korolev *et al.*, 2002). However, only six of these amino acid residues are conserved in the corresponding nematode sequence 189-SSDPVGPAE-197. Moreover, an amino acid residue corresponding to T^{175} of the *T. maritima* loop is lacking in the *C. elegans* spermidine synthase (Dufe *et al.*, in press). The residues near the putrescine binding site in rat are Y^{79}, D^{173}, S^{174}, D^{176} and Y^{241}. D^{173} is the negatively charged site that can bind to the protonated amine group of putrescine, and is located in the “gatekeeping” loop which is also present in rat enzyme. The “gatekeeping” loop is dynamic

and is suggested to cover putrescine substrate. The presence of the “gatekeeping” loop indicates the dynamic nature of the putrescine-binding site, hence the difficulty in defining the best fitting compound into the site.

The sequence alignment also shows that the *C. elegans* spermidine synthase contains an insertion of about 27 amino acids close to the N-terminus. This insertion is not present in mammalian, bacterial and protozoan spermidine synthases and is a particular feature of only nematode spermidine synthases. The amino acid sequences of these insertions reflect the phylogenetic relationship of the nematodes according to the taxonomic classification by Blaxter *et al* (1998). *C. briggsae*, *A. caninum*, *A. suum* and *H. contortus* are members of the same class as *C. elegans.* In the three-dimensional structure of the *C. elegans* spermidine synthase most of the nematode-specific sequence spatially is unfortunately disordered. However, it is clear that this loop is located in close proximity to the gatekeeper loop. It is unclear whether these two structural elements interact and how this interaction may contribute to the reaction cycle (Dufe *et al*., in press). However, deletion of the nematode specific sequence had no effect on the specific activity of the *C. elegans* enzyme under standard assay conditions.

Apart from the nematode-specific insertion, a unique feature of *C. elegans* spermidine synthase is a coiled region A^{278} and T^{282}, which in the other structures is a short helix (P^{262}-F^{267}) while in the adjacent region the coil between R^{265} and E^{270} in *T. maritima* is substituted by helix in *C. elegans*. The high conservation of the structure of the active site and the absence of bound substrate in the present structure of the enzyme does not allow any straightforward explanation for the high K_m value found for dcAdoMet.

In conclusion, the similar primary amino acid sequence and structure prediction supports the suggestion of a universal catalytic mechanism for all spermidine synthases (Korolev *et al*., 2002). All the spermidine synthases possess a similar gate-keeping loop near the active site. The unique features of the nematode spermidine synthases are that they possess a nematode specific insertion and have two coiled regions of unknown function.

4.1.2 Localization of the *P. falciparum* and *C. elegans* spermidine synthase

The amino acid sequence of the *P. falciparum* spermidine synthase contains an N-terminal extension. Remarkably, the recombinant expression of *P. falciparum* spermidine synthase in *E. coli* was achieved only when the first 29 amino acids were omitted. *In silico* analyses with the full-length *P. falciparum* sequence using several prediction programs gave ambiguous results. Several programs predict a cytoplasmatic localization of the spermidine synthase protein, whereas others suggested that the *P. falciparum* N-terminal extension resembles a signal peptide. In particular, the tool PlasmoAP (www.plasmodb.org/restricted/ PlasmoAPcgi.shtml) predicts a potential apicoplast targeting signal. The signal peptide-like character of the N-terminus might account for the lack of recombinant expression of the full-length spermidine synthase in *E. coli*. Western blot analyses of the native *P. falciparum* spermidine synthase, however, revealed that the protein is not processed in the erythrocytic stage of the parasite but exhibits the molecular mass that corresponds to the deduced full-length polypeptide of 36.6 kDa. Similar N-terminal extensions are also present in plant spermidine synthases such as in *A. thaliana* (Hanzawa *et al.*, 2000). In this regard it is noteworthy to state that the *P. falciparum* spermidine synthase is predicted to be more closely related to plant enzymes than to spermidine synthases from animal origin.
The IFA analyses, however, shows a cytoplasmatic localization of *P. falciparum* spermidine synthase. The nuclear genome of *P. falciparum* contains a number of genes that exhibit the highest degree of similarity to plant orthologues. In accordance with that, comparative genomic analysis of the malaria causative agent, *P. falciparum*, with other eukaryotes for which the complete genome is available, revealed that the genome from *P. falciparum* was more similar to the genome of a plant, *A. thaliana*, than to other non-apicomplexan taxa (Bastien *et al.*, 2004). It is suggested that these proteins were originally encoded by the genome of the apicoplast, a plastid-like organelle of *P. falciparum* derived from a secondary endosymbiotic red algae (Foth and McFadden, 2003). It occurs throughout the Apicomplexa and is an ancient feature of this group acquired by the process of endosymbiosis. Like plant

chloroplasts, apicoplasts are semi-autonomous with their own genome and expression machinery. During evolution most of these plastid genes have been transferred to the nucleus of the host cell. While proteins that function in the apicoplast have to be transported back into the organelle by the means of a classical signal peptide and a bipartite leader, it is likely that some proteins remain and fulfil their physiological roles in the cytoplasm of the parasite. The nuclear-encoded apicoplast genes are predicted to encode substantial N-terminal extensions when compared with the equivalent plastid and bacterial proteins. The extreme N-terminal regions (16-34 amino acids) of these extensions resemble classic signal peptides, containing a hydrophobic domain followed by a “von Heijne” cleavage site (Waller *et al.*, 2000).

These leader sequences are necessary and sufficient to direct import of the reporter protein into the plastid in both parasites. Further analysis of the leader sequences showed that they are, indeed, bipartite: the N-terminus starts with a typical hydrophobic signal peptide that can usually be recognized by a neural network (Nielsen *et al.*, 1997), while the remainder of the N-terminal extension represents a plastid transit peptide. Deletion of just the transit peptide caused proteins, that now only contained an N-terminal signal peptide, to be secreted from the cell (into the parasitophorous vacuole) (DeRocher *et al.*, 2000), while removal of the signal peptide alone led to accumulation of the protein in the cytosol (Waller *et al.*, 2000).

The spermidine synthase gene expression pattern was determined in *C. elegans* (Lüersen *et al.*, 2004) by microinjection of green fluorescent protein (GFP) reporter gene constructs. All transgenic worms exhibited a cytosolic GFP expression in their intestinal cells. Plant spermidine synthases have also been shown to be cytosolic, for example, in Chinese cabbage leaves (Sindhu and Cohen, 1984) and in alfalfa tissues (Bagga *et al.*, 1997). Mammalian spermidine synthase was also found in cytoplasmic extracts isolated from bovine brain (Raina *et al.*, 1984).

The *P. falciparum* spermidine synthase was predicted to have an apicoplast targeting sequence, however, the spermidine synthase gene from *Plasmodium* does not contain a classical signal peptide. In accordance with that the protein is therefore not targeted into any

organelles in the plasmodium cell. This could possibly be the reason for the cytoplasmic localization of the enzyme. It is possible that it lost parts of the signalling sequence during evolution or that some parts mutated and therefore are not functional anymore.

4.2 Uniqueness of polyamine metabolism in *P. falciparum*

During the erythrocytic schizogony *P. falciparum* spermidine synthase exhibits a stage specific expression with the amount of mRNA as well as protein peaking at the old trophozoite stage, which matches with the transcription rate of the bifunctional *P. falciparum* ODC/AdoMetDC (Müller *et al.*, 2000). Moreover, the coordinated expression correlates with the increasing polyamine levels concurring the parasite's maturation from the ring to the schizont stage (Assaraf *et al.*, 1984; DasGupta *et al.*, 2005) that by itself runs in parallel to the general metabolic activity of the parasite. Human erythrocytes contain only trace amounts of polyamines and lack the biosynthetic enzymes for their production but are capable of polyamine transport *in vitro*. During the asexual cycle of *P. falciparum*, the parasite induces a stage-dependent elevation in the levels of polyamines by increased metabolism and uptake of extracellular pools (Assaraf *et al.*, 1984; DasGupta *et al.*, 2005). It has been suggested that polyamines such as putrescine and/or spermidine are required for the synthesis of proteins in parasitized erythrocytes. One important protein is the eukaryotic translation initiation factor (eIF-5A), an essential protein that contains the unique amino acid hypusine (Molitor *et al.*, 2004). Hypusine is biosynthesized by a two-step enzymatic mechanism starting with the transfer of an aminobutyl moiety from the polyamine substrate spermidine to the amino group of a specific lysine residue in the eIF-5A precursor and is catalyzed by the enzyme deoxyhypusine synthase (Kaiser *et al.*, 2003). The second step of hypusinylation is completed by deoxyhypusine hydroxylase. Polyamines have been also suggested to be essential for the translation of the DNA polymerase mRNA and that polyamines play an important role in regulating the cell cycle of the malarial parasite (Bachrach and Abu-Elheiga, 1990). It appears that polyamines regulate the schizogony process of *P. falciparum* (Assaraf *et al.*, 1984; Wright *et al.*, 1991; DasGupta *et al.*, 2005).

The remarkable characteristic of the spermidine synthase from *P. falciparum* is that it also accepts spermidine as a substrate and converts it into spermine. It has been suggested that plant spermidine synthase from alfalfa may also produce spermine. These authors postulated that spermidine once formed remains bound to the active site and a second aminopropyl group is transferred to this, leading to the formation of spermine and other minor polyamines (Bagga *et al.*, 1997). A BLAST search using the *P. falciparum* genome data base (Plasmo DB) did not reveal any hint for a spermine synthase gene. Nevertheless, low amounts of spermine are found in *P. falciparum* infected RBC (Assaraf *et al.*, 1984; Bitonti *et al.*, 1987; DasGupta *et al.*, 2005). The level increases during the maturation of the parasite and, remarkably, spermine synthesis was found to be elevated especially under conditions when putrescine levels decline due to ODC inhibition (Assaraf *et al.*, 1987; Bitonti *et al.*, 1989; DasGupta *et al.*, 2005). Comparable to *P. falciparum* spermidine synthase, the *E. coli* enzyme could also replace putrescine with spermidine. Compared to putrescine the reaction rate was reported to be 10% (Bowman, 1973). However, spermine was not found in *E. coli* grown in minimal salt medium (Dion and Cohen, 1972). To a lower degree the *T. maritima* spermidine synthase also accepts spermidine as substrate, and based on the crystal structure it was proposed that the “putrescine” part of the polyamine fits to the acceptor binding site with the aminopropyl group extending out into the solvent (Korolev *et al.*, 2002). In contrast to that, stringent substrate specificity was demonstrated for mammalian and plant spermidine synthases that do not accept spermidine as amine acceptor. Here, spermine is exclusively formed by the second aminopropyl transferase, spermine synthase (Pegg *et al.*, 1981; Hashimoto *et al.*, 1998). The *C. elegans* spermidine synthase has a similar substrate specificity for putrescine and does not accept spermidine. Spermine synthase is absent in the genome of this nematode (Lüersen *et al.*, 2004). In conclusion, we suggest that *P. falciparum* spermidine synthase is responsible for the small but significant spermine synthesis found in the erythrocytic stages of the parasite. *P. falciparum* possesses a unique polyamine metabolism in that the two key enzymes ODC and AdoMetDC have a bifunctional organisation. The spermidine synthase enzyme is also exceptional in that it catalyzes the

formation of spermidine and spermine. There is no suggestion for an interconversion pathway. This leads to the following proposed scheme.

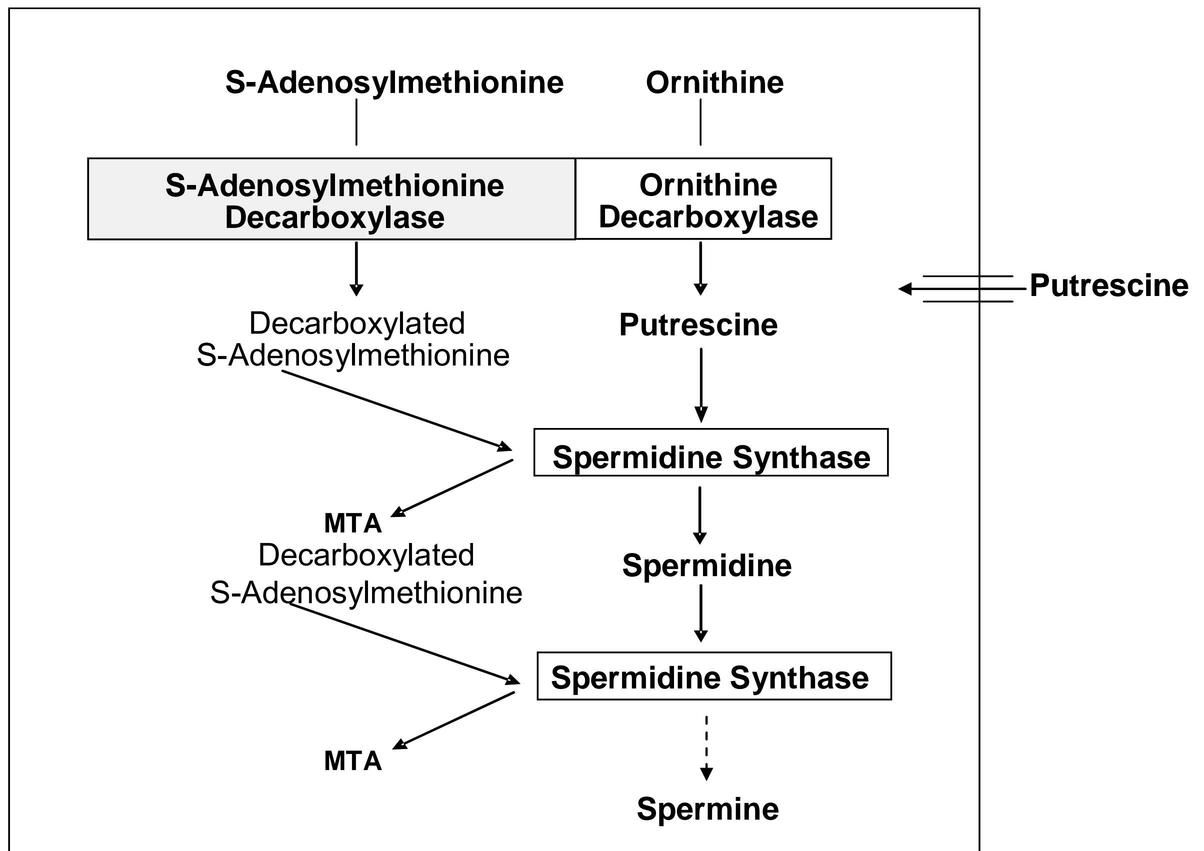


Figure 4.2 Schematic diagram of the polyamine metabolic pathway in *P. falciparum*

4.2.1 Protein-Protein Interactions

In *P. falciparum*, ODC and AdoMetDC activities are located on a single polypeptide, with the AdoMetDC domain in the N-terminal part connected to the C-terminal ODC domain through a hinge region (Müller *et al.*, 2000). There are numerous metabolic pathways in prokaryotes and eukaryotes which include such bifunctional enzymes. Most of these bifunctional enzymes catalyse consecutive reactions and two examples from *P. falciparum* include the bifunctional dihydrofolate reductase-thymidylate synthase (Chitnumsub et al. 2004) and the hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate syntheses (PPPK-DHPS) (Kasekarn, *et al.*, 2004).

One advantage of this bifunctional organisation is the possibility of substrate channelling. Substrate channeling is the process of direct transfer of an intermediate between the active sites of two enzymes that catalyze sequential reactions in a biosynthetic pathway (Srere, 1987; Spivey and Ovadi, 1999). The potential advantages of substrate channelling are that it prevents or impedes the loss of intermediates by diffusion, decreases the transit time required for an intermediate to reach the active site of the next enzyme and reduces the transit time for the system to reach the new steady state. Furthermore, it protects chemically labile intermediates, circumvents unfavourable equilibria and segregates the intermediates of competing chemical and enzymatic reactions and provides new means of metabolic regulation by the modulation of enzyme associations (Spivey and Ovadi, 1999; Ovadi and Srere, 2000; Miles *et al.*, 1999). The fusion of enzyme activities into one polypeptide as in tryptophan synthase (Miles, 2001) or fatty acid synthetase (Wakil, 1989) is the most highly evolved example of microenvironment control of substrate (intermediate) channelling.
In contrast to that, the bifunctional *P. falciparum* ODC/AdoMetDC catalyzes parallel reactions of the polyamine synthesis pathway. Hence, substrate channelling cannot occur within the *P. falciparum* ODC/AdoMetDC protein. Substrate channelling can also be achieved by multienzyme complexes of different polypeptides named metabolon such as in the α-keto acid dehydrogenase complexes (Mooney *et al.*, 2002). Therefore, it is possible that the dcAdoMet and putrescine formed by the *P. falciparum* ODC/AdoMetDC-catalyzed reactions may be channelled effectively to spermidine synthase via protein-protein interaction, modulating the formation of the product spermidine. However, in the present study a tight connection between *P. falciparum* ODC/AdoMetDC and spermidine synthase was not demonstrated under the chosen experimental conditions. The multicomplex transition state between *P. falciparum* ODC/AdoMetDC and spermidine synthase may be unstable or transient. In this regard, it should be recalled that some complexes are inherently weak with the possibility that both free and complexed enzymes exist within the cells (Srere and Ovadi, 1990). Another study showed experimental data indicating the existence of a metabolon carrying multiple enzymes from the polyamine biosynthesis pathway in *A. thaliana* (Panicot

et al., 2002). Moreover, the existence of a multifunctional enzyme involved in the synthesis of putrescine was proposed in *Lathyrus sativus* (Srivenugopal and Adiga, 1981).

4.3 *P. falciparum* spermidine synthase: a potential drug target

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today. Drug resistance has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. The economics of developing new pharmaceuticals for tropical diseases, including malaria are such that there is a great disparity between the public health importance of the disease and the amount of resources invested in developing new cures. Therefore considerable concentrated attention should be paid to the development of additional malaria drugs (Yeung, *et al.*, 2004).
Targeting the polyamine synthesis enzymes is an attractive chemotherapeutic strategy, since depletion of intracellular polyamine pools generally concurs with growth arrest. A major difficulty in exploiting enzymes of the polyamine metabolism as putative targets is that the intracellular concentrations of polyamines in mammalian cells are regulated by feedback mechanisms and involve multiple routes of synthesis and interconversion. These sophisticated regulatory mechanisms enable mammalian cells to adapt to considerable changes of extra- and intracellular polyamine concentrations.
The difficulties encountered in interfering with polyamine metabolism of mammalian cells are a source of optimism. This is because the parasitic polyamine pathway is much simpler and there is no alternative pathway by which the parasite can salvage itself if the polyamine synthesis is inhibited (Assaraf *et al.*, 1984). Moreover, the parasites depend much more on polyamines due to a higher proliferation rate compared to normal human cells. Therefore interfering with a key pathway should have more severe consequences for the parasite than for its host. Accordingly, the *P. falciparum* spermidine synthase still remains a good target for chemotherapy, although there are high degrees of similarity between the host and the

parasite enzymes that seems to hinder the development of specific inhibitors directed solely against the enzymes of the parasite.

Among the inhibitors tested against *P. falciparum* spermidine synthase, 4MCHA was found to be the most potent against the recombinant protein and the *P. falciparum* culture. When added to cultured *P. falciparum* for 48 h, it led to an 85 % growth arrest at a concentration of 100 µM. The IC_{50} values for the *C. elegans* spermidine synthase are in the same range as the IC_{50} values that have been reported for *Plasmodium* and rat spermidine synthases (8.1 µM for cyclohexylamine and 1.7 µM for 4MCHA (Shirahata *et al.*, 1991, 1993). In conclusion, the plasmodial and *C. elegans* spermidine synthases resemble the mammalian counterparts to a great extent. However, the nematode-specific insertion present close to the N-terminus of the protein as well as the low affinity for dcAdoMet and the feedback inhibitor MTA characterize *C. elegans* spermidine synthase.

4MCHA drastically lowers the spermidine concentration in HTC cells and in rat tissues (Shirahata *et al.*, 1993; Beppu *et al.*, 1995). However, the total polyamine concentration remains unchanged due to compensatory enhanced putrescine and spermine levels. In contrast to our results on *P. falciparum*, even a long term exposure of 250 µM 4MCHA for 8 days did not alter the growth rate of the mammalian cell line (Beppu *et al.*, 1995). In comparison, oral administration of 4MCHA for 10 days or 4 months drastically decreased spermidine concentrations, but did not affect the growth of treated rats (Shirahata *et al.*, 1993). In line with these data and results on other inhibitors, the spermidine synthase is not proposed to be a drug target in mammalian cells (Pegg *et al.*, 1995). In contrast to the mammalian system, spermidine synthase inhibitors have an adversely negative effect towards the *Plasmodium* culture and led to parasite death. The addition of spermidine to the culture medium did not significantly reverse the effect of the spermidine synthase inhibitors 4MCHA and APE on *P. falciparum* growth. This may be indicative for an inefficient uptake of spermidine by the parasitized erythrocytes. This finding is consistent with another study on AdoMetDC inhibitors (DasGupta *et al.*, 2005), where supplementation of the *P. falciparum* culture medium with putrescine or spermidine could not rescue the survival of the parasite,

but is in contrast to a previous report, where the addition of spermidine could, at least partially, circumvent the growth arrest by an AdoMetDC inhibitor (Wright *et al.*, 1991). DL-alpha-difluoromethylornithine (DFMO) inhibited ornithine decarboxylase (ODC) activity and arrested the growth of *P. falciparum* at the early trophozoite stage. When putrescine or spermidine was added to the parasites grown in culture, the arrest was reversed, and normal schizogony was completed even in the presence of DFMO (Assaraf *et al.*, 1987). Therefore, it has been proven that ODC inhibition can be reversed by supplementation with putrescine (Das Gupta *et al.* 2005).

The intraerythrocytic development of *P. falciparum* correlates with increasing levels of the polyamines putrescine, spermidine, and spermine in the infected red blood cells. The impact and effect of 4MCHA and APE has not been investigated on the polyamine levels within *Plasmodium* parasites. Therefore, to date we can not exclude that the spermidine synthase inhibitors 4MCHA and APE have additional targets than the spermidine synthase in the metabolism of *P. falciparum*.

Recently, several studies on spermidine synthase null mutants have demonstrated that spermidine synthesis is absolutely required by many lower eukaryotes including protozoan parasites (Hamasaki-Katagiri *et al.*, 1997; Guo *et al.*, 1999; Roberts *et al.*, 2001; Jin *et al.*, 2002). In this regard it is remarkable, that mammalian cells are relatively unaffected by spermidine depletion caused by spermidine synthase inhibitors (Pegg *et al.*, 1995; Shirahata *et al.*, 1993; Beppu *et al.*, 1995).

In addition, the current study shows evidence that the spermidine synthase from *Plasmodium* has some unusual properties when compared to the host enzyme. It can accept spermidine as a substrate and this suggests that perhaps it has a different active site cavity when compared to the human enzyme, which might be exploitable for the design of plasmodial specific inhibitors. The structural analogue of the substrate putrescine, 4MCHA is introduced as a lead compound that can perhaps be modified for the development of a more specific drug against the plasmodial enzyme.

In conclusion, some of these inhibitors for example, 4MCHA and to some extent APE were found to be potent plasmodicidals, whereby exogenous spermidine could not significantly reverse the growth arrest. Therefore, we suggest that *P. falciparum* spermidine synthesis represents a promising drug target.

Chapter 5 Summary

The naturally occurring polyamines spermidine and spermine are involved in numerous cellular processes and are found to be essential for cell proliferation and differentiation of prokaryotes and eukaryotes. In the polyamine synthesis pathway, spermidine is formed by the transfer of an aminopropyl group from decarboxylated *S*-adenosylmethionine (dcAdoMet) to a terminal amino group of putrescine. This reaction is catalyzed by spermidine synthase, where by the precursors putrescine and dcAdoMet are provided by the enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC), respectively. In contrast to spermidine synthases, the two key enzymes ODC and AdoMetDC have been extensively investigated in many organisms. In the current study, the spermidine synthase from the human malaria parasite *Plasmodium falciparum* and the model organism *Caenorhabditis elegans* was cloned, recombinantly expressed and characterized.
Malaria remains one of the most devastating tropical diseases with 300-500 million infections annually, resulting in 1-3 million deaths. There are only a few chemotherapeutics available that serve as treatment against malaria. This is compounded by the increasing prevalence of multi-resistant *P. falciparum* worldwide that has become a serious public health threat to the global control of the disease. Thus there is an urgent and pressing need for new drugs attacking novel targets in the metabolism of the malaria parasite. Inhibition of polyamine synthesis correlates with blockage of cell growth. Hence, the enzymes of the polyamine synthesis pathway represent attractive targets for the therapeutic intervention of rapidly proliferating cells such as tumour cells and parasites like *P. falciparum*. It is therefore conceivable that exploitation of this pathway could lead to the detection of inhibitors that will have a more adverse effect on the parasites than on the human host. The biochemical characterization of the polyamine metabolic pathway of *P. falciparum* is essential to illustrate differences between the parasite and its human host. Accordingly, previous studies have demonstrated that *P. falciparum* possesses a unique bifunctional ODC/AdoMetDC. However,

the third enzyme of the polyamine biosynthetic pathway, spermidine synthase, has not been investigated.

P. falciparum contains one copy of the spermidine synthase gene. Northern and Western blot analyses revealed a stage specific expression during the erythrocytic schizogony with the maximal amount of transcript and protein in mature trophozoites. The spermidine synthase polypeptide of 321 amino acids has a molecular mass of 36.6 kDa and contains an N-terminal extension that, similarly, is also found in certain plant but not in animal or bacterial orthologues. Immunofluorescence assays indicate a cytoplasmic localization of the spermidine synthase. Omitting the first 29 amino acids, a truncated form of *P. falciparum* spermidine synthase has been recombinantly expressed in *Escherichia coli*. The enzyme catalyses the transfer of an aminopropyl group from dcAdoMet onto putrescine with K_m values of 35 and 52 µM, respectively.

It is possible that the dcAdoMet and putrescine formed by the *P. falciparum* ODC/AdoMetDC-catalyzed reactions may be channelled effectively to spermidine synthase via protein-protein interaction, modulating the formation of the product spermidine. The three proteins were co-expressed and the supernatant analysed by Western blot analyses. However there was no suggestion for protein-protein interaction.

In contrast to mammalian spermidine synthases, spermidine can replace to some extent putrescine as the aminopropyl acceptor. Hence, *P. falciparum* spermidine synthase has the capacity to catalyse the formation of spermine that is found in small amounts in the erythrocytic stages of the parasite. By applying site-directed mutagenesis it was shown that the highly conserved amino acid residues Y^{102} and D^{196} of *P. falciparum* spermidine synthase are possibly important for substrate binding. Among the spermidine synthase inhibitors tested against *P. falciparum* spermidine synthase, *trans*-4-methylcyclohexylamine (4MCHA) was found to be most potent with a K_i value of 0.18 µM. In contrast to the situation in mammals, where inhibition of spermidine synthase has no or only little effect on cell proliferation, 4MCHA was an efficient inhibitor of *P. falciparum* cell growth *in vitro* with an IC_{50} of 35 µM, indicating that *P. falciparum* spermidine synthase represents a putative drug target.

Consistent with reports on other organisms, the free-living nematode *C. elegans* depends on polyamines, since ODC null mutants that do not have access to exogenous polyamines exhibit a stage-specific block of embryogenesis. The *C. elegans* ODC and AdoMetDC have been previously investigated at the protein level. Here, the spermidine synthase of *C. elegans* has been recombinantly expressed and biochemically characterised. The protein revealed a high degree of similarity to other eukaryotic spermidine synthases with the exception of a low affinity towards the substrate dcAdoMet (K_m = 110 µM) and a less pronounced feedback inhibition by the second reaction product 5'-methylthioadenosine (IC_{50} = 430 µM). The *C. elegans* protein that carries a nematode-specific insertion of 27 amino acids close to its N-terminus was used for crystallization in collaboration with Dufe *et al.* at the University of Lund, Sweden, leading to the first x-ray structure of a dimeric eukaryotic spermidine synthase. The 3-dimensional structure of this enzyme gave a better insight of the active site cavity of spermidine synthases.

6 REFERENCES

Abo-Dalo B, Ndjonka D, Pinnen F, Liebau E, Lüersen K. A novel member of the GCN5-related N-acetyltransferase superfamily from *Caenorhabditis elegans* preferentially catalyses the *N*-acetylation of thialysine (*S*-(2-aminoethyl)-L-cysteine). Biochem J 2004;384:129-37.

Abrahamsen MS, Morris DR. Cell type-specific mechanisms of regulating expression of the ornithine decarboxylase gene after growth stimulation. Mol Cell Biol. 1990;10:5525-8.

Achidi EA, Salimonu LS, Asuzu MC, Berzins K, Walker O. Studies on *Plasmodium falciparum* parasitemia and development of anemia in Nigerian infants during their first year of life. Am J Trop Med Hyg. 1996;55:138-43.

Alhonen-Hongisto L, Seppanen P, Janne J. Intracellular putrescine and spermidine deprivation induces increased uptake of the natural polyamines and methylglyoxal bis(guanylhydrazone). Biochem J. 1980;192:941-5.

Alhonen-Hongisto L. Regulation of S-adenosylmethionine decarboxylase by polyamines in Ehrlich ascites-carcinoma cells grown in culture. Biochem J. 1980;190:747-54.

Assaraf YG, Abu-Elheiga L, Spira DT, Desser H, Bachrach U. Effect of polyamine depletion on macromolecular synthesis of the malarial parasite, *Plasmodium falciparum*, cultured in human erythrocytes. Biochem J 1987;242:221-6.

Assaraf YG, Golenser J, Spira DT, Bachrach U. Polyamine levels and the activity of their biosynthetic enzymes in human erythrocytes infected with the malarial parasite, *Plasmodium falciparum*. Biochem J 1984; 222:815-19.

Bacchi CJ, Garofalo J, Mockenhaupt D, McCann PP, Diekema KA, Pegg AE, Nathan HC, Mullaney EA, Chunosoff L, Sjoerdsma A, Hutner SH. In vivo effects of alpha-DL-difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei brucei*. Mol Biochem Parasitol. 1983;7:209-25.

Bacchi CJ, Nathan HC, Hutner SH, McCann PP, Sjoerdsma A. Polyamine metabolism: a potential therapeutic target in trypanosomes. Science. 1980;210:332-4.

Bachrach U, Abu-Elheiga L. Effect of polyamines on the activity of malarial alpha-like DNA polymerase. Eur J Biochem. 1990;191:633-7.

Bagga S, Rochford J, Klaene Z, Kuehn GD, Phillips GC. Putrescine Aminopropyltransferase Is Responsible for Biosynthesis of Spermidine, Spermine, and Multiple Uncommon Polyamines in Osmotic Stress-Tolerant Alfalfa. Plant Physiol. 1997;114:445-454.

Bastien O, Lespinats S, Roy S, Metayer K, Fertil B, Codani JJ, Marechal E. Analysis of the compositional biases in *Plasmodium falciparum* genome and proteome using *Arabidopsis thaliana* as a reference. Gene. 2004;336:163-73.

Behm CA, Bendig MM, McCarter JP, Sluder AE. RNAi-based discovery and validation of new drug targets in filarial nematodes. Trends Parasitol. 2005;21:97-100.

Bello-Fernandez C, Packham G, Cleveland JL. The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci U S A. 1993;90:7804-8.

Beppu T, Shirahata A, Takahashi N, Hosoda H, Samejima K. Specific depletion of spermidine and spermine in HTC cells treated with inhibitors of aminopropyltransferases. J Biochem (Tokyo) 1995;117:339-45.

bifunctional ornithine decarboxylase, *S*-adenosyl-L-methionine decarboxylase, enables a well balanced polyamine synthesis without domain-domain interaction. J Biol Chem 2001;276:29651-6.

Birkholtz LM, Wrenger C, Joubert F, Wells GA, Walter RD, Louw AI. Parasite-specific inserts in the bifunctional S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum* modulate catalytic activities and domain interactions. Biochem J. 2004;377:439-48.

Birkholtz LM. Functional and structural characterization of the unique bifunctional enzyme complex involved in regulation of polyamine metabolism in *Plasmodium falciparum* (Thesis) 2002.

Bitonti AJ, Dumont JA, Bush TL, Edwards ML, Stemerick DM, McCann PP, Sjoerdsma A. Bis(benzyl)polyamine analogs inhibit the growth of chloroquine-resistant human malaria

parasites (*Plasmodium falciparum*) in vitro and in combination with alpha-difluoromethylornithine cure murine malaria. Proc Natl Acad Sci U S A 1989;86:651-5.

Bitonti AJ, Kelly SE, McCann PP. Characterization of spermidine synthase from *Trypanosoma brucei brucei*. Mol Biochem Parasitol. 1984;13:21-8.

Bitonti AJ, McCann PP, Sjoerdsma A. *Plasmodium falciparum* and *Plasmodium berghei*: effects of ornithine decarboxylase inhibitors on erythrocytic schizogony. Exp Parasitol 1987;64:237-43.

Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas WK. A molecular evolutionary framework for the phylum Nematoda. Nature. 1998;392:71-5.

Bloland PB. Drug Resistance in Malaria. Geneva, World Health Organization. 2001:1-32.

Bowman WH, Tabor CW, Tabor H. Spermidine biosynthesis. Purification and properties of propylamine transferase from *Escherichia coli*. J Biol Chem 1973;248:2480-6.

Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. (2003) *PLos Biology* 1, 85-100.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

Braun-Breton C, Rosenberry TL, Pereira da Silva LH. Glycolipid anchorage of *Plasmodium falciparum* surface antigens. Res Immunol 1990;141:743-55.

Breman JG, Alilio MS, Mills A. Conquering the intolerable burden of malaria: what's new, what's needed: a summary. Am. J. Trop. Med. Hyg. 2004; 71:1-15.

Breman JG. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg. 2001; 64:1-11.

Bruce BD. The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. Biochim Biophys Acta. 2001;1541:2-21.

Bürglin TR, Lobos E, Blaxter ML. *Caenorhabditis elegans* as a model for parasitic nematodes. Int J Parasitol. 1998;28:395-411.

Byers TL, Ganem B, Pegg AE. Cytostasis induced in L1210 murine leukaemia cells by the S-adenosyl-L-methionine decarboxylase inhibitor 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine may be due to hypusine depletion. Biochem J. 1992;287:717-24.

Cacciapuoti G, Porcelli M, Carteni-Farina M, Gambacorta A, Zappia V. Purification and characterization of propylamine transferase from *Sulfolobus solfataricus*, an extreme thermophilic archaebacterium. Eur J Biochem. 1986;161:263-71.

Casero RA Jr, Pegg AE. Spermidine/spermine N1-acetyltransferase - the turning point in polyamine metabolism. FASEB J. 1993;7:653-61.

Cerami C, Frevert U, Sinnis P, Takacs B, Clavijo P, Santos MJ, Nussenzweig V. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. Cell. 1992;70:1021-33.

Chitnumsub P, Yuvaniyama J, Vanichtanankul J, Kamchonwongpaisan S, Walkinshaw MD, Yuthavong Y. Characterization, crystallization and preliminary X-ray analysis of bifunctional dihydrofolate reductase-thymidylate synthase from *Plasmodium falciparum*. Acta Crystallogr D Biol Crystallogr. 2004;60:780-3.

Clos J, Westwood JT, Becker PB, Wilson S, Lambert K, Wu C. Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. Cell. 1990;63:1085-97.

Cohen SN, Chang AC, Hsu L. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci U S A. 1972; 69:2110-4.

Cohen SS. A guide to the polyamines. Oxford University Press, Oxford 1998.

Cohn MS, Tabor CW, Tabor H, Wickner RB. Spermidine or spermine requirement for killer double-stranded RNA plasmid replication in yeast. J Biol Chem. 1978;253:5225-7.

Coward JK, Motola NC, Moyer JD. Polyamine biosynthesis in rat prostate. Substrate and inhibitor properties of 7-deaza analogues of decarboxylated *S*-adenosylmethionine and 5'-methylthioadenosine. J Med Chem 1977;20:500-5.

Coward JK, Pegg AE. Specific multisubstrate adduct inhibitors of aminopropyltransferases and their effect on polyamine biosynthesis in cultured cells. Adv Enzyme Regul. 1987;26:107-13.

Coward JK. Aminopropyltransferases: mechanistic studies and the synthesis of specific inhibitors. Adv Exp Med Biol. 1988;250:111-5.

Cox FEG. Modern Parasitology: A textbook of parasitology. Blackwell Scientific, London 1993.

Da'Dara AA, Henkle-Duhrsen K, Walter RD. A novel trans-spliced mRNA from *Onchocerca volvulus* encodes a functional S-adenosylmethionine decarboxylase. Biochem J. 1996;320:519-30.

Da'dara AA, Walter RD. Molecular and biochemical characterization of S-adenosylmethionine decarboxylase from the free-living nematode *Caenorhabditis elegans*. Biochem J. 1998;336:545-50.

Das Gupta R, Krause-Ihle T, Bergmann B, Müller IB, Khomutov AR, Müller S, Walter RD, and Lüersen K. 3-Aminooxy-1-aminopropane and derivatives have an antiproliferative effect on cultured *Plasmodium falciparum* by decreasing intracellular polyamine concentrations. Antimicrob Agents Chemother 2005;49:2857-64.

Davis TM, Brown AE, Smith CD. Metabolic disturbances in *Plasmodium* coatneyi-infected rhesus monkeys. Int J Parasitol. 1993;23:557-63.

Dejima H, Kobayashi M, Takasaki H, Takeda N, Shirahata A, Samejima K. Synthetic decarboxylated *S*-adenosyl-L-methionine as a substrate for aminopropyl transferases. Biol Pharm Bull 2003;26:1005-8.

DeRocher A, Hagen CB, Froehlich JE, Feagin JE, Parsons M. Analysis of targeting sequences demonstrates that trafficking to the *Toxoplasma gondii* plastid branches off the secretory system. J Cell Sci. 2000;113:3969-77.

Desjardins RE, Canfield CJ, Haynes D, Chulay JD. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 1979;16:710-8.

Dion AS, Cohen SS. Polyamine stimulation of nucleic acid synthesis in an uninfected and phage-infected polyamine auxotroph of *Escherichia coli* K12. Proc Natl Acad Sci U S A 1972;69:213-7.

Dreyer G, Noroes J, Addiss D. The silent burden of sexual disability associated with lymphatic filariasis. Acta Trop. 1997;63:57-60.

Dufe VT, Lüersen K, Eschbach ML, Haider N, Karlberg T, Walter RD, Al-Karadaghi S. Cloning, expression, characterisation and three-dimensional structure determination of *Caenorhabditis elegans* spermidine synthase. FEBS Letters (in press).

Duke BO, Marty AM, Peett DL, Gardo J, Pion SD, Kamgno J, Boussinesq M. Neoplastic change in *Onchocerca volvulus* and its relation to ivermectin treatment. Parasitology. 2002;125:431-44.

Dyer M, Day KP. Commitment to gametocytogenesis in *Plasmodium falciparum*. Parasitol Today. 2000;16:102-7.

Emanuelsson O, Nielsen H, von Heijne G. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci. 1999;8:978-84.

Fillingame RH, Jorstad CM, Morris DR. Increased cellular levels of spermidine or spermine are required for optimal DNA synthesis in lymphocytes activated by concanavalin A. Proc Natl Acad Sci U S A. 1975;72:4042-5.

Foth BJ, McFadden GI. The apicoplast: a plastid in *Plasmodium falciparum* and other Apicomplexan parasites. Int Rev Cytol. 2003;224:57-110.

Gerner EW, Mamont PS. Restoration of the polyamine contents in rat hepatoma tissue-culture cells after inhibition of polyamine biosynthesis. Relationship with cell proliferation. Eur J Biochem. 1986;156:31-5.

Ghoda L, Phillips MA, Bass KE, Wang CC, Coffino P. Trypanosome ornithine decarboxylase is stable because it lacks sequences found in the carboxyl terminus of the mouse enzyme which target the latter for intracellular degradation. J Biol Chem. 1990;265:11823-6.

Giffin BF, McCann PP, Bitonti AJ, Bacchi CJ. Polyamine depletion following exposure to DL-alpha-difluoromethylornithine both in vivo and in vitro initiates morphological alterations and mitochondrial activation in a monomorphic strain of *Trypanosoma brucei brucei*. J Protozool. 1986;33:238-43.

Goda H, Watanabe T, Takeda N, Kobayashi M, Wada M, Hosoda H, Shirahata A, Samejima K. Mammalian spermidine synthase - identification of cysteine residues and investigation of the putrescine binding site. Biol Pharm Bull. 2004;27:1327-32.

Graser G, Hartmann T. Biosynthesis of spermidine, a direct precursor of pyrrolizidine alkaloids in root cultures of *Senecio vulgaris L*. Planta 2000;211:239-45.

Greenwood B, Mutabingwa T. Malaria in 2002. Nature 2002;415:670-672.

Grens A, Scheffler IE. The 5'- and 3'-untranslated regions of ornithine decarboxylase mRNA affect the translational efficiency. J Biol Chem. 1990;265:11810-6.

Guo K, Chang WT, Newell PC. Isolation of spermidine synthase gene (*spsA*) of *Dictyostelium discoideum*. Biochim Biophys Acta 1999;1449:211-6.

Hafner EW, Tabor CW, Tabor H. Mutants of *Escherichia coli* that do not contain 1,4-diaminobutane (putrescine) or spermidine. J Biol Chem. 1979 Dec 25;254(24):12419-26.

Hamasaki-Katagiri N, Tabor CW, Tabor H. Spermidine biosynthesis in *Saccharomyces cerevisae*: polyamine requirement of a null mutant of the SPE3 gene (spermidine synthase). Gene 1997;187:35-43.

Hanahan D. In: DNA cloning Vol. 1, a practical approach. IRL Press, Oxford, 1985;109-114.

Hannonen P, Raina A, Janne J. Polyamine synthesis in the regenerating rat liver: stimulation of S-adenosyl methionine decarboxylase, and spermidine and spermine synthases after partial hepatectomy. Biochim Biophys Acta. 1972;273:84-90.

Hanzawa Y, Takahashi T, Michael AJ, Burtin D, Long D, Pineiro M, Coupland G, Komeda Y. ACAULIS5, an *Arabidopsis* gene required for stem elongation, encodes a spermine synthase. EMBO J. 2000;19:4248-56.

Hashimoto T, Tamaki K, Suzuki K, Yamada Y. Molecular cloning of plant spermidine synthases. Plant Cell Physiol 1998;39:73-9.

Hasne MP, Ullman B. Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*. J Biol Chem. 2005;280:15188-94.

Hastings IM, D'Alessandro U. Modelling a predictable disaster: the rise and spread of drug-resistant malaria. Parasitol. Today 2000;16:340-347.

Heby O, Roberts SC, Ullman B. Polyamine biosynthetic enzymes as drug targets in parasitic protozoa. Biochem Soc Trans. 2003;31:415-9.

Heller JS, Fong WF, Canellakis ES. Induction of a protein inhibitor to ornithine decarboxylase by the end products of its reaction. Proc Natl Acad Sci U S A. 1976 ;73:1858-62.

Hibasami H, Borchardt RT, Chen SY, Coward JK, Pegg AE. Studies of inhibition of rat spermidine synthase and spermine synthase. Biochem J 1980;187:419-28.

Hibasami H, Kawase M, Tsukada T, Maekawa S, Sakurai M, Nakashima K. 2-Mercaptoethylamine, a competitive inhibitor of spermidine synthase in mammalian cells. FEBS Lett. 1988;229:243-6.

Hickok NJ, Seppanen PJ, Gunsalus GL, Janne OA. Complete amino acid sequence of human ornithine decarboxylase deduced from complementary DNA. DNA. 1987;6:179-87.

Higaki I, Matsui-Yuasa I, Terakura M, Kinoshita H, Otani S. Increased spermidine or spermine level is essential for hepatocyte growth factor-induced DNA synthesis in cultured rat hepatocytes. Gastroenterology. 1994;106:1024-31.

Hölttä E. Oxidation of spermidine and spermine in rat liver: purification and properties of polyamine oxidase. Biochemistry. 1977;16:91-100.

Hurta RA, Greenberg AH, Wright JA. Transforming growth factor beta 1 selectively regulates ornithine decarboxylase gene expression in malignant H-ras transformed fibrosarcoma cell lines. J Cell Physiol. 1993;156:272-9.

Hyvonen T. Excretion of acetylated and free polyamines by polyamine depleted Chinese hamster ovary cells. Int J Biochem. 1989;21:313-6.

Igarashi K, Kashiwagi K. Polyamine transport in bacteria and yeast. Biochem J. 1999;344:633-42.

Inselberg J. Gametocyte formation by the progeny of single *Plasmodium falciparum* schizonts. J Parasitol. 1983;69:584-91.

Jänne J, Alhonen L, Leinonen P. Polyamines: from molecular biology to clinical applications. Ann Med. 1991;23:241-59.

Jin Y, Bok JW, Guzman-de-Pena D, Keller NP. Requirement of spermidine for developmental transitions in *Aspergillus nidulans*. Mol Microbiol 2002;46:801-12.

Johnson JG, Epstein N, Shiroishi T, Miller LH. Factors affecting the ability of isolated *Plasmodium knowlesi* merozoites to attach to and invade erythrocytes. Parasitology. 1980;80:539-50.

Kaiser A, Gottwald A, Maier W, Seitz HM. Targeting enzymes involved in spermidine metabolism of parasitic protozoa - a possible new strategy for anti-parasitic treatment. Parasitol Res. 2003;91:508-16.

Kaiser A, Gottwald A, Wiersch C, Lindenthal B, Maier W, Seitz HM. Effect of drugs inhibiting spermidine biosynthesis and metabolism on the in vitro development of *Plasmodium falciparum*. Parasitol Res 2001;87:963-72.

Kajander EO, Harvima RJ, Kauppinen L, Akerman KK, Martikainen H, Pajula RL, Karenlampi SO. Effects of selenomethionine on cell growth and on S-adenosylmethionine metabolism in cultured malignant cells. Biochem J. 1990;267:767-74.

Kajander EO, Kauppinen LI, Pajula RL, Karkola K, Eloranta TO. Purification and partial characterization of human polyamine synthases. Biochem J 1989; 259:879-86.

Käpyaho K, Poso H, Janne J. Role of propylamine transferases in hormone-induced stimulation of polyamine biosynthesis. Biochem J. 1980;192:59-63.

Kasekarn W, Sirawaraporn R, Chahomchuen T, Cowman AF, Sirawaraporn W. Molecular characterization of bifunctional hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase from *Plasmodium falciparum*. Mol Biochem Parasitol. 2004;137:43-53.

Katz A, Kahana C. Transcriptional activation of mammalian ornithine decarboxylase during stimulated growth. Mol Cell Biol. 1987;7:2641-3.

Khomutov RM, Hyvonen T, Karvonen E, Kauppinen L, Paalanen T, Paulin L, Eloranta T, Pajula RL, Andersson LC, Poso H. 1-Aminooxy-3-aminopropane, a new and potent inhibitor of polyamine biosynthesis that inhibits ornithine decarboxylase, adenosylmethionine decarboxylase and spermidine synthase. Biochem Biophys Res Commun. 1985;130:596-602.

Kobayashi M, Watanabe T, Xu YJ, Tatemori M, Goda H, Niitsu M, Shirahata A, Samejima K. Control of spermidine and spermine levels in rat tissues by trans-4-methylcyclohexylamine, a spermidine-synthase inhibitor. Biol Pharm Bull. 2005;28:569-73.

Korhonen VP, Halmekyto M, Kauppinen L, Myohanen S, Wahlfors J, Keinanen T, Hyvonen T, Alhonen L, Eloranta T, Janne J. Molecular cloning of a cDNA encoding human spermine synthase. DNA Cell Biol. 1995;14:841-7.

Korolev S, Ikeguchi Y, Skarina T, Beasley S, Arrowsmith C, Edwards A, Joachimiak A, Pegg AE, Savchenko A. The crystal structure of spermidine synthase with a multisubstrate adduct inhibitor. Nat Struct Biol 2002 ;9:27-31.

Korpela H, Holtta E, Hovi T, Janne J. Response of enzymes involved in the metabolism of polyamines to phytohaemagglutinin-induced activation of human lymphocytes. Biochem J. 1981;196:733-8.

Kumar N, Aikawa M, Grotendorst C. *Plasmodium gallinaceum*: critical role for microtubules in the transformation of zygotes into ookinetes. Exp Parasitol. 1985;59:239-47.

Kumar S, Tamura K, Jakobsen IB, Nei M. *MEGA*2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17:1244-5.

Kyes S, Pinches R, Newbold C. A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. Mol Biochem Parasitol 2000;105:311-5.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.

Lindenthal C, Klinkert MQ. Identification and biochemical characterisation of a protein phosphatase 5 homologue from *Plasmodium falciparum*. Mol Biochem Parasitol. 2002;120:257-68.

Liu LX, Weller PF. Antiparasitic drugs. N Engl J Med. 1996;334:1178-84.

Lopatin AN, Shantz LM, Mackintosh CA, Nichols CG, Pegg AE. Modulation of potassium channels in the hearts of transgenic and mutant mice with altered polyamine biosynthesis. J Mol Cell Cardiol. 2000;32:2007-24.

Lucius R, Loos-Frank B. Parasitologie. Spektrum Akademischer Verlag, Berlin 1997.

Lüersen K, Eschbach ML, Liebau E, Walter RD. Functional GATA- and initiator-like-elements exhibit a similar arrangement in the promoters of *Caenorhabditis elegans* polyamine synthesis enzymes. Biol Chem. 2004; 385:711-21.

Macrae M, Kramer DL, Coffino P. Developmental effect of polyamine depletion in *Caenorhabditis elegans*. Biochem J. 1998;333:309-15.

Macrae M, Plasterk RH, Coffino P. The ornithine decarboxylase gene of *Caenorhabditis elegans*: cloning, mapping and mutagenesis. Genetics. 1995;140:517-25.

Marsh K, English M, Crawley J, Peshu N. The pathogenesis of severe malaria in African children. Ann Trop Med Parasitol. 1996;90:395-402.

Marsh K, Snow RW. Host-parasite interaction and morbidity in malaria endemic areas. Philos Trans R Soc Lond B Biol Sci. 1997;352:1385-94.

Marton LJ, Pegg AE. Polyamines as targets for therapeutic intervention. Annu Rev Pharmacol Toxicol 1995;35:55-91.

Mattila T, Honkanen-Buzalski T, Poso H. Reversible inhibition of bacterial growth after specific inhibition of spermidine synthase by dicyclohexylamine. Biochem J. 1984;223:823-30.

McCann PP, Bacchi CJ, Hanson WL, Cain GD, Nathan HC, Hutner SH, Sjoerdsma A. Effect on parasitic protozoa of α-difluoromethylornithine – an inhibitor of ornithine decarboxylase. In: Caldarera CM, Zappia, V and Bachrach, U, editors. Advances in Polyamine Research Vol 3, Raven Press, New York 1981;97-110.

Meis JF, Ponnudurai T. Ultrastructural studies on the interaction of *Plasmodium falciparum* ookinetes with the midgut epithelium of *Anopheles stephensi* mosquitoes. Parasitol Res. 1987;73:500-6.

Michael E, Bundy DA, Grenfell BT. Re-assessing the global prevalence and distribution of lymphatic filariasis. Parasitology. 1996;112:409-28.

Miles EW, Rhee S, Davies DR. The molecular basis of substrate channeling. J Biol Chem. 1999;274:12193-6.

Miles EW. Tryptophan synthase: a multienzyme complex with an intramolecular tunnel. Chem Rec. 2001;1:140-51.

Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature. 2002;415:673-9.

Miller LH, Usami S, Chien S. Alteration in the rheologic properties of *Plasmodium knowlesi*-infected red cells. A possible mechanism for capillary obstruction. J Clin Invest. 1971;50:1451-5.

Mitchell JL, Judd GG, Bareyal-Leyser A, Ling SY. Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells. Biochem J. 1994;299:19-22.

Molitor IM, Knobel S, Dang C, Spielmann T, Allera A, Konig GM. Translation initiation factor eIF-5A from *Plasmodium falciparum*. Mol Biochem Parasitol. 2004;137:65-74.

Mooney BP, Miernyk JA, Randall DD. The complex fate of alpha-ketoacids. Annu Rev Plant Biol. 2002;53:357-75.

Morgan DM. Polyamines. An overview. Mol Biotechnol. 1999;11:229-50.

Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V, Rodriguez A. Migration of *Plasmodium* sporozoites through cells before infection. Science. 2001;291:141-4.

Müller S, Coombs GH, Walter RD. Targeting polyamines of parasitic protozoa in chemotherapy. Trends Parasitol 2001;17:242-9.

Müller S, Da'dara A, Lüersen K, Wrenger C, Das Gupta R, Madhubala R, Walter RD. In the human malaria parasite *Plasmodium falciparum*, polyamines are synthesized by a bifunctional ornithine decarboxylase, *S*-adenosylmethionine decarboxylase. J Biol Chem 2000;275:8097-102.

Müller S, Wittich RM, Walter RD. The polyamine metabolism of filarial worms as chemotherapeutic target. Adv Exp Med Biol. 1988;250:737-43.

Murakami Y, Matsufuji S, Miyazaki Y, Hayashi S. Forced expression of antizyme abolishes ornithine decarboxylase activity, suppresses cellular levels of polyamines and inhibits cell growth. Biochem J. 1994;304:183-7.

Nagradova N. Interdomain communications in bifunctional enzymes: how are different activities coordinated? IUBMB Life 2003;55:459-66.

Ndjonka D, Da'dara A, Walter RD, Luersen K. *Caenorhabditis elegans* S-adenosylmethionine decarboxylase is highly stimulated by putrescine but exhibits a low specificity for activator binding. Biol Chem. 2003;384:83-91.

Newton CR, Warn PA, Winstanley PA, Peshu N, Snow RW, Pasvol G, Marsh K. Severe anaemia in children living in a malaria endemic area of Kenya. Trop Med Int Health. 1997;2:165-78.

Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 1997;10:1-6.

Nosten F, ter Kuile FO, Luxemburger C, Woodrow C, Kyle DE, Chongsuphajaisiddhi T, White NJ. Cardiac effects of antimalarial treatment with halofantrine. Lancet. 1993;341:1054-6.

Oka T, Perry JW, Kano K. Hormonal regulation of spermidine synthase during the development of mouse mammary epithelium in vitro. Biochem Biophys Res Commun. 1977;79:979-86.

Ovadi J, Srere PA. Macromolecular compartmentation and channeling. Int Rev Cytol. 2000;192:255-80.

Pajula RL, Raina A, Eloranta T. Polyamine synthesis in mammalian tissues. Isolation and characterization of spermine synthase from bovine brain. Eur J Biochem. 1979;101:619-26.

Pajula RL, Raina A. Methylthioadenosine, a potent inhibitor of spermine synthase from bovine brain. FEBS Lett. 1979;99:343-5.

Pajunen A, Crozat A, Janne OA, Ihalainen R, Laitinen PH, Stanley B, Madhubala R, Pegg AE. Structure and regulation of mammalian S-adenosylmethionine decarboxylase. J Biol Chem. 1988;263:17040-9.

Panicot M, Minguet EG, Ferrando A, Alcazar R, Blazquez MA, Carbonell J, Altabella T, Koncz C, Tiburcio AF. A polyamine metabolon involving aminopropyl transferase complexes in *Arabidopsis*. Plant Cell. 2002;14:2539-51.

Parchment RE. The implications of a unified theory of programmed cell death, polyamines, oxyradicals and histogenesis in the embryo. Int J Dev Biol. 1993;37:75-83.

Pegg AE, Kameji T, Shirahata A, Stanley B, Madhubala R, Pajunen A. Regulation of mammalian S-adenosylmethionine decarboxylase. Adv Enzyme Regul. 1988;27:43-55.

Pegg AE, McCann PP. Polyamine metabolism and function. Am J Physiol. 1982;243:212-21.

Pegg AE, Poulin R, Coward JK. Use of aminopropyltransferase inhibitors and of non-metabolizable analogs to study polyamine regulation and function. Int J Biochem Cell Biol 1995;27:425-42.

Pegg AE, Shuttleworth K, Hibasami H. Specificity of mammalian spermidine synthase and spermine synthase. Biochem J 1981;197:315-20.

Pegg AE, Williams-Ashman HG. On the role of *S*-adenosyl-L-methionine in the biosynthesis of spermidine by rat prostate. J Biol Chem 1969;244:682-93.

Pegg AE. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Res. 1988;48:759-74.

Pegg AE. Reactivity of analogues of S-adenosylmethionine in the enzymic synthesis of spermidine by mammalian tissues. Biochim Biophys Acta. 1969;177:361-4.

Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J. 1986;234:249–262.

Peltier JB, Friso G, Kalume DE, Roepstorff P, Nilsson F, Adamska I, van Wijk KJ. Proteomics of the chloroplast: systematic identification and targeting analysis of lumenal and peripheral thylakoid proteins. Plant Cell. 2000;12:319-41.

Pimenta PF, Touray M, Miller L. The journey of malaria sporozoites in the mosquito salivary glands. J Eukaryot Microbial. 1994;41:608-24.

Pistocchi R, Kashiwagi K, Miyamoto S, Nukui E, Sadakata Y, Kobayashi H, Igarashi K. Characteristics of the operon for a putrescine transport system that maps at 19 minutes on the *Escherichia coli* chromosome. J Biol Chem. 1993;268:146-52.

Pradel G, Garapaty S, Frevert U. Proteoglycans mediate malaria sporozoite targeting to the liver. Mol Microbiol. 2002;45:637-51.

Raina A, Hyvonen T, Eloranta T, Voutilainen M, Samejima K, Yamanoha B. Polyamine synthesis in mammalian tissues. Isolation and characterization of spermidine synthase from bovine brain. Biochem J. 1984;219:991-1000.

Raina A, Tuomi K, Pajula RL. Inhibition of the synthesis of polyamines and macromolecules by 5'-methylthioadenosine and 5'-alkylthiotubercidins in BHK21 cells. Biochem J. 1982;204:697-703.

Ridley RG. Medical need, scientific opportunity and the drive for antimalarial drugs. Nature 2002;415:686-693.

Roberts DJ, Pain A, Kai O, Kortok M, Marsh K. Autoagglutination of malaria-infected red blood cells and malaria severity. Lancet. 2000;355:1427-8.

Roberts SC, Jiang Y, Jardim A, Carter NS, Heby O, Ullman B. Genetic analysis of spermidine synthase from *Leishmania donovani*. Mol Biochem Parasitol 2001;115:217-26.

Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, Chandramohan D, Sharp B. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet 2003;361:1174-1181.

Sachs J, Malaney P. The economic and social burden of malaria. Nature 2002;415:680-5.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor Press, Cold Spring Habor, NY, 1989.

Samejima K, Yamanoha B. Purification of spermidine synthase from rat ventral prostate by affinity chromatography on immobilized S-adenosyl(5')-3-thiopropylamine. Arch Biochem Biophys. 1982;216:213-22.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977;74:5463-7.

Seiler N, Bolkenius FN, Rennert OM. Interconversion, catabolism and elimination of the polyamines. Med Biol. 1981;59:334-46.

Seiler N, Dezeure F. Polyamine transport in mammalian cells. Int J Biochem. 1990;22:211-8.

Seiler N, Knödgen B. Determination of di-and polyamines by high-performance liquid chromatographic separation of their 5-dimethylaminonaphthalene-1-sulfonyl derivatives. J Chromatogr 1978;145:29-39.

Seiler N. Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. Curr Drug Targets 2003;4:537-64.

Shantz LM, Holm I, Janne OA, Pegg AE. Regulation of S-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content. Biochem J. 1992;288:511-8.

Shantz LM, Pegg AE. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. Cancer Res. 1994;54:2313-6.

Shin SCJ, Vanderberg JP, Terzakis JA. Direct infection of hepatocytes of *Plasmodium berghei*. J Protozool. 1982;29:448-54.

Shirahata A, Morohohi T, Fukai M, Akatsu S, Samejima K. Putrescine or spermidine binding site of aminopropyltransferases and competitive inhibitors. Biochem Pharmacol 1991;41:205-12.

Shirahata A, Morohoshi T, Samejima K. Trans-4-methylcyclohexylamine, a potent new inhibitor of spermidine synthase. Chem Pharm Bull (Tokyo). 1988;36:3220-2.

Shirahata A, Takahashi N, Beppu T, Hosoda H, Samejima K. Effects of inhibitors of spermidine synthase and spermine synthase on polyamine synthesis in rat tissues. Biochem Pharmacol 1993;45:1897-903.

Silverman PH, Schooley JC, Mahlmann LJ. Murine malaria decreases hemopoietic stem cells. Blood. 1987;69:408-13.

Sinden RE, Hartley RH. Identification of the meiotic division of malarial parasites. J Protozool. 1985;32:742-4.

Sinden RE, Smalley ME. Gametocytes of *Plasmodium falciparum* phagocytosis by leucocytes *in vivo* and *in vitro*. Trans R Soc Trop Med Hyg. 1976;70:344-5.

Sinden, RE. Rodent malaria. Cell Biology. In: Killick-Kendrick R, Peters W, ed. London, Academic Press. 1978;85-168.

Sindhu RK and Cohen SS. Subcellular Localization of Spermidine Synthase in the Protoplasts of Chinese Cabbage Leaves. Plant Physiol. 1984;76: 219–223.

Singh S, Puri SK, Singh SK, Srivastava R, Gupta RC, Pandey VC. Characterization of simian malarial parasite (*Plasmodium knowlesi*)-induced putrescine transport in rhesus monkey erythrocytes. A novel putrescine conjugate arrests in vitro growth of simian malarial parasite (*Plasmodium knowlesi*) and cures multidrug resistant murine malaria (*Plasmodium yoelii*) infection in vivo. J Biol Chem. 1997;272:13506-11.

Spivey HO, Ovadi J. Substrate channeling. Methods. 1999;19:306-21.

Srere PA, Ovadi J. Enzyme-enzyme interactions and their metabolic role. FEBS Lett. 1990;268:360-4.

Srere PA. Complexes of sequential metabolic enzymes. Annu Rev Biochem. 1987;56:89-124.

Srivenugopal KS, Adiga PR. Enzymic conversion of agmatine to putrescine in *Lathyrus sativus* seedlings. Purification and properties of a multifunctional enzyme (putrescine synthase). J Biol Chem. 1981;256:9532-41.

Suh KN, Kain KC, Keystone JS. Malaria. CMAJ. 2004;170:1693-1702.

Suzuki T, Kashiwagi K, Igarashi K. Polyamine regulation of S-adenosylmethionine decarboxylase synthesis through the 5'-untranslated region of its mRNA. Biochem Biophys Res Commun. 1993;192:627-34.

Tabor CW, Tabor H, Xie QW. Spermidine synthase of *Escherichia coli*: localization of the speE gene. Proc Natl Acad Sci U S A. 1986;83:6040-4.

Tabor CW, Tabor H. Polyamines in microorganisms. Microbiol Rev. 1985;49:81-99.

Tabor CW, Tabor H. Polyamines. Annu Rev Biochem 1984;53:749-90.

Tabor CW, Tabor H. Putrescine aminopropyltransferase (*Escherichia coli*). Methods Enzymol. 1983;94:265-70.

Takechi M, Matsuo M, Ziba C, MacHeso A, Butao D, Zungu IL, Chakanika I, Bustos MD. (2001) Trop. Med. Int. Health 6, 429-434.

Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF, McFadden GI. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol. 2004;137:13-21.

Towbin H, Gordon J. Immunoblotting and dot immunobinding - current status and outlook. J Immunol Methods 1984;72:313-40.

Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976;193:673-5.

Tsuji M, Rodrigues EG, Nussenzweig RS. Progress toward a malaria vaccine: efficient induction of protective anti-malaria immunity. Biol Chem. 2001;382: 553-70.

van Vugt M, Ezzet F, Nosten F, Gathmann I, Wilairatana P, Looareesuwan S, White NJ. No evidence of cardiotoxicity during antimalarial treatment with artemether-lumefantrine. Am J Trop Med Hyg. 1999;61:964-7.

Vanderberg JP. *Plasmodium berghei*: Quantitation of sporozoites injected by mosquitoes feeding on a rodent host. Exp Parasitol. 1997;42:169-81.

von Heijne G, Nishikawa K. Chloroplast transit peptides. The perfect random coil? FEBS Lett. 1991;278:1-3.

Vujcic S, Diegelman P, Bacchi CJ, Kramer DL, Porter CW. Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. Biochem J. 2002;367:665-75.

Wahlfors J, Alhonen L, Kauppinen L, Hyvonen T, Janne J, Eloranta TO. 1990 Human spermidine synthase: cloning and primary structure. DNA Cell Biol. 1990;9:103-10.

Wahlgren M and Perlmann P. Malaria: Molecular and Clinical Aspects. Harwood Academic Pub., New York 1999.

Wakil SJ. Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry 1989;28:4523-30.

Wallace HM. Polyamines: specific metabolic regulators or multifunctional polycations? Biochem Soc Trans. 1998;26:569-71.

Waller RF, Keeling PJ, Donald RG, Striepen B, Handman E, Lang-Unnasch N, Cowman AF, Besra GS, Roos DS, McFadden GI. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. Proc Natl Acad Sci U S A. 1998;95:12352-7.

Waller RF, Reed MB, Cowman AF, McFadden GI. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. EMBO J. 2000;19:1794-802.

Weiss U. Malaria. Nature 2002;415:669.

White MW, Degnin C, Hill J, Morris DR. Specific regulation by endogenous polyamines of translational initiation of S-adenosylmethionine decarboxylase mRNA in Swiss 3T3 fibroblasts. Biochem J. 1990;268:657-60.

White NJ, Ho M. The pathophysiology of malaria. Adv Parasitol. 1992;31:83-173.

White NJ. Antimalarial drug resistance. J. Clin. Invest. 2004;113:1084-1092.

White NJ. Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia. 1999;41:301-8.

WHO report. 1995.

WHO report. 2001.

Wiest L, Pegg AE. Assay of spermidine and spermine synthases. Methods Mol Biol. 1998;79:51-7.

Winkel BS. Metabolic channeling in plants. Annu Rev Plant Biol. 2004;55:85-107.

Winstanley PA, Ward SA, Snow RW. Clinical status and implications of antimalarial drug resistance. Microbes Infect. 2002;4:157-64.

Winstanley PA. Chemotherapy for *falciparum* malaria: the armoury, the problems and the prospects. Parasitol. Today 2000;16:146-153.

Wood WB. Determination of pattern and fate in early embryos of *Caenorhabditis elegans*. Dev Biol. 1988;5:57-78.

Wrenger C, Lüersen K, Krause T, Müller S, Walter RD. The *Plasmodium falciparum*

Wright PS, Byers TL, Cross-Doersen DE, McCann PP, Bitonti AJ. Irreversible inhibition of *S*-adenosylmethionine decarboxylase in *Plasmodium falciparum*-infected erythrocytes: growth inhibition *in vitro*. Biochem Pharmacol 1991;41:1713-8.

Xie QW, Tabor CW, Tabor H. Deletion mutations in the speED operon: spermidine is not essential for the growth of *Escherichia coli*. Gene. 1993;126:115-7.

Yamanaka H, Kubota M, Carson DA. Synergistic inhibition of polyamine synthesis and growth by difluoromethylornithine plus methylthioadenosine in methylthioadenosine phosphorylase-deficient murine lymphoma cells. Cancer Res. 1987;47:1771-4.

Yamanoha B, Samejima K, Nakajima T, Yasuhara T. Differences between homogeneous spermidine synthases isolated from rat and pig liver. J Biochem (Tokyo). 1984;96:1273-81.

Yeung S, Pongtavornpinyo W, Hastings IM, Mills AJ, White NJ. Antimalarial drug resistance, artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. Am. J. Trop.Med. Hyg. 2004;71:179-186.

Yoon SO, Lee YS, Lee SH, Cho YD. Polyamine synthesis in plants: isolation and characterization of spermidine synthase from soybean (*Glycine max*) axes. Biochim Biophys Acta 2000 ;1475:17-26.

Zappia V, Cacciapuoti G, Pontoni G, Oliva A. Mechanism of propylamine-transfer reactions. Kinetic and inhibition studies on spermidine synthase from *Escherichia coli*. J Biol Chem 1980;255:7276-80.

Appendix

Acknowledgements

Published Articles:

1) Haider N, Eschbach ML, Dias S de S, Gilberger TW, Walter RD, Lüersen K. The spermidine synthase of the malaria parasite *Plasmodium falciparum*: molecular and biochemical characterisation of the polyamine synthesis enzyme. Mol Biochem Parasitol. 2005;142:224-36.

2) Cloning, expression, characterisation and three-dimensional structure determination of *Caenorhabditis elegans* spermidine synthase
Veronica T. Dufe, Kai Lüersen, Marie-Luise Eschbach, Nashya Haider, Tobias Karlberg, Rolf D. Walter, Salam Al-Karadaghi. FEBS Letters (in press).

Acknowledgements

I would like to begin by thanking Professor Rolf D. Walter for accepting me to do a PhD in the department of Biochemistry. I thank him for his forbearance, patience and for his guidance over the years, as he has always been very helpful during the course of this PhD.

I would also like to thank PD Dr. Sylke Müller for accepting to co-referee the dissertation.

My special thanks go to the Vereinigung der Freunde des Tropeninstituts for providing me with a fellowship to carry out the doctoral research project.

I am indebted to Dr. Kai Lüersen for his kindness and generosity. I would have been unable to complete this work without his help, foresight and formulations which were very important to finish the work.

Marie-Luise Eschbach, The best TA and lab-mate; the Biochemistry department would be lost without you!! Thanks for teaching me all the lab-techniques over the years, and thank you for your tolerant guidance and helping hand in the lab despite being extremely busy yourself.

Svenja and Stefanie, you are both great friends and you have both been there for me whenever I needed that extra help! You have always been willing to help in every way you could, even though you yourselves have overbearing and demanding careers.

I would like to thank the PhD laboratory colleagues Jana, Robin, Ingrid, Rosie and Caroline for making the work environment so friendly and warm. Thank you for being so understanding, for listening, always giving me a helping hand when it was required and not

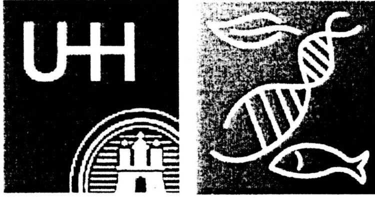
least for the great times inside and outside the lab. I will always treasure those wonderful memories at the concerts, and other special evenings during my stay in Hamburg.

Of course I would also like to thank Carsten the “big friendly giant” for his help with my work; Zita for her immense help and guidance; and Marzena, Bärbel and Sylke for their immense moral support throughout these years.

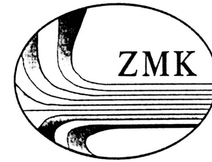
I am very grateful to be blessed with a wonderful, loving and understanding family. Abbu and Ammu you are the best parents ever! Without your moral support I would never have succeeded. I am indebted to you for all the long distance phone calls and the willingness to listen to me whenever I needed you to. Silwat and Moin, you guys are the best siblings someone could ask for. Thanks so much for always being there for me.

To my best friend Gigi Ho, you’re tremendous! I am really blessed to have a friend like you. Thank you for being a great moral support throughout the years, and the conversations and visits to pep me up!

I would also like to thank all my friends and acquaintances in Hamburg, through whom I have come to see a special city in which I will always feel welcome. Thank you for making these years exceptional.



University of Hamburg
Department of Biology



ZENTRUM FÜR MEERES-
UND KLIMAFORSCHUNG

October 24th, 2005

This is to certify that the quality of the English employed in PhD thesis submitted by the candidate Nashya Haider entitled "The characterization of the spermidine synthase from *Plasmodium falciparium* (Welch 1897) and *Caenorhabditis elegans* (Maupas) is of a standard sufficient to fulfill the language requirements of the University of Hamburg.

Sincerely

Prof. Dr. Michael St. John
Vice Director, Center for Marine and Climate Research
Institute for Hydrobiology and Fisheries Science
University of Hamburg
Olbersweg 24
22767 Hamburg
Germany
Tel +4940428386600
FAX +4940428386618
email michael.st.john @uni-hamburg.de