Parasite-specific inserts in the bifunctional S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum* modulate catalytic activities and domain interactions

Lyn-Marie BIRKHOLTZ*, Carsten WRENGER†, Fourie JOUBERT*, Gordon A. WELLS*, Rolf D. WALTER† and Abraham I. LOUW*[1]

*Department of Biochemistry, School of Biological Sciences, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa, and †Biochemical Parasitology, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, D-20359, Hamburg, Germany

Polyamine biosynthesis of the malaria parasite, *Plasmodium falciparum*, is regulated by a single, hinge-linked bifunctional PfAdoMetDC/ODC [*P. falciparum* AdoMetDC (S-adenosylmethionine decarboxylase)/ODC (ornithine decarboxylase)] with a molecular mass of 330 kDa. The bifunctional nature of AdoMetDC/ODC is unique to Plasmodia and is shared by at least three species. The PfAdoMetDC/ODC contains four parasite-specific regions ranging in size from 39 to 274 residues. The significance of the parasite-specific inserts for activity and protein–protein interactions of the bifunctional protein was investigated by a single- and multiple-deletion strategy. Deletion of these inserts in the bifunctional protein diminished the corresponding enzyme activity and in some instances also decreased the activity of the neighbouring, non-mutated domain. Intermolecular interactions between AdoMetDC and ODC appear to be vital for optimal ODC activity. Similar results have been reported for the bifunctional *P. falciparum* dihydrofolate reductase-thymidylate synthase [Yuvaniyama, Chitnumsub, Kamchonwongpaisan, Vanichtanankul, Sirawaraporn, Taylor, Walkinshaw and Yuthavong (2003) Nat. Struct. Biol. **10**, 357–365]. Co-incubation of the monofunctional, heterotetrameric ≈150 kDa AdoMetDC domain with the monofunctional, homodimeric ODC domain (≈180 kDa) produced an active hybrid complex of 330 kDa. The hinge region is required for bifunctional complex formation and only indirectly for enzyme activities. Deletion of the smallest, most structured and conserved insert in the ODC domain had the biggest impact on the activities of both decarboxylases, homodimeric ODC arrangement and hybrid complex formation. The remaining large inserts are predicted to be non-globular regions located on the surface of these proteins. The large insert in AdoMetDC in contrast is not implicated in hybrid complex formation even though distinct interactions between this insert and the two domains are inferred from the effect of its removal on both catalytic activities. Interference with essential protein–protein interactions mediated by parasite-specific regions therefore appears to be a viable strategy to aid the design of selective inhibitors of polyamine metabolism of *P. falciparum*.

Key words: malaria, *Plasmodium*-specific insert, polyamine, protein interaction.

INTRODUCTION

The polyamines, of which putrescine, spermidine and spermine are the most prevalent, are involved in numerous functions crucial to macromolecular syntheses, cell development and differentiation [1]. The synthesis of the polyamines is mainly under the control of the rate-limiting decarboxylases, ODC (ornithine decarboxylase) and AdoMetDC (S-adenosylmethionine decarboxylase) [1,2]. A variety of anti-tumour drugs have been developed targeting ODC activity in these highly proliferative cells [1,3]. Polyamine metabolism has recently attracted attention as a drug target in parasitic protozoa, since α-difluoromethylornithine, an inhibitor of ODC, has been successful in the clinical treatment of African sleeping sickness caused by *Trypanosoma gambiense* [4]. The *in vivo* efficacy of α-difluoromethylornithine in the treatment of malaria is only moderate, but erythrocytic schizogony is arrested *in vitro* and α-difluoromethylornithine was shown to be curative of rodent malaria when used in combination with polyamine analogues [5]. These results provide strong support for studies aimed at evaluating the anti-malarial potential of polyamine biosynthesis as a drug target.

Malaria remains one of the most devastating tropical infectious diseases affecting humankind. The disease currently affects 300–500 million people annually with 90 % of infections occurring in sub-Saharan Africa [6]. Resistance of *Plasmodium falciparum*, the most virulent parasite infecting humans, to current anti-malarials is widespread, while control programmes are impeded by insecticide resistance, and social and environmental factors [7]. In order to intervene with a disease of such magnitude, a multi-faceted strategy must be pursued. One approach is the biochemical characterization of parasite-specific properties of metabolic pathways and their constituent enzymes for rational drug-development strategies.

A distinguishing feature of polyamine metabolism in the malaria parasite is the linkage of AdoMetDC and ODC activities on a single, bifunctional polypeptide PfAdoMetDC/ODC [Pf (*P. falciparum*) AdoMetDC/ODC] [8]. The AdoMetDC domain at the N-terminus is connected to the C-terminal ODC domain via a hinge region. Monofunctional mammalian ODC in its active form is an obligate homodimeric protein, whereas monofunctional AdoMetDC isolated from various organisms presents as a post-translationally cleaved heterotetrameric protein [9,10]. The

Abbreviations used: ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; Pf, *P. falciparum*; DHFR-TS, dihydrofolate reductase/thymidylate synthase.

[1] To whom correspondence should be addressed (e-mail braam.louw@bioagric.up.ac.za).

bifunctional PfAdoMetDC/ODC polypeptide assembles into a functional 330 kDa heterotetrameric protein complex proposed to consist of a homodimeric ODC component and a heterotetrameric AdoMetDC component [8]. Another unique characteristic of PfAdoMetDC/ODC is that it contains several areas of inserted amino acids compared with homologous proteins of other organisms, which almost doubles the size of the malarial protein. Several other bifunctional *P. falciparum* proteins have been described, including DHFR (dihydrofolate reductase)-TS (thymidylate synthase; also found in other protozoa) [11,12], DHPS-PPPK (dihydropteroate synthetase/dihydrohydroxymethylpterin pyrophosphokinase) [13], glucose-6-phosphate dehydrogenase/6-phosphogluconolactonase [14] and guanylate cyclase/adenylate cyclase [15]. Parasite-specific inserts have been reported in at least seven other proteins of *P. falciparum* [11,16–21]. These characteristics raise intriguing questions as to the evolutionary origin and functional advantages of the parasite-specific inserts and the bifunctional organization of these Plasmodial proteins.

Proposals for the origin and function of the bifunctional arrangement include gene-fusion or duplication events [14,15] to enable substrate channelling, co-ordinated regulation of protein activities/abundances and/or intramolecular communication and interaction [4,22]. Investigations of the bifunctional PfDHFR-TS have shown that the active sites of the DHFR and TS operate independently of each other. However, the catalytic activity of TS is dependent on distinct physical interactions with the DHFR domain as shown by mutational studies and a recently published crystal structure [23]. Studies have revealed that mutations of active site residues in one domain of the bifunctional PfAdoMetDC/ODC complex abolish the activity of that domain, but do not influence the activity of the neighbouring domain [8,24,25]. Similarly, inhibition of one domain with a specific inhibitor has a singular effect on that domain. It therefore seems that the individual decarboxylase activities can function independently from each other [24]. However, specific interdomain interactions for stabilization of the bifunctional complex PfAdoMetDC/ODC cannot be excluded. Both domains can be expressed in an active, monofunctional form with a partial hinge region (131 and 143 residues of the hinge for AdoMetDC and ODC, respectively). However, the ODC domain is almost 10 times less active in its monofunctional form than in association with AdoMetDC in the bifunctional complex [25]. This observation alludes to distinctive interactions of ODC with the AdoMetDC domain for optimal catalytic activity, similar to the dependence of TS activity on its association with DHFR [23,26,27].

The role of the parasite-specific inserts in Plasmodial proteins is uncertain. Proposals for their function include contribution to the antigenic repertoire of the parasite and interaction with as yet undefined, regulatory proteins [28–30]. However, these inserted regions appear to be structurally unconstrained and non-immunogenic. It has furthermore been shown that the parasite-specific inserts, which typically contain hydrophilic low-complexity regions, are species-specific, rapidly diverging areas most likely forming non-globular domains extruding from the core of the proteins [31]. The crystal structure of *P. falciparum* DHFR-TS has confirmed the predicted surface localization of the parasite-specific inserts and revealed some of their functions [23]. The first insert in DHFR extends away from the domain surface and does not interfere with the core structure of the protein, but appears to stabilize interdomain interactions between the DHFR and TS domains. Furthermore, a second insert is also located at the domain surface and was shown to interact with the junction region between the two domains [23]. Obvious questions therefore arise as to the importance of the parasite-specific inserts for the activity and/or heterotetrameric structure of PfAdoMetDC/ODC.

We investigated in this study the potential involvement of the parasite-specific regions in the catalytic activities as well as physical interactions of the two decarboxylase domains of the bifunctional PfAdoMetDC/ODC by following a single and multiple deletion mutagenesis strategy. The smallest, most structured 39 amino acid residue insert (this study and [32]), which is conserved between at least three Plasmodial species, could be shown to be essential for both decarboxylase activities as well as for inter- and intramolecular protein–protein interactions. The proposed hinge region between the two domains appears to be more important for interdomain interactions and only indirectly for enzyme activities. The remaining large, less conserved inserts appear to be mostly unstructured and important for enzymic activities.

EXPERIMENTAL

Sequence and structural analyses

Amino acid sequence alignments for PfAdoMetDC/ODC (GenBank accession number AF094833) and the corresponding enzymes from human, mouse, *Leishmania donovani* and *Trypanosoma brucei* were performed with Clustal W [33] using the default parameters. GenBank accession numbers for AdoMetDCs were as follows: human (M21154), mouse (D12780), *T. brucei* (U20092), *L. donovani* (LDU20091); for ODCs, human (M31061), mouse (J03733), *T. brucei* (J02771) and *L. donovani* (M81192). Bifunctional AdoMetDC/ODC was also identified in other *Plasmodium* species through analyses of the PlasmoDB database (http://www.plasmoDB.org) by disabling low-complexity filtering and repeat masking. Open reading frames were identified for *Plasmodium berghei* (berg_296a09.q1c) and *Plasmodium yoelii* (chrPyl_cpy1465) with getorf and plotorf from the Emboss package. These sequences were also included in the multiple alignment. Analyses of low-complexity regions were performed with the SEG programme [34]. Secondary structure predictions, antigenic profiles and Kyte and Doolittle hydrophobicity plots were obtained with the PredictProtein server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html).

Deletion mutagenesis

Deletion mutants were created for all the major inserts present in both the PfAdoMetDC and PfODC domains, as well as for the central core of the hinge region (180 amino acids, residues 573–752) connecting these domains in the bifunctional enzyme. Oligonucleotides used for the site-directed deletion mutagenesis are indicated in Table 1. A typical deletion mutagenesis reaction (50 μl final volume) contained 10 ng of the expression plasmids with the specific inserts (pASK-IBA3 for bifunctional PfAdoMetDC/ODC; pASK-IBA7 with either the PfAdoMetDC or PfODC domains; Institut für Bioanalytik, Göttingen, Germany) [8,24,25], 150 ng of both the mutagenic sense and antisense mega-primers (Table 1), 1 × *Pfu* DNA polymerase reaction buffer, 2.5 mM of each dNTP and 3 units of *Pfu* DNA polymerase (Promega). The cycling parameters were 95 °C for 50 s, 55 °C for 1 min and 68 °C for 12 min (bifunctional construct) or 9 min (individual domains) repeated for 18 cycles in total after an initial denaturation step of 95 °C for 3 min. After nine cycles, a further 1 unit of *Pfu* DNA polymerase was added to amplifications of the bifunctional constructs. The PCR products were then treated with 20 units of *Dpn*I (New England Biolabs) for 3 h at 37 °C, followed by removal of the digested parental DNA templates using the standard protocols described in the High Pure PCR Product

Table 1 Mutagenic mega-primer oligonucleotides used for deletion mutagenesis of PfAdoMetDC/ODC

Sites where deletion occurred are indicated with a dash. The number of nucleotides removed in each of the deletion mutant primers is shown.

Primer	Sequence (5′ → 3′)	No. of nucleotides deleted
ΔA_1 sense	GAC GGA TAT AGC TTC TAC GTT T-AA TGA ATT TTA TTT TAC ACC TTG TGG	591
ΔA_1 antisense	CCA CAA GGT GTA AAA TAA AAT TCA TT-A AAC GTA GAA GCT ATA TCC GTC	591
ΔH sense	GTG TAG AAA AAG AAA CTT TG-G AAA AAA TGA AAG ATT ATA TAA GTG	540
ΔH antisense	CAC TTA TAT AAT CTT TCA TTT TTT C-CA AAG TTT CTT TTT CTA CAC	540
ΔO_1 sense	GGA GGG GGA TAT CCA GAA GAA TTA GAA TAT GAT-AGT TTT GAA AAA ATA TCA TTG GC	117
ΔO_1 antisense	GC CAA TGA TAT TTT TTC AAA ACT-ATC ATA TTC TAA TTC TTC TGG ATA TCC CCC TCC	117
ΔO_2 sense	GAC CAT TAC GAT CCT TTA AAT TTT T-TC TCA TAT TAT GTA AGC GAT AGT ATA TAT GG	435
ΔO_2 antisense	CCA TAT ATA CTA TCG CTT ACA TAA TAT GAG AA-A AAA TTT AAA GGA TCG TAA TGG TC	435

Purification Kit (Roche Diagnostics). The purified mutated constructs with nicks were then ligated with 6 units of T4 DNA ligase (Promega) at 4 °C for 16 h and subsequently transformed into competent DH5α *Escherichia coli* cells (Invitrogen). All mutations were verified by nucleotide sequencing using the Sanger dideoxy chain-termination reaction for double-stranded DNA or terminator cycle sequencing using BigDye™ Terminator v2.0 Cycle Sequencing Ready Reaction mix on an automated ABI Prism 377 DNA Automatic Sequencer (PE Applied Biosystems).

Recombinant expression and purification of wild-type and mutant proteins

Strep-tag II fusion-protein purifications

The entire coding region of the PfAdoMetDC/ODC was directionally cloned in-frame into the *Bsa*I site of the expression vector pASK-IBA3 (Institut für Bioanalytik) [8]. The constructs containing PfAdoMetDC/ODC or the mutant forms of this protein were transformed into the AdoMetDC- and ODC-deficient competent *E. coli* cell line, EWH331, kindly provided by Dr H. Tabor (National Institutes of Health, Bethesda, MD, U.S.A.) [35]. The individual AdoMetDC and ODC domains (wild-type forms) containing parts of the hinge region (88 and 92 residues for AdoMetDC and ODC, respectively) were cloned into the pASK-IBA7 vector and expressed in BL21-CodonPlus™ (DE3)-RIL *E. coli* (Stratagene) [24,25]. A single colony was picked from an overnight culture on solid medium and grown for 16 h at 37 °C in Luria–Bertani medium containing 50 μg/ml ampicillin. Thereafter, the culture was diluted 1:10 and grown at 37 °C until the D_{600} reached 0.5 unit. The expression of proteins was initiated by induction with 200 ng/ml anhydrotetracycline (Institut für Bioanalytik). The cells were then grown for a further 16 h at 22 °C for the bifunctional enzymes and for 4 h at 30 °C for the individual domains before harvesting. The cell pellets were resuspended in cold 100 mM Tris/HCl, pH 8.0/1 mM EDTA containing 0.1 mM PMSF. The cells were incubated on ice for 30 min with 0.1 mg of lysozyme, sonicated and pelleted by ultracentrifugation (100000 ***g*** for 60 min at 4 °C). The isolated protein extracts after the ultracentrifugation steps were added to StrepTactin® Affinity columns (1 ml bed volume; Institut für Bioanalytik) and isolated using the manufacturers' guidelines.

His-tag fusion-protein purifications

The PfODC domain (residues 804–1419), lacking the N-terminal hinge region that connects it to PfAdoMetDC, was cloned into the expression plasmid pJC40 for expression as a fusion protein with an N-terminal His-tag, as described previously [25]. The concentrations of the purified proteins were determined with the Bradford method. Purified proteins were analysed by SDS/PAGE and visualized by silver staining.

Protein–protein interactions

Protein–protein interactions of wild-type or mutant hinge-linked bifunctional proteins were determined by size-exclusion FPLC. Furthermore, protein–protein interactions between separate, wild-type or mutant monofunctional PfODC and PfAdoMetDC were analysed by determining their ability to form hybrid, bifunctional heterotetrameric complexes. Separately expressed and purified proteins (100–200 μg) were allowed to interact (1:1 molar ratio) by co-incubation for 10 min in buffer at room temperature. Protein mixtures were subjected to FPLC on a Superdex S-200 column (2.6 cm × 60 cm) equilibrated with 40 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 0.1 mM PMSF and 0.02 % Brij-35 at a flow rate of 2 ml/min. The column was calibrated with Dextran Blue (2000 kDa), BSA (≈145 kDa dimeric form), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa).

Protein was detected in the collected fractions with Coomassie Brilliant Blue G-250, dot-blot Western immunodetection and enzyme activity determination as follows. The collected fractions were dot-blotted on to nitrocellulose membranes using a BioDot apparatus (Bio-Rad). The membranes were blocked in 3 % low-fat milk powder in PBS at 4 °C for 16 h, followed by incubation with polyclonal Strep-tag II rabbit antiserum raised against Strep-tag II peptide conjugated to keyhole limpet haemocyanin (Institut für Bioanalytik) for 1 h at room temperature. After three washes with 0.05 % Tween-20 in PBS, the membrane was incubated with horseradish-peroxidase-conjugated anti-rabbit donkey whole IgG (Amersham Biosciences) for 1 h at room temperature in 1 % low-fat milk powder in PBS. The membrane was again washed three times in 0.05 % Tween-20 in PBS. Chemiluminescent proteins were visualized with the ECL Plus™ Western Blotting system (Amersham Biosciences).

Enzyme assays

PfAdoMetDC and PfODC activities were assayed by trapping $^{14}CO_2$ released from either *S*-adenosyl-L-[^{14}C]methionine for PfAdoMetDC or L-[1-^{14}C]ornithine for ODC, as described previously [8]. Recombinant proteins were dialysed overnight at 4 °C against 50 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, 0.02 % Brij-35 and 0.1 mM PMSF.

PfAdoMetDC enzyme reactions (250 μl final volume) contained 50 mM KH_2PO_4, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM *S*-adenosylmethionine and 62.5 nCi of *S*-adenosyl-L-[^{14}C]methionine (57 mCi/mmol; Amersham Biosciences) for the bifunctional enzyme or 125 nCi for the individual PfAdoMetDC activity. A 250 μl reaction for PfODC activity contained 40 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 40 μM pyridoxal 5-phosphate, 0.1 mM ornithine and 25 nCi of L-[1-^{14}C]ornithine (for the bifunctional enzyme; 52 mCi/mmol; Amersham Biosciences) or 250 nCi for the individual PfODC domain. Reactions were incubated at 37 °C for 30 min in the presence of enzyme. Enzyme activities are expressed as either total activity (nmol · min^{-1}) or specific activity (nmol · min^{-1} · mol of protein^{-1}) as indicated in the Figure or Table legends.

RESULTS

Parasite-specific regions in PfAdoMetDC/ODC

The parasite-specific inserts in the bifunctional PfAdoMetDC/ODC were defined based on a multiple alignment of the PfAdoMetDC/ODC amino acid sequence with the corresponding sequences of human, mouse, *T. brucei* and *L. donovani* enzymes and from previously published results [8]. The inserts in PfAdoMetDC/ODC have no sequence homologues in any other organism and are interspersed between conserved sequence blocks shared with other homologous proteins (Figure 1). Three major inserts of more than 10 residues each were identified: an area of 197 residues in the PfAdoMetDC domain (insert A_1, residues 214–410) and two areas in the PfODC domain: a 39 residue insert close to the PfAdoMetDC domain (insert O_1, residues 1047–1085) and a large 147 residue insert near the C-terminus of the protein (insert O_2, 1156–1302). The hinge region (residues 530–804) that connects the PfAdoMetDC and PfODC activities was defined by Müller et al. ([8] and Figure 1).

Without the inserts and the central part of the hinge region (H, residues 573–752, 180 residues), the individual decarboxylase domains show moderate identity with the respective proteins of other organisms. In the AdoMetDC domain, the malarial sequence shares a sequence identity of 18 % with the human and murine enzymes, 15 % with *T. brucei* and 14 % with *L. donovani* proteins. More pronounced identities of 28 % (human), 27 % (mouse), 31 % (*T. brucei*) and 17 % (*L. donovani*) are evident in the ODC domain.

Analyses of the PlasmoDB revealed partial sequences of AdoMetDC and ODC for *Plasmodium chabaudi* and *Plasmodium knowlesi* (results not shown). Full open reading frames were identified for bifunctional AdoMetDC/ODC in two murine *Plasmodium* species, *P. berghei* and *P. yoelii* (Figure 1), suggesting conservation of the bifunctional nature in Plasmodia, but not in other protozoa. Sequence alignments of AdoMetDC/ODC between the Plasmodia show large variations in sequence composition and length of the large inserts, A_1 and O_2, in both domains (Figure 1). The AdoMetDC domains of the murine Plasmodial

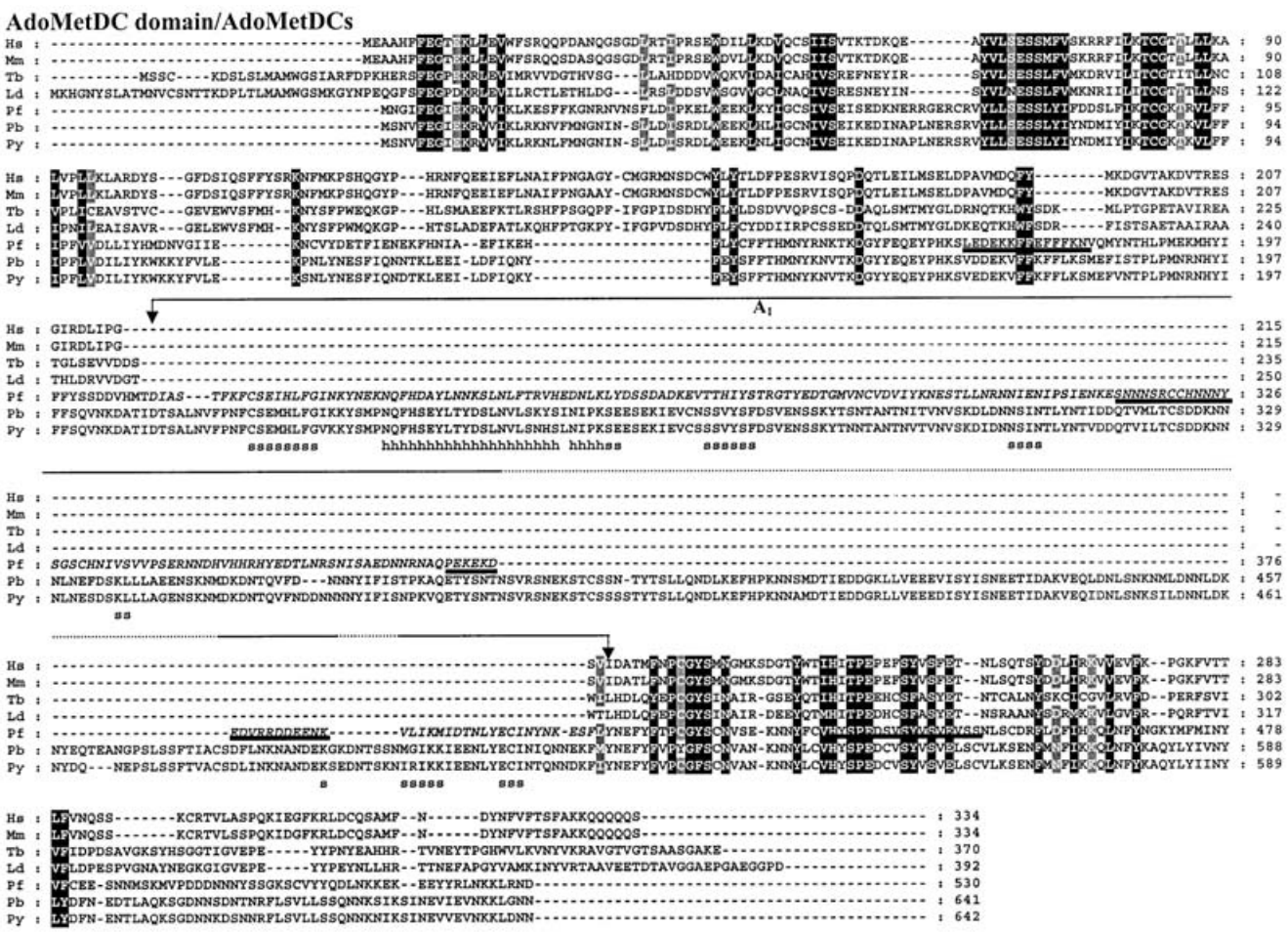

Figure 1 For legend see facing page

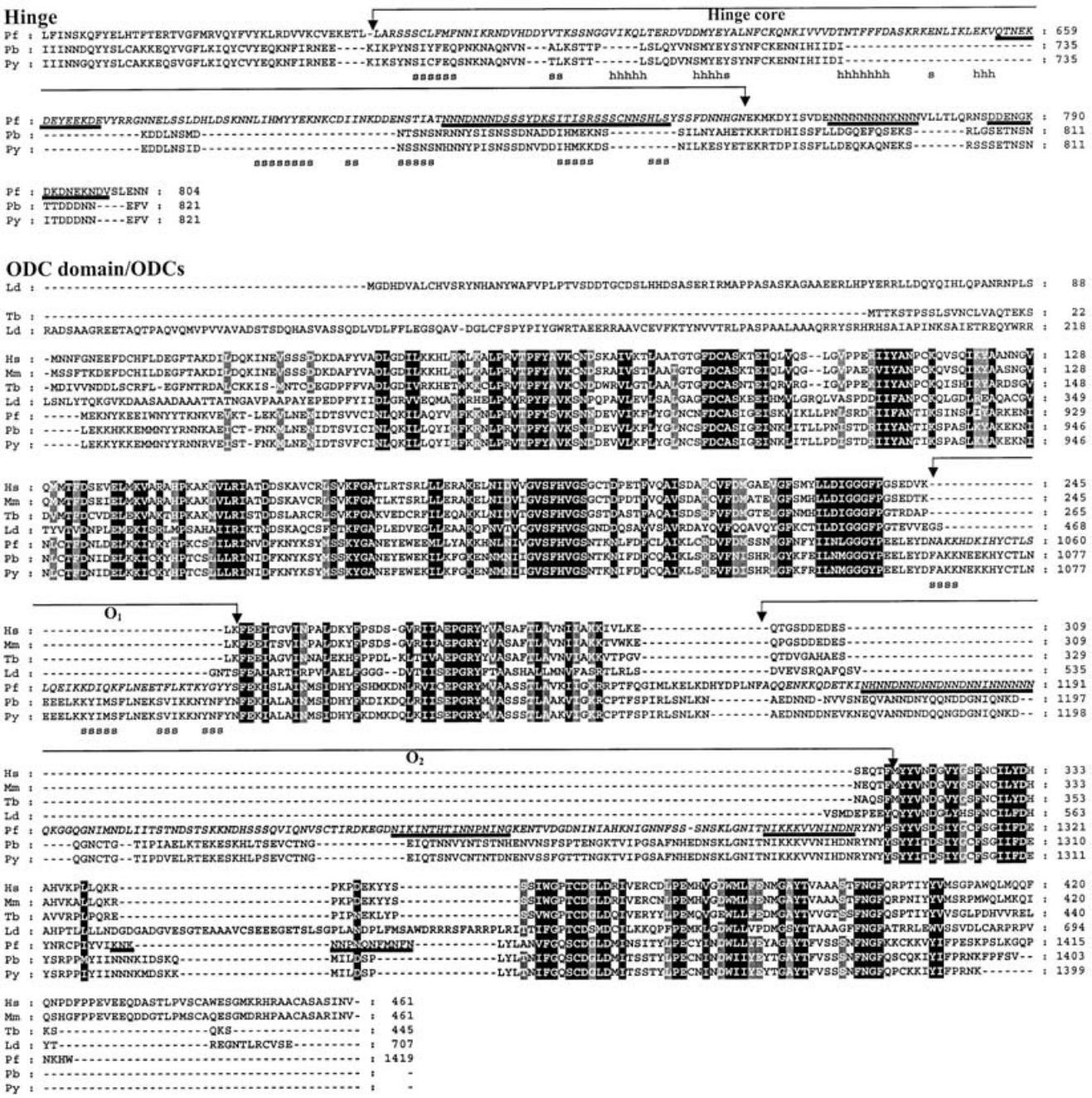

Figure 1 Multiple alignment of the deduced amino acid sequence of the bifunctional PfAdoMetDC/ODC with the corresponding individual sequences for AdoMetDC or ODC from *Homo sapiens* (Hs), *Mus musculus* (Mm), *T. brucei* (Tb) and *L. donovani* (Ld)

Bifunctional AdoMetDC/ODC open reading frames were also identified for *P. berghei* (Pb) and *P. yoelii* (Py). Left-hand panel: AdoMetDC domain; right-hand panel: hinge region and ODC domain. Clustal W alignment was performed for the individual AdoMetDC and ODC proteins using default parameters. The alignment was manipulated to maximize similarity and compensate for different chain lengths. The putative PfAdoMetDC, hinge and PfODC domains are indicated. Amino acids shown in black boxes are > 80 % conserved and > 60 % conserved residues are shown in grey boxes. The parasite-specific inserts that were deleted are indicated in italics and horizontal brackets (A_1, 214–410; H, 573–752; O_1, 1047–1085; O_2, 1156–1302). Underlined sequence indicates low-complexity areas in PfAdoMetDC/ODC. Predicted secondary structures of the parasite-specific inserts and hinge region in *P. falciparum* are indicated; s, β-sheets; h, α-helices.

enzymes are ≈100 residues longer than in the *P. falciparum* enzyme. In contrast, the ODC domain is longer in *P. falciparum* due to the ≈26-residue-longer insert O_2. The amino acid sequences of both large inserts (A_1 and O_2) seem to be more conserved between the murine Plasmodial sequences but differ from *P. falciparum*, specifically in the distribution of Asn and $(Asn\text{-}Asn\text{-}Asp)_x$ repeats in the *P. falciparum* sequence. The hinge region is also smaller in the murine Plasmodial bifunctional enzymes. The smallest insert O_1 in the ODC domain is better conserved between the Plasmodial species in terms of both sequence identity (50 %) and length suggesting functional specificity compared with the other larger, but more variable inserts.

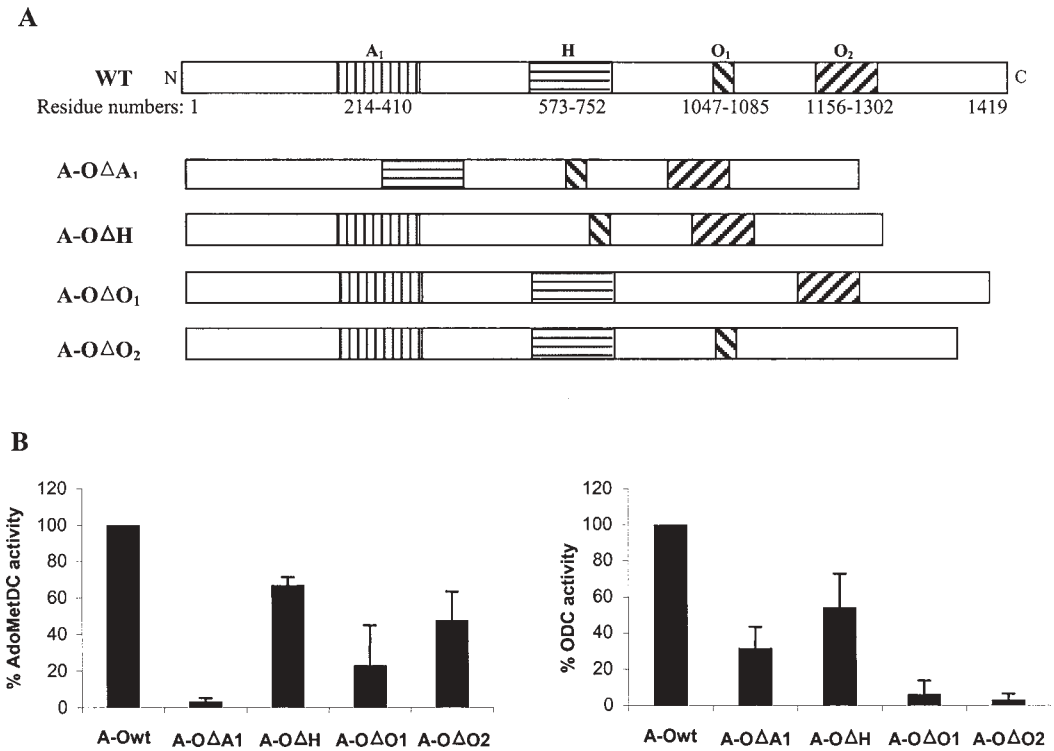

Figure 2 Deletion mutagenesis of parasite-specific inserts and hinge region in the bifunctional PfAdoMetDC/ODC

(**A**) Schematic representation of the deletion strategy used. Wild-type (WT) PfAdoMetDC/ODC is shown at the top with the positions and residue numbers of the specific inserts (A_1, H, O_1 and O_2) and the various deletion mutants are shown at the bottom. (**B**) Activity analyses of wild-type (A-Owt) and mutated bifunctional PfAdoMetDC/ODC. Results are means ± S.D. from three independent experiments performed in duplicate and specific activities were normalized to the wild-type activity.

Sequence and structure analyses of the parasite-specific regions

The defined parasite-specific areas in the bifunctional AdoMetDC/ODC specifically of *P. falciparum* were analysed for various sequence and structural properties to obtain some indication of possible functions. The parasite-specific areas in PfAdoMetDC/ODC are rich in charged residues, predominantly Asn, Asp, Lys, Ser and Glu, as well as Leu and Ile (Figure 1). Noticeably, both the large inserts (A_1 and O_2) and the central part of the hinge region in PfAdoMetDC/ODC are Asn-rich with insert A_1 containing 16%, the hinge region 15% and insert O_2, 29%. However, the smallest insert in the PfODC domain (O_1) is composed of 21% Lys residues. Kyte and Doolittle hydrophobicity analyses of the parasite-specific inserts indicated that all the inserts have a pronounced hydrophilic nature and are not highly antigenic (results not shown; Hopp–Woods equation [36]). The Wootton and Federhen algorithm (SEG algorithm) [34] predicted low-complexity areas in all the inserts and the central part of the hinge region, except for insert O_1 in the PfODC domain (Figure 1). The latter insert seems to be the only area containing significant secondary structure with four β-sheets arranged in an anti-parallel manner as indicated in a comparative structural model of the PfODC domain (Figure 1 and [32]). The other inserts are predicted to be non-globular with a tendency towards unstructured loops interspersed in the majority of cases with β-sheets (Figure 1). Significant α-helical areas were only predicted for insert A_1 and the central part of the hinge region, whereas no secondary structures were evident in insert O_2. All the inserts are predicted to have a surface localization and not to be involved in the core structures of the proteins as evident from comparative structural models of the independent domains ([32], and G.A. Wells, L.-M. Birkholtz, R.D. Walter and A.I. Louw, unpublished work).

Deletion mutagenesis of parasite-specific regions in PfAdoMetDC/ODC

The bifunctional organization of the PfAdoMetDC/ODC provides a unique opportunity to establish the role of the parasite-specific inserts in complex formation and enzyme activities. Individual removal of the parasite-specific inserts and the central part of the hinge region from the bifunctional enzyme resulted in four deletion-mutants (mutants A-OΔA_1, A-OΔH, A-OΔO_1 and A-OΔO_2; Figure 2A). The different mutant proteins were expressed under the same conditions as the wild-type protein, isolated by affinity chromatography and analysed by SDS/PAGE. The expressed mutant proteins all showed the expected decrease in molecular mass after SDS/PAGE and did not have a major influence on the levels of protein obtained, except for the hinge-region deletion, where expression levels were 40% lower than that of the wild type (results not shown).

Decarboxylase activities of deletion mutants of mono- and bi-functional enzymes

AdoMetDC and ODC activities in the bifunctional enzyme are markedly reduced ($\approx$95%) when the deletion of the specific insert occurs within the respective domain (Figure 2B). Significantly, deletion of the A_1 and O_1 inserts, both closer in the linear amino acid sequence to the neighbouring domain and hinge region, also influences the activity of the neighbouring domain. For instance,

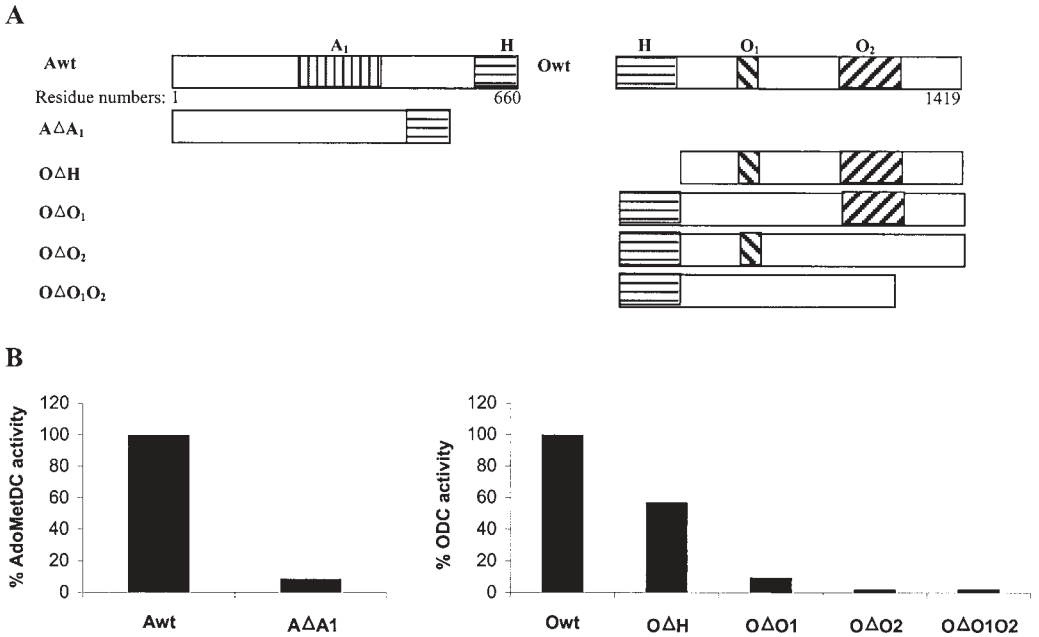

Figure 3 Effects of deletions on the individual activities of monofunctional PfAdoMetDC and PfODC domains

(**A**) Schematic representation of the deletion mutants created in the individual decarboxylase domains. Wild-type monofunctional PfAdoMetDC and PfODC (Awt and Owt) are shown at the top with the positions of the specific inserts (A_1, H, O_1 and O_2) and the various deletion mutants are shown at the bottom. Numbering is as for the bifunctional enzyme complex. (**B**) AdoMetDC and ODC activities. Experiments were performed in duplicate and specific activities were normalized to the wild-type activity. The OΔH mutant data are from Krause et al. [25].

deletion of O_1 reduces ODC activity by 94 % and also decreases AdoMetDC activity by 77 %. Deletion of A_1 reduces AdoMetDC activity by 96 % and ODC activity by 69 %. This is not as pronounced for the O_2 deletion mutant of the ODC domain, which results in only a 52 % reduction in AdoMetDC activity but a 97 % reduction in ODC activity. The AdoMetDC activity is decreased by 33 % in the hinge deletion mutant, but almost half of the ODC activity is lost (Figure 2B). These results indicate that the parasite-specific inserts, especially A_1 and O_1, mediate specific physical interactions between the two domains that are essential for both decarboxylase activities.

The monofunctional PfAdoMetDC and PfODC domains can be separately expressed as active proteins [24,25]. The direct contribution of the parasite-specific inserts to the activities of the monofunctional domains was investigated with single and combined deletion of inserts in the separately expressed domains (Figure 3). As expected from the results obtained with the bifunctional protein, deletion mutants of the monofunctional enzymes had significantly lower residual decarboxylase activities (Figure 3B). Mutant $A\Delta A_1$ resulted in a 91 % decrease of AdoMetDC activity (Figure 3B) compared with a 96 % decrease of mutant A-OΔA_1 in PfAdoMetDC/ODC (Figure 2B). Even more pronounced activity loss was evident in the ODC domain deletion mutants: OΔO_1 decreased ODC activity by 91 %, whereas OΔO_2 or the double-deletion mutant OΔO_1O_2 both led to 98 % less ODC activity (Figure 3B). The reductions in monofunctional ODC activities were similar to those observed for the bifunctional deletion mutants (Figure 3B). Deletion of insert A_1 in the monofunctional PfAdoMetDC domain, and double deletion of the O_1 and O_2 inserts in the monofunctional PfODC domain result in equivalent primary sequence lengths and moderate identity to homologues of these proteins in other organisms (Figure 1). Since the mutants $A\Delta A_1$ and OΔO_1O_2 of the respective monofunctional decarboxylases are essentially inactive, these areas appear to be important for the appropriate conformation of the active-site centres of the respective domains and to reflect parasite-specific properties.

Previous studies have indicated that the specific activity of the monofunctional PfODC domain with the hinge region is decreased by nearly 90 % by comparison with its activity in the bifunctional complex. Removal of the hinge region (residues 661–804) from the monofunctional protein results in a 43 % loss of activity in the ODC domain (Figure 3B and [25]) compared with a 46 % loss in activity of the bifunctional protein after removal of the central part (residues 573–752) of the hinge region (Figure 2B). These results suggest that physical interactions of ODC with the AdoMetDC domain are relatively more important than the hinge region for the catalytic activity of ODC.

Oligomeric state of the deletion mutants of the bifunctional PfAdoMetDC/ODC

The single ≈160 kDa heterodimeric form of the protein is expected if the mutants are unable to assemble into a bifunctional hybrid complex. Studies showed that mutants A-OΔA_1, A-OΔH, A-OΔO_1 and A-OΔO_2 were all able to form heterotetrameric protein complexes of ≈330 kDa, as determined with size-exclusion chromatography (results not shown).

Hybrid complex formation between monofunctional PfAdoMetDC and PfODC domains

The separate domains and their mutant forms were analysed for their ability to interact and form heterotetrameric complexes of 270–330 kDa through intermolecular protein–protein interactions. The monofunctional PfAdoMetDC and PfODC domains can be stably expressed as a heterotetrameric PfAdoMetDC of ≈150 kDa (subunits of 9 and ≈70 kDa) and obligate homodimeric PfODC of ≈180 kDa, respectively (L.-M. Birkholtz,

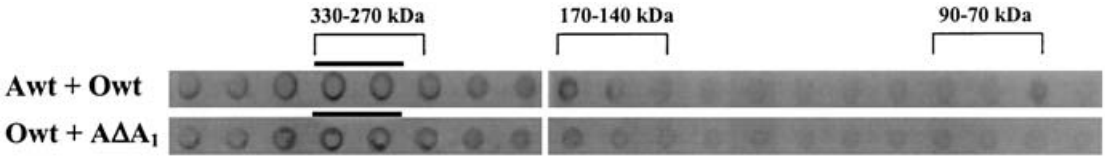

Figure 4 Protein–protein interactions between the separately expressed wild-type AdoMetDC and ODC domains or wild-type ODC and the mutant $A\Delta A_1$

Size-exclusion chromatography fractions were analysed at the different sizes corresponding to the heterotetrameric bifunctional complex size (330–270 kDa), the homodimeric size of PfODC or the heterotetrameric PfAdoMetDC (170–140 kDa) and the monomeric forms of the domains (90–70 kDa). Awt and Owt activities detected in the different fractions are indicated with a horizontal bar.

Table 2 Protein–protein interactions between the separate, monofunctional wild-type and mutant forms of AdoMetDC and ODC domains

The catalytic activities (nmol · min^{-1}) associated with the hybrid bifunctional complexes (330–270 kDa), monofunctional heterotetrameric AdoMetDC and homodimeric ODC (170–140 kDa) and monomeric proteins (90–70 kDa) were calculated as percentages of the combined activities in the three size-exclusion fractions. nd, not detectable. Details of the wild type and mutants are given in Figure 3.

	Catalytic activity (% of total activity)					
	330–270 kDa		170–140 kDa		90–70 kDa	
	AdoMetDC	ODC	AdoMetDC	ODC	AdoMetDC	ODC
Awt + Owt	62	98	30	2	8	nd
Awt + OΔH	8	nd	89	nd	3	nd
Awt + $O\Delta O_1$	18	nd	43	nd	39	nd
Owt + $A\Delta A_1$	nd	88	nd	12	nd	nd

unpublished work; [24,25]). Co-incubation of wild-type monofunctional PfAdoMetDC and PfODC resulted in the formation of the expected ≈330 kDa bifunctional, heterotetrameric complex as determined by size-exclusion chromatography and enzyme activity assays (Figure 4 and Table 2). This is consistent with the assembly of the domains in a heterotetramer consisting of an obligate homodimeric ODC (≈180 kDa) and a heterotetrameric AdoMetDC (≈150 kDa). The new hybrid AdoMetDC + ODC complex was stable enough to survive size-exclusion chromatography, indicating considerable interactions between the domains. These results show that the monofunctional AdoMetDC and ODC domains are able to reassemble in a stable, active complex that reflects the natural state of the bifunctional protein (Figure 4 and Table 2).

The potential role of the parasite-specific inserts in physical interactions between the domains was investigated by co-incubation of the wild-type form of one domain with a deletion mutant of the second domain (Table 2). Hybrid bifunctional complex formation was analysed by size-exclusion chromatography, enzymic assays of the wild-type partner protein and Western blots. Wild-type AdoMetDC was co-incubated with the ODC hinge deletion (OΔH [25]) or with the $O\Delta O_1$ deletion mutant. Bifunctional complex formation was still evident after removal of the hinge region from the ODC domain (OΔH; 8 % of total protein activity), although most of the activity was associated with the single heterotetrameric AdoMetDC (≈150 kDa). Deletion of the smallest O_1 insert ($O\Delta O_1$) in the ODC domain also resulted in a modest amount of bifunctional complex (18 % of total protein activity; Table 2) with most of the activity associated with the single heterotetrameric AdoMetDC (≈150 kDa). The wild-type ODC domain and the AdoMetDC deletion mutant $A\Delta A_1$ still associated as a hybrid heterotetrameric bifunctional complex of ≈330 kDa (Figure 4 and Table 2). Wild-type ODC and wild-type AdoMetDC or the AdoMetDC deletion mutant, $A\Delta A_1$ are therefore capable of forming stable hybrid bifunctional complexes. Insert O_1 or the hinge region in wild-type ODC, in contrast, appears to be important for the formation of the hybrid complex.

DISCUSSION

Polyamine metabolism is regulated by AdoMetDC and ODC. In all organisms except for *Plasmodium*, the decarboxylases are smaller, monofunctional proteins. Although both decarboxylase activities reside on one polypeptide, the two activities of the bifunctional PfAdoMetDC/ODC were suggested to function independently of each other [8,24]. Another unique feature of the bifunctional protein is that it contains several parasite-specific sequences with, as yet, no known functions. Here we show that a bifunctional PfAdoMetDC/ODC is also a characteristic of other Plasmodia and provide evidence that the parasite-specific inserted amino acids mediate intra- and interdomain interactions that are important for bifunctional complex formation and decarboxylase activities.

To date, a bifunctional AdoMetDC/ODC has not been described for any other organism and it therefore seems to be a unique property of Plasmodia. All the human malaria species normally cluster with the primate parasites while the rodent parasites form a single clade [37]. The conservation of bifunctional AdoMetDC/ODC in at least the diverse human and murine *Plasmodium* species indicates that its origin is not due to a neutral event, but must confer a distinct evolutionary advantage to malaria parasite metabolism. Explanations for this could include adjustment to the different host environments and/or unique regulation of polyamine levels at different development stages in these rapidly proliferating parasites.

Comparative structural models of both the decarboxylase domains show the parasite-specific inserts to project outwards from the surface of these proteins ([32], and G. A. Wells, L.-M. Birkholtz, R. Walter and A. I. Louw, unpublished work), similar to the parasite-specific inserts of PfDHFR-TS [23]. Deletion mutagenesis of the parasite-specific inserts in the bifunctional PfAdoMetDC/ODC and monofunctional domains indicated that the inserts are essential for the activity (and by inference the inherent conformation of the active site centre) of the particular domain. However, in the bifunctional protein, inserts O_1, O_2 and A_1 also influence the activity/conformation of the neighbouring domain, suggesting their participation in intra- as well as interdomain interactions. The likely location of these inserts at the protein surface implies that their effects are brought about by long-range interactions, which are propagated throughout the respective and neighbouring domains to the active site [23,38,39].

The monofunctional ODC domain with only a partial hinge region (wild-type ODC in this study) was shown to have the same K_m but only one-tenth of its activity in the native bifunctional protein. In contrast, the AdoMetDC domain with a partial hinge

region had a similar K_m and activity as in the native bifunctional complex [25]. Removal of the central part of the hinge region of the bifunctional protein reduced ODC activity only 2-fold suggesting that interdomain interactions are more important for ODC activity than the hinge region. Presumably, this is still possible in the hinge deletion mutant of the bifunctional protein although the lesser activities of both domains of this mutant indicate the disturbance of specific interdomain interactions. The lower activity but significantly higher K_m of the hinge-deletion mutant of monofunctional ODC compared with the wild-type enzyme, however, also indicates the interruption of discrete physical associations between the hinge region and the ODC domain [25].

Reassociation of the individually expressed, monofunctional PfAdoMetDC and PfODC domains is in part mediated by the smallest parasite-specific insert (insert O_1) in the ODC domain as well as the hinge region. Insert O_1 is the only highly conserved insert between *Plasmodium* species and is predicted to form four anti-parallel β-sheets [32]. This region is also more important for both decarboxylase activities in the bifunctional PfAdoMetDC/ODC. The large parasite-specific insert (A_1) in AdoMetDC does not seem to mediate physical interactions between the two domains, which are important for bifunctional complex formation. However, the associated conformational changes as evidenced by the loss of catalytic activities after removal of insert A_1 indicate the interruption of discrete interdomain interactions.

Deletion of O_1 in monofunctional ODC results in the inability of ODC monomers to dimerize (results not shown), indicating that intact homodimeric ODC is needed for its association with AdoMetDC. The dimer interface appears to be conserved between ODC of *P. falciparum* and *T. brucei* [32]. Mutagenesis of single residues at the dimer interface of *T. brucei* ODC was shown not to affect its dimerization, although activity was lost in many instances [38]. It has been proposed that the energetics of subunit interaction of ODC are distributed throughout the dimer interface and that long-range energetic coupling of interface residues to the active site is essential for enzyme function. Protein–protein interaction in this case therefore may be optimized for catalytic function and not only for high-affinity monomer associations. The inactive monomeric PfODC resulting from deletion of insert O_1 suggests significant conformational changes at the dimer interface of the deletion mutant in order to surmount the energetics associated with interactions between the subunits.

Several studies have established that monofunctional TS of *P. falciparum* is only active once the DHFR-TS hybrid complex is re-established [26,27]. Furthermore, even partial deletion of the 5′-end of the gene of PfDHFR-TS resulted in the simultaneous loss of both DHFR and TS activities. Several amino acid residues have been identified to be involved in interdomain interactions including two α-helices respectively localized in insert 1 of DHFR and the junction region [23]. The results obtained in this study indicate that the parasite-specific inserts in PfAdoMetDC/ODC have similar roles to those of PfDHFR-TS. It is therefore conceivable that the bifunctional nature of at least DHFR-TS and AdoMetDC/ODC of *P. falciparum* is a common strategy of the parasite to obtain optimum catalytic activity and/or stability of the C-terminal domains of these proteins through interdomain interactions mediated by the junction or hinge region, respectively.

The evidently comparable structural properties of PfDHFR-TS and PfAdoMetDC/ODC raise the interesting possibility that the prevalence of parasite-specific inserts in malaria parasite proteins compared with their homologues in other organisms is a general requirement to enable beneficial interdomain interactions in bifunctional proteins and perhaps also between monofunctional proteins. One additional benefit gained by the bifunctional arrangement of PfDHFR-TS is to enable substrate channelling of dihydrofolate molecules from TS to DHFR before exchange with bulk medium [12,23]. Currently there is no evidence for substrate channelling in PfAdoMetDC/ODC [4]. Moreover, unlike PfDHFR-TS the enzymes of PfAdoMetDC/ODC function in two linked, but separate, metabolic pathways. Since the mammalian equivalents of proteins that regulate polyamine metabolism (interconversion pathway enzymes, antizyme) appear to be absent from the parasite genome it is possible that one purpose of the bifunctional arrangement of PfAdoMetDC/ODC is to enable regulation of polyamine turnover in the parasite.

To date, no drug could be shown to selectively inhibit either AdoMetDC or ODC activity in *P. falciparum*. The structural conservation of the active sites between malarial and mammalian decarboxylases, as revealed by comparative homology models of the ODC and AdoMetDC domains, further complicates the discovery of selective inhibitors ([32] and G.A. Wells, L.-M. Birkholtz, R.D. Walter and A.I. Louw, unpublished work). However, the bifunctional nature of some *P. falciparum* proteins compared with their monofunctional homologues in mammals represents a significant difference between host and parasite biochemistry. Thus beyond active-site-directed strategies, an alternative and potentially powerful strategy for selective chemotherapy against malaria may be to inhibit interdomain interactions. The use of low-molecular-mass peptidomimetics that disrupt protein–protein interactions has been explored for drug development in many systems, including *Lactobacillus casei* thymidylate synthase [40], HIV-1 protease [41], herpes simplex virus DNA polymerase and human glutathione reductase [42]. Recently, synthetic peptides were also shown to prevent dimerization of and to effectively inactivate *P. falciparum* triosephosphate isomerase [43].

One obvious target site to prevent interdomain interactions in PfAdoMetDC/ODC is the structured parasite-specific insert O_1 of the ODC domain, which is highly conserved between the human and at least two murine parasite species. The other parasite-specific inserts and the hinge region is rich in charged residues, especially Asp and Asn. Interestingly, Asn-/Glu-rich areas have also been described as 'prion domains' that are involved in protein–protein interactions between functional domains of some proteins [44,45]. Perutz et al. [46] have suggested that poly-Glu and poly-Asn repeats and regions rich in other polar residues behave as modular mediators, termed 'polar zippers', due to formation of hydrogen-bonded networks between the side chains of these amino acids. Further studies are in progress to investigate this possibility and to identify the specific areas and/or amino acids in the parasite-specific inserts and the hinge region involved in interdomain interactions and catalytic activities of PfAdoMetDC/ODC.

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REFERENCES

1 Cohen, S. S. (1998) A Guide to the Polyamines, Oxford University Press, New York
2 Tabor, C. W. and Tabor, T. (1984) Polyamines. Annu. Rev. Biochem. **53**, 749–790
3 McCann, P. P. and Pegg, A. E. (1992) Ornithine decarboxylase as an enzyme target for therapy. Pharmacol. Ther. **54**, 195–215
4 Müller, S., Coombs, G. H. and Walter, R. D. (2001) Targeting polyamines of parasitic protozoa in chemotherapy. Trends Parasitol. **17**, 242–249

5 Bitonti, A. J., Dumont, J. A., Bush, T. L., Edwards, M. L., Stemerick, D. M., McCann, P. P. and Sjoerdsma, A. (1989) 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. **86**, 651–655
6 Greenwood, B. and Mutabingwa, T. (2002) Malaria in 2002. Nature (London) **415**, 670–672
7 Winstanley, P. A., Ward, S. A. and Snow, R. W. (2002) Clinical status and implications of antimalarial drug resistance. Microbes Infect. **4**, 157–164
8 Müller, S., Da'dara, A., Luersen, K., Wrenger, C., Das Gupta, R., Madhubala, R. and Walter, R. D. (2000) In the human malaria parasite *Plasmodium falciparum*, polyamines are synthesized by a bifunctional ornithine decarboxylase, S-adenosylmethionine decarboxylase. J. Biol. Chem. **275**, 8097–8102
9 Ekstrom, J. L., Mathews, I. I., Stanley, B. A., Pegg, A. E. and Ealick, S. E. (1999) The crystal structure of human S-adenosylmethionine decarboxylase at 2.25 A resolution reveals a novel fold. Structure Fold Des. **7**, 583–595
10 Kern, A. D., Oliveira, M. A., Coffino, P. and Hackert, M. L. (1999) Structure of mammalian ornithine decarboxylase at 1.6 A resolution: stereochemical implications of PLP-dependent amino acid decarboxylases. Structure Fold Des. **7**, 567–581
11 Bzik, D. J., Li, W. B., Horri, T. and Inselburg, J. (1987) Molecular cloning and sequence analysis of the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene. Proc. Natl. Acad. Sci. U.S.A. **84**, 8360–8364
12 Ivanetich, K. M. and Santi, D. V. (1990) Bifunctional thymidylate synthase-dihydrofolate reductase in protozoa. FASEB J. **4**, 1591–1597
13 Triglia, T. and Cowman, A. F. (1994) Primary structure and expression of the dihydropteroate synthase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. **91**, 7149–7153
14 Clarke, J. L., Scopes, D. A., Sodeinde, O. and Mason, P. J. (2001) Glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase. A novel bifunctional enzyme in malaria parasites. Eur. J. Biochem. **268**, 2013–2019
15 Carucci, D. J., Witney, A. A., Muhia, D. K., Warhurst, D. C., Schaap, P., Meima, M., Li, J.-L., Taylor, M. C., Kelly, J. M. and Baker, D. A. (2000) Guanylyl cyclase activity associated with putative bifunctional integral membrane proteins in *Plasmodium falciparum*. J. Biol. Chem. **275**, 22147–22156
16 Gilberger, T. W., Schirmer, R. H., Walter, R. D. and Müller, S. (2000) Deletion of the parasite-specific insertions and mutation of the catalytic triad in glutathione reductase from chloroquine-sensitive *Plasmodium falciparum* 3D7. Mol. Biochem. Parasitol. **107**, 169–179
17 Giesecke, H., Barale, J. C., Langsley, G. and Cornelissen, A. W. (1991) The C-terminal domain of RNA polymerase II of the malaria parasite *Plasmodium berghei*. Biochem. Biophys. Res. Commun. **180**, 1350–1355
18 Kappes, B., Doerig, C. D. and Graeser, R. (1999) An overview of *Plasmodium* protein kinases. Parasitol. Today **15**, 449–454
19 Bonnefoy, S., Attal, G., Langsley, G., Tekaia, F. and Mercereau-Puijalon, O. (1994) Molecular characterisation of the heat shock protein 90 of the human malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. **67**, 157–170
20 Luersen, K., Walter, R. D. and Müller, S. (1999) The putative γ-glutamylcysteine synthetase from *Plasmodium falciparum* contains large insertions and a variable tandem repeat. Mol. Biochem. Parasitol. **98**, 131–142
21 Rozmajzl, P. J., Kimura, M., Woodrow, C. J., Krishna, S. and Meade, J. C. (2001) Characterisation of the P-Type ATPase 3 in *Plasmodium falciparum*. Mol. Biochem. Parasitol. **116**, 117–126
22 Miles, E. W., Rhee, S. and Davies, R. M. (1999) The molecular basis of substrate channeling. J. Biol. Chem. **274**, 12193–12196
23 Yuvaniyama, J., Chitnumsub, P., Kamchonwongpaisan, S., Vanichtanankul, J., Sirawaraporn, W., Taylor, P., Walkinshaw, M. D. and Yuthavong, Y. (2003) Insights into antifolate resistance from malarial DHFR-TS structures. Nat. Struct. Biol. **10**, 357–365
24 Wrenger, C., Luersen, K., Krause, T., Müller, S. and Walter, R. D. (2001) The *Plasmodium falciparum* bifunctional ornithine decarboxylase, S-adenosylmethionine decarboxylase enables a well balanced polyamine synthesis without domain-domain interaction. J. Biol. Chem. **276**, 29561–29656
25 Krause, T., Luersen, K., Wrenger, C., Gilberger, T. W., Müller, S. and Walter, R. D. (2000) The ornithine decarboxylase domain of the bifunctional ornithine decarboxylase/S-adenosylmethionine decarboxylase of *Plasmodium falciparum*: recombinant expression and catalytic properties of two different constructs. Biochem. J. **352**, 287–292
26 Wattanarangsan, J., Chusacutanachai, S., Yuvaniyama, J., Kamchonwongpaisan, S. and Yuthavong, Y. (2003) Effect of N-terminal truncation of *Plasmodium falciparum* dihydrofolate reductase on dihydrofolate reductase and thymidylate synthase activity. Mol. Biochem. Parasitol. **126**, 97–102
27 Shallom, S., Zhang, K., Jiang, L. and Rathod, P. K. (1999) Essential protein-protein interactions between *Plasmodium falciparum* thymidylate synthase and dihydrofolate reductase domains. J. Biol. Chem. **274**, 37781–37786
28 Schofield, L. (1991) On the function of repetitive domains in protein antigens of *Plasmodium* and other eukaryotic parasites. Parasitol. Today **7**, 99–105
29 Reeder, J. C. and Brown, G. V. (1996) Antigenic variation and immune evasion in *Plasmodium falciparum*. Immunol. Cell Biol. **74**, 546–554
30 Kemp, D. J., Coppel, R. L. and Anders, R. F. (1987) Repetitive genes and proteins in malaria. Annu. Rev. Microbiol. **41**, 181–208
31 Pizzi, E. and Frontali, C. (2001) Low-complexity regions in *Plasmodium falciparum* proteins. Genome Res. **11**, 218–229
32 Birkholtz, L., Joubert, F., Neitz, A. W. H. and Louw, A. I. (2003) Comparative properties of a three-dimensional model of *Plasmodium falciparum* ornithine decarboxylase. Proteins Struct. Funct. Genet. **50**, 464–473
33 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673–4680
34 Wootton, J. C. and Federhen, S. (1996) Analysis of compositionally biased regions in sequence databases. Methods Enzymol. **266**, 554–571
35 Hafner, E. W., Tabor, C. W. and Tabor, H. (1979) Mutants of *Escherichia coli* that do not contain 1,4-diaminobutane (putrescine) of spermidine. J. Biol. Chem. **254**, 12419–12426
36 Geourjon, C., Deleage, G. and Roux, B. (1991) ANTHEPROT: an interactive graphics software for analysing protein structures from sequences. J. Mol. Graph. **9**, 188–190
37 Ayala, F. J., Escalante, A. A., Lal, A. A. and Rich, S. M. (1998) Evolutionary relationships of human malaria parasites. In Malaria: Parasite Biology, Pathogenesis and Protection (Sherman, I. W., ed.), pp. 285–300, ASM Press, Washington, D.C.
38 Myers, D. P., Jackson, L. K., Ipe, V. D., Murphy, G. E. and Phillips, M. A. (2001) Long-range interactions in the dimer interface of ornithine decarboxylase are important for enzyme function. Biochemistry **40**, 13230–13236
39 Peterson, C. B. and Schachman, J. K. (1992) Long range effects of amino acid substitutions in the catalytic chain of aspartate transcarbamoylase. Localized replacements in the carboxyl-terminal alpha-helix cause marked alterations in allosteric properties and intersubunit interactions. J. Biol. Chem. **267**, 2443–2450
40 Prasanna, V., Bhattacharjya, S. and Balaram, P. (1998) Synthetic interface peptides as inactivators of multimeric enzymes: inhibitory and conformational properties of three fragments from *Lactobacillus casei* thymidylate synthase. Biochemistry **37**, 6883–6893
41 Schramm, H. J., Boetzel, J., Buttner, J., Fritsche, E., Gohring, W., Jaeger, E., Konig, S., Thumfart, O., Wenger, T., Nager, N. E. and Schramm, W. (1996) The inhibition of human immunodeficiency virus by 'interface peptides'. Antiviral Res. **30**, 155–170
42 Zutshi, R., Brickner, M. and Chmielewski, J. (1998) Inhibiting the assembly of protein-protein interfaces. Curr. Opin. Chem. Biol. **2**, 62–66
43 Singh, S. K., Maithal, K., Balaram, H. and Balaram, P. (2001) Synthetic peptides as inactivators of multimeric enzymes: inhibition of *Plasmodium falciparum* triosephosphate isomerase by interface peptides. FEBS Lett. **501**, 19–23
44 Michelitsch, M. D. and Weissman, J. S. (2000) A sensus of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. Proc. Nat. Acad. Sci. U.S.A. **97**, 11910–11915
45 Wickner, R. B., Taylor, K. L., Edskes, H. K. and Maddelein, M.-L. (2000) Prions: portable prion domains. Curr. Biol. **10**, R335–R337
46 Perutz, M. F., Johnson, T., Suzuki, M. and Finch, J. T. (1994) Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc. Natl. Acad. Sci. U.S.A. **91**, 5355–5358

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