In the human malaria parasite *Plasmodium falciparum* (Pf), polyamines are synthesized by a bifunctional enzyme that possesses both ornithine decarboxylase (ODC) and S-adenosyl-L-methionine decarboxylase (AdoMetDC) activities. The mature enzyme consists of the heterotetrameric N-terminal AdoMetDC and the C-terminal dimeric ODC, which results in the formation of a heterotetrameric complex. For the native bifunctional protein a half-life longer than 2 h was determined, which is in contrast to the extreme short half-life of its mammalian monofunctional counterparts. The biological advantage of the plasmodial bifunctional ODC/AdoMetDC might be that the control of polyamine synthesis is achieved by only having to regulate the abundance and activity of one protein. An interesting feature in the regulation of the bifunctional protein is that putrescine inhibits P/ODC activity ~10-fold more efficiently than the mammalian ODC activity, and in contrast to the mammalian AdoMetDC the activity of the P/AdoMetDC domain is not stimulated by the diaminopentane. To analyze post-translational processing, polymerization, and domain-domain interactions, several mutant proteins were generated that have single mutations in either the P/ODC or P/AdoMetDC domains. The exchange of amino acids essential for the activity of one domain had no effect on the enzyme activity of the other domain. Even prevention of the post-translational cleavage of the AdoMetDC domain or ODC dimerization and thus the interference with the folding of the protein hardly affected the activity of the partner domain. In addition, inhibition of the activity of the P/ODC domain had no effect on the activity of the P/AdoMetDC domain and vice versa. These results demonstrate that no domain-domain interactions occur between the two enzymes of the bifunctional P/ODC/AdoMetDC and that both enzymatic activities are operating as independent catalytic sites that do not affect each other.

The polyamines putrescine, spermidine, and spermine are ubiquitous and essential cellular components involved in various metabolic processes including the proliferation and differentiation of bacteria, plants, and animals (1, 2). The two rate-limiting enzymes of polyamine synthesis are ornithine decarboxylase (ODC, EC 4.1.1.17) and S-adenosyl-L-methionine decarboxylase (AdoMetDC, EC 4.1.1.50), which provide putrescine and decarboxylated S-adenosyl-L-methionine (AdoMet) for the synthesis of spermidine. Interference with polyamine biosynthesis is considered as an antitumor and antiparasitic strategy, although the success of polyamine-related cancer therapeutic approaches has been modest (3–5). However, 2,3-difluoromethylornithine (DFMO), an inhibitor of ODC originally designed as an anticancer agent, has proved to be clinically successful in the treatment of African sleeping sickness caused by the protozoan *Trypanosoma gambiense* (6). The selective toxicity of DFMO on *T. gambiense* is complex and discussed to depend on various metabolic differences between parasite and mammalian host including the presence of trypanothione, altered levels of AdoMet, and also the longer half-life of the parasite ODC when compared with the mammalian enzyme (7). This success has reinforced the view that the polyamine metabolism of parasitic protozoa might be suitable for chemotherapeutic exploitation. We suggest that because of a higher polyamine requirement of rapidly proliferating cells, the interference with their synthesis has more severe consequences for the parasite compared with the host organism.

Malaria is the most important tropical disease in the developing countries; rough estimates indicate 500 million clinical cases per annum causing more than 1 million deaths. Malaria is undergoing resurgence, and the control of *Plasmodium falciparum* malaria has become a severe problem. Because of rapidly spreading drug resistance there is an urgent and pressing need for new antimalarial drugs.

ODC and AdoMetDC are usually derived from separate genes and act individually as highly regulated monofunctional enzymes. In *P. falciparum*, however, 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 by a hinge region (8). Corresponding to the respective dimeric and heterotetrameric structures of the mammalian ODC and AdoMetDC (9, 10), in *Plasmodium* a heterotetrameric enzyme complex is formed consisting of two bifunctional polypeptides post-translationally cleaved in the AdoMetDC domain. Here we report on the expression and mutational analyses of the recombinant bifunctional *P. falciparum* (Pf)ODC/AdoMetDC and address possible functional consequences and the potential significance of the bifunctional
nature of this parasite protein, which may help to validate this protein as a target for a chemotherapeutic intervention of malaria.

**EXPERIMENTAL PROCEDURES**

*Culture of P. falciparum—P. falciparum* 3D7 cells were maintained in continuous culture according to Trager and Jensen (11). Parasites were grown in human erythrocytes (A+) in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, 5% heat-inactivated human plasma (A), and 5% AlbuMAX II (Life Technologies, Inc.) at 5% (v/v) hematocrit in 150-cm² flasks at 37 °C with a gaseous phase of 90% N₂, 5% O₂, and 5% CO₂.

To determine the half-life of the *PfODC/AdoMetDC*, a synchronized *Plasmodium* culture at the trophozoite stage was incubated with cyclohexamide (50 μg/ml, Sigma) to prevent protein de novo synthesis. The decay of ODC and AdoMetDC activities was measured after 30, 60, and 120 min. aliquots of infected erythrocytes enriched with gelfunctioned Bio-Beads (Buchler Melsungen) (12, 13). Synchronization of the culture was performed by incubation of cells in 2 volumes of 0.3 M alanine, 10 mM HEPES, pH 7.4, for 5 min at 37 °C (14). The percentage of infected erythrocytes was determined by light microscopy of Giems-stained thin smears, and the number of the erythrocytes was determined in a Coulter Max M cell counter.

**Determination of AdoMet and Ornithine Contents in *P. falciparum*—** Trophozoite-infected erythrocytes of a synchronized *Plasmodium* culture were separated from noninfected erythrocytes by discontinuous Percoll/alanine gradient centrifugation (15). The number of trophozoite-infected erythrocytes was determined as described above, and subsequently *Plasmodium* parasites were isolated from the host erythrocytes by saponin lysis (16). For the determination of AdoMet, samples of infected erythrocytes were deproteinized by adding 0.2 M trichloroacetic acid for 12 h at 4 °C. After centrifugation (10,000 × g, 4 °C, 20 min), aliquots of the supernatant were analyzed by reversed phase HPLC on a Spherisorb ODS II column (5 μm, 250 × 3 mm, Machery-Nagel) according to Guattari (17) with minor modifications. Separation was performed isocratically at a flow rate of 0.55 ml/min using 40 mM ammonium dihydrogenophosphate, 6 mM o-phthalaldehyde, and 6% (v/v) methanol, pH 4.2, as mobile phase. The effluent was monitored by UV detection at 254 nm (diode array detector 400, Kontron). The ornithine content of *P. falciparum* was determined by the method of Patchett et al. (18) with minor modifications. Isolated *P. falciparum* cells were deproteinized by heating at 95 °C for 5 min before being centrifugated at 10,000 × g for 20 min at 4 °C. Aliquots of the supernatant were derivatized with equal volumes of o-phthalaldehyde reagent (40 mM o-phthalaldehyde, 0.2 M potassium borate buffer, pH 9.4, 0.14 M 2-mercaptoethanol, and 10% methanol) and subjected to a Spherisorb ODS II (5 μm, 125 × 3 mm, Machery-Nagel) reversed phase HPLC for analysis. The mobile phase consisted of solvent A (10 mM potassium dihydrogenophosphate, pH 5.9) and solvent B (acetonitrile/methanol/water, 4:3:3, v/v/v). Separation was performed at a flow rate of 0.3 ml/min analyzing the following gradient (percentage of solvent B: 0 min, 20%; 4 min, 27%; 11.5 min, 27%; 28 min, 100%; 31 min, 100%; 40 min, 20%; 58 min, 20%). o-Phthalaldehyde-derivatized ornithine was detected by fluorescence photometer (excitation 338 nm, emission 425 nm, SPM 25, Kontron).

**Recombinant Expression of the *PfODC/AdoMetDC*—** The entire coding sequence of the *PfODC/AdoMetDC* wild-type and mutants cloned into the vector pASK-IBA3 (Institut für Bioanalytik, Göttingen, Germany) were expressed in the AdoMetDC- and ODC-deficient Escherichia coli line EWH331, kindly provided by Dr. H. Tabor (19), and subsequently purified as described previously (8). The purified recombinant protein was subjected to fast protein liquid chromatography on a calibrated Superdex 5/200 column (2.6 × 60 cm) equilibrated with 40 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02% Brij-35 at a flow rate of 2 ml/min. The homogeneity of the enzyme preparation was analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were revealed by Coomassie staining (20). The concentration of the purified recombinant protein was determined according to Bradford (21).

**Caking, Expression, and Purification of the *PfODC/AdoMetDC Domain—**

The AdoMetDC domain comprises the amino acids 1–660 including 131 amino acids of the hinge region of the bifunctional protein (Fig. 1). Base pair 1–1980 of the coding region of the ODC/AdoMetDC was amplified by polymerase chain reaction (PCR) from genomic *P. falciparum* DNA using the sense oligonucleotide 5'-GGCGGGCGCTTCCGGCGCAT-GAACGGAATTTTGGAAGG-3' and the antisense oligonucleotide 5'-GCCGCCGCTTCTATCATATATTCTTCTTGTGTTAACC-3'.

The PCR product was cloned into pCR®2.1-TOPO using a TOPO TA cloning kit (Invitrogen) and transformed into *E. coli* DH5α cells. Recombinant plasmid DNA was isolated and digested with BsaI (5 units/μl, New England Biolabs). The insert was gel-purified and cloned into BsaI-cut pASK-IBA7 expression vector (Institut für Bioanalytik). The chimeric *PfODC/AdoMetDC* was expressed in *E. coli* BL21-CodonPlusTM (DE3)-RIL (Stratagene) and purified by anion exchange chromatography according to manufacturer recommendations (Stratagene).

**Enzyme Assays—** Both AdoMetDC and ODC activities were assayed by trapping released 14CO₂ from S-adenosyl-l-(methyl-14C)methionine and I-[1-14C]ornithine (57 and 52 mCi/mmol, respectively, Amersham Pharmacia Biotech) as described previously (22, 23). The values of the bifunctional rPfODC/AdoMetDC and rPfAdoMetDC domains for the respective substrates were determined by varying the concentrations of the proteins. *PfODC/AdoMetDC* as well as wild type and the equivalent Gly721-Hinge-ODC were subjected to gel filtration, and dot blot assays were performed simultaneously, and the reaction mixture contained (in a final volume of 250 μl) 40 mM KH₂PO₄ or Tris/HCl buffer, pH 7.5, for determination of AdoMetDC and ODC activities, respectively, 1 mM dithiothreitol, 1 mM EDTA, 40 mM pyridoxal 5-phosphate, 100 μM ornithine, and 100 μM AdoMet. The activity rates of ODC and AdoMetDC were determined by trapping 14CO₂ derived from the respective added labeled substrates 50 nCi [14C]ornithine or [14C]AdoMet. The competitive inhibitors CGP40215A and CGP54169A (PfODC/AdoMetDC and ODC inhibitors synthesized and provided by Novartis Pharma (24, 25)). Both inhibitors, when used at a concentration of 100 μM, totally inhibited the respective domain activity (Ref. 26).

**Oligonucleotides and Site-directed Mutagenesis—** Oligonucleotides were designed to replace amino acid residues potentially involved in *PfODC/AdoMetDC* activity (Table I). The putative active site residues of the ODC domain Lys660, Lys770, Cys1355, Asp1356, and Asp1359 were targeted for mutagenesis. Site-directed mutagenesis was performed by in vitro mutagenesis in the *PfODC/AdoMetDC* domain, Ser723 was replaced by alanine. In addition, the amino acid residue Gly723 (equivalent to Gly1386) of the PfHinge-ODC (26) was mutated into tyrosine. 35 ng of the double-stranded supercoiled expression plasmid pASK-IBA3/FODC/AdoMetDC and 100 ng of mutagenic sense and antisense primers were used in a 50-μl PCR containing deoxyribonucleotides, reaction buffer, and Pfu DNA polymerase according to manufacturer recommendations (Stratagene). The PCR amplification parameters were 95 °C for 50 s, 55 °C for 60 s, and 68 °C for 12 min for 17 cycles. The linear amplification product was treated with endonuclease DpnI (10 units/μl, New England Biolabs) for 1 h to eliminate the parental template. An 8-μl aliquot from each PCR was used for the transformation of competent *E. coli* DH5α cells. All mutations were verified by nucleotide sequencing using the Sanger dye-exchange chain termination reaction for double-stranded DNA (20). Approximately 95% of the analyzed colonies contained the desired mutation, and one clone of each construct was transformed for the expression in competent *E. coli* EWH331 cells. The expressed proteins were purified by Strep-tactin affinity chromatography according to manufacturer recommendations (Institut für Bioanalytik).

**Dot Blot Analyses—** The recombinant wild-type and Gly1386-Tyr mutant *PfODC/AdoMetDC* as well as wild type and the equivalent Gly723-Tyr mutant *PfHinge-ODC* were subjected to gel filtration, and dot blot analyses of the collected fractions were performed. The fractions were transferred to nitrocellulose membranes with a BioDot apparatus (Bio-Rad). The membrane was blocked in 3% low fat milk powder in PBS (20) overnight at 4 °C. Subsequently the membrane was incubated with polyclonal rabbit antisera raised against the rPfODC/AdoMetDC protein (1: 5000) for 1 h. After three washes with 0.05% Tween 20 in PBS, the membrane was incubated for 1 h with 1:10,000 diluted alkaline phosphatase-coated anti-rabbit IgG (Dianova) in PBS containing 1% low fat milk powder. After washing the membrane three times in 0.05% Tween 20 in PBS, the proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 0.1 M Tris/HCl, 0.1 mM NaCl, and 20 mM MgCl₂, pH 9.5.

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2 R. D. Walter, unpublished data.
**RESULTS AND DISCUSSION**

Characterization of the rPf ODC/AdoMetDC and AdoMetDC Domain—To characterize and determine the steady-state kinetic parameters of ODC and AdoMetDC activities, the bifunctional P/ODC/AdoMetDC was recombiantly expressed as a Strep-tactin fusion protein in the E. coli strain EWI1331 and purified according to Müller et al. (8). The yield was 250 µg liter⁻¹ of bacterial cell culture.

Likewise, the AdoMetDC domain of the bifunctional *P. falciparum* ODC/AdoMetDC consisting of the amino acids 1–529 and additional 131 amino acids of the hinge region (Fig. 1) was separately expressed as a Strep-tactin fusion protein in the *E. coli* BL21-CodonPlus™(DE3)-RIL and purified according to that described in Ref. 8. 1 liter of culture yielded 350 µg of recombinant P/AdoMetDC domain. The rPfAdoMetDC was found to be catalytically active, just as it has been reported for the separately expressed rP/ODC (26). The P. falciparum ODC/AdoMetDC exhibited ODC and AdoMetDC activity optimal between pH 7.0 and 8.5, peaking at pH 8.0 and 7.5, respectively, when tested with potassium phosphate and Tris/HCl between pH 7.0 and 8.5, peaking at pH 8.0 and 7.5, respectively. These data are comparable with those for both separately expressed ODC do-

| Primer | Direction | Sequence (5’→3’) |
|-------|-----------|-----------------|
| WT    | sense     | GTGTATTGCTGACAGCAGATCTTATAATCATTTTGTG |
| S73A  | sense     | CATCAAAATGTAATAAGATGCCCTGACAAATAAGATGAG |
| S73A  | antisense | CTTCACTTATTTGTGAAAGT |
| WT    | sense     | GATCTTCCCATTGAGAGTTG |
| R135A | sense     | ACATCCCAAGCCTGACAGACATAGTTGAGTAGAG |
| R135A | antisense | CATATCCCAAGCCTGACAGACATAGTTGAGTAGAG |
| WT    | sense     | GCTTGGACAAATGATGGAGTAGAG |
| D1356A| sense     | GCTTGGACAAATGATGGAGTAGAG |
| D1356A| antisense | GCTTGGACAAATGATGGAGTAGAG |
| WT    | sense     | CATATCCCAAGCCTGACAGACATAGTTGAGTAGAG |
| D1359A| sense     | CATATCCCAAGCCTGACAGACATAGTTGAGTAGAG |
| D1359A| antisense | CATATCCCAAGCCTGACAGACATAGTTGAGTAGAG |
| WT    | sense     | GCTTGGACAAATGATGGAGTAGAG |
| G1382Y| sense     | GCTTGGACAAATGATGGAGTAGAG |
| G1382Y| antisense | GCTTGGACAAATGATGGAGTAGAG |

**FIG. 1.** Schematic organization of the P/ODC/AdoMetDC and the positions of the changed amino acid residues. The numbers correspond to the mutated amino acids. The AdoMetDC domain consists of amino acid residues 1–529 and the hinge region of 530–804, and the ODC domain is formed by the amino acid residues 805–1101.

- **Table I:** Mutagenic oligonucleotides for site-directed mutagenesis of P/ODC/AdoMetDC

Nucleotides that were exchanged in comparison to the wild type (WT) are underlined and in bold letters.
cine synthesis considering intracellular concentrations of 81 μM for putrescine and 29 μM for ornithine in *Plasmodium*. In contrast, the mammalian ODC is hardly inhibited by putrescine; the *K*<sub>m</sub> value of 600 μM (32) is about 10-fold higher, a value that makes it unlikely that putrescine at a physiological concentration exerts an effect on the mammalian ODC activity.

Another important feature in the regulation of polyamine synthesis in *Plasmodium* is that PfAdoMetDC is not stimulated by putrescine in a concentration range between 10 μM and 2 mM, which shows that *P. falciparum* lacks the regulatory mechanism proposed for mammalian cells to relate putrescine abundance to the synthesis of spermidine (33).

To investigate whether the active sites of the bifunctional ODC/AdoMetDC affect each other and/or depend on a catalytically active partner site for catalysis, we used an assay system in which both reactions were performed simultaneously. The mixture contained the substrates of both enzyme reactions as described under "Experimental Procedures." The activity rates of the individual enzymes were determined by the trapping of 14CO<sub>2</sub> derived from radiolabeled ornithine and AdoMet, respectively. As shown in Table III, the specific activities of ODC and AdoMetDC under these assay conditions were 34.4 and 20.6 nmol min<sup>−1</sup> mg<sup>−1</sup>, respectively. When the substrates of the "partner site reaction" was omitted, the respective activity rates of ODC and AdoMetDC were not affected. Further, the addition of the specific ODC inhibitor CGP52623A at a concentration that leads to total loss of ODC activity (26) did not influence the AdoMetDC activity; similarly, the inhibition of the AdoMetDC site by CGP40215A did not result in alterations of the ODC activity. These data clearly suggest that both active sites of the bifunctional ODC/AdoMetDC act independently of each other. In contrast to our finding on the plasmoidal bifunctional ODC/AdoMetDC, a domain-domain interaction was reported for the leishmanial bifunctional dihydrofolate reductase/thymidylate synthase, in which the DHFR activity is enhanced when the TS site is occupied (34).

**Mutagenic Analyses of the rPD/ODC/AdoMetDC—**The comparison of the deduced amino acid sequence from the bifunctional rPD/ODC/AdoMetDC with those of the respective mammalian proteins revealed that the amino acids involved in catalytic activities, protein processing, and cofactor binding are conserved in the *Plasmodium* sequence (8). To confirm that these amino acids are essential for processing of the AdoMetDC domain and for ODC domain activity and polymerization, mutagenic analyses of the residues Ser<sup>73</sup>, Lys<sup>466</sup>, Lys<sup>970</sup>, Cys<sup>1356</sup>, Asp<sup>1356</sup>, and Asp<sup>1359</sup> were performed (their positions are shown in Fig. 1).

The PfAdoMetDC domain contains the putative cleavage site Leu-Ser-Glu-Ser-Ser<sup>74</sup> which, when the wild-type protein was processed into a large subunit and a small subunit, was expected to occur between Glu<sup>72</sup> and Ser<sup>73</sup> (8). The mutation of Ser<sup>73</sup> into alanine prevented the post-translational cleavage into the β- and α-subunit. As shown by SDS-polyacrylamide gel electrophoresis analysis, the size of the unprocessed mutant compared with the wild-type protein was increased by 9 kDa (Fig. 2). In addition, the mutant PfAdoMetDC was inactive. The cleavage of the proenzyme is essential to form the pyruvoyl prosthetic group derived from Ser<sup>73</sup> and is essential for the enzymatic activity of PfAdoMetDC, similar to the mammalian AdoMetDC (35). Interestingly the activity of the P/ODC domain was not affected by this mutation (Table IV) despite the fact that part of the recombinant protein did not fold properly anymore.

In the ODC domain of the bifunctional protein the amino acids Lys<sup>466</sup> and Lys<sup>970</sup>, which are thought to be involved in binding of the cofactor pyridoxal 5-phosphate and to be necessary for the conformation of the active site (29, 36), were ex-

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**Table II**

|                      | Spec. act. (nmol min<sup>−1</sup> mg<sup>−1</sup>) | Spec. act. (nmol min<sup>−1</sup> mg<sup>−1</sup>) | Spec. act. (nmol min<sup>−1</sup> mg<sup>−1</sup>) |
|----------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
|                      | r/PfODC/AdoMetDC                                 | r/PfHinge-ODC/AdoMetDC                           | Mammalian proteins                               |
| ODC                  | 38                                               | 4.3                                              | 12800                                            |
|                      | 41 ± 9 (n = 3)                                   | 47.3                                             | 30–200                                           |
|                      | 68 ± 13 (n = 3)                                  | 50.4                                             | 600                                              |
| AdoMetDC             | 20                                               | 51 ± 10 (n = 3)                                  | 24                                               |
|                      | 58 ± 8 (n = 4)                                   | 43 ± 16 (n = 6)                                  | 78                                               |

**Table III**

**Functional analysis of the active sites of the bifunctional rPD/ODC/AdoMetDC**

The activity rates of ODC and AdoMetDC were determined in the presence of the enzymatically active partner sites, respectively. The complete assay mixture contained the substrates for both enzyme reactions. Omission of the respective substrates AdoMet and ornithine led to nonactive partner sites, and likewise, the inhibition by CGP40215A and CGF52623A at 100 μM concentration. Results are the means of duplicate determinations of three independent recombinant expres- sions under assay conditions as described under "Experimental Procedures." Spec. act., specific activities.

| Assay conditions | Spec. act. (nmol min<sup>−1</sup> mg<sup>−1</sup>) |
|------------------|--------------------------------------------------|
| ODC              | 34.4 ± 2.2                                       |
|                  | 32.7 ± 3.2                                       |
|                  | 29.7 ± 2.1                                       |
|                  | 20.6 ± 2.2                                       |
|                  | 19.6 ± 2.5                                       |
|                  | 19.3 ± 1.3                                       |

![Fig. 2. SDS-polyacrylamide gel electrophoresis analysis of the rPD/ODC/AdoMetDC wild type and Ser<sup>73</sup>-Ala mutant proteins.](http://www.jbc.org/)

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The activity rates of ODC and AdoMetDC were determined in the presence of the enzymatically active partner sites, respectively. The complete assay mixture contained the substrates for both enzyme reactions. Omission of the respective substrates AdoMet and ornithine led to nonactive partner sites, and likewise, the inhibition by CGP40215A and CGF52623A at 100 μM concentration. Results are the means of duplicate determinations of three independent recombinant expres- sions under assay conditions as described under "Experimental Procedures." Spec. act., specific activities.
changed by alanine. Consistent with the results reported on the monofunctional ODC of mammals and Trypanosoma brucei, the recombinant mutants of PfODC/AdoMetDC did not show any residual ODC activity, and their AdoMetDC activity was not affected by these mutations within the ODC domain. Three additional amino acid residues (Cys1355, Asp1356, and Asp1359) within the motif 1356-1358 Gly-Gln-Ser-Cys-Asp-Gly-Leu-Asp of the P. falciparum ODC domain encompassing the proposed region responsible for DFMO binding (29, 37) were exchanged by alanine. The ODC activity of the Cys1355, Asp1356, and Asp1359 mutants were found to be reduced by 91, 92, and 97%, respectively, a result consistent with the former reports on the mammalian ODC. Again, AdoMetDC activities were not significantly affected by these mutations (Table IV). In conclusion, these data support the results presented in Table III, which show that there is no functional interaction between the two enzymatic activities and strongly suggest that no domain-domain interactions between the PfODC and PfAdoMetDC exist. Both domains are independently acting enzymic sites of a bifunctional protein.

Gly1382 (equivalent to Gly1382 in P/ODC/AdoMetDC), although not located at the dimer interface, has been reported to be important for the dimerization of the mouse ODC monomer (9, 38). To investigate the role of this residue in the organization of the heterotetrameric ODC/AdoMetDC, we changed Gly1382 into tyrosine, expecting that this mutation would prevent dimerization of the ODC domain and possibly interfere with the formation of the heterotetrameric complex. The mutation resulted in the total inactivation of ODC activity, whereas the AdoMetDC activity was reduced by only 27% (Table IV). Interestingly the mutation of Gly1382 did not prevent the formation of the 330-kDa heterotetrameric ODC/AdoMetDC complex as shown in Fig. 3A. Recombinant PfODC/AdoMetDC wild type and the Gly1382-Tyr mutant protein were analyzed by gel filtration and subsequent dot-blot analyses. Should the mutation of Gly1382 obstruct the formation of this complex structure, one would expect the formation of heterotetramers of about 160 kDa. However, as shown by dot-blot analysis, the mutant protein eluted from the gel-sizing column in the range of 330 kDa, clearly consistent with the size of a heterotetrameric complex. This result does not exclude the participation of Gly1382 in the dimerization of the ODC domains but indicates that additional residues take part in the polymerization process, possibly located in the AdoMetDC domain or the hinge region. To show that Gly1382 is essential for the dimerization of the ODC domains, we mutated this residue in the separately expressed rPfHinge-ODC domain (26) and analyzed the organization of the resulting mutant protein. Gel filtration of the rPfHinge-ODC domain wild type and the mutant Gly721-Tyr (equivalent to Gly1382 in P/ODC/AdoMetDC) revealed proteins in the range of 160 and 80 kDa, which demonstrates dimeric and monomeric organization of the expressed ODC mutant proteins, respectively (Fig. 3B). This result confirms that this glycine residue is involved in the dimerization process of the plasmodial ODC.

It has been discussed that the organization of bifunctional compared with monofunctional proteins might have a biological advantage. For the leishmanial DHFR/TS, which catalyzes sequential reactions, substrate channeling and domain-domain interactions (34, 39) have shown for the P. falciparum ODC/AdoMetDC, substrate channeling can be excluded because the spermidine synthase is not part of the bifunctional enzyme complex (8). Another possible advantage of a bifunctional enzyme could be domain-domain interactions, which would facilitate the synthesis of the respective products or possibly exhibit regulatory functions on the activity of the partner domains. Shallom et al. (39) have shown for the plasmodial bifunctional DHFR/TS that physical interaction between the DHFR and TS domains is necessary to obtain a catalytically active TS. Data obtained from the mutagenesis experiments suggest, however, that PfODC and PfAdoMetDC domains do not interact directly and operate independently of each other. Inactivation of the PfODC and PfAdoMetDC domains by specific enzyme inhibitors and exchange of essential residues did not deplete or enhance the activity of the other domains but indicates that additional residues take part in the polymerization process, possibly located in the AdoMetDC domain or the hinge region. To show that Gly1382 is essential for the dimerization of the ODC domains, we mutated this residue in the separately expressed rPfHinge-ODC domain (26) and analyzed the organization of the resulting mutant protein. Gel filtration of the rPfHinge-ODC domain wild type and the mutant Gly721-Tyr (equivalent to Gly1382 in P/ODC/AdoMetDC) revealed proteins in the range of 160 and 80 kDa, which demonstrates dimeric and monomeric organization of the expressed ODC mutant proteins, respectively (Fig. 3B). This result confirms that this glycine residue is involved in the dimerization process of the plasmodial ODC.

TABLE IV
Specific activities of the rPfODC/AdoMetDC wild-type and mutant proteins

| Mutant      | Spec. activity of AdoMetDC | Spec. activity of ODC |
|-------------|----------------------------|-----------------------|
| Wild type   | 18.5 ± 1.3                 | 24.2 ± 2.8            |
| S73A        | n.d.                       | 22.0 ± 1.8            |
| K586A       | 17.9 ± 1.7                 | n.d.                  |
| K970A       | 19.8 ± 0.4                 | n.d.                  |
| C1355A      | 18.1 ± 4.9                 | 2.1 ± 0.1             |
| D1356A      | 17.0 ± 3.8                 | 1.9 ± 0.1             |
| D1359A      | 19.9 ± 1.8                 | 0.8 ± 0.2             |
| G1382Y      | 13.5 ± 0.9                 | n.d.                  |

Fig. 3. Dot-Blot analyses. Purified recombinant protein was applied to gel filtration on Superdex S-200. The eluted fractions were blotted on a nitrocellulose membrane as described under “Experimental Procedures” and analyzed by immunodetection. The bound antibodies were detected by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate staining. A, fractions of the rPfODC/AdoMetDC wild type (I) are shown compared with the rPfODC/AdoMetDC Gly1382-Tyr mutant (II). B, fractions of the rPfHinge-ODC wild type (I) are compared with the rPfHinge-ODC Gly721-Tyr mutant (II). The numbers of the fractions are given on the top line, and the molecular masses of the eluted proteins are shown on the bottom line.

Fig. 4. Half-life of the rPfODC/AdoMetDC protein. The turnover of the bifunctional protein was determined by measuring the decay of ODC and AdoMetDC after the addition of 50 μg/ml cycloheximide to the culture to inhibit protein synthesis. Aliquots were drawn at the time points indicated, and the ODC and AdoMetDC activities were determined in the lysate of the isolated parasitized erythrocytes as described under “Experimental Procedures.” , cycloheximide-treated culture;  ■ , control culture.
domain in the mutant compared with the wild-type protein. Our results with the separately expressed P/ODC and P/AdoMetDC domains support the data obtained with the bifunctional mutant proteins. Steady-state kinetic analyses of both separately expressed domains are in the same range as those determined for the entire bifunctional protein (26). A biological advantage of the plasmodial bifunctional ODC/AdoMetDC might be that the control of polyamine synthesis is achieved by only having to regulate the abundance and activity of one protein.

Turnover of P. falciparum ODC/AdoMetDC—An important aspect when thinking about targeting a protein with drugs is its half-life in the cell. In mammalian cells ODC and AdoMetDC have a very short half-life (40–43). The availability of DFMO, the specific inhibitor of ODC, did not have the anticipated success in cancer therapy, possibly because the target enzyme is so rapidly degraded within the cell. The rapid degradation of mammalian ODC is conferred by its C-terminal PEST region, a domain that is characterized by the high abundance of the amino acids proline, glutamic acid, serine, and threonine (44). This PEST region is not found in T. brucei ODC, which accordingly has a longer half-life than its mammalian counterpart (45). Interestingly ODC of the related insect parasite Cricthida fasciculata possesses a PEST region, and the half-life of the protein is as short as that of the mammalian enzyme (46). Analysis of the C-terminal part of the plasmodial ODC/AdoMetDC sequence reveals a relatively high abundance of these amino acids, which we previously considered to be a potential PEST region (8). Incubating trophozoite-infected erythrocytes with cycloheximide for up to 2 h and determining the specific activities of ODC and AdoMetDC after the respective incubation periods showed, however, that the enzyme activities remained almost unchanged during the incubation time (Fig. 4). These data indicate that P/ODC/AdoMetDC is a rather stable protein in these developmental stages of the parasite and seems to have a half-life of several hours similar to the T. brucei ODC (45).

Even though the half-life of the plasmodial protein is rather long, DFMO has only moderate effects on the survival of the erythrocytic stages of P. falciparum in vitro and on the development of Plasmodium berghei in an experimental animal model (47). There are several explanations for this inefficiency of DFMO including the possible poor uptake into the infected cells; after all, the drug has to cross three membranes to reach its target. Another possibility could be the occurrence of alternative pathways that supply the required putrescine. To evaluate the precise role of the ODC in the synthesis of polyamines in P. falciparum and to validate its potential as a drug target, a selective knockout using gene replacement techniques will be necessary.

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The *Plasmodium falciparum* Bifunctional Ornithine Decarboxylase, S-Adenosyl-l-methionine Decarboxylase, Enables a Well Balanced Polyamine Synthesis without Domain-Domain Interaction

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