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In the Human Malaria Parasite *Plasmodium falciparum*, Polyamines Are Synthesized by a Bifunctional Ornithine Decarboxylase, *S*-Adenosylmethionine Decarboxylase*

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The polyamines putrescine, spermidine, and spermine are crucial for cell differentiation and proliferation. Interference with polyamine biosynthesis by inhibition of the rate-limiting enzymes ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) has been discussed as a potential chemotherapy of cancer and parasitic infections. Usually both enzymes are individually transcribed and highly regulated as monofunctional proteins. We have isolated a cDNA from the malaria parasite *Plasmodium falciparum* that encodes both proteins on a single open reading frame, with the AdoMetDC domain in the N-terminal region connected to a C-terminal ODC domain by a hinge region. The predicted molecular mass of the entire transcript is 166 kDa. The ODC/AdoMetDC coding region was subcloned into the expression vector pASK IBA3 and transformed into the AdoMetDC- and ODC-deficient *Escherichia coli* cell line EWH331. The resulting recombinant protein exhibited both AdoMetDC and ODC activity and co-eluted after gel filtration on Superdex S-200 at ~333 kDa, which is in good agreement with the molecular mass of ~326 kDa determined for the native protein from isolated *P. falciparum*. SDS-polyacrylamide gel electrophoresis analysis of the recombinant ODC/AdoMetDC revealed a heterotetrameric structure of the active enzyme indicating processing of the AdoMetDC domain. The data presented describe the occurrence of a unique bifunctional ODC/AdoMetDC in *P. falciparum*, an organization which is possibly exploitable for the design of new antimalarial drugs.

Polyamines are ubiquitous and play a pivotal role in cell growth and differentiation (1, 2). The biosynthesis of polyamines depends on the decarboxylation of ornithine to putrescine and the subsequent attachment of aminopropyl groups to its terminal amino substituents to form spermidine and spermine, respectively. Ornithine decarboxylase (ODC,[1] EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50), the latter provides the aminopropyl group, are rate-limiting enzymes in this pathway. Usually the level and the activities of both of these enzymes are individually regulated on the transcriptional, translational as well as the post-translational level (3–6).

Plasmodium falciparum is the causative agent of severe malaria. The rapidly spreading resistance against existing drugs has led to an urgent need for the development of new antimalarials, which attack novel targets in the metabolism of *P. falciparum*.

In previous studies it was shown that inhibition of polyamine biosynthesis as well as the use of polyamine analogues that interfere with polyamine functions have antitumor and antiparasitic effects (7–9). The specific ODC inhibitor difluoromethylornithine blocks the erythrocytic schizogony of *P. falciparum* in culture and reduces the parasitemia in *Plasmodium berghei*-infected mice (10–13). Likewise, inhibition of AdoMetDC by MDL 73811 has a plasmodicidal effect *in vitro* (14). A combination of difluoromethylornithine and bis(benzyl)polyamines were curative in rodent malaria (15). These data provide evidence that the polyamine biosynthesis might be a potential target for a chemotherapeutic attack of *P. falciparum* blood stage forms.

We have identified a protein of unusual size from *P. falciparum* exhibiting both AdoMetDC and ODC activity. Further, we have isolated the corresponding transcript that encodes a bifunctional plasmodial AdoMetDC and ODC in a single open reading frame. Recombinant expression of the entire transcript resulted in the formation of a protein that exhibited both enzyme activities supporting the existence of a novel bifunctional ODC/AdoMetDC in *P. falciparum*.

EXPERIMENTAL PROCEDURES

Culture of P. falciparum—P. falciparum FCBR and 3D7 were maintained in continuous culture according to Trager and Jensen (16). Parasites were grown in human erythrocytes (blood group A^+) in RPMI 1640 medium (Life Technologies, Inc.), supplemented with 25 mM HEPES, 20 mM sodium bicarbonate, 40 μg ml^{-1} gentamicin, and 10% heat-inactivated human A^+ plasma at 5% (v/v) hematocrit in 150-cm^2 flasks at 37 °C with a gaseous phase 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 smears. Synchronization of the parasites was achieved by incubation of cells in two volumes of 0.3 M alanine, 10 mM HEPES, pH 7.4, for 5 min at 37 °C according to Braun-Breton *et al.* (17). For saponin lysis *Plasmodium* infected erythrocytes at a hematocrit of 25% were incubated for 10 min at 4 °C in Earle's balanced salt solution containing 0.15%

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF094833.

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[1] The abbreviations used are: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

(w/v) saponin before the addition of 4 volumes of Earle's balanced salt solution. The reaction mixture was centrifuged at 1500 × *g* for 5 min at 4 °C, and the resulting pellet was washed twice in ice-cold Earle's balanced salt solution.

Native Ornithine Decarboxylase and S-Adenosylmethionine Decarboxylase from P. falciparum—Isolated parasites were homogenized by sonification in 1 volume of 40 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). The homogenate was centrifuged at 100,000 × *g* for 1 h (TFT 55.38, Centrikon T-1065, Kontron), and the resulting supernatant was used for a partial purification of the native *P. falciparum* ODC/AdoMetDC. The extract was loaded onto a DEAE-cellulose column (2.0 cm × 10 cm) at a flow rate of 40 ml h^{-1}. The column was washed with 2 volumes of buffer A, and elution was performed with a linear gradient of 0–0.5 M NaCl in buffer A. The eluant containing the enzyme activities was concentrated to 2 ml using a Centricon 50 microconcentrator (Amicon) and subjected to fast protein liquid chromatography on a calibrated Superdex S-200 column (1.6 cm × 60 cm) equilibrated with buffer A at a flow rate of 2 ml min^{-1}. The Superdex S-200 column was calibrated with apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and ovalbumin (45 kDa) all from Sigma.

Enzyme Assays—Both AdoMetDC and ODC activities were assayed by determining the release of CO_2 from *S*-adenosyl-L-[methyl-^{14}C]-methionine (57 mCi $mmol^{-1}$, Amersham Pharmacia Biotech) and L-[1-^{14}C]-ornithine (57 mCi $mmol^{-1}$, Amersham Pharmacia Biotech) as described previously (18, 19). The reaction mixtures contained in a final volume of 0.25 ml were: 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 50 μM *S*-adenosylmethionine for the AdoMetDC assay or 50 μM ornithine and 100 μM pyridoxal 5-phosphate for the ODC assay. The reactions were initiated by the addition of enzyme and incubated for 30 min at 37 °C. Results are the mean of three independent experiments.

cDNA Libraries of P. falciparum—The λ-Zap *Eco*RI library of *P. falciparum* K1 was kindly provided by Dr. B. Kappes (Heidelberg, Germany) and the pcDNA II plasmid library of *P. falciparum* 3D7 was provided by Dr. D. Kaslow (Bethesda, MD).

Isolation of RNA and Northern Blot Analysis—Total RNA of *P. falciparum* 3D7 was extracted from asynchronous as well as from highly synchronized cultures from different age stages (12–18 h, 24–30 h, and 36–42 h) using Trizol according to the manufacturer's instructions (Life Technologies, Inc.). Twenty five μg of total RNA from asynchronous parasites or of total RNA of each developmental stage were separated on an agarose formaldehyde gel and transferred to positively charged nylon membranes (Roche Molecular Biochemicals). The membranes were hybridized consecutively with a radiolabeled probe of the AdoMetDC domain (nucleotides 1–1586), the ODC domain (nucleotides 2413–4260), and the hinge region (nucleotides 1601–2402) in 50% formamide, 5× SSC, 5× Denhardt's solution, and yeast tRNA at 42 °C overnight. Subsequently, the membranes were washed in 2× SSC, 0.1% SDS at room temperature for 20 min followed by two washes in 1× SSC, 0.1% SDS, and 0.5× SSC, 0.1% SDS for 20 min at 60 °C. As a loading control the stage specific blot was reprobed with a *P. falciparum* rRNA probe (20). The signals were visualized by exposure to a BIOMAX film (Sigma) overnight.

Isolation of ODC/AdoMetDC—Genomic DNA of *P. falciparum* (FCBR) was prepared using the Stratagene DNA extraction kit according to the manufacturer's recommendations and used for polymerase chain reaction (PCR). To amplify a portion of the ODC/AdoMetDC gene, primers sense, 5′-TTTGAAAAAATATCATTG-3′; and antisense, 5′-CAAGCCATCACATGATTGTCC-3′, were synthesized based on two independent expressed sequence tags published by the *Plasmodium* genome project which showed similarity to ODCs from other organisms (expressed sequence tag 0236c3, accession number T02591; and expressed sequence tag 1291c3, accession number N97807). The PCR was performed with 100 ng of *P. falciparum* genomic DNA under the following conditions: 95 °C for 2 min, 45 °C for 2 min, 72 °C for 2 min for 30 cycles using PCR-Supermix (Life Technologies, Inc.). The amplified DNA product was resolved by agarose gel electrophoresis and visualized using ethidium bromide. The product was isolated (Gene Clean II kit, Bio 101) and ligated into the pCRII plasmid vector (TA-Cloning System, Invitrogen), and the nucleotide sequence was determined and analyzed.

Screening of cDNA Libraries—Two different cDNA libraries cloned in λ-Zap and pcDNA II were screened using the radiolabeled [α-^{32}P]ATP-PCR products as probes according to standard plaque and colony hybridization procedures (21).

To isolate the 5′-region of the ODC/AdoMetDC mRNA Marathon cDNA (CLONTECH) obtained from reverse transcribed *P. falciparum* total RNA was used. A PCR using the specific antisense oligonucleotide AS 1 (5′-CTCCTTTAATTTGATCACAACCC-3′) and the adapter primer AP 1 (CLONTECH) was performed. An aliquot of this first PCR was used for a second, nested PCR with the oligonucleotides AS 2 (5′-CCGTTCATTATATGAGATGTTAAG-3′) and AP 2 (CLONTECH). The resulting product was gel-purified, subcloned into pCRII (Invitrogen), and sequenced.

Sequence Analysis—The nucleotide sequences of both strands of the PCR products and the cDNAs were determined by the dideoxy chain termination method (21) or by terminator cycle sequencing using Ampli *Taq* DNA polymerase (Applied Biosystems) on an ABI PRISM™ automated sequencer (Perkin-Elmer). DNA and amino acid sequences were analyzed by DNASIS/PROSIS computer software (Hitachi).

Recombinant Expression of the ODC/AdoMetDC Coding Region—The entire coding region of the ODC/AdoMetDC from *P. falciparum* was amplified with the sense oligonucleotide 5′-GCGCGCGGTCTCCAATG-AACGGAATTTTTGAAGG-3′ and the antisense oligonucleotide 5′-GC-GCGCGGTCTCCGCGCTCCAATGTTTGTTTGGTTGCCCC-3′ using *Pfu* polymerase (Stratagene) and genomic DNA of *P. falciparum* as a template. The PCR product was digested with *Bsa*I and cloned into a *Bsa*I cut pASK-IBA3 expression vector (Institut für Bioanalytik, Göttingen). The vector containing the *P. falciparum* ODC/AdoMetDC was transfected into the AdoMetDC- and ODC-deficient *Escherichia coli* line EWH331, kindly provided by Dr. H. Tabor (22). A single colony was picked and grown overnight in Luria-Bertani medium. The bacterial culture was diluted 1:50 and grown at 37 °C until the A_{600} reached 0.5. The expression was initiated with 200 ng/ml of anhydrotetracycline, and the cells were grown an additional 12 h at 20 °C before being harvested. The cell pellet was resuspended in 100 mM Tris-HCl buffer, pH 8, containing 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, sonified, and then centrifuged at 100,000 × *g* for 1 h. The supernatant was purified using Strep-tactin resin according to the manufacturer's recommendation (Institut für Bioanalytik, Göttingen) and subsequently assayed for both AdoMetDC and ODC activity. The eluant of the affinity chromatography was applied to the calibrated Superdex S-200 for gel filtration as described above. The homogeneity of the enzyme preparation was analyzed by SDS-PAGE and Tricine-SDS-PAGE, and the proteins were revealed by silver staining (21, 23).

RESULTS

Native P. falciparum ODC and AdoMetDC—Activities of both ODC and AdoMetDC were determined in the 100,000 × *g* supernatant of *P. falciparum*. Both activities co-purified on DEAE-cellulose and gel filtration. The specific activities after these chromatographic steps were determined to be 93.2 ± 26.5 pmol min^{-1} mg^{-1} for ODC and 14.8 ± 5.6 pmol min^{-1} mg^{-1} of protein for AdoMetDC. Further, the K_m values for ODC and AdoMetDC for their respective substrates L-ornithine and *S*-adenosylmethionine were determined to be 42.4 ± 8.9 μM and 33.5 ± 14.6 μM, respectively. Performing chromatography on the calibrated Superdex S-200 column resulted in a single, symmetrical peak of AdoMetDC and ODC activity corresponding to a molecular mass of 326 ± 42 kDa (n = 3) as shown in Fig. 1, indicating either complex formation of both proteins during the isolation or the presence of a bifunctional protein, which contains both enzyme activities on a single polypeptide chain.

Characterization of P. falciparum ODC/AdoMetDC cDNA—Attempts to amplify part of the *P. falciparum* ODC using genomic DNA, sense, and antisense primers based on the two expressed sequence tags published by the *Plasmodium* genome project resulted in a PCR product of 729 bp. The deduced amino acid sequence of this PCR product showed similarity to the C-terminal region of ODC sequences from other organisms but was found to be enlarged by an insertion of 148 amino acids compared with the ODCs of mammals and other organisms (Fig. 2). This PCR fragment was used to screen a *P. falciparum* K1 λ-Zap cDNA library. Numerous putative ODC cDNA clones were identified, and sequence analysis of 12 of these clones showed that they were identical except for the length of their 5′-end. The largest clone (AD 10) isolated contained an insert of 3515 bp, which consisted of an open reading frame of 1151 amino acids. The last 57 bp of AD 10 encompass the 3′-untranslated region with a typical polyadenylation signal sequence

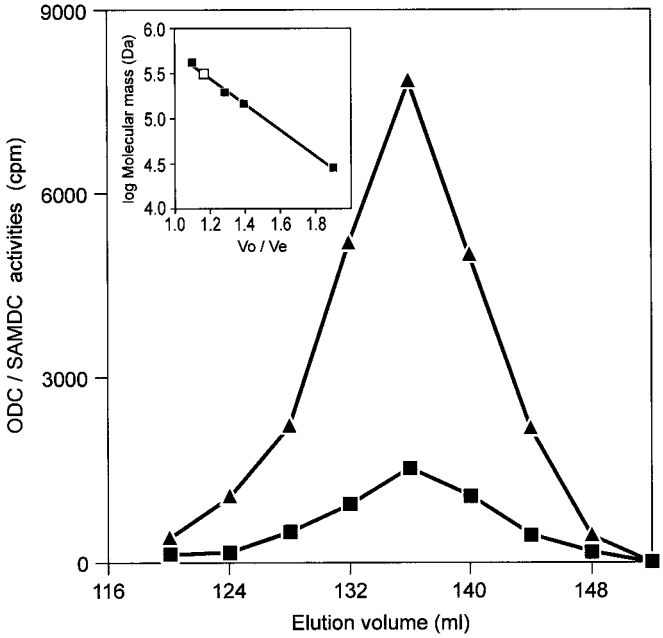

FIG. 1. **Elution profile of native ODC and AdoMetDC activities after gel filtration on Superdex S-200 and determination of the molecular mass of the bifunctional protein.** ODC (▲) and AdoMetDC (■) activities were determined as described under "Experimental Procedures." The molecular mass of the bifunctional protein was determined to be ~326 kDa, as indicated in the *inset*. *Inset,* molecular mass markers: apoferritin (443 kDa); β-amylase (200 kDa); alcohol dehydrogenase (150 kDa); ovalbumin (45 kDa). □, apparent molecular mass of ODC/AdoMetDC activities (326 kDa).

(AATAAA) located 17 bp upstream of the poly(A)$^+$ tail. To identify the 5′-region of the putative ODC/AdoMetDC cDNA, a PCR product based on the 5′-end of AD 10 was used as a hybridization probe to screen the *P. falciparum* 3D7 pcDNA II plasmid library and a cDNA clone (1A1) containing an additional 831 bp upstream of AD 10 and an overlap of 168 bp was isolated. However, because this clone did not contain a stop codon upstream of the potential first in frame methionine, we verified the translational start site by performing a 5′-rapid amplification of cDNA ends (RACE). The merged nucleotide sequence of 4383 bp, which is identical with the recently reported coding region of the ODC/AdoMetDC gene (accession number AF 112367), contains an open reading frame of 1419 amino acids, which appears to consist of three domains: residues 1–529 show similarity to AdoMetDCs of different organisms, residues 530–804 seem to function as a connecting region, and the C-terminal part (residues 805–1419) shows homology to known ODC sequences (Fig. 2). Both the AdoMetDC and the ODC domain contain large insertions that interrupt the regions of homology. The predicted molecular mass of the resulting protein was calculated to be 166,434 Da and strongly supports the suggestion that *P. falciparum* AdoMetDC and ODC are located on a single polypeptide chain encoded by a single transcript. These results imply that ODC and AdoMetDC form a bifunctional protein in *P. falciparum*.

Analysis of the Deduced Amino Acid Sequence in P. falciparum ODC/AdoMetDC cDNA—Pairwise sequence comparisons of the predicted plasmodial amino acid sequences of the AdoMetDC and ODC domains with the AdoMetDC and ODC sequences of the respective monofunctional proteins of mammals (24, 25), nematodes (18, 19), and protozoa (26, 27) exhibit a moderate degree of identity. However, within the amino acid sequences of AdoMetDC and ODC from mammals, there are some highly conserved amino acids and regions, which are reported to be essential for catalytic activity, dimerization, and pro-enzyme processing (28–33), and these are also found in the putative AdoMetDC and ODC domains of the plasmodial bifunctional protein (Fig. 2).

The plasmodial AdoMetDC domain (residues 1–529) is remarkably enlarged compared with the 334 amino acids containing human AdoMetDC (25). Significant homology between the plasmodial AdoMetDC domain and the AdoMetDCs from other organisms is shown in Fig. 2. The consensus region LSESS in the AdoMetDC domain contains the putative cleavage site between Glu-72 and Ser-73 for the processing of the proenzyme, equivalent to Glu-67 and Ser-68 in the mammalian protein. The cleavage of the pro-enzyme into a small β and large α subunit has been shown to be essential to form the prosthetic group derived from the serine residue and to generate the enzymatically active AdoMetDC (34) and is also essential for the activity of the recombinantly expressed AdoMetDC domain from *P. falciparum*.[2] Other amino acids reported from the human AdoMetDC to be responsible for activation of processing and enzyme activity are Glu-8, Glu-11, Cys-87, Glu-178, and Glu-256 (29, 32). These residues are represented in the plasmodial AdoMetDC domain by Glu-6, Glu-9, Cys-87, Glu-170, and Glu-447, respectively. The connecting region joining the ADoMetDC and the ODC domains is defined from amino acids 530 to 804. This region of 274 amino acids shows no homology to AdoMetDC and ODC enzymes from other organisms and has no similarity to the N-terminal 220–260 amino acids extension of the ODCs from *Leishmania donovani* and *Crithidia fasciculata* (26, 27). The putative ODC domain (residues 805–1419) consists of 614 amino acids and thus is larger than ODCs from mammals and smaller than ODCs from *L. donovani* and *C. fasciculata* (24, 26, 27, 35). The residues that are essential for dimerization and catalytic activity of the ODC of mammals and other eukaryotes (28, 30, 31, 33, 36) are found to have equivalents in the ODC domain of the *P. falciparum* sequence. Namely, the consensus sequence PFYAVKCN at position 64–71 of the mammalian ODC with the cofactor pyridoxal 5-phosphate binding Lys-69 was found at position 863–870 of the *P. falciparum* ODC domain although with changes of alanine and cysteine to serines. The region GQSCDGLD in position 1352–1359 of the plasmodial ODC domain is most likely equivalent to the consensus sequence GPTCDGLD of ODC sequences of various other eukaryotes. Thus the sequence contains the Cys-360 of the mammalian ODC, which has been proposed as the major binding site for difluoromethylornithine (30). The substitution in this region of threonine by serine is also known from *Drosophila melanogaster* (37). Further, Gly-387, reported from the mouse ODC to be involved in the dimerization process, may be represented in the *P. falciparum* sequence by Gly-1382 (38).

In conclusion, the overall amino acid homologies of the *P. falciparum* AdoMetDC and ODC domains to the mammalian counterparts are rather low. However, the occurrence of homology boxes surrounding amino acids responsible for catalytic activity, structural features, and co-factor binding in AdoMetDCs and ODCs of other organisms strongly suggests that the isolated transcript encodes indeed a bifunctional *P. falciparum* ODC/AdoMetDC.

At the C terminus, the *P. falciparum* sequence resembles the ODCs from the other invertebrates and lacks the extension with the PEST region responsible for the short half-life of mammalian ODCs. However, closer analysis reveals a higher abundance of the three amino acids Pro, Glu, and Ser within that region, possibly representing a PEST region in the *Plas-*

[2] C. Wrenger, K. Lüersen, S. Müller, and R. D. Walter, unpublished data.

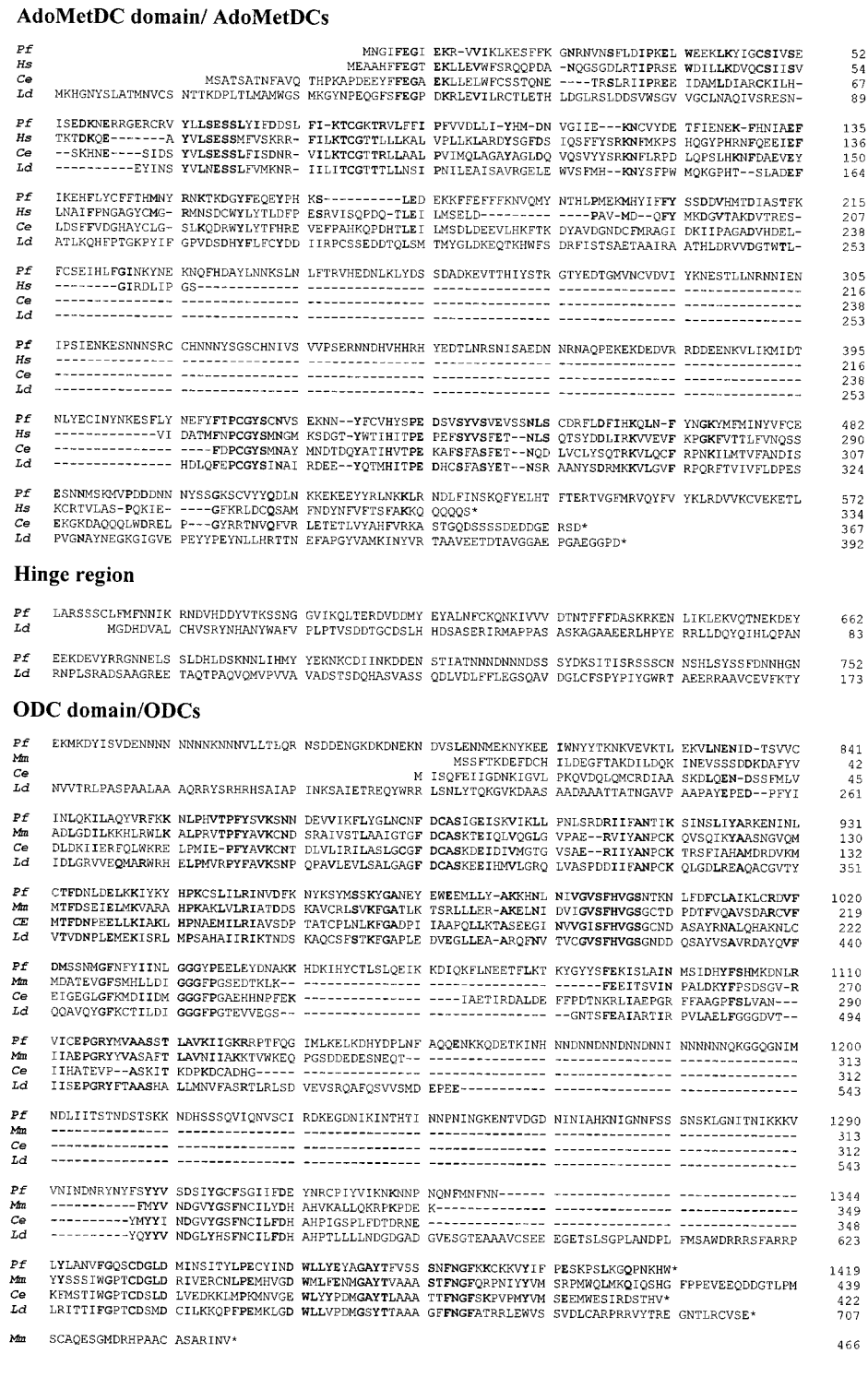

FIG. 2. **Alignment of the deduced amino acid sequences of the bifunctional ODC/AdoMetDC from *P. falciparum* (*Pf*) with the respective human AdoMetDC (*Hs*) and the mouse ODC (*Mm*), the *L. donovani* AdoMetDC and ODC (*Ld*), and the *Caenorhabditis elegans* AdoMetDC and ODC (*Ce*).** Identical amino acid residues are shown in *bold type*. Gaps (−) were introduced into the sequence to maximize homology and to compensate for the different chain lengths. The putative AdoMetDC and ODC domains and the connecting region are indicated.

modium protein.

Expression of ODC/AdoMetDC during the Erythrocytic Life Cycle of P. falciparum—To show that both the AdoMetDC domain and the ODC domain as well as the connecting region of the bifunctional protein are part of the same transcript, total RNA from *P. falciparum* 3D7 was analyzed by Northern blot hybridization using AdoMetDC cDNA, ODC cDNA, and hinge cDNA as probes, respectively. A single transcript of approximately 7 kilobases, thus nearly one and half times the size of the *Pf*ODC/AdoMetDC cDNA clone was identified (Fig. 3*A*). Further, to find out if the ODC/AdoMetDC is stage specifically expressed, mRNAs from various developmental stages of a highly synchronized *P. falciparum* culture were probed with the AdoMetDC cDNA. The result presented in Fig. 3*B* clearly demonstrates a stage-specific expression with abundant ODC/AdoMetDC mRNA in the late trophozoite stage (24–30 h after invasion).

Production of an Enzymatically Active Recombinant P. falciparum ODC/AdoMetDC—To determine whether the *P. falciparum* ODC/AdoMetDC cDNA clone encodes a bifunctional protein with ODC and AdoMetDC activity, it was expressed as fusion protein using the expression vector pASK-IBA3 in the *E. coli* strain EWH 331, which is a mutant lacking both enzyme activities (22). The *E. coli* extract was purified by affinity chromatography and applied to gel filtration on Superdex S-200. Both activities, ODC and AdoMetDC, co-eluted in the molecular mass range of ~333 ± 10 kDa (n = 3). The purified

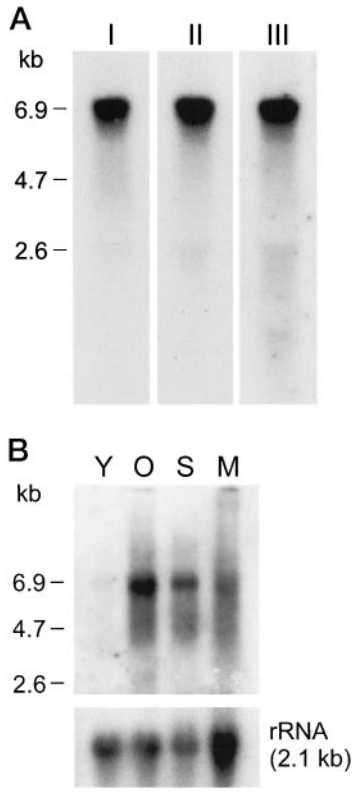

FIG. 3. **Northern blot analysis.** Total RNA of *P. falciparum* 3D7 was isolated using Trizol according to the manufacturer's recommendations (Life Technologies, Inc.). For the identification of the ODC/AdoMetDC transcript 25 μg of total RNA were separated on a 1% formaldehyde agarose gel and subsequently blotted to a positively charged nylon membrane (21). *A,* the blot was hybridized with radiolabeled probes encompassing the AdoMetDC domain (*I*), the hinge region (*II*), and the ODC domain (*III*), respectively. *B,* the stage-specific expression of the ODC/AdoMetDC transcript was shown by separating 25 μg of total RNA isolated from parasites of different age stages (*Y,* 12–18 h; *O,* 24–30 h; *S,* 36–42 h; *M,* mixed staged) of highly synchronized *P. falciparum* probed with the radiolabeled AdoMetDC cDNA. As a loading control the blot was reprobed with a radiolabeled rRNA from *P. falciparum*.

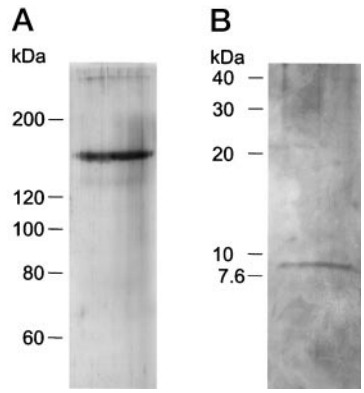

FIG. 4. **Analysis of the purified recombinant *P. falciparum* ODC/AdoMetDC by SDS-PAGE.** The active recombinant ODC/AdoMetDC was purified as described under "Experimental Procedures" and analyzed by SDS-PAGE and Tricine-SDS-PAGE (21, 23). The processed subunits of ~160 and 9 kDa are shown in *A* and *B*, respectively. The proteins were revealed by silver staining. Sizes of the molecular mass standards are shown.

recombinant bifunctional protein revealed specific activities of 38 ± 2 nmol min^{-1} mg^{-1} and 20 ± 7 nmol min^{-1} mg^{-1} of protein for ODC and AdoMetDC, respectively. Further, SDS-PAGE and Tricine-SDS-PAGE analyses show apparent homogeneity of the preparation. Two protein subunits were identified, an ~160 kDa subunit containing the C-terminal Strep-tag, the ODC domain, the hinge region, and the processed α-AdoMetDC domain and a 9-kDa subunit that corresponds to the cleaved β-subunit of the processed AdoMetDC domain (Fig. 4).

DISCUSSION

Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase represent the rate-limiting enzymes of polyamine biosynthesis. Their abundance, activity, and stability is regulated individually on the transcriptional, translational, and post-translational level in all organisms investigated so far. We have identified a transcript in the malaria parasite *P. falciparum* that shows homology to ODC sequences of other organisms in the C-terminal region and to AdoMetDC sequences in the N-terminal region demonstrated by the occurrence of residues essential for catalytic activity, dimerization, and pro-enzyme processing (28–30, 32, 33, 38).

Bifunctional proteins are not unusual in *P. falciparum*. Within the folate metabolic pathway of *Plasmodia* but also of other protozoa, two bifunctional proteins were identified that are thought to coordinate consecutive reactions in this pathway (39–42). The best known example is that of thymidylate synthase and dihydrofolate reductase, which form a bifunctional protein containing the dihydrofolate reductase domain in the N-terminal region joined to thymidylate synthase at the C terminus by a junction peptide of varying size depending on the organism (39, 43, 44). The other bifunctional protein identified in *P. falciparum* consists of an N-terminal dihydropteroate synthetase joined to a C-terminal dihydro-6-hydroxymethylpterin pyrophosphokinase (41). It has been speculated that the biological advantage of bifunctional proteins is a metabolic channeling of substrates and thus an optimized formation of products without any further regulatory processes involved (40).

However, that seems not to be likely for the particular bifunctional ODC/AdoMetDC because the products decarboxylated AdoMet and putrescine require the activity of a third enzyme, spermidine synthase, to form spermidine. Coordinated regulation on the transcriptional and translational level of ODC/AdoMetDC synthesis appears to be advantageous to ensure that AdoMet, which may be in short supply, is only decarboxylated when putrescine is available for the formation of spermidine. This hypothesis is in agreement with the predominant transcription of the ODC/AdoMetDC gene at the trophozoite stage, demonstrating that at this time of parasite development the requirement for polyamines is high due to rapid growth and the onset of DNA synthesis. In addition, it can be envisaged that in the bifunctional protein the catalytic activity of the ODC and AdoMetDC are directly affected by each other, their substrates, and products.

The deduced polypeptide chain of the bifunctional *Plasmodium* enzyme has a predicted molecular mass of 166 kDa, whereas the enzymatically active native enzyme as well as the recombinantly expressed ODC/AdoMetDC exhibited a molecular mass of about 330 kDa. Further, SDS-PAGE analysis of the recombinant bifunctional ODC/AdoMetDC revealed that the enzyme consists of subunits of 160 and 9 kDa, suggesting a heterotetrameric structure, derived from two proproteins, post-translationally processed and cleaved within the AdoMetDC domain.

The monofunctional mammalian ODC consists of two identical subunits and forms a homodimer (45). Monofunctional AdoMetDC isolated from several organisms, including parasitic worms and protozoa, is post-translationally cleaved and two low and two high molecular weight subunits form the heterotetrameric active protein. During this post-translational processing the essential pyruvate prosthetic group is formed

from a serine residue at the cleavage site (19, 25, 34, 46). The region surrounding the potential cleavage site is almost completely conserved within the plasmodial sequence. Assuming that a similar process occurs in *P. falciparum*,[2] the native ODC/AdoMetDC most likely is an enzyme complex consisting of a homodimeric ODC part and a heterotetrameric AdoMetDC part separated by a hinge region that may facilitate the formation of such a complex structure. However, the results obtained on the organization of the bifunctional protozoan dihydrofolate reductase-thymidylate synthase revealed that it is a homodimeric protein, consistent with the occurrence of a homodimeric monofunctional thymidylate synthase but in contrast to the monofunctional dihydrofolate reductase, which is usually a monomer in other organisms (47, 48).

The unusual size of the *P. falciparum* ODC/AdoMetDC results also from the fact that a number of large insertions interrupt the regions of amino acid homology within both the ODC and AdoMetDC domains. Insertions of different sizes have been identified in several plasmodial proteins such as protein kinases, HSP 90, RNA polymerases, and the γ-glutamylcysteine synthetase (20, 49–51). These insertions are characterized by a high abundance of charged amino acids like Asp, Asn, Gln, Glu, Ser, and Lys. Conspicuously, both insertions in the putative bifunctional *Pf*ODC/AdoMetDC are enriched in the amino acid asparagine, which represents about 18% in the AdoMetDC insertion and 30% in the insert of the ODC domain. The function of such insertions is still unknown.

Both ODC and AdoMetDC are proteins of low stability in mammalian cells. Their half-lives range between 10 min and 1 h. Rapid degradation is conferred by antizyme (in the case of ODC) and the presence of the so called PEST region in their protein sequences (52, 53). Such PEST regions are characterized by a high abundance of the amino acids proline, glutamic acid, serine, and threonine and are found to be correlated with the short half-life of proteins (54). Such sequences are not only found in mammalian ODC and AdoMetDC but also in the sequence of *C. fasciculata* ODC (27), which is surprising because other trypanosomatids contain an ODC protein with a much longer half-life of several hours. Accordingly, the sequence of *Trypanosoma brucei* ODC does not contain a region enriched in the amino acids PEST (55). Analysis of the C-terminal part of the *Pf*ODC domain revealed a relatively high abundance of Pro, Glu, and Ser, which may indicate a rapid turnover of the plasmodial protein. However, it has been reported previously that plasmodial ODC is rather stable (56).

Preliminary steady-state kinetic parameters for both enzyme activities were determined using partially purified ODC/AdoMetDC. The K_m values of both enzyme activities were comparable to those of the AdoMetDC and ODC monofunctional proteins from mammals (32, 46, 57, 58).

The data presented here describe the occurrence of a unique bifunctional ODC/AdoMetDC in *P. falciparum*. This organization suggests differences in comparison to the mammalian proteins, which may be exploitable for the design and synthesis of parasite-specific inhibitors. Using such inhibitors and applying molecular methods for the validation of the essential role of this protein for the survival of *P. falciparum* we will further assess this novel bifunctional protein as a target for the design of new antimalarials.

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