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Biochemical characterisation and novel classification of monofunctional *S*-adenosylmethionine decarboxylase of *Plasmodium falciparum*

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ABSTRACT

Plasmodium falciparum like other organisms is dependent on polyamines for proliferation. Polyamine biosynthesis in these parasites is regulated by a unique bifunctional *S*-adenosylmethionine decarboxylase/ornithine decarboxylase (PfAdoMetDC/ODC). Only limited biochemical and structural information is available on the bifunctional enzyme due to the low levels and impurity of an instable recombinantly expressed protein from the native gene. Here we describe the high level expression of stable monofunctional PfAdoMetDC from a codon-harmonised construct, which permitted its biochemical characterisation indicating similar catalytic properties to AdoMetDCs of orthologous parasites. In the absence of structural data, far-UV CD showed that at least on secondary structure level, PfAdoMetDC corresponds well to that of the human protein. The kinetic properties of the monofunctional enzyme were also found to be different from that of PfAdoMetDC/ODC as mainly evidenced by an increased K_m. We deduced that complex formation of PfAdoMetDC and PfODC could enable coordinated modulation of the decarboxylase activities since there is a convergence of their k_{cat} and lowering of their K_m. Such coordination results in the aligned production of decarboxylated AdoMet and putrescine for the subsequent synthesis of spermidine. Furthermore, based on the results obtained in this study we propose a new AdoMetDC subclass for plasmodial AdoMetDCs.

1. Introduction

The polyamines putrescine, spermidine and spermine are aliphatic, low-molecular weight nitrogenous bases that can form electrostatic interactions with anionic molecules such as nucleic acids, proteins and lipids [1]. They are involved in many growth processes including cell differentiation, proliferation [2,3] and apoptosis [4,5]. Polyamine concentrations are strictly regulated on transcriptional, translational and post-translational levels as well as by uptake and efflux processes [1]. Erythrocytes infected by *Plasmodium falciparum* (the causative agent of fatal malaria) contain high levels of polyamines during stages of rapid growth [6,7]. In *Plasmodium* spp, polyamine biosynthesis is uniquely characterised by the bifunctional arrangement of the rate-limiting decarboxylases, *S*-adenosylmethionine decarboxylase (PfAdoMetDC) and ornithine decarboxylase (PfODC) in a single protein, PfAdoMetDC/ODC [8]. The PfAdoMetDC and PfODC domains within PfAdoMetDC/ODC catalyze the synthesis of decarboxylated *S*-adenosyl-L-methionine (dcAdoMet) from AdoMet and putrescine from L-ornithine, respectively. dcAdoMet then provides an aminopropyl moiety for the synthesis of spermidine from putrescine in a reaction catalysed by spermidine synthase.

The *PfAdometdc/Odc* gene encodes a 1419 residue polypeptide that is maintained as a single, bifunctional protein with a hinge region (275 residues) connecting the 530-residue PfAdoMetDC domain (N-terminus) to the 614-residue PfODC domain (C-terminus) [8]. Both of these decarboxylase activities function independently of each other within the bifunctional complex [9], although it was shown that inter- and intradomain interactions modulate both activities [10]. In contrast to their single-enzyme orthologues in other organisms, the PfODC activity in the bifunctional enzyme is strongly feedback-inhibited by putrescine, whereas PfAdoMetDC activity is not stimulated by putrescine

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; CGP48664, 4-amidinoindan-1-one-2′-amidinohydrazone; dcAdoMet, decarboxylated *S*-adenosyl-L-methionine; DLS, dynamic light scattering; far-UV CD, far ultraviolet circular dichroism; *harmA*/*wtO*, harmonised *S*-adenosylmethionine decarboxylase/wild-type ornithine decarboxylase; Hsp70, *E. coli* heat shock protein 70; MDL73811, 5′-([(Z)-4-amino-2-butenyl]methylamino)-5′-deoxyadenosine); ODC, ornithine decarboxylase; SEC, size-exclusion chromatography.

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[9,11]. In the absence of known regulatory mechanisms that control polyamine levels in other organisms, the presence of a single PfAdoMetDC/ODC polypeptide has been suggested to allow regulation of polyamine pools in plasmodia [9].

AdoMetDC from various organisms are classified into five distinct subclasses based on their oligomeric structure, mechanism of autocatalytic processing and activation factors [12,13]. Of these, only the crystal structures of human (*Homo sapiens*), plant (*Solanum tuberosum*) and prokaryotic (*Thermotoga maritima*) AdoMetDCs have been solved [14–16]. The quaternary structure of the human monomeric protein revealed a novel four-layer αββα-sandwich domain, which exists as an $(\alpha\beta)_2$ dimer where the α- and β-subunits are formed by an autocatalytic processing event (non-hydrolytic serinolysis). Processing results in the simultaneous formation of the active site pyruvoyl cofactor at Ser68 [16]. In the case of plasmodial AdoMetDC, homology models predicted a similar α/β-fold to that of the human protein for the monofunctional PfAdoMetDC [11] but other properties e.g. oligomeric status or catalytic mechanism were not elucidated.

In this study, soluble and active monofunctional PfAdoMetDC was expressed from a codon-harmonised construct in sufficient purity and quantity for its biochemical and biophysical characterisation. We show that the monofunctional enzyme is able to dimerise but this is not essential for activity of the enzyme and that monofunctional PfAdoMetDC has similar kinetic properties compared to homodimeric kinetoplastid AdoMetDCs. These results offer a novel insight into a likely mechanism where the enzyme activities of PfAdoMetDC and PfODC are concurrently modulated in the bifunctional protein to align the rate of delivery of the two substrates required for downstream biosynthesis of spermidine. In addition, a comparison to the structurally characterised AdoMetDCs from other organisms, illustrates differences in the oligomeric status, mechanism of regulation and autocatalytic processing of PfAdoMetDC, which necessitates the introduction of a novel subclass for plasmodial AdoMetDC, namely 2b-III.

2. Materials and methods

2.1. Cloning of codon-harmonised monofunctional PfAdometdc and bifunctional PfAdometdc/Odc

Codon harmonisation was used for production of heterologously expressed monofunctional PfAdoMetDC in *E. coli*. The codon harmonisation algorithm is implemented in a PHP-script driven web interface and is available at http://www.sami.org.za/equalize. Synonymous codons were chosen to ensure that the positional codon frequency of low/intermediate and high usage codons are similar to the frequency used by that of the *E. coli* host [17]. Codon-harmonised nucleotides 1–1461 (GeneArt, Regensburg, Germany) encoding the core of PfAdoMetDC (487 residues) [11] was used to replace the corresponding wild-type (wt) sequence within *PfAdometdc/Odc* (*wtA/wtO*, GENBANK ID: Q8IJ77) in pASK-IBA3 [8] to create a full-length, partially harmonised *PfAdometdc/Odc* construct (*harmA/wtO*). From this construct the first 1461 harmonised nucleotides and an additional 255 wild-type nucleotides were amplified with sense (5′-ATGGTAGGTCTCAAATGAATGGCATTTTCGAAGGCATTGAAA-3′) and antisense (5′-ATGGTAGGTCTCAGCGCTCAAAGTTTCTTTTTCTACACA-TTTAAC-3′) primers. The resultant fragment was subcloned into the *Bsa*I site of pASK-IBA3 (C-terminal Strep-tag) to create a construct encoding monofunctional PfAdoMetDC (572 residues). Protein expression from this harmonised construct was compared to the expression of a protein from the wild-type gene (construct provided by Dr. C. Wrenger [9]), which encodes half of the hinge region (wtPfAdoMetDC-hinge, 660 residues) with an N-terminal Strep-tag. In addition, expression of the full-length PfAdoMetDC/ODC proteins from the pASK-IBA3 constructs containing *harmA*/*wtO* and *wtA*/*wtO* [8] were also compared. All sequences were verified by Sanger dideoxy nucleotide sequencing.

2.2. Protein expression and purification

The monofunctional PfAdoMetDC and wtPfAdoMetDC-hinge [9] as well as the bifunctional PfAdoMetDC/ODC (encoded by *harmA*/*wtO* and *wtA*/*wtO*) proteins were expressed in BL21 Star™ cells (Invitrogen). Protein purification using Strep-*Tactin* affinity matrix (IBA) was performed as described previously [10]. Protein bands separated by SDS-PAGE and visualised with Colloidal Coomassie or silver staining were identified with LC–MS/MS [18]. The monofunctional proteins separated with SDS-PAGE were also identified with Western immunodetection using monoclonal Strep-tag II mouse antiserum conjugated to horseradish peroxidase (Acris antibodies) [10].

The affinity-purified PfAdoMetDC protein sample was further separated with size-exclusion chromatography (SEC) using an Äkta Explorer System (Amersham Pharmacia Biotech). A Superdex®-S200 10/300GL SE column (Tricorn, GE Healthcare) was calibrated with the Gel Filtration Standard kit (BioRad) and equilibrated with wash buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA). Protein samples (500 μL) were loaded and fractions corresponding to the $(\alpha\beta)_2$ dimeric (estimated size of 140 kDa) and (αβ) monomeric (∼70 kDa) PfAdoMetDC were collected, pooled and subsequently concentrated with Amicon Ultra centrifugal filter devices (MWCO 3000, Millipore). Enzyme concentration was determined by UV absorbance measurements at 280 nm with an extinction coefficient of 69110 M^{-1} cm^{-1}.

2.3. PfAdoMetDC activity assays

The PfAdoMetDC and wtPfAdoMetDC-hinge proteins were assayed for enzyme activity directly after purification and after two weeks of storage at 4 and −20 °C. The assays contained 5 μg enzyme, 100 μM *S*-adenosyl-L-methionine chloride (Sigma–Aldrich) and 50 nCi *S*-[carboxy-^{14}C]adenosyl-L-methionine (55 mCi/mmol, Amersham Biosciences) in a total volume of 250 μL assay buffer (50 mM KH_2PO_4 pH 7.5, 1 mM EDTA, 1 mM DTT) and were performed as previously described [8]. Experiments were performed in duplicate for three individual experiments and specific enzyme activities were expressed as the amount of CO_2 produced in nmol/min/mg or nmol/min per nmol protein when constructs with different sizes were compared. Statistical analysis was performed using the paired Student's *t*-test with $P < 0.05$ indicating statistical significance.

2.4. Oligomeric status analyses with SEC

The oligomeric status of affinity-purified PfAdoMetDC at concentrations of 1 mg/mL and 4 mg/mL was analysed with SEC under non-reducing conditions. Reducing SEC was also performed by equilibrating the SEC column with wash buffer containing 10 mM DTT. Monomeric and dimeric protein fractions collected from SEC were analysed in terms of their elution volumes (V_e) relative to the void volume (V_o) of the column (V_e/V_o). The SEC-purified fractions were also visualised with reducing (10 mM DTT added to the sample buffer immediately prior to gel loading and electrophoresis) and non-reducing SDS-PAGE. Peptide mass fingerprinting with MALDI-MS was performed on the bands corresponding to the monomeric and dimeric proteins. Protein sample extraction, dehydration and preparation for MALDI-MS as well as sample derivatisation with 55 mM iodoacetamide to identify Cystines possibly involved in disulphide-bond formation, was performed as described [19,20].