Biochemical characterization of Plasmodium falciparum CTP:phosphoethanolamine cytidylyltransferase shows that only one of the two cytidylyltransferase domains is active

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The intra-erythrocytic proliferation of the human malaria parasite Plasmodium falciparum requires massive synthesis of PE (phosphatidylethanolamine) that together with phosphatidylcholine constitute the bulk of the malaria membrane lipids. PE is mainly synthesized de novo by the CDP:ethanolamine-dependent Kennedy pathway. We previously showed that inhibition of PE biosynthesis led to parasite death. In the present study we characterized P/ECT (P. falciparum CTP:phosphoethanolamine CT (cytidylyltransferase)), which we identified as the rate-limiting step of the PE metabolic pathway in the parasite. The cellular localization and expression of P/ECT along the parasite life cycle were studied using polyclonal antibodies. Biochemical analyses showed that the enzyme activity follows Michaelis–Menten kinetics. P/ECT is composed of two CT domains separated by a linker region. Activity assays on recombinant enzymes upon site-directed mutagenesis revealed that the N-terminal CT domain was the only catalytically active domain of P/ECT. Consequently, three-dimensional homology modelling of P/ECT showed critical amino acid differences between the substrate-binding sites of the two CT domains. P/ECT was predicted to fold as an intramolecular dimer suggesting that the inactive C-terminal domain is important for dimer stabilization. Given the absence of PE synthesis in red blood cells, P/ECT represents a potential antimalarial target opening the way for a rational conception of bioactive compounds.

Key words: CTP:phosphoethanolamine cytidylyltransferase (ECT), EC 2.7.7.14, malaria, phosphatidylethanolamine.

INTRODUCTION

In eukaryotic cells PE (phosphatidylethanolamine) and PC (phosphatidylcholine) constitute the most abundant structural phospholipids. PE is synthesized by the de novo Kennedy pathway, also named CDP-ethanolamine pathway and by phosphatidylserine decarboxylase, which mediates the transformation of phosphatidylserine into PE (Figure 1). In the de novo Kennedy pathway, Etn (ethanolamine) is phosphorylated by an EK (Etn kinase) followed by a ECT (CTP:Etnt cytidylyltransferase; EC 2.7.7.14), which transfers a cytidyl group from CTP to the newly formed P-Etn leading to CDP-Etn. The final step involves a transmembrane ethanolaminephosphotransferase, producing PE [1,2] (Figure 1).

Within the Kennedy pathway, ECT is considered to be the rate-controlling step [3]. In mammalian cells, the rate of PE biosynthesis has been shown to be related to not only the supply of CDP-Etn, but also to the availability of diacylglycerol suggesting that the metabolic flux is governed by these two metabolites [4]. Genes coding for ECT have been identified in several eukaryotic organisms such as plants [5,6], yeast [7], the protozoan parasite Trypanosoma brucei [8] and mammalian cells [9,10]. In the rat and human a single gene expresses two isoforms of ECT, Pcyt2α and Pcyt2β, that differ significantly in their kinetic parameters [1,11]. It has been suggested that ECT activity is regulated by the expression levels of the two distinct isoforms [12]. All known ECTs possess two CT domains of approximately 140 amino acids that probably result from an internal duplication. They are separated by a variable-sized linker region. Both CT domains of ECTs exhibit the characteristic CTP-binding motif HxGH. The second motif R(T/S)xG(V/I)STT, the signature of the CT superfamily, is less conserved in the C-terminal CT domain. Therefore the functionality of the C-terminal domain has often been questioned [1,13]. The X-ray three-dimensional structure of hECT (human ECT) has been solved by a Structural Genomics consortium and is available in the PDB (PDB code 3ELB).

In Plasmodium falciparum, the deadliest human malaria parasite, the synthesis of PE is of crucial importance since the parasite membranes are almost exclusively comprised of PE and PC with quasi-absence of other structural lipids such as cholesterol. PE is supplied by the parasitic machinery consisting of the de novo Kennedy pathway and, to a lesser extent, in phosphatidylserine decarboxylation [14], like in mammalian cells [15] (Figure 1). The production of membrane lipids is essential for the asexual intraerythrocytic proliferation of the parasite which corresponds to the symptomatic phase of the disease. Because of their critical role in Plasmodium development and proliferation, phospholipid biosynthetic pathways are potential antimalarial targets.

Abbreviations used: CHT, CTP:phosphocholine cytidylyltransferase; CEPT, CDP-ethanolamine; 1,2-diacyl-sn-glycerol ethanolamine-phosphotransferase; CT, cytidylyltransferase; ECT, CTP:phosphoethanolamine CT; EK, ethanolamine kinase; Etn, ethanolamine; FBS, fetal bovine serum; GCT, CTP:glycerol-3-phosphate CT; HECT, human ECT; IPTG, isopropyl β-D-thiogalactopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P-Etn, phospho-Etn; PTAld, P. falciparum aldolase; PECT, P. falciparum ECT; PFK, P. falciparum FK; RBC, red blood cell; IRBC, infected RBC.

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pharmacological targets. An antimalarial strategy blocking the rate-limiting steps of PC biosynthesis by the CDP-choline-dependent pathway has been successfully developed through the use of choline analogues [16–18]. The choline analogue T3 (renamed Albitiazolium) [16] is currently in clinical development for the treatment of severe malaria by parenteral route. De novo PE synthesis also appears essential for the parasite. Etn structural analogues have a deleterious impact on Plasmodium growth at the blood stage [19,20]. The antimalarial activity of Etn analogues has been correlated with a specific inhibition of PE biosynthesis, whereas the synthesis of PC remained unaffected [20,21]. Genetic manipulations in the rodent malaria parasite Plasmodium berghei revealed that the Ect gene is necessary for parasite survival [22].

In the present study we identified and characterized the limiting enzymatic step of the CDP-Etn-dependent pathway in P. falciparum. Quantification of parasite metabolites and kinetic studies of enzymes involved within the pathway show that P. falciparum ECT plays a crucial role, as has been observed previously for its mammalian counterpart. Using polyclonal antibodies we show that P/ECT is mostly localized within the malaria parasite cytoplasm and its expression profile along the parasite life cycle reveals an increase at the later stages. Taking advantage of various recombinant P/ECT constructs, we demonstrate that, despite the presence of two CT domains, only the N-terminal one is catalytically active. Finally, the homology three-dimensional structural model of P/ECT brings insight into the molecular basis of the different catalytic behaviours of CT domains.

**EXPERIMENTAL**

**Parasite culture and preparation of parasite extracts**

The *P. falciparum* 3D7 strain [MRA102 from MR4 (Malaria Research and Reference Reagent Resource Center)] was cultured in A⁺ or O⁺ human RBCs (red blood cells) obtained from the local blood bank (Etablissement Français du Sang, France) at 5% haematocrit in RPMI 1640 (Gibco) supplemented with 0.5% albumax I (Gibco), 10 μg/ml hypoxanthine (Sigma) and 50 μg/ml gentamycin (Gibco). Suspensions were incubated at 37°C in a culture gas chamber under a gaseous mixture of 5% O₂, 5% CO₂ and 90% N₂. Parasites were synchronized with 5% sorbitol at two consecutive parasite life cycles [23]. For the enzymatic activity assays mature stage parasites were enriched from parasite cultures by the gelatin floatation method using Plasmin® (Fresenius Kabi) [24]. The parasites were then lysed by adding an equal volume of water containing a cocktail of anti-proteases (Roche) prior to performing the activity assays.

**Analysis of PE pathway metabolites**

Erythrocyte suspensions, either infected with *P. falciparum* or not, were washed twice in RPMI 1640. The erythrocytes were incubated at 20% haematocrit in 500 μl of RPMI 1640 containing 25 mM Heps buffer (pH 7.4). Etn incorporation was measured by increasing concentrations of [¹⁴C]Etn (4.8 Ci/mol) (16–500 μM; American Radiolabeled Chemicals), either at 37°C or 4°C for 1 h. Reactions were stopped at 4°C and the cells were washed twice with ice-cold 0.9% NaCl solution. The cellular lipids were extracted according to the procedure of Folch et al. [25] modified by Rock [26]. The organic and aqueous phases of Folch extracts were evaporated and dissolved in 100 μl of chloroform/methanol [2:1 (v/v)] or ethanol/water [1:1 (v/v)] respectively. The organic phase was fractionated by silica gel TLC developed with chloroform/methanol/acetic acid/water [65:43:1:3 (v/v/v/v)] and water-soluble metabolites, from the aqueous supernatant, by TLC developed with methanol/0.5% NaCl/30% ammonia [50/50/1 (v/v/v)]. Radioactivity spots were revealed by autoradiography and identified by migration and revelation with iodine vapour and ninhydrin reagent of the appropriate standards. The radioactive spots were scraped and radioactivity was determined by scintillation counting. The amounts of [¹⁴C]Etn incorporated into water-soluble metabolites and lipids were determined on the basis of the incorporated radioactivity and specific activity of the precursor in the incubation medium and were expressed as nmol/10⁸ IRBC (infected RBC)/h. The values for infected erythrocytes were corrected by subtraction of the activity of uninfected cells. Data were processed using a non-linear regression model as a function of increasing Etn concentrations. The relationships between the end-product PE (x) and the intermediate metabolites, P-Etn (y) and CDP-Etn (y), were established with linear regression models. The correlation coefficients (r or r²) and P values were calculated from statistical analyses. All analyses were performed with GraphPad Prism4® software. The values represent the mean of triplicates.

**Enzyme activity assays**

ECT activity from *P. falciparum* extracts or from recombinant CDP-Etn from its radiolabelled substrate [¹⁴C]P-Etn (55 mCi/mmoll) (American Radiolabeled Chemicals). EK activity from parasite extract was measured by following the formation of CDP-Etn from P-Etn at 37°C (v/v/v/v) and water-soluble metabolites, from the aqueous supernatant, by TLC developed with methanol/0.5% NaCl/30% ammonia [50/50/1 (v/v)]. Radioactivity spots were revealed by autoradiography and identified by migration and revelation with iodine vapour and ninhydrin reagent of the appropriate standards. The radioactive spots were scraped and radioactivity was determined by scintillation counting. The amounts of [¹⁴C]Etn incorporated into water-soluble metabolites and lipids were determined on the basis of the incorporated radioactivity and specific activity of the precursor in the incubation medium and were expressed as nmol/10⁸ IRBC (infected RBC)/h. The values for infected erythrocytes were corrected by subtraction of the activity of uninfected cells. Data were processed using a non-linear regression model as a function of increasing Etn concentrations. The relationships between the end-product PE (x) and the intermediate metabolites, P-Etn (y) and CDP-Etn (y), were established with linear regression models. The correlation coefficients (r or r²) and P values were calculated from statistical analyses. All analyses were performed with GraphPad Prism4® software. The values represent the mean of triplicates.
mix at 100°C for 5 min. For each assay, a sample of 20 μl was spotted on to a TLC silica-gel plate pre-activated at 100°C for 1 h. Radiolabelled products and substrates of PfECT and PfEK were separated using as developing solvent ethanol/0.5% NaCl/30% ammonia [50:50:1 (v/v/v)] and ethanol/2% ammonia [1:1 (v/v)] respectively. Radioactive spots corresponding to CDP-Etn or P-Etn were quantified as described above. All enzyme assays were conducted three times in duplicates. Kinetic data were fitted with the Michaelis–Menten equation and kinetic parameters were calculated from the obtained curves (GraphPad Prism).

Production of polyclonal antisera and Western blotting
Female BALB/c mice (8-week-old; Charles River Laboratories) were immunized by subcutaneous injections of 20–50 μg of purified recombinant PfECT (residues 130–262) emulsified in Freund adjuvant. PfECT antisera were collected after the third boost, aliquoted and stored at −20°C. Distribution of the endogenous PfECT enzyme in the soluble and insoluble fractions of parasite lysates was analysed from schizont stage parasites synchronized as mentioned above. Parasite cultures fractions of parasite lysates was analysed from schizont stage parasites synchronized as mentioned above. Parasite cultures were fixed for 4 h at room temperature (20°C). Uninfected RBC extracts were used as controls. For the three stages of the parasite life cycle, parasites were released from highly synchronized cultures. A total of 15 μg of proteins were loaded per stage, separated by SDS/PAGE (12% gel) and transferred on to nitrocellulose membrane (Protran, Whatman). PfAld monoclonal IgG were used at a 1:300 dilution and anti-PfECT antisera were used at 1:6000 dilution; Promega). Secondary antibody was alkaline phosphatase-conjugated anti-(mouse IgG) (1:1000) in 2% FBS (fetal bovine serum) and 40% Triton X-100 in PBS for 1 h. Cells were washed three times and then incubated with ECT sequences of P. falciparum (UniProtKB/Swiss-Prot accession number Q8IDM2), Saccharomyces cerevisiae (UniProtKB/Swiss-Prot accession number P33412), rat (UniProtKB/Swiss-Prot accession number O88637), bovine (UniProtKB/Swiss-Prot accession number Q5EA75) and human (UniProtKB/Swiss-Prot accession number Q99447) were aligned using MUSCLE [27]. The linker region (amino acids 152–186) was removed to reflect the homology modelling of the hECT N-terminal and C-terminal CT domains was generated on the basis of the structure of the hECT N-terminal and C-terminal CT domains sharing 38% and 40% sequence identity respectively. MODELLER 9v8 [28] was used to perform the homology modelling of the PfECT intramolecular dimeric complex using the hECT structure (PDB code 3ELB) as template. The linker region (amino acids 152–186) was removed to reflect the structurally characterized hECT intramolecular dimer. The hECT co-crystallized ligand CMP was used as a constraint in MODELLER to keep the binding site in an appropriate conformation. The final model was chosen based on its low

Immunofluorescence assays for subcellular localization
To immunolocalize PfECT in IRBCs, non-synchronized cultures were fixed for 4 h at room temperature (20°C) in 4% paraformaldehyde in PBS followed by two washes with PBS. The cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min, blocked in 2% FBS (fetal bovine serum) and incubated with PfECT antisera (1:400 dilution) in 2% FBS for 1 h. After three washes, cells were then incubated with the secondary antibody anti-(mouse IgG) Alexa Fluor Red 594 (Invitrogen) (dilution 1:1000) in 2% FBS for 1 h. Cells were then washed three times and then mounted on to slides using a Vectashield mounting medium containing DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories). Parasites were observed with a Zeiss Axioimager epifluorescence microscope equipped with ApoTome (Carl Zeiss). Images were acquired using a 63× oil-immersion objective lens and DIC (differential interference contrast) for transmitted light. Images were processed with Axiovision software (Carl Zeiss) and ImageJ (MacBiophotonics).

Cloning, expression and purification of recombinant PfECTs
The PfECT cDNA sequence (PF3D7_1347700) was codon-optimized for expression in *Escherichia coli* (GenScript) and cloned as a Ndel/BamHI fragment in pET-15b expression vector (Novagen) for production of the N-terminal His₆-tagged protein. Site-directed mutagenesis (QuickChange, Stratagene) was performed to introduce mutations of histidine residues His₁⁴₆ and His₂⁶₉ replaced with alanine. *E. coli* strain BL21(DE3) cells carrying the plasmids with the PfECT constructs were grown at 37°C to mid-log phase in LB (Luria–Bertani) medium and protein expression was induced with 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside; Invitrogen) for ∼24 h at 16°C. Bacteria were harvested by centrifugation at 6000 g for 15 min at 4°C and resuspended in a buffer containing 20 mM Tris/HCl (pH 8), 400 mM NaCl, 2 mM 2-mercaptoethanol, 0.5% Triton X-100, 1 mg/ml lysozyme and a protease inhibitor cocktail (Roche). The cells were lysed by sonication on ice at 50% power for 5 min, incubated with 0.5 units/ml lysozyme and 1 mg/ml DNAse. The lysates were clarified by centrifugation at 10 000 g for 30 min at 4°C. Uninfected RBC extracts were analysed with CD. For each assay, a sample of 20 μl was spotted on to a TLC silica-gel plate pre-activated at 100°C for 1 h. Radioactive spots corresponding to CDP-Etn or P-Etn were analyzed as described above. All enzyme assays were conducted three times in duplicates. Kinetic data were fitted with the Michaelis–Menten equation and kinetic parameters were calculated from the obtained curves (GraphPad Prism).

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CD analysis
Purified recombinant proteins (wild-type and mutated PfECTs) concentrated to 0.3 mg/ml were used for UV-CD analysis in a buffer containing 20 mM sodium phosphate (pH 7.4) and 2 mM 2-mercaptoethanol. CD spectra were recorded from 195 to 260 nm at 20°C on a Chirascan CD Spectrophotometer (Applied Photophysics) with a 0.5 cm path-length quartz cell. Data were collected at 0.5 nm intervals and an accumulation time of 1 s. All protein spectra were measured three times and corrected by subtraction of respective buffer spectra. The PfECT spectra were analysed with CDNN CD Spectra Deconvolution Software (Applied Photophysics).

**Homology modelling**

ECT sequences of *P. falciparum* (UniProtKB/Swiss-Prot accession number Q8IDM2), *Saccharomyces cerevisiae* (UniProtKB/Swiss-Prot accession number P33412), rat (UniProtKB/Swiss-Prot accession number O88637), bovine (UniProtKB/Swiss-Prot accession number Q5EA75) and human (UniProtKB/Swiss-Prot accession number Q99447) were aligned using MUSCLE [27]. The structural model for PfECT N- and C-terminal CT domains was generated on the basis of the structure of the hECT N-terminal and C-terminal CT domains sharing 38% and 40% sequence identity respectively. MODELLER 9v8 [28] was used to perform the homology modelling of the PfECT intramolecular dimeric complex using the hECT structure (PDB code 3ELB) as template. The linker region (amino acids 152–186) was removed to reflect the structurally characterized hECT intramolecular dimer. The hECT co-crystallized ligand CMP was used as a constraint in MODELLER to keep the binding site in an appropriate conformation. The final model was chosen based on its low
value of the MODELLER objective function, Ramachandran plot (MolProbity) [29], ProQ [30] scores and visual inspection.

RESULTS AND DISCUSSION

Dynamics of the de novo PE biosynthesis pathway in Plasmodium falciparum-infected erythrocytes

The disruption of the essential PE pathway through the use of a pharmacological agent produces an antimalarial effect [20,21]. In order to determine the most critical step of the pathway, we characterized its three enzymatic steps. The parasite obtains Etn from the plasma or from serine through decarboxylation [14] (Figure 1). To characterize the dynamics of the pathway, we quantified the incorporation of radiolabelled Etn in the intermediate metabolites P-Etn, CDP-Etn and the end-product PE at the initial linear phase. We incubated IRBCs with increasing Etn concentrations and subsequently measured the metabolite levels. The amounts of P-Etn, CDP-Etn and PE in the IRBCs increased before reaching a plateau at Etn concentrations above 200 μM (Figure 2). The labelling of CDP-Etn was much lower than that of P-Etn and PE (Figure 2) and the maximal Etn incorporation was measured by following the formation of CDP-Etn from radio-

Characterization of parasite PECT activity and comparison with PIEK activity

The parasitic ECT enzymatic activity in P. falciparum extracts was measured by following the formation of CDP-Etn from radiolabelled P-Etn. The substrates and the product were separated by TLC. The rate of catalysis showed a characteristic Michaelis-Menten saturation curve with a $K_m$ value of 373 ± 123 μM for P-Etn and a $V_{max}$ value of 4.3 ± 0.3 pmol/min/10^7 parasites (Figure 3A). For comparative purpose, we characterized the endogenous phosphorylation of Etn into P-Etn performed by PIEK which is the first step of the CDP-Etn-dependent pathway [21,31]. The affinity of PIEK for its substrate Etn was 71 ± 4 μM and the specific activity ($V_{max}$) was 39.6 ± 0.1 pmol/min/10^7 parasites (Figure 3B). The affinity of PIEK for P-Etn was thus approximately 5-fold lower than the affinity of PIEK for Etn and under optimal conditions PIEK was ∼4-fold less active than PIEK (Table 1). The comparison of cellular catalytic activities of PIEK, PECT and P. knowlesi CEPT (choline/ethanolaminephosphotransferase 1) [32] revealed that PECT exhibited the lowest affinity for its substrate and the lowest specific activity of the enzymes of de novo PE pathway under optimal conditions (Table 1). In other terms, the first and the last enzymatic reactions of the Kennedy pathway were more efficient than the cytidylation of P-Etn catalysed by PIEK. This finding is in agreement with a regulatory role of this enzyme within the pathway.

However, the availabilities within the parasite of the metabolites involved in the pathway have to be taken into consideration. The concentration of Etn in adult plasma has been described to be from 5 to 20 μM [33,34]. In addition, in Plasmodium Etn can also be provided through decarboxylation of serine [14,35]. This latter pathway supplies less than 5% of the total pool of P-Etn in the parasite [35] and has therefore not been considered for the following calculation. We quantified the Etn metabolites in the same way as described for Figure 2, though this time using Etn at physiological concentrations (Supplementary Figure S1). For an Etn concentration of 10 μM, the observed P-Etn and CDP-Etn levels incorporated from Etn in the parasite were found to be ∼55 nmol/10^10 IRBCs and ∼5 nmol/10^10 IRBCs respectively (Supplementary Figure S1). Under these conditions and based on the volume of 50 fl for a mature parasite [36], the concentrations of P-Etn and CDP-Etn within the parasite were calculated to be ∼100 μM and ∼10 μM respectively. The P-Etn concentration was thus in the range of the $K_m$ value of PECT (373 μM). For CTP, the second substrate of the

### Table 1: Kinetic parameters for endogenous PECT, PIEK, P. knowlesi CEPT and recombinant PIEK

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/10^7 parasites)</th>
<th>$k_{cat}$ (s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECT</td>
<td>P-Etn</td>
<td>373 ± 123</td>
<td>4.0 ± 0.3</td>
<td>3.4 ± 0.3</td>
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<tr>
<td>PIEK</td>
<td>Etn</td>
<td>71 ± 4</td>
<td>39.6 ± 0.1</td>
<td>42 ± 0.6</td>
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<tr>
<td>H422A-PECT</td>
<td>CDP-Etn</td>
<td>18 ± 3</td>
<td>12.6 ± 5</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>wt-PECT</td>
<td>CTP</td>
<td>123 ± 4</td>
<td>3.7 ± 0.6</td>
<td>12.6 ± 0.6</td>
</tr>
<tr>
<td>H146A-PECT</td>
<td>P-Etn</td>
<td>374 ± 72</td>
<td>&lt;0.04 (μmol/min/mg)</td>
<td></td>
</tr>
</tbody>
</table>

* [21]. † [32].

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enzyme, the parasite concentration is likely to be lower than the \( K_m \) value (465 ± 70 \( \mu M \)) of \( P/EKT \) (see below). This suggests that the rate of \( P/EKT \) activity in the parasite grown under standard conditions (without excess of Etn) is mainly limited by the availability of CTP, in addition to the intrinsic kinetic parameters of the enzyme. In mammalian cells, the amount of DAG (diacylglycerol) has also been found to be a limiting factor for PE biosynthesis suggesting that the regulation of the metabolic flux is a multi-factor-dependent process [4]. However, amongst the three enzymatic steps of the PE pathway, the preponderant role of \( P/EKT \) in the metabolic flux as evidenced by the Etn incorporation data and kinetic parameters drove us to thoroughly characterize this enzyme.

**Sequence analysis of \( P/EKT \)**

The *P. falciparum* genome database PlasmoDB (http://plasmodb.org/plasmo/) [37] predicts the presence of one gene (PF3D7_1347700) encoding a putative \( P/EKT \). Amino acid sequence alignment with human, *S. cerevisiae* and *Trypanosoma brucei* ECT sequences confirmed the presence of two CT domains in \( P/EKT \) separated by a linker region (Figure 4 and Supplementary Figure S2 at http://www.biochemj.org/bj/450/bj4500159add.htm). The \( P/EKT \) N-terminal and C-terminal CT domains (approximately 135 amino acids) share 34 % sequence identity, whereas the sequences of the N-terminal CT domain of \( P/EKT \) and of human ECT were found to be 38 % identical. Two conserved motifs, the CTP-binding motif HxGH and the cytidylyltransferase signature motif R(S/T)xG(V/I)STT form the active site of the cytidylyltransferase superfamily. In \( P/EKT \) and other aligned sequences, the CTP-binding motif is present in both CT domains whereas the C-terminal CT signature motif lacks some conserved residues of the R(S/T)xG(V/I)STT consensus motif (Figure 4). The X-ray three-dimensional structure of hECT (PDB code 3ELB) shows that the protein contains one polypeptide chain, but fold as an intramolecular dimer. The N-terminal CT domain of human ECT interacts with the C-terminal one through the conserved dimerization motifs K\(^{144}\)WVDEVV\(^{190}\) and R\(^{276}\)YVSEVV\(^{285}\). In \( P/EKT \), both sequences are present (K\(^{193}\)WDEYV\(^{198}\) and K\(^{470}\)VVDDV\(^{476}\)) (Supplementary Figure S2). The \( P/EKT \) sequence exhibits two peculiarities: (i) a 130-amino acid N-terminal sequence and (ii) a non-conserved extended region of ∼140 amino acids linking the two CT cores (Supplementary Figure S2).

**Expression and purification of recombinant \( P/EKT \)**

The coding sequence of \( P/EKT \) as annotated in PlasmoDB was confirmed by cDNA sequencing. A codon-optimized sequence was cloned into pET15b for expression as a N-terminal His\(_7\)-tagged recombinant protein. The soluble protein fraction of the IPTG-induced bacterial culture showed a prominent band on the SDS/PAGE at ~90 kDa. This molecular mass was higher than the expected one at 68 kDa (Supplementary Figure S2). The peculiar mobility of \( P/EKT \) could result from a very high content of polar residues and/or the presence of a flexible linker containing atypical amino-acid compositions (stretches of aspartic acid and asparagine residues) [38,39] (Supplementary Figure S2). A two-step protocol consisting of Ni\(^{2+}\)-affinity chromatography followed by gel-filtration chromatography was performed to obtain pure protein at a final concentration of 0.3 mg/ml (Supplementary Figure S3 at http://www.biochemj.org/bj/450/bj4500159add.htm). Recombinant purified \( P/EKT \) was used for cellular and further kinetic characterizations.
**Cellular localization and expression of PfECT along the *P. falciparum* blood stages**

We raised antibodies against the recombinant *Pf*ECT protein by immunization of mice. Pre-immune serum did not show any detectable signal with infected erythrocytes by Western blot analysis (Supplementary Figure S4 at http://www.biochemj.org/bj/450/bj4500159add.htm). Immune sera revealed a band at an apparent molecular mass of approximately 90 kDa while this band was not visible with uninfected erythrocytes (Supplementary Figure S4). We thus observed the same aberrant migration of *Pf*ECT from parasite extract and from recombinant protein (see above). When infected erythrocytes were sub-fractionated using saponin treatment followed by differential centrifugation, the bulk of *Pf*ECT was recovered in the soluble cytosolic fraction of parasite extracts (Figure 5A). To analyse the expression of *Pf*ECT along the parasite life cycle immunodetections were performed on highly synchronized cultures. The enzyme appeared to be mainly expressed at the late stages (trophozoite and schizont) (Figure 5B). Similar expression profiles were found for *Pf*EK, *Pf*CK (*P. falciparum* choline kinase) [21] and *Pf*CCT (*P. falciparum* CCT; CTP:phosphocholine CT); R. Cerdan, A. Contet and H. Vial, unpublished work) involved in the CDP-Etn and in its counterpart CDP-choline pathways. The increase of several enzymes of these biosynthetic pathways is probably connected to the need of phospholipids for membrane formation at the late stages of the parasite life cycle in order to produce up to 32 daughter cells which will subsequently invade new erythrocytes. Apart from its essential role in membrane formation, PE is also involved in other biological processes in eukaryotic cells. For example, PE is the donor of the P-Etn group in glycosyl-phosphatidylinositol anchors [8,40,41]. During autophagy, PE is conjugated to the ATG8 protein on autophagosomal membranes [42]. Although no direct evidence of such roles for PE in *Plasmodium* has been found so far, it cannot be excluded that a part of the synthesized PE could have other functions than the formation of membranes.

The subcellular localization of *Pf*ECT was also determined by immunofluorescence assays. The signal of the *Pf*ECT antisera was detected at all parasite stages showing a diffuse and dotted pattern (Figure 5C). These data suggested that *Pf*ECT is present in the cytoplasm. *Pf*ECT localization was in agreement with the localizations of rat [43] and *T. brucei* ECT [8]. The immunofluorescence analyses also revealed some regions of higher concentration for *Pf*ECT. We hypothesize that *Pf*ECT could be localized near the endoplasmic reticulum membrane in close proximity to the following enzyme within the Kennedy pathway, the transmembrane protein CEPT.

**Kinetic characterization of recombinant *Pf*ECT**

Purified recombinant *Pf*ECT protein incubated with radio-labelled P-Etn and CTP showed a high CT activity which increased linearly with time for at least 20 min. *Pf*ECT was active over a broad pH range from 6 to 9 and the activity was optimal at pH 8 (Supplementary Figure S5 at http://www.biochemj.org/bj/450/bj4500159add.htm). The enzymatic activity followed Michaelis–Menten kinetics with respect to both substrates with an apparent *Km* of 452 ± 86 μM for P-Etn and of 465 ± 70 μM for CTP. The maximal velocity of the enzyme reaction was found to be 3.0 ± 0.3 μmol/min/mg of *Pf*ECT corresponding to a turnover rate (*kcat*) of 3.4 ± 0.3 s⁻¹ (Figure 6 and Table 1). When 2 mM of radioactive phosphocholine was added as a substrate instead of 2 mM P-Etn, CDP-choline could not be detected (<0.04 μmol/min/mg) attesting that *Pf*ECT was specific for P-Etn.

**Only one CT domain of *Pf*ECT is catalytically active**

All currently known ECT sequences possess two CT domains, but the R(S/T)xG(V/I)STT signature motif of their N- and C-terminal CT domain differ substantially (Figure 4). To our knowledge, although the lack of key residues in the C-terminal signature motif sequence has already been mentioned for hECT [13], no experimental data related to the enzymatic activity of the...
of the purified recombinant PfECTs were measured by following the formation of CDP-Etn with varying concentration of one substrate, whereas the second substrate was fixed at 2 mM.

For CTP and the Pf wild-type values of this mutated enzyme were thus similar to the ones of NaCl/25 % ammonia [50:50:1 (v/v/v)]. The Pf CT domains of the full-length recombinant pET vector and expressed in E. coli. All attempts resulted in the production of insoluble protein fragments. We then opted for an alternative approach aiming at mutating the individual CT domains of the full-length recombinant PfECT enzyme.

Several previous biochemical and structural studies indicated the crucial role of the histidine residues of the CTP-binding motif HxGH for the CT activity [13,44,45]. For example, for the Bacillus subtilis GCT (CTP:glycerol-3-phosphate CT), mutations of these histidine residues led to a dramatic loss in activity [46]. Therefore we mutated individually the second histidine residue of each P/ECT CTP-binding motif HxGH to alanine, generating the mutated proteins H146A-PfECT and H422A-PfECT. CD analysis of both mutated and wild-type enzymes showed that the spectra were overlapping, reflecting the same composition in secondary structures and indicating that the overall folding of P/ECT was likely to be maintained after the mutation of the histidine residues (Supplementary Figure S6 at http://www.biochemj.org/bj/450/bj4500159add.htm). ECT enzymatic activity was evaluated on both mutated and the wild-type enzymes under identical conditions. Noticeably, high activity was detected with H422A-PfECT, whereas H146A-PfECT didn’t show any measurable activity up to 2 mM of P-Etn (Figure 6A). The H422A-PfECT activity was linear before reaching a saturation plateau as the substrate concentration increased (Figures 6B and 6C). The affinity ($K_m$) of the H422A-PfECT was found to be 565 ± 60 µM for P-Etn and 364 ± 72 µM for CTP and the $k_{cat}$ value was 4.1 ± 0.6 s$^{-1}$ (Table 1). The kinetic values of these mutated enzymes were thus similar to the ones of wild-type PfECT (Table 1) suggesting that PfECT relies solely on its N-terminal CT domain for its catalytic activity. Strikingly, all ECTs known to date possess two CT domains like PfECT, probably resulting from an early event of duplication during evolution. For all the analysed ECT sequences, the CTP-binding motif is conserved in both the N- and C-terminal domains unlike the CT signature motif (Figure 4). In the light of our results, it is probable that the C-terminal domain of other eukaryotic ECTs is also catalytically inactive. Within the CT superfamily, bacterial GCT and eukaryotic CCT contain only one CT domain. The X-ray structure of B. subtilis and Staphylococcus aureus GCT [44,45,47] and rat CCT [13] revealed that the enzymes fold as homodimers. P/ECT, and probably other ECTs bearing an active N-terminal domain and an inactive C-terminal domain, likely exhibit a unique mechanism for their catalytic function.

Three-dimensional structural model of PfECT

The finding that the C-terminal CT domain was catalytically inactive prompted us to question the role of this domain in P/ECT function. To this end, we built a homology model of the P/ECT structure based on the X-ray structure of the hECT (residues 18–356), crystallized in presence of CMP (PDB code 3ELB). The hECT structure lacks the co-ordinates of the linker region between the N- and C-terminal CT domains. The three-dimensional structural model of P/ECT showed a global folding similar to human ECT. The enzyme folded as an intramolecular dimer, both CT domains being in contact. The P/ECT structure model adopted an $\alpha/\beta$-fold and each CT domain formed a Rossman fold commonly found in nucleotide-binding proteins (Figure 7A). Our particular interest was to compare the substrate binding sites of the N- and C-terminal CT domains of P/ECT. In depth examination of the substrate-binding site of the P/ECT model revealed important amino acid variations between the N- and the C-terminal domains. Two examples are described here. The arginine residue (Arg$^{269}$) of the N-terminal CT signature motif R(T/S)xG(V/I)STT probably mediates $\Pi$-stacking interactions with the cytosine ring, like in the hECT crystal structure. The replacement of this arginine residue by a serine (Ser$^{528}$) in the C-terminal CT domain of other eukaryotic ECTs is also catalytically inactive. Within the CT superfamily, bacterial GCT and eukaryotic CCT contain only one CT domain. The X-ray structure of B. subtilis and Staphylococcus aureus GCT [44,45,47] and rat CCT [13] revealed that the enzymes fold as homodimers. P/ECT, and probably other ECTs bearing an active N-terminal domain and an inactive C-terminal domain, likely exhibit a unique mechanism for their catalytic function.
length (Figure 7B). Critical residues for substrate binding differed between the N- and C-terminal site, whereas the amino acids at the dimerization interface were rather conserved in both domains. All available three-dimensional crystal structures of CTs show either homo- or pseudo-dimeric (for internal duplication of hECT) oligomerization states, highlighting the importance of dimerization for this superfamily. Thus the C-terminal CT domain of PfECT might allow the enzyme to dimerize by intramolecular contacts. The effect of disruption of the intramolecular dimerization will be interesting to evaluate for PfECT function.

In conclusion, the present study showed the essential role played by PfECT within the PE biosynthesis pathway of *P. falciparum* and revealed that only one of both CT domains of PfECT is catalytically active. As a follow-up to the present study, a new chemotherapeutic approach could be developed in order to impede the PE pathway by targeting its essential and rate-controlling enzyme. A compound screening might be a route to discover new biologically active molecules against this deadly disease.

**AUTHOR CONTRIBUTION**

Sweta Maheshwari, Marina Lavigne, Alicia Contet and Blandine Alberge performed the experimental work. Emilie Pihan and Dominique Douguet built the model structure and performed the structure analysis. Rachel Cerdan, Clemens Kocken and Henri Vial planned the strategy. Rachel Cerdan and Henri Vial designed the experiments. Sweta Maheshwari, Kai Wengelnik and Rachel Cerdan analysed and interpreted the data. Rachel Cerdan and Kai Wengelnik wrote the paper.

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**REFERENCES**


SUPPLEMENTARY ONLINE DATA
Biochemical characterization of Plasmodium falciparum CTp:phosphoethanolamine cytidylyltransferase shows that only one of the two cytidylyltransferase domains is active

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Figure S1 Etn metabolism in P. falciparum-infected erythrocytes

Etn incorporations in the intermediate metabolites and in the end product are reported for low Etn concentrations. Increasing concentrations of [14C]Etn were added to parasite culture and the levels of radio-labelled phospho-Etn (○), CDP-Etn (▲) and PEP (□) were quantified after a 1 h incubation. Results are from one typical experiment of three performed in triplicate and are expressed as nmol/1010 IRBC/h ± S.E.M.

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Figure S2  Sequence alignments of ECT

Primary sequences of PfECT (NCBI Reference Sequence XP_001350190.1; hECT), S. cerevisiae ECT (GenBank® accession number BAA09310.1; ScECT) and T. brucei ECT (GenBank® accession number EAN80358.1; TbECT) were aligned with CLUSTAL W2. Identical and similar residues are shaded in black and in grey respectively using BOXSHADE 3.21. N- and C-terminal CT domains are indicated by thick bars. Both CTP-binding (HxGH) sites and CT signature [R(S/T)xG(I/V)STT] of the N-terminal domain are indicated and boxed in red. The localization of the non-conserved C-terminal CT signature motif is indicated by a dashed red box. The eight-residue dimerization motifs described in homologous GCT and CCT three-dimensional structures are boxed in green for both CT domains. The two mutated histidine residues are labelled with a red asterisk. His221, Arg249 and Ser528 referred to in the text are labelled with #.

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Figure S3  Purification of recombinant PfECT

SDS/PAGE (12 % gel). Lane 1, total extracts of bacteria before IPTG induction; lane 2, after IPTG induction; lane 3, pellet; lane 4, supernatant after bacteria lysis; lane 5, 250 mM Ni-NTA (Ni²⁺–nitrilotriacetate) elution; and lane 6, sample eluted after gel filtration G200 (GE Healthcare). M, molecular mass marker (in kDa). PfECT is indicated by the arrows.

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Figure S4  Characterization of polyclonal anti-PfECT

Left-hand panel, IRBCs with pre-immune serum. Right-hand panel, uninfected RBCs and IRBCs revealed with an anti-PfECT antibody. PfECT is indicated by the arrow. M, molecular mass marker (in kDa).
Characterization of \textit{P. falciparum ECT}

**Figure S5** CT activity as a function of pH

The assay medium contained the recombinant PfECT protein incubated with buffers at various pH values. The amount (nmol) of synthesized CDP-Etn is given as a function of the pH value.

**Figure S6** Characterization of wild-type and mutated PfECT

CD spectra of wild-type PfECT (solid line), H146A-PfECT (dotted line) and H422A-PfECT (dashed line).

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