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Plasmodium falciparum CTP:phosphocholine cytidylyltransferase expressed in *Escherichia coli*: purification, characterization and lipid regulation

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The *Plasmodium falciparum* CTP:phosphocholine cytidylyltransferase (PfCCT) has been isolated from an overexpressing strain of *Escherichia coli*. The plasmid pETPfCCT mediated the overexpression of the full-length polypeptide directly. The recombinant protein corresponded to 6–9% of the total cellular proteins and was found essentially in the insoluble membrane fraction. Urea at 6 M was used to solubilize the recombinant protein from the insoluble fraction. The CCT activity was restored upon the removal of urea, and the protein was subsequently purified to homogeneity on a Q-Sepharose column. Approx. 1.4 mg of pure enzyme was obtained from a 250 ml culture of *E. coli*. Biochemical properties, including *in vitro* substrate specificity and enzymic characterization, were assessed. The lipid regulation of the recombinant plasmodial CCT activity was characterized for the first time. The K_m values were 0.49 ± 0.03 mM (mean $\pm$ S.E.M.) for phosphocholine and 10.9 ± 0.5 mM for CTP in the presence of lipid activators (oleic acid/egg phosphatidylcholine vesicles), whereas the K_m values were 0.66 ± 0.07 mM for phosphocholine and 28.9 ± 0.8 mM for CTP in the absence of lipid activators. The PfCCT activity was stimulated to the same extent in response to egg phosphatidylcholine vesicles containing anionic lipids, such as oleic acid, cardiolipin and phosphatidylglycerol, and was insensitive or slightly sensitive to PC vesicles containing neutral lipids, such as diacylglycerol and monoacylglycerol. Furthermore, the stimulated enzyme activity by oleic acid was antagonized by the cationic aminolipid sphingosine. These lipid-dependence properties place the parasite enzyme intermediately between the mammalian enzymes and the yeast enzyme.

INTRODUCTION

Plasmodium falciparum, a haematozoan parasite, causes human malaria, one of the most prevailing parasitic diseases worldwide. Malaria chemotherapy is increasingly hampered by the dramatic appearance of *P. falciparum* parasites that are polypharmacoresistant to conventional drugs as well as recently discovered drugs. At present, the phospholipid metabolic pathway is being evaluated as a very attractive antimalarial drug target [1,2]. The most promising drug strategy is to block the choline transporter which provides the intra-erythrocytic parasite with choline, a precursor required for the synthesis of phosphatidylcholine (PC) [3].

PC is the major membrane phospholipid in eukaryotes. In addition to its important structural role, PC serves as a major source of second messengers for signal-transduction molecules [4,5]. Furthermore, the *de novo* PC synthesis is essential to normal cell growth, particularly to progress of G1 to S phase during the cell cycle [6]. CTP:phosphocholine cytidylyltransferase (CCT; EC 2.7.7.15) is the rate-limiting step enzyme in *de novo* PC synthesis and catalyses the conversion of phosphocholine and CTP into CDP-choline and pyrophosphate [7–9]. This enzyme has been the subject of recent intensive studies because it has been shown to play a key role in the regulation of PC synthesis in eukaryotic cells [9,10]. In mammalian cells, the CCT activity can be regulated by interconversion between an active membrane-bound form and an inactive cytosolic form [9,11]. The lipid composition of the membrane is a main determinant of the distribution of these two forms. For mammalian CCTs, lipid regulators of the enzyme activity have been identified: (1) lipid activators including anionic phospholipids, fatty acids and neutral lipids; and (2) inhibitory cationic lipids such as sphingosine [12–16].

The primary sequences of CCTs have been determined for several mammalian CCTs [17–22], yeast CCT [23], and plasmodial CCT [24]. These recent advances in molecular cloning have spurred the investigation of regulation mechanisms at the molecular level. In particular, there is much evidence that an amphipathic α-helical region between residues 236 and 293 of the rat CCT sequence is responsible for the membrane-binding function [25–27]. Deletion of this α-helix, using limited chymotrypsin proteolysis [27] or truncation mutants [25,26], led to a constitutively active enzyme which could be no further stimulated by lipid activators. It has also been suggested that the multiple phosphorylation sites in the C-terminal domain of rat CCT could be involved in the enzyme activation [28–30]. Whereas much is known about mammalian CCTs, little is known about yeast CCT, a lower-eukaryotic enzyme. The lipid regulation mode of yeast CCT is somewhat different from that of rat CCT: (1) yeast CCT is activated only by negatively charged lipids, whereas rat CCT is modulated both by charged and neutral lipids; and (2) yeast CCT is activated within a small concentration range of charged lipids, while rat CCT activation reaches a plateau [31]. These metabolic differences among CCT molecules led us to ask whether or not plasmodial CCT resembles other CCTs with regard to the regulation by lipids. In this respect, the lipid

Abbreviations used: (Pf)CCT, (*Plasmodium falciparum*) CTP:phosphocholine cytidylyltransferase; CL, cardiolipin; DG, dioleoylglycerol; DTT, dithiothreitol; IPTG, isopropyl thiogalactopyranoside; LB, Luria–Bertani broth; MG, mono-oleoylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TBS, Tris-buffered saline.

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regulation of plasmodial CCT was characterized for the first time.

Moreover, in an attempt to evaluate CCT as a rational drug target in the malaria chemotherapy, we have been attracted to study the functional mechanism of this enzyme in the *Plasmodium* parasite. A characterization of the recombinant PfCCT is of certain importance to our knowledge of regulation modes of phospholipid metabolism and potential physiological roles of this enzyme in the malaria parasite. Indeed, this characterization is essential to help to decipher its intrinsic properties so that this enzyme could be used as a specific target. Hence the present paper describes the purification of the recombinant PfCCT, its biochemical properties and the lipid regulation of this key regulatory enzyme in the PC metabolic pathway.

MATERIALS AND METHODS

Chemicals

PC (type XI-E from egg yolk), oleic acid, sphingosine (from bovine brain cerebrosides), phosphatidylglycerol (PG), cardiolipin (CL) (from bovine heart), 1,2-dioleoylglycerol (DG), monooleoylglycerol (MG), phosphocholine, CTP, ATP, dCTP, CDP, GTP and PMSF were from Sigma. Phospho[*methyl*-^{14}C]choline was purchased from Amersham France. Q-Sepharose HP was obtained from Pharmacia. Triton X-100 was a product of Merck. DTT and isopropyl thiogalactopyranoside (IPTG) were purchased from Euromedex. All the chemicals were of the highest purity grade available.

Expression plasmid and bacterial strain

The protocols concerning the construction of the recombinant *P. falciparum* CCT have been previously described [24]. The PfCCT gene was inserted into the expression vector pET3d (termed pETPfCCT) and expressed in the *Escherichia coli* strain BL21(DE3)pLysS, which expresses the T7 RNA polymerase under the inducible *lac* UV5 promoter [32].

Plasmodial CCT production in *E. coli*

Single colonies from *E. coli* BL21(DE3)pLysS cells harbouring the plasmid pETPfCCT were used to inoculate 10 ml of Luria–Bertani broth (LB) containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and were grown overnight at 37 °C. The saturated cell culture was used to inoculate a large culture (to a dilution of 1:100) of fresh LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. This culture was grown at 37 °C until the absorbance at 550 nm reached about 0.7. IPTG was added to a final concentration of 0.5 mM and the culture was further grown for 3 h at 37 °C. Cells were then harvested by a centrifugation at 5000 ***g*** and 4 °C for 10 min. Pelleted cells were washed once with ice-cold 50 mM Tris/HCl buffer, pH 7.5. Following centrifugation at 10000 ***g*** and 4 °C for 10 min, the washed pellet was frozen and kept at −20 °C until use.

Solubilization of PfCCT from inclusion bodies and refolding

Frozen cells were resuspended in lysis buffer [20 mM Tris/HCl (pH 7.4)/1 mM EDTA/50 mM NaCl/1 mM DTT/1 mM PMSF] and broken by five cycles of 30 s sonication. All the purification steps were performed at 4 °C. The insoluble fraction was isolated from the cell lysate by centrifugation at 20000 ***g*** for 15 min. The inclusion-body pellet was resuspended in 20 vol. of ice-cold 50 mM Tris/HCl, pH 7.4, containing 1 % (w/v) Triton X-100 and 2 mM EDTA, incubated for 15 min on ice, and centrifuged at 20000 ***g*** for 15 min. This pellet was washed twice with 20 vol. of lysis buffer and solubilized in 15 ml (for 500 ml cell culture) of 6 M urea in 50 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 1 mM DTT, by rotary stirring for 6 h. The solution containing denatured protein was then clarified by ultracentrifugation at 100000 ***g*** for 1 h and submitted to dialysis: a first dialysis against 1.5 litres of TED buffer [20 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM DTT] for 12 h, followed by two further dialyses against 1.5 litres of TED buffer for 4 h each, in order to ensure the refolding of the proteins. Finally, this refolded extract was ultracentrifuged at 100 000 ***g*** for 1 h in order to eliminate protein aggregates.

Purification of PfCCT

A column (2.5 cm × 20 cm) packed with Q-Sepharose HP anion-exchanger was equilibrated with TED buffer. After loading 8 ml of the refolded extract, the column was washed with 1 vol. of the same buffer and the bound proteins were eluted with a linear gradient of 0.3–0.9 M NaCl in TED buffer at a flow rate of 0.8 ml/min. The eluent was monitored at 280 nm with a UV detector. Fractions (4 ml each) were collected and analysed by SDS/PAGE, and the CCT enzyme activity was assayed. Fractions containing the active PfCCT (48 kDa protein) were pooled then dialysed against TED buffer. The sample was concentrated by ultrafiltration through a Centricon 30 filter (Amicon) to a final concentration of 0.5 mg/ml. For storage, the purified PfCCT was supplemented with glycerol to a final concentration of 10 %, divided into aliquots of 0.5 ml and stored at −20 °C until use.

Protein determination and SDS/PAGE

The concentration of protein at each step was determined by Coomassie Blue G-250 binding assay (Pierce) as described by Bradford [33] using BSA as a standard. SDS/PAGE was performed as described by Laemmli [34]; usually, a 10 % polyacrylamide running gel with a 5 % polyacrylamide stacking gel was used. The molecular mass of proteins on SDS/PAGE was determined by comparison with the relative mobilities of the standard proteins (Sigma) comprising myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa).

N-terminal sequencing

N-terminal sequencing was performed using an Applied Biosystems (model 470) sequencing system. The purified PfCCT (100 pmol) was subjected to SDS/10 %-PAGE then blotted on to PVDF membrane (Schleicher and Schuell). After revealing the protein with Ponceau S Red, the band was excised and destained. The protein sample was loaded on to the glass filter and the sequence run was performed according to the manufacturer's instructions.

Western blotting

Electrophoretic transfer of proteins from SDS/PAGE gels to nitrocellulose and PVDF membrane was performed according to the method of Burnette [35], with minor modification. Transfer was performed in 25 mM Tris/HCl, pH 8, containing 192 mM glycine, 0.1 % SDS and 10 % (v/v) methanol for 2 h at a constant current of 150 mA. Following transfer, the membrane was washed three times with Tris-buffered saline (TBS) [20 mM Tris/HCl (pH 7.4)/0.15 M NaCl] containing 0.5 % (w/v) Tween

20 (TBS-T) (5 min each time). The membrane was then saturated with 0.5 % BSA in TBS-T for 1 h at room temperature. After three washings with TBS-T as described above, the membrane was incubated with antibodies raised against a synthetic peptide corresponding to residues 1–16 of PfCCT (H.-J. Yeo, M. P. Larvor and H. J. Vial, unpublished work) to a dilution 1:50 000 for 1 h. Then the membrane was washed with TBS-T and incubated with a goat anti-rabbit–peroxidase conjugate (Sigma; dilution 1:40000) for 30 min. Finally, the membrane was washed with TBS-T and developed with an ECL detection system (Amersham France) according to established procedures.

CCT assay

The standard reaction mixture for CCT assay contained 50 mM Tris/HCl, pH 7.4, 1.1 mM phospho[*methyl*-^{14}C]choline (sp. radioactivity 45 nCi/nmol), 20 mM CTP, 40 mM $MgCl_2$, PfCCT (2–4 μg) and 100 μM oleic acid/egg PC (1:1, mol/mol) vesicles as lipid activators in a final volume of 20 μl. The reaction mixture was incubated for 15 min at 37 °C, stopped by heating at 85 °C for 10 min, and then kept on ice. Under these conditions, CCT assays were linear with time and protein quantity. CDP-[^{14}C]choline formation was quantified by spotting 12 μl of the reaction mixture on to a silica-gel 60 TLC plate (Merck) that had previously been activated by heating at 100 °C for 1 h. The TLC plate was developed with ethanol/2 % ammonia (1:1, v/v). The radioactive spots were revealed by exposing the TLC plate to X-OMAT film (Kodak) for 10 h, and the compounds were identified by comparison with the migration of appropriate standards. CDP-[^{14}C]choline spots were scraped directly into scintillation vials and radioactivity was quantified using a Beckman LS5000 liquid-scintillation spectrometer. All the assays were done in duplicate at least twice.

Preparation of liposomes

The method for preparation of sonicated vesicles has been described previously [36]. Lipids were mixed with chloroform or chloroform/methanol (with the exception of oleic acid, which was mixed with hexane) and the solvents were evaporated under nitrogen at room temperature. The lipids were hydrated in 50 mM Tris/HCl buffer, pH 7.5, and vesicles were formed by sonication using a Vibra Cell sonicator (Bioblock) at 50 % power output at 4 °C for 12 min. Vesicles prepared by this method had mean diameters ranging from 112 nm to 138 nm, depending on lipids, determined by photon correlation spectroscopy using a Coultronics N4S Sub-Micron Particle Analyzer. The liposome suspension (daily prepared) remained stable until use.

RESULTS

Expression, solubilization and purification of the recombinant PfCCT

The PfCCT gene was inserted into the expression vector pET3d and the plasmid pETPfCCT was introduced in the *E. coli* strain BL21(DE3)pLys [24]. SDS/PAGE analysis of cellular proteins from IPTG-induced *E. coli* cells harbouring the plasmid pETPfCCT indicated the presence of a prominently stained band corresponding to an apparent molecular mass of 48 kDa (Figure 1, lane 2). The time course of induction of PfCCT revealed that the expression of PfCCT was induced rapidly (after 30 min) following the addition of IPTG, increased with time of incubation with IPTG, but did not increase further after 3 h (results not shown). Densitometric analysis suggested that the recombinant protein constituted about 6–9 % of the total cellular proteins. However, in addition to the major 48 kDa band, there were

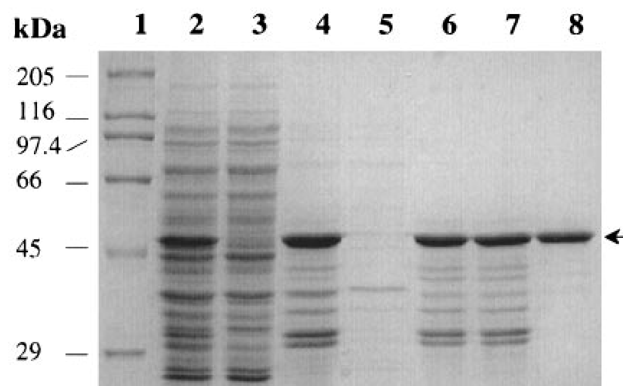

Figure 1 SDS/PAGE analysis of purification of the recombinant PfCCT overexpressed in *E. coli*

Proteins were separated on a 10%-polyacrylamide gel and stained with Coomassie Blue R-250. Lane 1, molecular-mass markers (kDa); lane 2, total cell lysate; lane 3, soluble fraction of the cell lysate; lane 4, insoluble fraction of the cell lysate; lane 5, supernatant obtained after detergent treatment of the insoluble fraction; lane 6, pellet recovered after detergent treatment of the insoluble fraction (inclusion bodies); lane 7, refolded proteins of the urea-solubilized inclusion bodies; lane 8, purified recombinant PfCCT from Q-Sepharose column. The recombinant PfCCT is indicated by the arrow.

additional bands of apparent molecular masses 30 kDa and 32 kDa related to the 48 kDa PfCCT (see below).

Many recombinant proteins overexpressed in bacterial cells often form insoluble inclusion bodies. Thus it was not surprising to find the recombinant PfCCT predominantly in the pelleted membrane fraction, as indicated by SDS/PAGE analysis (Figure 1, lanes 3 and 4). Our previous efforts to recover the recombinant protein from inclusion bodies, by solubilization of the insoluble fraction in the presence of 8 M urea and renaturation by dialysis, failed to obtain clear soluble protein extract. Most of the protein precipitated during refolding, leading to a cloudy solution. However, a preliminary treatment of the membrane fraction with 1 % (w/v) Triton X-100 did release numerous membrane-associated proteins, helping further purification (Figure 1, lane 5). The detergent-treated insoluble fraction (Figure 1, lane 6) was washed twice with lysis buffer in order to eliminate the detergent, solubilized with 6 M urea and refolded (Figure 1, lane 7), as described in the Materials and methods section. This procedure gave rise to a significant enrichment of the recombinant PfCCT from the contaminating proteins, and the recombinant PfCCT appeared to be about 60 % pure at this step according to the SDS/PAGE analysis.

The refolded extract was applied on to a Q-Sepharose anion-exchange column, as described in the Materials and methods section. The elution profile at 280 nm (Figure 2A) showed that most of the proteins bound to the Q-Sepharose column. Elution with a linear 0.3–0.9 M NaCl gradient resulted in an elution pattern characterized by a major peak between 470 and 510 mM NaCl (fractions 32–36), a second peak between 380 and 460 mM NaCl (fractions 23–31), and other minor peaks. SDS/PAGE analysis of the individual fractions (Figure 2B) indicated that the recombinant protein of 48 kDa was eluted in fractions 32–36 as a pure single band, whereas fractions 23–31 contained two proteins of 30 kDa and 32 kDa respectively. The Western blot (Figure 2C) showed that these 30 kDa and 32 kDa forms, like the 48 kDa protein, could be recognized on the one hand by antibodies elicited against a peptide corresponding to residues 1–16 of PfCCT (H.-J. Yeo, M. P. Larvor and H. J. Vial,

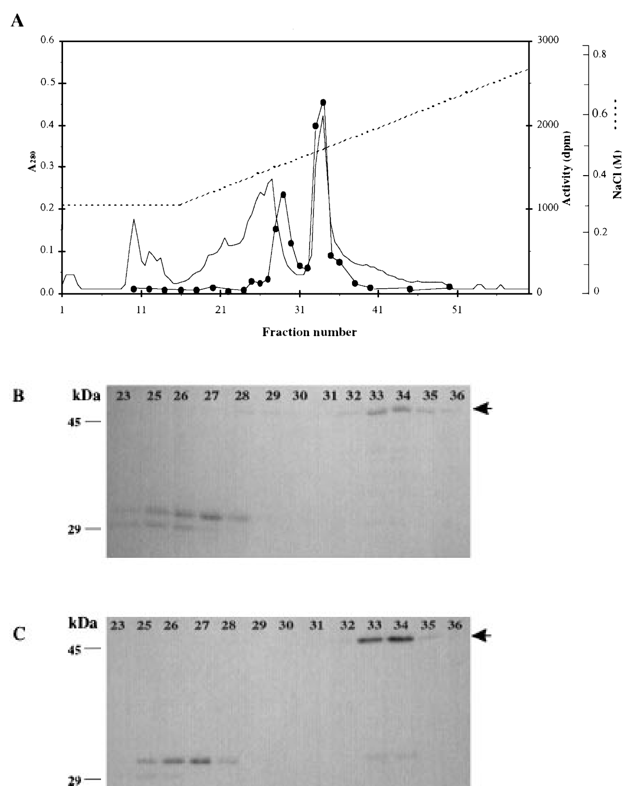

Figure 2 Q-Sepharose HP anion-exchange chromatography of refolded proteins

(**A**) Refolded proteins extracted from the inclusion bodies (see the Materials and methods section) were applied on to Q-Sepharose HP anion-exchange column. Elution was achieved with a linear 0.3–0.9 M NaCl gradient (––––). Fractions (4 ml each) were collected and assayed for the PfCCT activity in the presence of 100 μM oleic acid/egg PC (1:1, mol/mol) vesicles (—●—). The absorbance at 280 nm of proteins eluted from the column is shown by a continuous line (——). (**B**) SDS/PAGE analysis of individual fractions from the Q-Sepharose column (23–36). (**C**) Western-blot analysis of individual fractions from the Q-Sepharose column. SDS/PAGE in (**B**) was transferred on to a nitrocellulose membrane. PfCCT was identified with antibodies raised against a synthetic peptide corresponding to residues 1 to 16 of PfCCT (see the Materials and methods section).

unpublished work), and on the other hand by antibodies raised against a peptide corresponding to residues 164–177 of rat CCT (kindly given by Dr. D. E. Vance, Department of Medicine, University of Alberta, Edmonton, Canada) (results not shown). Moreover, the N-terminal sequence of these proteins (30, 32 and 48 kDa) was identical with that of PfCCT [24], indicating that the 30 kDa and 32 kDa fragments were proteolytic products of PfCCT. The PfCCT activity profile presented two peaks (Figure 2A). The major one, which was eluted between 470 and 510 mM NaCl, precisely overlapped that of the absorption profile. This peak constituted the intact purified PfCCT (48 kDa), which was stored. It represented 72 % of the total recovered activity. The minor peak, which was eluted between 410 and 460 mM NaCl, was slightly shifted in comparison with the corresponding peak of the absorption profile. This strongly suggested that the 32 and 30 kDa proteins corresponded to an active fragment and an inactive fragment of the PfCCT respectively.

Table 1 summarizes the purification steps. Typically, isolation of inclusion bodies and refolding alone gave rise to 4.5 mg of 60 %-purified protein (19-fold enrichment with 94 % yield) from a 250 ml bacterial culture. After the additional Q-Sepharose column step, the enzyme was purified 22-fold with a 34 % recovery (1.4 mg from 250 ml culture). The refolded extract and the Q-Sepharose-purified enzyme could be stored at −20 °C for at least 2 months without significant loss of activity in the presence of 10 % glycerol. At 4 °C, the refolded extract was stable for 1 week with a loss of only 10 % activity, whereas the enzyme purified with Q-Sepharose showed a loss of more than 80 % for the same period.

Table 1 Purification of recombinant PfCCT

Step	Volume (ml)	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
Bacterial lysate	250	90	716	7.96	1	100
Refolded extract	7.5	4.5	679	151	19	94
Q-Sepharose	8	1.4	246	175	22	34

Properties of recombinant PfCCT

The purified recombinant PfCCT was submitted to protein sequencing. The sequence of the N-terminus was MDSSNY-FHDCK, as predicted from the DNA sequence [24]. For enzymological characterization, the partially purified enzyme, i.e. the refolded extract, was used, since it was stable longer than the enzyme purified with Q-Sepharose (see the subsection above) and since the two enzyme sources led to identical results.

At saturating substrate concentrations, the reaction proceeded linearly at 37 °C for about 15 min and then progressively reached a plateau. Optimal activity of an enzyme depends on several factors, including the pH and concentrations of specific ions. The recombinant PfCCT activity was cation-dependent. In the presence of 5 mM CTP, maximum activity of PfCCT was reached with 10–40 mM Mg^{2+} (results not shown). Mn^{2+} could partially replace Mg^{2+} within a narrow range of optimal concentrations around 5 mM. At concentrations higher than their optimal concentrations, both of these ions caused a strong inhibition of the enzyme activity. Maximal activity of the recombinant PfCCT, obtained in the presence of $MgCl_2$ at the concentration of 20 mM, was 1.4-fold higher than in the presence of $MnCl_2$ at the concentration of 5 mM (results not shown). The recombinant PfCCT activity had broad optimal pH, ranging from 6.5 to 8. Furthermore, the profile of the effect of pH on the enzyme activity was similar in the presence and in the absence of lipid activators (e.g. oleic acid/egg PC vesicles; see below). The effect of ionic strength on the PfCCT activity was also examined using various concentrations of NaCl (results not shown). The PfCCT activity was optimal up to 0.2 M NaCl, and higher concentrations of NaCl inhibited the PfCCT activity. For instance, only 61 % and 33 % of activity remained in the presence of 0.3 M NaCl and 0.5 M NaCl respectively.

To investigate the specificity of the CTP substrate, dCTP, GTP, ATP and CDP were examined as possible substrates. Only dCTP was a slightly active substrate: the activity in the presence of 5 mM dCTP was only 22 % of that obtained with 5 mM CTP (results not shown).

To determine the K_m values of the PfCCT for CTP and phosphocholine, the enzyme reaction was assayed under standard conditions with phosphocholine varying from 0.1 to 5 mM and CTP ranging from 0.5 to 40 mM. The saturation kinetics with phosphocholine, as determined by Lineweaver–Burk analysis, indicated K_m values of 0.49 ± 0.03 mM (mean ± S.E.M., $n = 2$) and 0.66 ± 0.07 mM ($n = 2$) in the presence and in the absence of

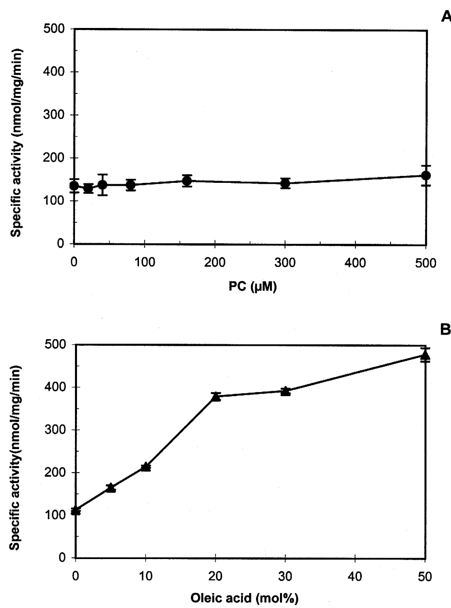

Figure 3 Dependence of the PfCCT activity on pure egg PC vesicles or on the mol% of oleic acid in egg PC vesicles

(**A**) PfCCT (2.5 μg) was assayed in the presence of the indicated concentration of pure egg PC vesicles. (**B**) PfCCT was assayed in the presence of 100 μM egg PC vesicles containing the indicated mol% of oleic acid. Results are expressed as means ± S.E.M.

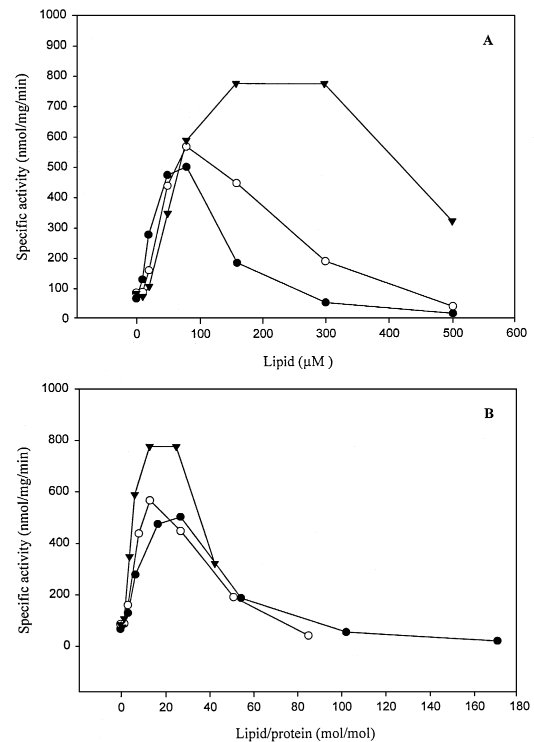

Figure 4 Dependence of the PfCCT activity on the concentration of oleic acid/egg PC vesicles

(**A**) The specific activity was determined in the presence of the indicated concentration of oleic acid/egg PC (1 : 1, mol/mol) vesicles and various amounts of PfCCT in the reaction mixture (●, 2.5 μg; ○, 5 μg; ▼, 10 μg). (**B**) Specific activity as a function of the lipid/protein mol ratio.

lipid activators respectively. The presence of lipid activators significantly increased the affinity of the parasite enzyme for CTP, with K_m values of 10.9 ± 0.5 mM ($n = 3$) and 28.9 ± 0.8 mM ($n = 3$) in the presence and in the absence of lipid activators respectively. No inhibition was observed at high concentrations of the substrates CTP and phosphocholine. V_{max} in the presence of lipids (550 nmol/min per mg) was 5.5-fold higher than in the absence of lipids.

Lipid regulation of PfCCT

Our initial experiments showed that the activity of the recombinant PfCCT was clearly increased in the presence of oleic acid incorporated into egg PC vesicles. This prompted us to investigate the effects of selected lipids. For this study we first examined whether our enzyme preparations were free of endogenous lipids. Following extraction of lipids from PfCCT preparations [37], the total phosphorus content of the organic phase was determined as described by Bartlett [38]. By this analysis, the quantity of endogenous phospholipids detected in our assays was not significant (less than 0.8 μM of total phospholipids, $n = 6$). Moreover, the enzyme activity was not modified when assayed in the presence of high concentrations (up to 30 μM) of fatty-acid-free BSA [39], indicating a negligible quantity or the absence of endogenous fatty acids in the assays. The water-insoluble lipids were included into egg PC unilamellar sonicated vesicles (liposomes), since the presence of detergents such as Triton X-100 or octyl glucoside in mixed micelles was shown to prevent the activation of CCT by neutral lipids [13]. The sonicated vesicles had a mean diameter ranging from 112 to 138 nm, depending on the lipids involved.

Oleic acid dispersions alone (up to 500 μM) (results not shown) or pure egg PC vesicles (Figure 3A) had no effect on the PfCCT activity, in agreement with previous studies [13,16]. However, the equimolar incorporation of oleic acid into egg PC vesicles increased the PfCCT activity up to about 5-fold (Figure 3B). A steep dependence curve was observed with the activation of PfCCT up to 20 mol% of oleic acid in egg PC vesicles, followed by a further moderate activation. Since maximal activation of PfCCT was obtained with 50 mol% of oleic acid in egg PC vesicles, sonicated unilamellar vesicles composed of oleic acid/egg PC (1 : 1, mol/mol) were then used in standard lipid activation assays. The effect of the concentration of oleic acid/egg PC (1 : 1, mol/mol) vesicles on the PfCCT activity was characterized by biphasic profiles (Figure 4A). Maximal specific activities were observed for lipid concentrations of 50, 100 and 180 μM, depending on the total amount of CCT (2.5, 5 and 10 μg respectively) that was added to the incubation medium. Interestingly, the dependence of the PfCCT activity on the lipid concentration gave rise to quite similar profiles when the specific activities were plotted as a function of the lipid/protein ratio (Figure 4B). This indicated that the optimal lipid concentration was strictly related to the amount of enzyme in the assay. At lipid concentrations higher than those mentioned above, the activation curve declined sharply: PfCCT was inhibited with a lipid/protein ratio of higher than 50 : 1 (mol/mol)

The anionic phospholipids CL and PG were tested as a function of lipid concentration (Figure 5A) or as a function of the mol% anionic phospholipids incorporated into egg PC vesicles (Figure 5B). The concentration-dependence of the PfCCT activity reached a plateau at 10 μM CL and at 50 μM PG, and nearly the same maximal activity was obtained as with oleic acid/egg PC vesicles. Higher concentrations, of up to 100 μM for each anionic

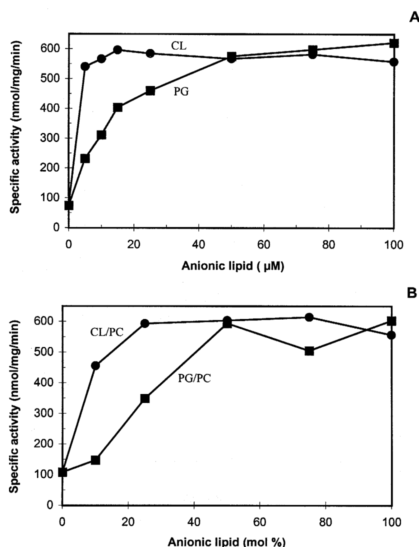

Figure 5 Dependence of the PfCCT activity on anionic phospholipids

(**A**) Dependence of the PfCCT activity on the concentration of anionic phospholipids. PfCCT was assayed in the presence of vesicles containing only CL (●) or PG (■) at the indicated concentrations. (**B**) Dependence of the PfCCT activity on the mol% of anionic phospholipids in egg PC vesicles. PfCCT was assayed in the presence of 100 μM egg PC vesicles containing the indicated mol% of a given anionic phospholipid.

phospholipid, did not affect the maximal enzyme activity. To study the dependence of the PfCCT activity on the mol% of anionic phospholipids in egg PC vesicles, PfCCT was assayed with the optimal lipid concentration of 100 μM, determined from the results of Figure 5(A). The PfCCT activity reached a plateau at 25% CL and 50% PG incorporated into egg PC vesicles (Figure 5B).

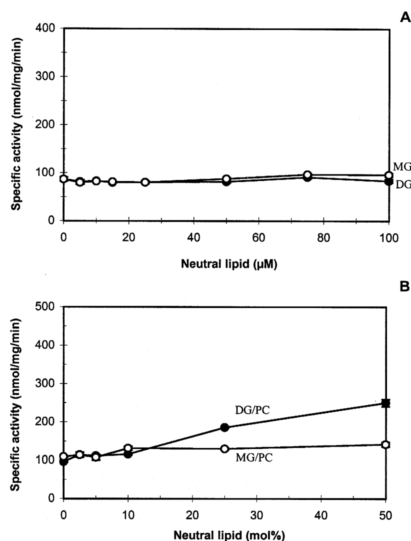

Figure 6 Dependence of the PfCCT activity on neutral lipids

(**A**) Dependence of the PfCCT activity on the concentration of neutral lipids. PfCCT was assayed in the presence of the indicated concentration of monoacylglycerol (○) and diacylglycerol (●). (**B**) PfCCT was assayed in the presence of 100 μM egg PC vesicles containing the indicated mol% of the neutral lipid.

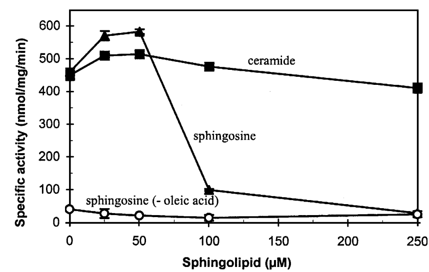

Figure 7 Antagonism between oleic acid and sphingosine for PfCCT activation

Effects of sphingosine (▲) and ceramide (■) on the PfCCT activity were examined. Oleic acid/egg PC (1:1, mol/mol) vesicles at a constant concentration (100 μM) and sphingosine/egg PC or ceramide/egg PC (1:1, mol/mol) vesicles at various concentrations were added to the reaction mixture. As a negative control, the effect of sphingosine/egg PC (1:1, mol/mol) vesicles alone (— oleic acid) was also tested (○).

When the neutral lipids alone, MG and DG, were assayed as sonicated dispersions, the PfCCT activity was not substantially affected even by 100 μM of these neutral lipids (Figure 6A). Similarly, the PfCCT activity was not affected by MG/egg PC vesicles (Figure 6B). Besides, DG/egg PC liposomes had a moderately stimulatory effect (maximal activity: 2.1-fold at 50 mol%) on the PfCCT activity, compared with the 5.5-fold enzyme stimulation obtained with other lipid activators. It should be noted that the concentrations of neutral lipids incorporated into 100 μM egg PC vesicles did not exceed 50%, since beyond this value no lipid bilayer is formed [31].

Sphingosine is a cationic aminolipid which has been reported to inhibit the mammalian CCT activity as well as the yeast CCT activity [14,31]. The effect of sphingosine on the PfCCT activity was examined with a constant concentration of oleic acid/egg PC (100 μM, 1:1, mol/mol) vesicles and various concentrations of egg PC vesicles containing equimolar lysosphingolipid (sphingosine) or sphingolipid (ceramide) (Figure 7). The result showed that 50 mol% (100 μM) of sphingosine/egg PC vesicles reduced by 80% the enzyme activity obtained in the presence of oleic acid/egg PC vesicles alone. At 250 μM of sphingosine/egg PC vesicles, a complete inhibition of the activation was observed: the specific activity was the same as that obtained in the absence of oleic acid/egg PC vesicles. On the other hand, the uncharged sphingolipid (ceramide) had no significant effect on the PfCCT activity.

DISCUSSION

In *Plasmodium*, as in yeast, PC can be synthesized via two distinct pathways, the CDP-choline pathway (Kennedy pathway) and the sequential methylation of phosphatidylethanolamine (PE), the latter originating either from the CDP-ethanolamine pathway or from phosphatidylserine decarboxylation [2,40]. On the basis of *in vitro* data, 40–60% of PC derives from the CDP-choline pathway, the remainder coming from the PE-*N*-methyltransferase pathway [2], whereas in mammalian cells the CDP-choline pathway is generally the essential route of the PC synthesis [41]. The reaction catalysed by CCT is the rate-limiting step of the CDP-choline pathway in *Plasmodium* [7] as well as in other eukaryotes [9]. In the malaria parasite, however, little is known about phospholipid regulation in general and, in particular, nothing is known regarding the means by which this key enzyme, CCT, is controlled. To our knowledge, CCT is the only enzyme

of the plasmodial phospholipid metabolic pathway [24] to have been cloned. Thus the present study was firstly aimed at the production of active PfCCT in order to investigate biochemical characteristics of the plasmodial CCT and its regulation by lipids.

Isolation of PfCCT was facilitated by overexpression of the full-length plasmodial CCT gene in *E. coli*. The recombinant protein constituted 6–9 % of the total cellular proteins and was produced as inclusion bodies, as in many other overexpression systems. The subsequent purification of PfCCT to homogeneity was achieved using a rapid and reproducible protocol including three main steps: (1) a preliminary treatment of the membrane fraction with Triton X-100, which eliminated numerous other membrane-associated proteins; (2) denaturation of the membrane fraction in the presence of 6 M urea, followed by dialysis (the functional protein was recovered upon the removal of urea); and (3) chromatography on a Q-Sepharose anion-exchange column. The enzyme was purified 22-fold with a 34 % yield. Other purification methods, including hydroxyapatite, mono-Q and Mono-P chromatographies, were ineffective at improving this yield. Most of the active fractions corresponded to a 48 kDa protein whose N-terminal sequence was exactly the one predicted from the DNA sequence of PfCCT [24]. Indeed, this 48 kDa protein was recognized by antibodies elicited against a synthetic peptide corresponding to residues 1–16 of PfCCT (H.-J. Yeo, M. P. Larvor and H. J. Vial, unpublished work). The protein was also recognized by antibodies raised against a 14-residue peptide (164–177) of rat CCT (kindly given by Dr. D. E. Vance), a fragment which is well conserved among CCTs of various organisms [17,18,23,24]. Besides, the fact that antibodies raised against short peptides (16 and 14 residues) derived from a protein can recognize the intact protein suggests that these peptides probably bear strong antigenic determinants.

The characteristics of the recombinant PfCCT, such as cation-dependence, pH-dependence and K_m value for phosphocholine, are reminiscent of the characteristics reported for the purified rat CCT [16]. In particular, the K_m value for phosphocholine (about 0.5 mM) was not significantly affected by lipid activators. Besides, the presence of lipid activators significantly increased the affinity of PfCCT for CTP by decreasing the K_m value from 28.9 mM to 10.9 mM (about 3-fold). Similarly, Yang et al. [26] reported for the rat liver recombinant CCT a decrease in the K_m value for CTP of about 35-fold (24.7 mM in the absence of lipid activators in comparison with 0.7 mM in their presence). Yeast CCT presents an apparent K_m for CTP of 1.4 mM, which is 2-fold higher than the cellular concentration of CTP [42]. In *P. falciparum*, the CTP concentration is lower than 0.1 mM [43] which is much lower than the K_m value for CTP of PfCCT. In addition, in *Plasmodium*, we have previously shown the endogenous phosphocholine concentration to be around 35 μM [7]. This implied, therefore, that PfCCT is saturated neither by CTP nor by phosphocholine, in agreement with its rate-limiting feature.

In the present paper the regulation of the PfCCT activity by lipids was investigated for the first time. Despite considerable differences among CCT sequences of various organisms, the PfCCT activity was also dependent on lipids. Previous studies indicated that completely lipid-depleted rat CCT had no significant activity, but that it could be re-activated up to 40-fold upon addition of lipid activators [13,39]. Another study reported 10–50-fold activation for both yeast CCT and rat CCT depending on the added lipids in the assays [31]. In our study, however, the PfCCT activity was easily detected without added lipids (approx. 100 nmol/min per mg). It could be further increased up to 5.5-fold by oleic acid/PC vesicles, and to the same extent by anionic phospholipids (CL and PG). This difference in the activation amplitude between rat CCT and PfCCT seems to be an inherent property of the enzyme, since we ensured that our enzyme preparations were free of endogenous lipids, which are potential activators. It is noteworthy that the maximal activation of PfCCT was obtained with a concentration of CL (charge of -2), which was lower than that of PG (charge of -1) (Figures 5A and 5B). Furthermore, we also observed an antagonism between the effect of oleic acid and that of sphingosine on the PfCCT activity, as previously reported for both rat CCT and yeast CCT [14,31]. As a cationic lipid, sphingosine likely neutralizes the negative charge of oleic acid, thereby attenuating the binding to the vesicles.

It is certainly important to study the regulation of the PfCCT activity by lipids, since specific properties among the CCTs of evolutionary distant organisms could reflect a number of important structural, functional and mechanistic differences. Our investigation showed certain similarities between the parasite enzyme and its mammalian or yeast counterparts. We found that the PfCCT activity increased with the concentration of oleic acid, but decreased when the lipid/protein (mol/mol) exceeded 50 (i.e. above 150 μM lipid under standard conditions). Thus the PfCCT activity shows a profile similar to that of the yeast CCT activity, but not to that of the mammalian enzyme, which was not inhibited by high oleic acid concentrations [31]. On the other hand, the dependence of the PfCCT activity on anionic phospholipids (CL and PG), with regard to both the activation curve shape and the optimal concentration ranges, resembles the one observed with rat CCT, since the activation of yeast CCT by anionic phospholipids was biphasic, with a clear decline of activity at high concentrations of anionic phospholipids [31]. Finally, the most striking difference between yeast CCT and rat CCT has been reported for their responses to neutral lipids: whereas yeast CCT was insensitive, rat CCT was activated by MG and DG, to the same extent as by anionic lipids [31]. In our assays, only DG/egg PC vesicles moderately stimulated the PfCCT activity (about 2-fold), compared with the stimulation obtained with other lipid activators. Taken together, the parasite enzyme presents lipid-dependence properties differing from those of the mammalian and yeast enzymes. These substantial differences led us to conclude that the CCT could interact with membranes in more than one way, and each CCT from various organisms possesses intrinsic properties.

In mammalian cells, the CCT translocation between the cytosol and membranes is a well-established mechanism for *in situ* enzyme activation. The lipid composition of the membrane, particularly the ratio of the bilayer- (e.g. PC) to the non-bilayer- (e.g. PE and DG) forming lipids, could modulate this reversible translocation of CCT which would be regulated by the phosphorylation state of the enzyme [44–48]. The process of mammalian CCT activation is linked to the physical binding of the enzyme to membranes, via both electrostatic and hydrophobic interactions [13,39,49]. An amphipathic α-helix of the enzyme is selectively stabilized by anionic membranes, with a clear correlation between the lipids inducing the α-helix conformation and those allowing the enzyme activation. The negative surface potential of the membrane would serve to concentrate a cationic membrane-binding domain of CCT on the membrane surface [13,14,50]. In fact, the membrane-binding domain would be involved in the interaction with both classes of lipid activators (anionic and neutral), as shown by analysis of proteolytic fragments lacking this region and synthetic fragments derived from this domain [26,27,51]. On the other hand, the yeast CCT activity is modulated only by anionic lipids, suggesting that the electrostatic interactions dominate in its activation process [31].

In addition, although two α-helical portions exist in the corresponding regions of yeast CCT, including a high density of charged residues, these helices are not amphipathic, with hydrophobic moment values lower than 0.2. As for PfCCT, we previously showed that two corresponding regions are predicted to be α-helical and highly amphipathic (with α-helical hydrophobic moment values of 0.33 and 0.46) [24]. They both contain a cluster of charged residues, with a predominance of cationic residues. This suggests interactions of these domains with the negatively charged membrane surface. Furthermore, they could interact with the membrane surface in such a way that the hydrophobic face of the amphipathic α-helices could intercalate into the lipid hydrophobic core. The PfCCT would therefore interact with the membrane in the same two ways as rat CCT. However, the moderate effect of neutral lipids on PfCCT, distinct from the effect observed on rat CCT could be explained by the existence of specific lipid-binding sites on PfCCT. Experiments are in progress in our laboratory to determine whether these regions adopt an α-helical conformation in solution and if they play a role in the interaction of PfCCT with the membrane.

Mechanisms by which the phospholipid synthesis is regulated are different among various organisms. In yeast, co-ordinated regulations of the phospholipid synthesis have been well documented [8,52–56]. The regulatory mechanisms in yeast and in malaria parasites have been expected to share some common features, since these two lower eukaryotes follow similar PC-synthetic pathways. Surprisingly, our recent data (M.-L. Ancelin, D. Martin, N. Elabaddi, J. E. Surgou and H. J. Vial, unpublished work) have shown that both pathways providing PC to the intracellular parasites are quite distinct and are not subjected to the co-ordinated control by *myo*-inositol and choline, as observed in yeast [55]. Much information on the regulation modes remains to be determined. One important question that needs answering is how the plasmodial enzymes of the phospholipid metabolism can provide a dynamic adaptation to phospholipid synthesis and turn over during the parasite life cycle. Hopefully the achievement of the characterization of plasmodial CCT, the key regulating enzyme in the CDP-choline pathway, will be very useful to decipher co-ordinated regulations with other phospholipid metabolism enzymes in the future.

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REFERENCES

1 Ancelin, M. L. and Vial, H. J. (1986) Antimicrob. Agents Chemother. **29**, 814–820
2 Vial, H. J. and Ancelin, M. L. (1992) in the Malarial Lipids, An Overview: Subcellular Biochemistry (Avila, J. L. and Harris, J. R., eds.), pp. 259–306, Plenum Press, New York
3 Vial, H. J., Calas, M., Bourguignon, J. J., Ancelin, M. L. and Giral, L. (1994) French Patent no. F9412301, Group VIRBAC
4 Exton, J. H. (1990) J. Biol. Chem. **265**, 1–4
5 Billah, M. M. and Anthes, J. C. (1990) Biochem. J. **269**, 281–291
6 Jackowski, S. (1994) J. Biol. Chem. **269**, 3858–3867
7 Ancelin, M. L. and Vial, H. J. (1989) Biochim. Biophys. Acta **1001**, 82–89
8 Carman, G. M. and Henry, S. A. (1989) Annu. Rev. Biochem. **58**, 635–669
9 Vance, D. E. (1989) in Phosphatidylcholine Metabolism (Vance, D. E., ed.), pp. 225–239, CRC Press, Boca Raton, FL
10 Pelech, S. L. and Vance, D. E. (1989) Trends Biochem. Sci. **14**, 28–30
11 Vance, D. E. and Pelech, S. L. (1984) Trends Biochem. Sci. **9**, 17–20
12 Cornell, R. B. and Vance, D. E. (1987) Biochim. Biophys. Acta **919**, 26–36
13 Cornell, R. B. (1991) Biochemistry **30**, 5873–5880
14 Sohal, P. S. and Cornell, R. B. (1990) J. Biol. Chem. **265**, 11746–11750
15 Weinhold, P. A., Rounsifer, M. E., Williams, S. E., Brubaker, P. G. and Feldman, D. A. (1984) J. Biol. Chem. **259**, 10315–10321
16 Weinhold, P. A., Rounsifer, M. E. and Feldman, D. A. (1986) J. Biol. Chem. **261**, 5104–5110
17 Kalmar, G. B., Kay, R. J., LaChance, A., Aebersold, R. and Cornell, R. B. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6029–6033
18 Kalmar, G. B., Kay, R. J., LaChance, A. and Cornell, R. B. (1994) Biochim. Biophys. Acta **1219**, 328–334
19 Rutherford, M., Tessner, T., Jackowski, S. and Rock, C. O. (1992) GenBank Accession no. Z12302
20 MacDonald, J. and Kent, C. (1993) Protein Expression Purif. **4**, 1–7
21 Sweitzer, T. D. and Kent, C. (1994) Arch. Biochem. Biophys. **311**, 107–116
22 Hogan, M., Zimmerman, L. J., Wang, J., Kuliszewski, M., Liu, J. and Post, M. (1994) Am. J. Physiol. **267**, L25–L32
23 Tsukagoshi, Y., Nikawa, J.-I. and Yamashita, S. (1987) Eur. J. Biochem. **169**, 477–486
24 Yeo, H.-J., Sri Widada, J., Mercereau-Puijalon, O. and Vial, H. J. (1995) Eur. J. Biochem. **233**, 62–72
25 Wang, Y. and Kent, C. (1995) J. Biol. Chem. **270**, 18948–18952
26 Yang, W., Boggs, K. P. and Jackowski, S. (1995) J. Biol. Chem. **270**, 23951–23957
27 Craig, L., Johnson, J. E. and Cornell, R. B. (1994) J. Biol. Chem. **269**, 3311–3317
28 Yang, W. and Jackowski, S. (1995) J. Biol. Chem. **270**, 16503–16506
29 Wang, Y. and Kent, C. (1995) J. Biol. Chem. **270**, 17843–17849
30 Cornell, R. B., Kalmar, G. B., Kay, R. J., Johnson, M. A., Sanghera, J. S. and Pelech, S. L. (1995) Biochem. J. **310**, 699–708
31 Johnson, J. E., Kalmar, G. B., Sohal, P. S., Walkey, C. J., Yamashita, S. and Cornell, R. B. (1992) Biochem. J. **285**, 815–820
32 Studier, F. W. (1991) J. Mol. Biol. **219**, 37–44
33 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
34 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
35 Burnette, W. N. (1981) Anal. Biochem. **112**, 195–203
36 Weinhold, P. A. and Feldman, D. A. (1992) Methods Enzymol. **209**, 248–258
37 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
38 Bartlett, G. R. (1959) J. Biol. Chem. **234**, 446–468
39 Cornell, R. B. (1991) Biochemistry **30**, 5881–5888
40 Nikoloff, D. M. and Henry, S. A. (1991) Annu. Rev. Genet. **25**, 559–583
41 Kennedy, E. P. (1986) in The Lipids and Membranes: Past, Present and Future (Op den Kamp, J. A. F., Roelofsen, B. and Wirtz, K. W. A., eds.), pp. 171–206, Elsevier Science Publishers B. A., Amsterdam
42 McDonough, V. M., Buxeda, R. J., Bruno, M. E. C., Ozier-Kalogeropoulos, O., Adeline, M.-T., McMaster, C. R., Bell, R. M. and Carman, G. M. (1995) J. Biol. Chem. **270**, 18774–18780
43 Hammond, D. J., Burchell, J. R. and Pudney, M. (1985) Mol. Biochem. Parasitol. **14**, 97–109
44 Jamil, H., Hatch, G. M. and Vance, D. E. (1993) Biochem. J. **291**, 419–429
45 Hatch, G. M., Jamil, H., Utal, A. K. and Vance, D. E. (1992) J. Biol. Chem. **267**, 15751–15758
46 Houweling, M., Jamil, H., Hatch, G. M. and Vance, D. E. (1994) J. Biol. Chem. **269**, 7544–7551
47 Watkins, J. D. and Kent, C. (1991) J. Biol. Chem. **266**, 21113–21117
48 Wang, Y., MacDonald, J. I. S. and Kent, C. (1993) J. Biol. Chem. **268**, 5512–5518
49 Arnold, R. S. and Cornell, R. B. (1996) Biochemistry **35**, 9917–9924
50 Feldman, D. A. and Weinhold, P. A. (1987) J. Biol. Chem. **262**, 9075–9081
51 Johnson, J. E. and Cornell, R. B. (1994) Biochemistry **33**, 4327–4335
52 Carman, G. M. and Zeimetz, G. M. (1996) J. Biol. Chem. **271**, 13293–13296
53 McGee, T., Skinner, H. and Bankaitis, V. A. (1994) J. Bacteriol. **176**, 6861–6868
54 McMaster, C. R. and Bell, R. M. (1994) J. Biol. Chem. **269**, 14776–14783
55 Greenberg, M. L. and Lopes, J. M. (1996) Microbiol. Rev. **60**, 1–20
56 Trotter, P. J. and Voelker, D. R. (1995) J. Biol. Chem. **270**, 6062–6070

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