Characterization of the lipid-binding domain of the *Plasmodium falciparum* CTP:phosphocholine cytidyltransferase through synthetic-peptide studies

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Phospholipid biosynthesis plays a key role in malarial infection and is regulated by CCT (CTP:phosphocholine cytidyltransferase). This enzyme belongs to the group of amphitropic proteins which are regulated by reversible membrane interaction. To assess the role of the putative membrane-binding domain of *Plasmodium falciparum* CCT (PfCCT), we synthesized three peptides, K21, V20 and K54 corresponding to residues 274–294, 308–327 and 274–327 of PfCCT respectively. Conformational behaviour of the peptides, their ability to bind to liposomes and to destabilize lipid bilayers, and their insertion properties were investigated by different biophysical techniques. The intercalation mechanisms of the peptides were refined further by using surface-pressure measurements on various monolayers at the air/water interface. In the present study, we show that the three studied peptides are able to bind to anionic and neutral phospholipids, and that they present an α -helical conformation upon lipid binding. Peptides V20 and the full-length K54 intercalate their hydrophobic parts into an anionic bilayer and, to a lesser extent, a neutral one for

V20. Peptide K21 interacts only superficially with both types of phospholipid vesicles. Adsorption experiments performed at the air/water interface revealed that peptide K54 is strongly surface-active in the absence of lipid. Peptide V20 presents an atypical behaviour in the presence of phosphatidylserine. Whatever the initial surface pressure of a phosphatidylserine film, peptide V20 and phosphatidylserine entities seem linked together in a special organization involving electrostatic and hydrophobic interactions. We showed that PfCCT presents different lipid-dependence properties from other studied CCTs. Although the lipid-binding domain seems to be located in the C-terminal region of the enzyme, as with the mammalian counterpart, the membrane anchorage, which plays a key role in the enzyme regulation, is driven by two α -helices, which behave differently from one another.

Key words: CTP:phosphocholine cytidyltransferase, malaria, membrane-binding domain, phospholipid bilayer, *Plasmodium falciparum*.

INTRODUCTION

Plasmodium falciparum, the most pathogenic human malaria parasite, has become pharmaco-resistant to conventional, as well as newly discovered, drugs. One of the most striking features of malarial-infected erythrocytes is a marked increase in phospholipid and neutral lipid content. This makes the phospholipid biosynthetic pathways a very attractive drug target for new anti-malarials [1]. Phosphatidylcholine (PC) is the major membrane phospholipid in eukaryotes, and in plasmodial membranes, in particular. CCT (CTP:phosphocholine cytidyltransferase; EC 2.7.7.15) catalyses the conversion of phosphocholine and CTP into CDP-choline and phosphate [2–4]. It is the rate-limiting-step enzyme in the *de novo* PC synthesis and this conversion plays a key role in the regulation of PC synthesis in both eukaryotic cells [4] and the malaria parasite [2].

In an attempt to evaluate CCT as a rational drug target for malaria chemotherapy, the CCT from *P. falciparum* (PfCCT) has been cloned, expressed and purified in our laboratory [5]. We have characterized the kinetic parameters and the lipid regulation of the recombinant plasmodial enzyme [6]. The PfCCT activity was stimulated by PC vesicles containing oleic acid and to the same extent by anionic phospholipid, but not by PC vesicles alone or vesicles containing neutral lipids. Moreover, the enzyme activity stimulated by anionic lipid was antagonized by the cationic aminolipid sphingosine. The parasite enzyme

presents lipid-dependence properties, differing from those of the mammalian and yeast enzymes: yeast CCT is activated only by negatively charged lipids, whereas rat CCT is modulated both by charged and neutral lipids; and yeast CCT is activated within a small concentration range of charged lipids, whereas rat CCT activation reaches a plateau [7,8]. More recently, the activation of *Caenorhabditis elegans* CCT has been shown to be similar to the one of mammalian CCT [9]. Our results place the parasite enzyme intermediately between the mammalian and the yeast enzyme.

To date, several cDNAs encoding CCTs have been cloned from various organisms, such as mammals (human, rat, mouse, Chinese hamster), plants (*Arabidopsis thaliana*, pea, rapeseed), the nematode *C. elegans*, the yeast *Saccharomyces cerevisiae*. All sequences are highly similar in the central region (the central region of PfCCT shares 48 % and 55 % sequence identity with those of rat and yeast CCTs respectively). The identification of a homologous enzyme, GCT (CTP:glycerol-3-phosphate cytidyltransferase) from *Bacillus subtilis*, not longer than the central region of CCT, strongly suggested that this domain was the catalytic core of the CTP-transfer reaction [10,11]. The crystal structure of GCT at 2.0 Å (1 Å = 0.1 nm) resolution [12] clearly demonstrated that CTP interacts directly with two conserved motifs: HXGH and RTXGISTT, both present in the plasmodial sequence (residues 104–107 and 229–236 respectively).

However, the significant conservation of the catalytic domains in the functionally related CCT absolutely forbids its use as a

Abbreviations used: CCT, CTP:phosphocholine cytidyltransferase; GCT, CTP:glycerol-3-phosphate cytidyltransferase; PC, phosphatidylcholine; PfCCT, *Plasmodium falciparum* CCT; PS, phosphatidylserine; PTFE, polytetrafluoroethylene; TFE, trifluoroethanol.

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target in malaria chemotherapy, since the host, as well as the parasite, could be attacked. We therefore focused on the membrane-binding domain, which is much less conserved than the catalytic domain, but plays a key role in the regulation of the enzyme.

CCT belongs to the group of the amphitropic proteins [13,14]: its activity can be regulated by interconversion between an inactive cytosolic form and an active membrane-bound form [4]. It has also been suggested that both active and inactive CCTs are bound to membranes, and that the activation of CCT involves conversion of a loosely bound form into a more tightly membrane-bound form [15]. In mammalian CCT α (human isoforms CCT β 1 and CCT β 2 have been identified [16,17]), the region following the catalytic central domain forms an amphipathic α -helix [18,19]. Biophysical data show that this amphipathic α -helix presents selectivity for anionic membranes, and would be surface-oriented [20]. The lipid-activation region of the CCT from *C. elegans* is also predicted to be an amphipathic α -helix [9]. This type of structure is known to mediate membrane binding in many other proteins, such as blood-clotting Factor VIII [21], vinculin [22] and DnaA protein [23,24]. When this region is removed by proteolysis [25], changed by mutation [9,26–28], or blocked by an antibody [29], the enzyme cannot translocate to membranes and subsequently is not lipid-activated. Therefore the amphipathic helical domain is responsible both for membrane binding *in vivo* and lipid activation *in vitro*. Moreover, a CCT α devoid of this lipid-binding domain is constitutively active [30]. This observation supports a model whereby the membrane-binding domain functions as an inhibitor of the catalytic domain in the absence of lipids [9,14]. This model is even more complex, since membrane binding *in vivo* is accompanied by dephosphorylation of the mammalian enzyme [31], but dephosphorylation is not required for binding to membranes [32,33]. Phosphorylation of rat CCT α takes place at the extreme C-terminus on 16 serine residues [34]. The kinases and phosphatases involved remain unidentified. It has been proposed that phosphorylation may be a mechanism to co-ordinate CCT activity with the cell cycle [35] or to increase the enzyme half-life time within the cell [33]. Furthermore, a second activation domain has been identified at the C-terminal end of the CCT α that interacts only with anionic lipids [36]. More recently, different groups have shown that the activity of the CCT is also regulated by physical properties of the membrane [37–39]. Indeed, negative curvature strain is one of the physical properties that enhances the activity of CCT [38,39].

From the results reported above, it is obvious that the membrane-binding mechanism plays a key role in the regulation of the mammalian enzyme activity and/or its localization in the cell. Considering PfCCT, the membrane-binding mechanism could thus be envisaged as a potential target of choice for malaria chemotherapy. Indeed, the PfCCT sequence does not show the three C-terminal 11-mer repeats found in mammalian CCT. Additionally, the secondary structure of this region is not predicted as a long unbroken α -helix structure, but revealed two potential α -helices in the regions of residues 274–294 and 308–327 [5,6].

In the present study, three peptides corresponding to the above-described sequences, plus the overall sequence of 274–327, were synthesized and analysed. CD experiments showed that the three peptides adopt an α -helical conformation in the presence of phosphatidylserine (PS) liposomes. Membrane binding and intercalation of the three peptides were assessed by fluorescence experiments. The ability of the peptides to disorganize lipid bilayers was analysed by means of liposome-lytic experiments. Their insertion properties were refined using surface-pressure measurements on mixed phospholipid monolayers at the air/water interface. This study proves that the three peptides are able to

bind to anionic and neutral phospholipids, but intercalation into anionic lipids occurs only with two peptides. The electrostatic and hydrophobic contributions to the binding are evaluated and discussed.

EXPERIMENTAL

Peptides

All peptides were synthesized on a MilliGen/Bioscience 9050 synthesizer using the solid-phase synthesis method [40]. The Fmoc (fluorenylmethoxycarbonyl) strategy was used with TBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] and HOBt (1-hydroxybenzotriazole) as coupling agents. They were subsequently purified by HPLC. Peptide purity was checked by analytical HPLC and MS. Peptides were freeze-dried and stored at -20°C . The concentrations of peptides K21 and V20 containing a tryptophan residue, and of peptide K54 containing two tryptophan residues, were determined by absorbance measurements at 280 nm. The absorption coefficient used was $\epsilon_{\text{tryptophan}} = 5600 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The concentration of peptide N16 used as a control and containing a tyrosine residue, was determined by absorbance measurements at 274 nm. The absorption coefficient used was $\epsilon_{\text{tyrosine}} = 1400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.4.

Chemicals

Egg PC and bovine brain PS were purchased from Sigma. Stock solutions of lipids in CHCl_3 were stored at -20°C .

Preparation of liposomes

Liposomes were prepared daily as previously described [6]. Briefly, dried lipid films were hydrated in 50 mM Tris/HCl buffer, pH 7.4, and were vortex-mixed for 20 min. This turbid liposome solution was used for liposome-lytic experiments. For other experiments, the solution was sonicated with ice-cooling for at least 15 min by using a titanium-tip sonicator. Titanium debris was removed by centrifugation at 12 000 g for 15 min.

CD measurements

CD measurements were carried out on a Jobin-Yvon Mark V dichrograph at a scan speed of 20 nm/min using quartz cells with a 1 mm path width. Samples contained 16, 40 or 43 μM peptide in PBS, pH 7.4. Spectra were smoothed and corrected for background absorption. Background samples were composed of peptide-free preparations. Values are expressed as mean residue molar ellipticity $[\theta]$. The percentage of helix was estimated from θ_{222} values [41].

Fluorescence experiments

Emission spectra of tryptophan were recorded on a Aminco-Bowman series 2 fluorescence spectrophotometer. Samples contained 10 μM peptide in PBS, pH 7.4. Lipid concentration varied from 0 to 4 mM. Samples were pre-incubated at 25°C at least 15 min before recording spectra. The excitation wavelength was 280 nm and the fluorescence emission was recorded from 300 nm to 440 nm. Fluorescence anisotropy experiments were carried out on a LS 50 PerkinElmer luminescence spectrometer. Samples contained 10 μM peptide in PBS, pH 7.4. Lipid concentration varied from 0 to 3.25 mM. The excitation and emission wavelengths

Table 1 Primary structures of the synthetic peptides

Tryptophan residues used in fluorescence experiments are underlined. Numbering corresponds to the amino acid sequence of PfCCT reported by Yeo et al. [5]. Peptides K21 and V20 correspond to the two potential α -helices, and peptide K54 encompasses the two peptides and an 11-residue linker.

Peptide	Peptide Sequence	Molecular mass (Da)	Net charge
K21	K ²⁷⁴ MKKNLIRWGEKVTDELTKV ²⁹⁴	2515	+3
V20	V ³⁰⁸ ENLQVKFKELFKIWKNASN ³²⁷	2302	+2
K54	K ²⁷⁴ MKKNLIRWGEKVTDELTKVTLTDKPLGTDQGVENLQVKFKELFKIWKNASN ³²⁷	6186	+3
N16	¹ MDSSNYFHDKTMLSE ¹⁶	1906	-2

were 295 nm and 350 nm respectively. Experiments were reproducible within 10%.

Liposome-lytic experiments

Turbid liposomes, prepared as described above, after vortex-mixing were diluted to a concentration of 500 μ M PS in PBS, pH 7.4. Peptides were then added to a final concentration of 100 μ M, and the samples were incubated at 25 °C. The transmittances of sample solutions were recorded at 400 nm and were accurate within $\pm 2\%$ transmittance.

Studies at the air/water interface

Surface-pressure measurements were performed at 18 °C according to the Wilhelmy method using a thermostatically controlled box and a custom-made film balance (accuracy of 0.5 mN/m) [42]. Lipid solutions in chloroform (egg PC or bovine brain PS) were spread at the air/subphase interface at constant film area using a circular Teflon trough (6 cm diameter; total volume of 50 ml), and concentrations were chosen so as to lead to various surface pressures (10–35 mN/m). The subphase was continuously stirred for mixing, but was slow enough in order to avoid disturbance of the lipid film. Peptides at concentrations ranging from 0.1 to 9 μ M, were injected in the subphase (PBS, pH 7.4), and surface-tension changes were recorded after 30 min. Surface pressure/area isotherms were obtained with a custom-made Langmuir PTFE (polytetrafluoroethylene) trough (535 cm² in area) equipped with a PTFE barrier [43]. Peptide V20 and PC were mixed and were spread at the air/subphase interface. After 20 min, evaporation of the solvent was almost complete, and the films were compressed at a speed ranging from 5 to 20 Å²/mol per min. The adsorption isotherm γ versus log (peptide concentration) shown in Figure 6(A) is derived from the Gibbs equation: $\Gamma = -d\gamma/[RTd \ln(C)]$, where γ is the surface tension (mN/m), C is the molar concentration of peptide, T is the absolute temperature (K), R is the perfect gas constant (8.31 J/K per mol) and Γ is the surface molar excess of adsorbed species in mol/m² from which an estimate of the molecular surface area in the surface-adsorbed state can be deduced. Recorded surface pressure values were accurate within ± 1 mN/m.

RESULTS

Properties and conformational studies of the peptides

The sequences of three peptides corresponding to the putative membrane binding region of PfCCT are presented in Table 1. Peptides K21 and V20 correspond to the first and second potential α -helices respectively. Amphipathic distributions between polar

and hydrophobic residues of these α -helices were obvious when these regions were plotted on a helical wheel projection [5]. The hydrophobic moment μ values [44] are high throughout these regions, with mean values of 0.46 for peptide K21 and of 0.33 for peptide V20. The amphipathic nature of these putative helices suggests a possible interaction with the outer monolayer of a membrane, their axes being parallel to the bilayer surface. All peptides show a positive net charge, which could be compatible with an interaction with anionic lipids. Peptides K21 and V20 contain a tryptophan residue localized on the hydrophobic face of the putative helix. Peptide K54 corresponds to both helices joined by a sequence of 13 residues, including a proline residue. Peptide N16 corresponds to the first 16 N-terminal residues of PfCCT and was synthesized in order to be used as a negative control, since its properties are completely different from those of the other peptides: its net charge is negative, its conformation is predicted to be random coil, and it is quite distant from the putative membrane-binding domain.

CD experiments were performed to determine the secondary structure of the peptides. In PBS buffer alone, CD spectra of all peptides were characteristic of a random-coil structure with a single negative extremum around 200 nm (Figure 1A). However, in the presence of 50% trifluoroethanol (TFE), which promotes secondary structure in peptides by inducing intramolecular hydrogen bonds [45], the spectrum of each peptide was indicative of an α -helical structure, characterized by two negative minima around 210 nm and 220 nm, and a positive effect below 200 nm (Figure 1A). The helical content was estimated at 50% for peptides K21 and K54 and at 78% for peptide V20. This proves the strong propensity of all peptides to adopt α -helical structures in a hydrophobic medium and confirms the secondary structure predictions [5]. To determine if the peptides can form amphipathic α -helices in the presence of membrane bilayers, the influence of liposomes on the peptide secondary structure was monitored by CD. For technical reasons, mainly due to scattering, the investigations were restricted to PS liposomes. When added to a suspension of PS liposomes in PBS, the peptides underwent a conformational transition characterized by two minima around 207 nm and 222 nm in the CD spectra (Figure 1B). This demonstrates that, upon binding to PS liposomes, all peptides adopt mainly an α -helical structure. The helical content of peptides K21 and K54 was 42% in the presence of 1.7 mM PS, while that of peptide V20 reached 56%.

Fluorescence studies

Since the CD investigations were restricted to anionic lipids only, and in order to extend the study to lipids with neutral (zwitterionic) polar head groups, we analysed the ability of the peptides to bind to PS or to PC liposomes by fluorescence anisotropy changes and fluorescence spectral shifts due to the modifications of the peptide tryptophan environment.

Figure 1 CD spectra of peptides K21, V20 and K54

(A) CD spectra of the peptides in buffer (thin lines) and in a 50% TFE solution (thick lines). (B) CD spectra of the peptides in the presence of 1.5 mM bovine brain PS liposomes. Peptide concentrations were 40 μM , 43 μM and 16 μM in both experiments for peptides K21, V20 and K54 respectively.

Figure 2 shows that the anisotropy of each peptide increased with the concentration of phospholipids, thus demonstrating a direct association between the peptides and liposomes. The nature of the phospholipid (anionic or zwitterionic) constituting the liposomes had no significant effect on the association with peptide K21 (Figure 2A), V20 (Figure 2B) and peptide K54 (Figure 2C). However, peptide V20 seemed to interact slightly better with PS liposomes than with PC liposomes, as the anisotropy increase is a little higher in the former case.

The positioning of the peptides with respect to the phospholipids still remained to be identified. To determine if the hydrophobic face of the helical peptides intercalates into the hydrophobic membrane core, we used the fluorescence properties provided by a single tryptophan residue located in position 282 of peptide K21, and in position 322 of peptide V20 (Table 1). Peptide K54 possesses both tryptophan residues. Addition of PS liposomes to peptides V20 and K54 generated blue shifts to 332 nm and 336 nm respectively (Figure 3). Spectral blue shifts of the emission increasing with increasing lipid concentration are indicative of a tryptophan residue going from a polar to a non-polar environment, i.e. from water to a lipid bilayer core. Although the K54 peptide interacts with anionic and neutral phospholipids, the tryptophan residues seem to be buried only with anionic phospholipids. These results suggest that the intercalation of the hydrophobic portion of α -helices into the bilayer core was driven by electrostatic interactions. The case of peptide V20 is somewhat more complicated. A neutral phospholipid, such as PC, did not induce important blue shifts, since the recorded difference is much smaller compared with that found with PS (Figure 3). In other terms, even if the tryptophan residue of peptide V20 was affected by the binding to both phospholipids, a better intercalation occurred with anionic phospholipids. No blue shift was observed for peptide K21, either in the presence of PS or PC (results not shown). Since the fluorescence anisotropy experiments did show an association between these lipids and all the peptides studied, the absence of blue shift with K21 means that the interaction is somehow superficial: no actual intercalation into the bilayer core occurs.

Liposome-lytic activity of peptides

The ability of peptides to disorganize acidic lipid bilayers was analysed by means of liposome-lytic experiments. Interaction of the peptides with acidic liposomes was monitored by change

Figure 2 Fluorescence anisotropy of peptide K21 (A), peptide V20 (B) and peptide K54 (C) as a function of liposome concentration

The excitation and emission wavelengths were 295 nm and 350 nm respectively. Egg PC liposomes and bovine brain PS liposomes were prepared as described in the Experimental section. Peptide concentrations were 10 μM in PBS, pH 7.4.

Figure 3 Blue shifts of the maximum emission wavelength of tryptophan as a function of liposome concentration

The tryptophan fluorescence of peptide K54 (circles) and peptide V20 (squares) was recorded in the presence of increasing concentrations of PC liposomes (open symbols) or PS liposomes (closed symbols). The excitation wavelength was 280 nm. Egg PC liposomes and bovine brain PS liposomes were prepared as described in the Experimental section. Peptide concentrations were 10 μ M in PBS, pH 7.4.

Figure 4 Kinetics of PS liposome-lytic activity of the peptides

Peptides (100 μ M) were incubated with turbid liposomes (500 μ M PS) prepared by vortex-mixing hydrated lipid films in PBS, pH 7.4. The transmittances of sample solutions were recorded at 400 nm. Transmittance of turbid PS liposomes (standard solution) was 13 %.

in the turbidity of PS liposomes over time after incubation of the peptides at 25 °C (Figure 4). Peptides V20 and K54 were very effective in clearing the liposome turbidity: around 85 % of transmittance were recovered after 5 h for peptide V20 and after 25 h for peptide K54. Contrary to this, the liposome turbidity did not change in the presence of peptides K21 or N16 (approx. 13 % transmittance). There was a fairly good correspondence between these results and those observed with the fluorescence spectral-shift experiments: intercalation into the bilayer core and a subsequent disorganization of PS liposomes occurred with peptides V20 or K54, but not with peptide K21. The kinetics of the

Figure 5 Logarithm of relative transmittance against time for peptide K54 (circles) and peptide V20 (squares)

Relative transmittance represents the ratio of the difference between %T at time t and %T at time ∞ , to the difference of %T at time 0 and %T at time ∞ . The first-order rate constants of the slow phases are given by the slopes of each straight part of the curves.

liposome-lytic activity of peptides V20 and K54 were analysed further by plotting the logarithm of relative transmittance versus time (Figure 5). Both kinetics were compatible with multiphasic phenomena. The terminal slow phases were characterized by comparable rates given by the slopes in Figure 5. Values of $(2.2 \pm 0.2) \times 10^{-3} \text{ min}^{-1}$ and $(3.4 \pm 0.2) \times 10^{-3} \text{ min}^{-1}$ were obtained for peptides K54 and V20 respectively. The lysis occurring during the initial complexed phases seemed more rapid for peptide V20 than for peptide K54.

Adsorption to monolayers and insertion properties of peptides

In order to determine the nature of the lipid/peptide interactions, the binding and insertion properties of the peptides with phospholipid monolayers at the air/water interface were investigated. The ability of peptide K54 (the longest peptide) to behave as a surfactant and to orient at the air/water interface in the absence of a lipid monolayer was examined by measuring the surface-tension decrease as a function of peptide concentration increase in the subphase. The surface tension decreased and did not change any more at values corresponding to concentrations higher than 3 μ M (Figure 6A). For those concentrations, the surface-layer concentration reached adsorption saturation and, presumably, peptide aggregates were formed in the solution above this threshold concentration. Thus peptide K54, which represents the entire binding domain of PfCCT, is strongly surface-active in the absence of lipids, since it can decrease the surface tension of the air/water interface by almost 25 mN/m.

Insertion experiments were achieved by injection of the different peptides into the subphase of a PC or a PS monolayer, the starting monolayer surface pressure being lower than or of the same order as the value of the surface-tension change, due to the peptide adsorption at the same concentration in the subphase. Peptide N16 was used as a negative control. Figure 6(B) presents the peptide-dependent surface-pressure change ($\Delta\pi$) as a function of the initial PC monolayer surface pressure (π_i). Pressure increases were the greatest for peptide K54 and varied in the sequence peptide K54 > peptide V20 > peptide N16. The slopes of the plots were -0.73 , -0.49 and -0.19 for peptides K54, V20 and N16 respectively. Thus all peptides interact with PC at all

Figure 6 Adsorption experiments of the peptides at the air/water interface

(A) Relative variations of the air/water surface tensions as a function of the logarithm of the peptide K54 concentration in the subphase in the absence of lipids. (B) Variation of the maximum surface-pressure increase as a function of the initial surface pressure of an egg PC monolayer. (C) Variation of the maximum surface-pressure increase as a function of the initial surface pressure of a bovine brain PS monolayer. Peptide concentrations were $1.2 \mu\text{M}$ in each case.

π_i values, but at different levels. Peptide N16, which has neither polar nor hydrophobic characteristics, interacts the least with PC, whereas peptide K54, which possesses two hydrophobic helical portions, interacts the most with PC. The abscissa intercept gives the critical exclusion surface pressure above which no peptide penetration occurs. For peptides K54, V20 and N16, the critical pressures for insertion into PC monolayers were 36, 33 and 32 mN/m respectively. Thus insertion of peptides into monolayers, as long as they are relatively compact, is facilitated by their length and their hydrophobicity.

For PS monolayers, the plots of $\Delta\pi$ against π_i (Figure 6C) were still linear, but showed significant and unexpected differences in comparison with those obtained for PC monolayers, demonstrating that the charges of the polar head groups of phospholipids were involved in the interaction. As expected for peptide N16, the slope was null and the ordinate intercept was very low (3 mN/m), indicating that peptide N16 did not interact strongly with PS monolayers, and had no intrinsic capacity to penetrate the lipid monolayer at the air/water interface, as the surface-pressure change when N16 was added to the subphase did not depend on the initial pressure of the monolayer film. This is not surprising considering that the net two negative charges of this peptide may induce electrostatic repulsion with the negatively charged head groups of PS. For peptides K54 and V20, the ordinate intercepts were very similar to those obtained with PC monolayers (27.5 and 13.7 mN/m respectively), and could provide evidence for intercalation of both peptides within anionic monolayers. However, unanticipated results were obtained: whereas the slope of the plot was still > -1 for peptide K54 (-0.41), it was null for peptide V20. Even if the space between the polar headgroups is wider for PS than for PC (because of the electrostatic repulsions generated by the negative charges of the dissociated carboxylic acid groups on PS), peptide V20 could not incorporate infinitely into the PS monolayer without changing $\Delta\pi$, as the packing of the monolayer tends to the maximum. The following explanation could be advanced: (i) being understood that peptide V20 interacts with PS via electrostatic and hydrophobic interactions, and (ii) as long as $\Delta\pi$ reaches the limit value of 13.7 mN/m, the excess molecules of PS would associate in such a way that the interaction between V20 and PS moieties remains constant. A possible explanation for that is a preferential location of V20 at the interface, forcing the molecules of PS to form a special organization around peptide V20 in a manner similar to

a mixed bi-dimensional network where PS molecules are located and immobilized at fixed distances.

The results reported above show that peptide V20 can penetrate into PC as well as into PS bilayers. In order to evaluate the hydrophobic contribution of the interaction of peptide V20 with phospholipids, we investigated the miscibility of peptide V20 with PC monolayers. Peptide V20 was mixed with PC and spread at the air/subphase interface, instead of being injected alone in the subphase. Pressure/area isotherms of PC monolayers with increasing concentrations of peptide V20 are presented in Figure 7(A). They showed that peptide V20 was miscible with PC, since the shapes of the mixed monolayers were not very different from those of pure PC monolayers. In the absence of peptide ($x=0$), the isotherm gives two particular values for egg-PC: (i) the plateau observed at high surface pressures gives the collapse pressure (47 mN/m), for which the area occupied by a molecule of PC is minimal (approx. 0.6 nm^2 ; hydrocarbon chains of the lipid are ordered and closely packed); and (ii) the abscissa intercept (zero surface pressure), approx. $1.8 \text{ nm}^2/\text{molecule}$, corresponds to the threshold above which molecules of PC are not constrained (no close contact with each other; hydrocarbon chains laid close to the surface of the subphase). In the presence of peptide V20 ($x > 0$) and for any surface pressure $> 6 \text{ mN/m}$, the molecular area was lower than that in the absence of peptide. This reduction of molecular area means that the peptide molecules behaved like additional entities, the area of which is lower than that of lipid. This is compatible with PC molecules that promote the folding of peptide V20 from random coil to a more compact α -helix. No surface pressure was detected above $2.8 \text{ nm}^2/\text{molecule}$, which was more than in the absence of peptide, thus indicating that peptide V20 was indeed localized at the interface. The shape of the pressure/area isotherms for the mixed film was similar to that of the pure lipid when the molar fraction x of peptide V20 was not higher than 0.25, suggesting that the film was an actual mixed peptide/lipid film. For higher molar fractions of V20, a surface-pressure increase was observed for molecular areas lower than $2.8 \text{ nm}^2/\text{molecule}$, showing a kind of plateau at surface-pressure values in the range 5–7 mN/m. The surface-pressure value at this plateau decreased as the peptide molar fraction increased. This observation suggests that the peptide could be rejected from the surface monolayer because of aggregate formation, which is consistent with the data shown in Figure 6(A) (break of the surface-tension variation for high values of the peptide

Figure 7 Compression isotherms of pure and mixed lipid-peptide monolayers, and evolution of the molecular area of mixed monolayers versus lipid-peptide mole fraction

(A) Pressure/area isotherms of egg PC monolayers with increasing concentrations of peptide V20 spread at the surface. The aqueous subphase used was PBS, pH 7.4. (B) Mean molecular area plotted against X . The molar fraction of the peptide V20, X_{V20} , is defined as the molar ratio $n_{V20}/(n_{PC} + n_{V20})$, and $X_{V20} + X_{PC} = 1$.

concentration). Figure 7(B) represents the mean molecular area variation of the mixed films sampled at constant surface pressure (20 mN/m) plotted against the molar fraction of the peptide. The continuously decreasing variation is indicative of the possible existence of a miscible state between peptide V20 and PC. Since the decrease of the mean molecular area occupied by both molecules was not a straight line (slight concave curvature), the interactions between both components in the monolayer were of attractive nature, as the mean area was lower than the sum of the areas of each component balanced by their respective molar fraction. This can be viewed as the result of the folding of peptide V20 in an α -helix upon binding to lipids, since this process reduces the molecular area in the monolayer. The extrapolation of the plot to $x = 1$ gives the molecular area occupied by the peptide alone (approx. 0.6 nm² at the considered surface pressure). Considering that peptide V20 adopts an α -helical conformation, the area in contact with the external membrane monolayer will be higher than those estimated from the monolayer experiment, suggesting that the positioning of peptide V20 at the interface was actually different from the case of real insertion in between the lipid molecules of the film.

DISCUSSION

Contrary to mammalian CCT, PfCCT does not contain a series of 11-mer repeats that adopt an unbroken α -helical conformation upon lipid binding [18–20], but more likely does contain two distinct helical regions separated by a link including an unconserved proline residue. Recent work has shown that lipid activation of *C. elegans* CCT is driven by a 21-residue segment predicted to be α -helical and localized in the same region of the enzyme [9].

Three peptides (K54, K21 and V20) respectively corresponding to the entire putative membrane-binding domain of PfCCT (residues 274–327) or its fragments (274–294) and (308–327),

were synthesized. These regions were predicted to adopt amphipathic α -helical structures in the enzyme [5]. The peptides preferentially shifted from a random-coil structure to an α -helical conformation in the presence of 50 % TFE (hydrophobic medium) or in the presence of PS liposomes, mimicking an anionic membrane environment. The propensity of peptide V20 to adopt an α -helical conformation was 25 % higher than that of peptide K21. Here, fluorescence anisotropy experiments showed direct association of all the studied peptides with neutral, as well as with anionic, liposomes. It was noticed that peptide V20 bound slightly more (by approx. 15 %) to PS liposomes than to PC liposomes.

Fluorescence spectral-shift experiments showed that Trp²⁸² of peptide V20, but not Trp³²² of peptide K21, reached a hydrophobic environment upon association with PS liposomes. The blue shift observed with peptide K54 was mainly due to Trp²⁸². On the other hand, no modification was observed in the environment of tryptophan residues upon association with PC liposomes. This suggested that intercalation of the hydrophobic face of the V20 α -helix into the bilayer core of PS liposomes was driven by electrostatic interactions, whereas the K21 α -helix interacted only with the surface of the bilayer membrane. The fact that both peptides behave differently in the presence of PS liposomes was unexpected, since their length and their hydrophobic character are similar. These results were confirmed by liposome-lytic experiments. Penetration of the α -helix present in peptides V20 and K54 into PS liposomes led to the disorganization of the bilayer core, and consequently to the lysis of PS liposomes. The kinetics of lysis by peptide V20 and peptide K54 were multiphasic. Slow phases presented comparable rates for both peptides. Interestingly, the initial rapid phase of PS liposomes lysis by peptide V20 was faster than that observed with peptide K54. This suggested that the N-terminal sequence, present in peptide K54, but not in peptide V20, slowed down the interaction and lysis of liposomes. The fact that peptide K21, containing this sequence, interacted only at the surface level of PS liposomes, but did not induce any lysis, was consistent with this hypothesis.

Peptides V20 and K54 bound to PC monolayers via hydrophobic interactions, confirming the intrinsic property of peptide K54 to locate at the air/water interface by adsorption (Figure 6A). These peptides also bound to PS monolayers, but in a different fashion, thus demonstrating the influence of electrostatic interactions between the cationic portions of α -helices and the anionic head groups of PS. However, the binding to anionic monolayers was more complex than the binding to neutral monolayers, since it involved electrostatic and hydrophobic interactions at the same time. The binding curves not only testify to the interaction of peptides and PS at the interface, but also to a possible formation of other types of structures in the surface film: they could form aggregated structures in the case of peptide K54, or they could form a thicker monolayer sequestering the amphipathic peptide in the case of peptide V20. The difficulty is to connect the results obtained with monolayers to those obtained with bilayers, and even more to cellular membranes.

Our results demonstrate the miscibility of peptide V20 with PS, and give an estimation of the minimal area that interacts with the outer monolayer of a membrane. This area is smaller than that expected if the peptide lay parallel to the membrane. We cannot exclude the possibility of a transbilayer arrangement, but this seems less than probable, considering the amphiphilicity of the α -helix. This discrepancy could be explained by the fact that the estimated area was obtained by extrapolation of the curve when peptide V20 was alone and adopted a random-coil conformation. The molecular area occupied by the peptide is thus not the same when it shifts to an α -helical conformation in the presence of PS.

Taken together, these results show that a lipid-binding domain of the PfCCT seems to be located at the C-terminal end of the enzyme catalytic domain. This membrane-binding domain has been identified and well studied in mammalian CCT α [18,20,26,28,46] and in *C. elegans* CCT [9]. In contrast with the other members of the groups, the binding to the phospholipids is driven by two amphipathic α -helices for PfCCT instead of a long unbroken α -helix [19,20]. The detailed analysis of this sequence in PfCCT has demonstrated that two peptides corresponding to the two α -helices behaved differently upon lipid binding. Our hypothesis is that the first helix (peptide K21) will bind superficially to the membrane via hydrophobic interactions with anionic or neutral lipids, whereas the second helix (peptide V20) will provide a deeper binding with penetration of the hydrophobic face of the helix only into anionic membranes. This second helix will then be stabilized by hydrophobic interactions with the aliphatic chains of the lipids and electrostatic interactions with polar headgroups. The implication of such a mechanism in the membrane selectivity of the PfCCT *in vivo* has still to be elucidated. Recently, Johnson et al. [47] showed that the membrane affinity and lipid selectivity for anionic membranes of mammalian CCT is not only driven by the positive charge of the amphipathic helix and the three serine residues interrupting the non-polar face [20], but also by the protonation of three glutamate residues in the 11-mer repeats. The membrane-binding domain of PfCCT does not contain these three 11-mer repeats (VEEKS subdomains) of the mammalian CCT, and these serine and glutamate residues are not conserved in PfCCT. The fine regulation of PfCCT probably involves a different mechanism where the two amphipathic helices are implicated at a different level. This enzyme is a rate-limiting enzyme in the *de novo* biosynthesis of PC, which is itself of crucial importance for the parasite growth [1,48,49].

Considering the peculiarity of the PfCCT relative to the mammalian enzyme, mechanistic differences are probably involved in the fine regulation of the regulatory lipid-binding domain, and effectors might differentially alter the function of this crucial en-

zyme. Most current reported effectors of CCT are cationic lipids, such as sphingosine, which antagonize the enzyme activation by anionic lipids by neutralizing the negative surface charge of the membrane, thereby attenuating the signal for binding [50,51].

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