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Redox- and pH-dependent association of plastocyanin with lipid bilayers: effect on protein conformation and thermal stability

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Abstract

The effect of electrostatic interactions on the conformation and thermal stability of plastocyanin (Pc) was studied by infrared spectroscopy. Association of any of the two redox states of the protein with positively charged membranes at neutral pH does not significantly change the secondary structure of Pc. However, upon membrane binding, the denaturation temperature decreases, regardless of the protein redox state. The extent of destabilization depends on the proportion of positively charged lipid headgroups in the membrane, becoming greater as the surface density of basic phospholipids increases. In contrast, at pH 4.8 the membrane binding-dependent conformational change becomes redox-sensitive. While the secondary structures and thermal stabilities of free and membrane-bound oxidized Pc are similar under acidic conditions, the conformation of the reduced form of the protein drastically rearranges upon membrane association. This rearrangement does not depend on electrostatic interactions to occur, since it is also observed in the presence of uncharged lipid bilayers. The conformational transition, only observed for reduced Pc, involves the exposure of hydrophobic regions that leads to intermolecular interactions at the membrane surface. Membrane-mediated partial unfolding of reduced Pc can be reversed by readjusting the pH to neutrality, in the absence of electrostatic interactions. This redox-dependent behavior might reflect specific structural requirements for the interaction of Pc with its redox partners.

Keywords: Plastocyanin–membrane interaction; Infrared spectroscopy; Protein conformation

1. Introduction

Plastocyanin (Pc) is a 10.5 kDa blue copper protein found in the inner side of the photosynthetic thylakoid membrane. In higher plants and green algae, Pc functions as a mobile electron shuttle between two membrane-bound protein complexes, cytochrome b_6/f and Photosystem (PS) I [1]. As with other physiological electron-transfer chains, membrane association is believed to tune up the structure of redox proteins for efficient functioning. The high resolution structures of oxidized [2] and reduced [3] Pc have revealed the existence of two different regions in the protein molecule, which have been proposed to be involved in the recognition of and bind-

Abbreviations: EYL, egg yolk lecithin; $DODAC^+$, dioctadecyldimethylammonium bromide; $DOTAP^+$, 1,2-dioleoyl-3-trimethylammonium propane; IR, infrared spectroscopy; Pc, plastocyanin; T_m, midpoint denaturation temperature

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ing to its physiological partners [4,5]. One is the 'northern' hydrophobic patch surrounding the copper ligand His87, and the other is the broad 'eastern' acidic patch around Tyr83. This negatively charged region can be further divided into a large (residues 42–45) and a small (59–61) acidic cluster.

In spite of the substantial body of experimental results, the pathway used for electron transfer from cytochrome *f* to Pc, and from Pc to PS I remains as yet unclear. Based on kinetic measurements on Pc mutants, it has been proposed that Tyr83 was part of the route used electron transfer from cytochrome *f* [6–8]. However, a recent nuclear magnetic resonance (NMR) and molecular dynamic study has suggested that this redox reaction proceeds through a shorter pathway, via His87 [9], as it has been proposed for electron transfer from Pc to PS I [10]. The initial protein–protein interaction is governed by electrostatic interactions, although it is becoming evident that once formed the electrostatic complex rearranges, adopting a conformation competent for electron transfer [11]. In this context, and based on recent experimental evidences, it has been postulated that although the interaction between oppositely charged residues of Pc and PS I or cytochrome *f* ensures docking, a subsequent conformational 'activation', under the influence of short-range forces, allows a closer contact between the 'northern' hydrophobic surface around His87 of Pc and its redox partners [9,10,12]. This conformational rearrangement would involve protein–protein contacts with exclusion of water from the interface area, and would bring the redox centers close enough for a rapid and efficient electron transfer reaction [9,13].

Regarding the initial electrostatic event, solution NMR studies on the plastocyanin–cytochrome *c* complex have shown that subtle conformational changes at the protein surface, brought about by the interaction between oppositely charged residues, are transmitted deep into the protein core [14]. The origin of these conformational rearrangements was postulated to be mainly based on a change in electrostatic potential. Supporting this proposal is the fact that similar effects were observed in other electrostatic protein–protein complexes, i.e., cytochrome *c*–cytochrome *c* peroxidase complex, and that cytochrome *c* adsorbed to a negative electrode shows similar changes to those observed upon complex formation [15]. Furthermore, a change in the electrostatic potential has been demonstrated to exert a pronounced effect on the conformation of cytochrome *c* bound to negatively charged lipid vesicles [16]. Similar considerations have been taken into account in this study, where as a model system to characterize the initial conformational transition induced by electrostatic interactions, we have studied the interaction of Pc with lipid bilayers containing different proportions of positively charged phospholipids. Unlike the case of cytochrome *c*, there are relatively few reports in the literature on model studies of Pc–membrane interactions. Flash photolysis studies have revealed that the reactivity of Pc bound to positively charged bilayers increased by as much as 6-fold [17]. However, the cause of such an increase in reactivity remains unclear and requires the conformational characterization of Pc in solution and membrane-bound. Due to the location of Pc on the inside of the thylakoid membrane, it should experience a decrease in pH upon illumination of about 2–3 pH units [18]. Therefore it is reasonable to propose that pH could also modulate the conformation of Pc. In order to address these points, we have used Fourier-transform infrared spectroscopy to probe the effect of electrostatic interactions and pH on the conformation and thermal stability of Pc. Studies on protein thermal stability often reveal important aspects of their conformation [19,20]. This is indeed the case of Pc, as will be shown below.

2. Materials and methods

Egg yolk lecithin (EYL; grade 1) was purchased from Lipid Products (South Nutfield, UK). The positively charged detergent dioctadecyldimethylammonium bromide ($DODAC^+$), and phospholipid 1,2-dioleoyl-3-trimethylammonium propane ($DOTAP^+$) were from Fluka and Avanti Polar Lipids, respectively. Cacodylic acid and deuterium oxide (99.8% purity, D_2O) were supplied by Sigma. Plastocyanin *a* was purified from leaves of poplar, *Populus nigra* var. *Italica*, as previously reported [21]. Protein concentration was determined by measuring the absorbance of oxidized Pc at 597 nm, using an extinction coefficient of 4.65 $mM^{-1}\,cm^{-1}$ [22].

Large unilamellar vesicles (LUV) were prepared by

the extrusion method of Mayer et al. [23], using polycarbonate membranes of a pore size of 0.1 μm (Nucleopore, Pleasanton, CA, USA). $DODAC^+$ and varying amounts of $DOTAP^+$ were incorporated into EYL bilayers to produce positively charged lipid vesicles. For preparation of samples used to study the conformation of reduced and oxidized Pc bound to lipid vesicles, LUV suspensions and protein solutions were mixed at a starting lipid to protein molar ratio of 50 in the presence of 5 mM ascorbic acid or 5 mM potassium ferricyanide. Either Hepes (50 mM; pH 7.0) or cacodylate (50 mM; pH 4.8), 50 mM NaCl were used as the suspending medium. The pH of each sample was checked before incubating them at room temperature for 60 min.

Prior to infrared measurements, the lipid–protein complexes prepared in D_2O based buffer were collected by centrifugation (184 000 × *g*, 45 min). Centrifugation rendered a band containing the complexes floating onto the buffer which contained the unbound protein. Samples, at a protein concentration of ≈0.3 mM were assembled between two calcium fluoride windows separated by a 50-μm-thick teflon spacer. Infrared spectra were recorded in a Nicolet 520 spectrometer at 25°C. A total of 1000 scans were average for each spectrum, using a shuttle device. Thermal studies were carried out following a step-heating method with ≈3°C steps. Samples were left to stabilize for 5 min before recording the spectra, and the temperature was monitored with a thermocouple in contact with the windows. Spectra were analyzed in a personal computer, where solvent subtraction, Fourier-self deconvolution and band position determination were performed as described previously [24]. Deconvolution was performed using a Lorentzian with a half-bandwidth of 18 cm^{-1} and a resolution enhancement factor $k = 2$.

3. Results

3.1. Interaction of Pc with positively charged membranes at neutral pH

We have examined the effect of Pc binding to positively charged lipid bilayers on the secondary structure and thermal stability of both redox states of the protein. The infrared spectrum of the membrane-associated protein displays in the 1800–1500 cm^{-1} region three broad absorption bands at 1740, 1647 and 1567 cm^{-1}, which can be assigned to phospholipid ester groups, amide I and carboxylate groups of aspartic and glutamic residues, respectively (Fig. 1A and B, solid traces). Due to the presence of very weak spectral features in the amide I region (1700–1615 cm^{-1}) of the samples containing only liposomes (Fig. 1A and B, broken traces), these spectra were used as reference to cancel out their contribution to the protein absorption bands (Fig. 1C). Deconvolution resolves otherwise overlapping absorption bands within the 1700–1500 cm^{-1} spectral region (Fig. 1C, upper trace). The amide I band of Pc in solution and bound to $EYL:DODAC^+$ membranes is composed

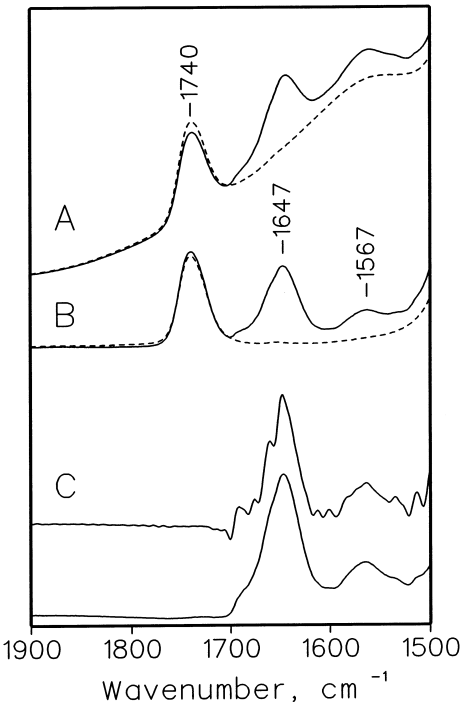

Fig. 1. Infrared spectra of free and membrane-bound Pc. (A) Original infrared spectra corresponding to oxidized Pc bound to $EYL:DOTAP^+$ (1:4 molar ratio) large unilamellar vesicles (——), and to the same liposome suspension in the absence of protein (- - -). (B) IR difference spectra obtained after subtracting the buffer contribution from the spectra shown in A. (C) IR difference spectrum derived from the subtraction of the traces displayed in A, before (bottom trace) and after (upper trace) deconvolution. Deconvolution was performed using a Lorentzian with a half-bandwidth of 18 cm^{-1} and a resolution enhancement factor $k = 2$. Measurements were carried out at 25°C.

of several underlying band components, clearly evidenced after deconvolution of their IR spectra (Fig. 2), that can be assigned to specific secondary structure elements of the protein. The assignment takes into account the crystal structure of Pc [3], theoretical [25] and experimental [26,27] studies on model polypeptides and globular proteins, and requires to analyze the spectra of the protein recorded in H_2O and D_2O. This analysis has been recently reported [28], and the tentative assignment of the amide I band components can be summarized as follows. The component bands at 1635 and 1624 cm^{-1} represent protein segments adopting β-structure. Those appearing above 1660 cm^{-1} indicate the presence of turns, although a small contribution from β-structures must be considered in this spectral region. Finally, the assignment of the dominant band component at 1646 cm^{-1} is not straightforward. Similar spectral features have been described for proteins that share the presence of irregular conformations (loops, turns) in their structures [20,29,30]. The small downshift (1–2 cm^{-1}) observed for this component upon deuteration, together with the fact that it is the main spectral feature modified during the cooperative thermal unfolding of Pc (data not shown), indicate that it represents polypeptide segments that are integral parts of the three-dimensional protein structure. This component most likely contains contributions from irregular structures and protein extended chains, whose backbone C=O groups could form weaker hydrogen bonds. This would lead to a shift of their corresponding amide I mode (C=O stretching) to frequencies higher than those usually found for β-structures (1640–1620 cm^{-1}) [26,27].

It should be noted here that special care has been taken in this study to ensure that essentially all the protein present in samples containing lipid vesicles is bound to the membrane. Therefore, any difference found between the solution and membrane-bound states of Pc can be directly related to protein conformational changes brought about by membrane-association rather than to modifications of the relative proportions of free and bound protein. As seen in Fig. 2, binding of either reduced or oxidized Pc to lipid vesicles made of EYL:DODAC$^+$ (40 mol%) does not significantly change their infrared spectra. Minor alterations of the relative intensity of band components located above 1670 cm^{-1} might reflect

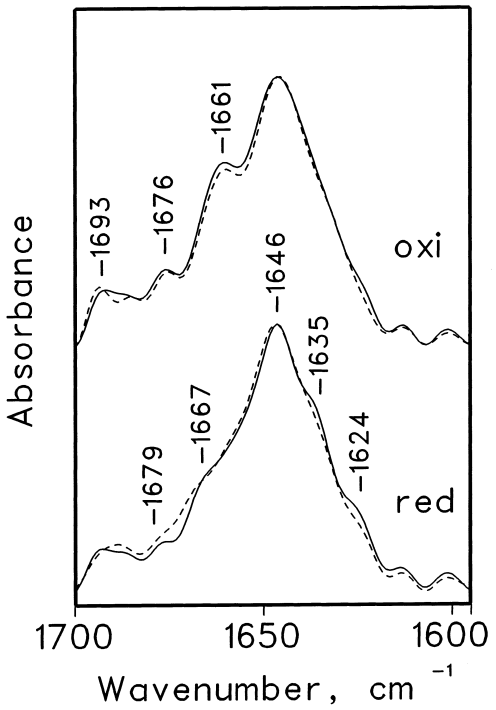

Fig. 2. Amide I band of Pc in solution and membrane-bound. Deconvoluted infrared spectra of oxidized (upper) and reduced (lower) Pc in solution (- - -) and bound to lipid vesicles made of EYL:DODAC$^+$ (60:40 mol%) (———). Protein concentration was ≈0.4 mM; 60 mM phosphate, pH 7.0. Other details as in Fig. 1.

that changes in the conformation of turn structures could be sufficient to accommodate the protein on the membrane interface. A comparison between the reduced and oxidized membrane-bound Pc reveals that they do maintain the spectral differences characteristic of their soluble forms (see band components at 1667, 1661 and 1624 cm^{-1}) [28]. To further characterize the conformational consequences of membrane binding, we have also measured the thermal stability of free and membrane-bound Pc. As found with other soluble and membrane proteins [16,19], thermal denaturation of Pc induces an increase in its amide I bandwidth (data not shown) [28]; therefore, the thermal stability of both, free and membrane-associated Pc can be easily characterized by following the amide I bandwidth as a function of temperature. The 'melting profiles' shown in Fig. 3 reveal that membrane binding substantially decreases the thermostability of both redox states of the protein. Interestingly, the difference observed between the thermal stability of reduced and oxidized

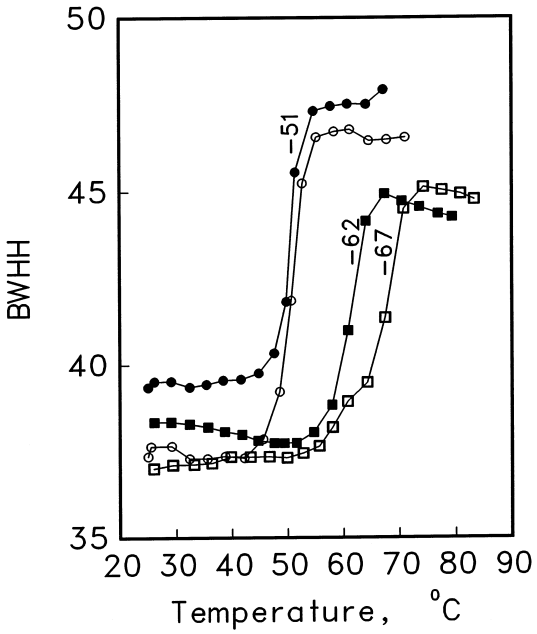

Fig. 3. Effect of membrane binding on the thermal stability of Pc. Temperature dependence of the amide I bandwidth at half-height (BWHH) of free (squares) and membrane-bound (circles), reduced (empty symbols) and oxidized (filled symbols) plastocyanin. Spectra were recorded in 60 mM phosphate, pH 7.0; lipid vesicles were made of EYL:DODAC$^+$ (60:40 mol%).

Pc in solution is abolished when membrane-bound. The midpoint denaturation temperature (T_m) values obtained for the thermal unfolding of reduced and oxidized Pc in solution are 67°C and 62°C, respectively. Considering the differences in protein concentration, these results are in good agreement with differential scanning calorimetry studies on oxidized Pc from spinach [31], and with CD data on both redox states of the same protein at neutral pH [32]. The T_m values for both oxidized and reduced membrane-bound states it is downshifted to 51°C. A detailed analysis of the thermal denaturation pathway of both redox states of the protein in solution has been recently reported using temperature-induced IR difference spectroscopy [28]. The differences described between the thermal unfolding pathway of reduced and oxidized Pc in solution are essentially maintained in their membrane-bound forms (data not shown). Therefore, membrane binding destabilizes Pc without significantly affecting its thermal denaturation pathway. Regardless of the redox state, Pc does not stably interact with neutral lipid vesicles at pH 7.0, since the centrifugation step used in sample preparation renders a lipid fraction that is virtually protein-free (data not shown).

The phospholipid DOTAP$^+$ was also used, instead of DODAC$^+$, to study the effect of the surface density of positively charged lipid headgroups on the secondary structure and stability of Pc. As seen for DODAC$^+$-containing vesicles, binding of Pc to EYL:DOTAP$^+$ model membranes does not substantially alter the infrared spectrum of the protein (data not shown). This suggests that, under these experimental conditions, membrane association does not involve significant rearrangements of the protein secondary structure. The similarity also applies to the spectral differences between the membrane-bound oxidized and reduced Pc and to the destabilization of both protein redox states observed upon membrane binding (Table 1). The extent of protein destabilization depends on the concentration of positively charged lipid in the bilayer, and is only observed at DOTAP$^+$ concentrations higher than 20 mol%. Above this concentration, the T_m value for mem-

Table 1
Effect of membrane surface potential and pH on the thermal stability of Pc in solution and bound to positively charged lipid vesicles

Sample	pH	T_m (°C)				
		Solution	EYL:DODAC$^+$ (60:40 mol%)	YL:DOTAP$^+$		
				20 mol%	40 mol%	80 mol%
Pc_{oxi}	7.0	61.8	50.4	62	58.2	55.3
Pc_{red}	7.0	66.7	51	–	–	55
Pc_{oxi}	4.8	48.2	50			
Pc_{red}	4.8	47.2	[a]			

T_m, midpoint denaturation temperature obtained from the temperature dependence of the amide I bandwidth.
[a]There was no evidence for a cooperative thermal unfolding of this sample. The same behavior was found with uncharged lipid vesicles, under these experimental conditions.

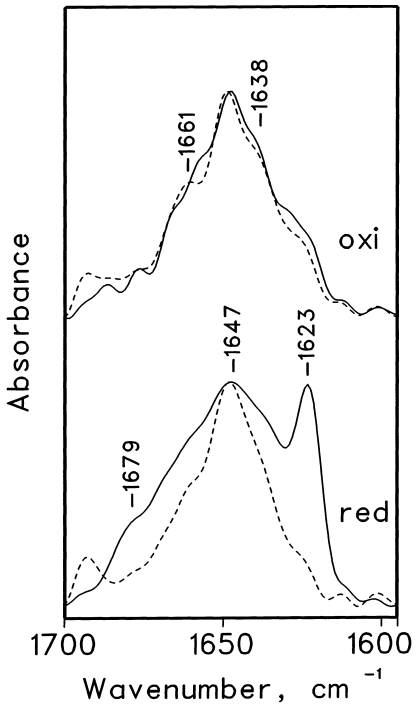

Fig. 4. Interaction of Pc with lipid vesicles made of EYL:DODAC$^+$ (60:40 mol%) at pH 4.8. Deconvoluted IR spectra of oxidized (upper) and reduced (lower) Pc in solution (- - -) and membrane-bound (———). Other details as in Fig. 1.

brane-bound Pc decreases as the amount of DOTAP$^+$ in the bilayer increases (Table 1).

3.2. Interaction of Pc with lipid vesicles at pH 4.8

Lowering the pH to 4.8 induces a destabilization of the protein that results in a decrease of the T_m values for oxidized and reduced Pc to 48.2°C and 47.2, respectively (Table 1) [28]. As expected for a protein with a p*I* of 3.92 [22,33], binding to positively charged lipid vesicles is still favored at pH 4.8. However, in contrast to what was found at neutral pH, the conformational consequences of the association are remarkably different for the oxidized and reduced states of plastocyanin (Fig. 4, top traces). While binding of oxidized Pc to EYL:DODAC$^+$ lipid vesicles only slightly modifies the position of the 1661 cm^{-1} band component (Fig. 4, top traces), suggesting that the secondary structure of the protein is essentially preserved, and has virtually no effect on the protein thermal stability (Table 1), membrane-association of the reduced protein causes a pronounced rearrangement of its conformation (Fig. 4, bottom traces). The spectroscopic evidence for such a conformational change is the increase in intensity of the 1623 and 1679 cm^{-1} bands observed upon membrane binding. Bands at similar positions have been related to intermolecular interactions between protein molecules that expose hydrophobic segments [16], although the possibility that these signals might be due to phospholipid–protein interactions cannot be discarded. The presence in the IR spectrum of membrane-bound, reduced Pc of component bands at similar positions to those observed for the protein in solution indicates that upon membrane association Pc retains some secondary structure elements (Fig. 4, bottom traces). The above finding raises the possibility that the initial electrostatic event, responsible for protein binding to the membrane interface, could trigger the structural transition that leads to the exposure of additional hydrophobic sites at the protein surface. To test this possibility we carried out similar experiments using electrically neutral lipid bilayers made of EYL. At pH 4.8, only reduced Pc was able to bind the uncharged lipid vesicles. The conformational consequences of the association are similar to those seen with charged vesicles, although the bands due to protein intermolecular interactions, i.e., 1681 and 1626 cm^{-1}, are more intense in the presence of neutral membranes (Fig. 5B). Therefore, electrostatic interactions between the protein and the membrane interface do not trigger the exposure of additional hydrophobic patches at the protein surface, but rather diminish protein intermolecular interactions under acidic conditions.

In order to detect whether the conformational change observed upon binding of reduced Pc to neutral lipid vesicles under acidic conditions was reversible, the pH of the sample was readjusted to 7.0 and it was centrifuged to separate the lipidic component, that appears as a band floating onto the buffer, from the unbound protein (see Section 2). In contrast to what was observed at pH 4.8, the IR spectrum of the lipid fraction shows absorption bands characteristic of phospholipid ester groups, and is virtually free from amide signals arising from the protein (Fig. 5C). Concentration of the water-soluble phase and its subsequent IR analysis (Fig. 5D), reveals that the protein has dissociated from the membrane and adopts a similar secondary structure to that de-

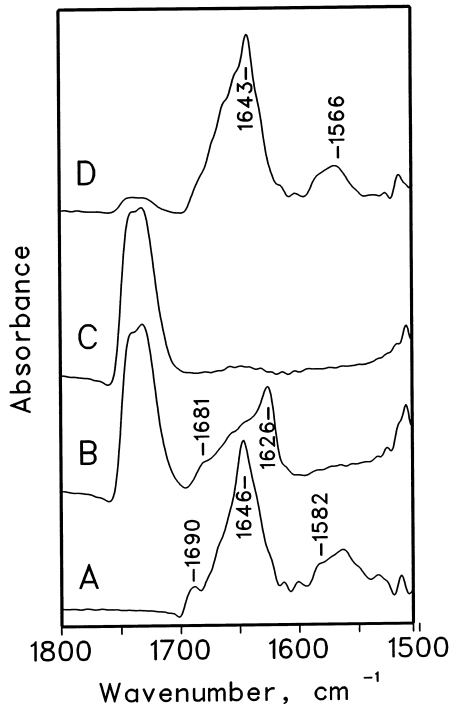

Fig. 5. Interaction of Pc with neutral lipid vesicles at pH 4.8. Deconvoluted infrared spectra of Pc in solution (A) and membrane-bound (B) at pH 4.8. Buffer composition was 50 mM cacodylate, 50 mM NaCl, pH 4.8. The pH of sample B was readjusted to 7.0, and after 30 min at room temperature the sample was centrifuged to separate the lipidic fraction (C) and the free, water-soluble protein which was concentrated (Amicon filters) for IR analysis (D). See other details in Fig. 1.

scribed in solution (Figs. 5A and 2). It should also be noted that as compared with the IR spectrum of the protein in solution, the main band component of Pc after membrane dissociation is slightly downshifted (2–3 cm^{-1}). This could be a consequence of solvent exchange of the unaccessible NH groups of the protein upon membrane binding, as can be deduced from the reduction of the amide II intensity (around 1550 and 1533 cm^{-1}; see spectra D and A in Fig. 5). The disappearance of the 1626 cm^{-1} infrared signal upon membrane dissociation indicates that the protein conformational change is reversible. However, if the same type of experiment is carried out in the presence of positively charged vesicles, the IR spectrum of the protein retains the low frequency (1623 cm^{-1}) signal, suggesting that the electrostatic interactions between the membrane interface and Pc act as a conformational 'trap' for the partially unfolded, membrane-bound protein (data not shown). We also attempted to follow the effect of reoxidation on the partition and conformational properties of membrane-bound, reduced Pc. At acidic pH, association of reduced Pc with neutral and positively charged membranes hampers its reoxidation by potassium ferricyanide, as can be inferred from the absence of the 597 nm absorption band characteristic of the oxidized protein (data not shown).

It is generally accepted that exposure of hydrophobic residues is linked to a destabilization of the protein tertiary structure which, in turn, has been associated in many proteins with the loss of the thermotropic cooperative transition [20,34]. This is indeed what is observed for reduced Pc when bound to both, positively charged and neutral lipid bilayers. The 'melting curves' obtained for both redox states of the protein in solution, at acidic pH, indicate that they do undergo a thermotropic conformational transition with similar T_m values (Table 1, Fig. 6). Under these experimental conditions, binding of oxidized Pc to positively charged membranes does not significantly modify its thermal stability (Table 1). In contrast, there is no evidence of thermal denaturation in the case of membrane-bound, reduced Pc

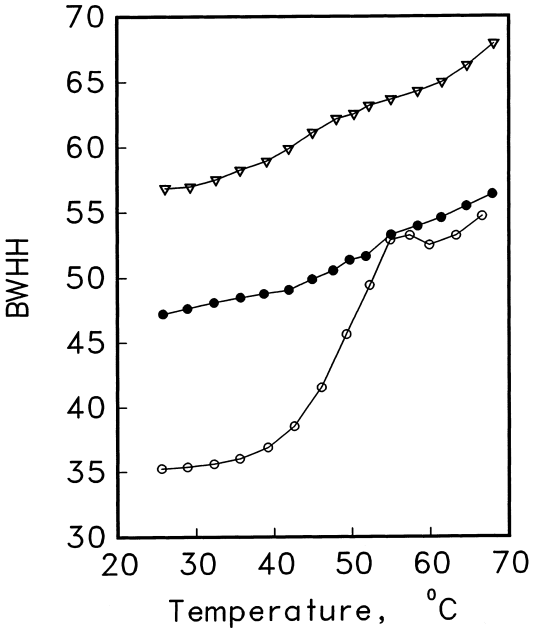

Fig. 6. Thermal stability of Pc in solution (○) and bound to neutral (●) and positively charged lipid vesicles made of EYL:$DODAC^+$ (60:40 mol%) (▽). Buffer was 50 mM cacodylate, 50 mM NaCl, pH 4.8.

(Table 1, Fig. 6). Instead, the width of its amide I band follows a progressive increase with temperature. The larger width observed for membrane-bound reduced Pc at 25°C is due to the appearance of band components at around 1626 and 1681 cm^{-1} which are only seen, at 1618 and 1679 cm^{-1}, in the spectra of the protein in solution at post-denaturation temperatures (i.e., above 48°C; data not shown). These observations indicate that a pH- and redox-dependent conformational change allows the association of reduced Pc with positively charged and neutral lipid bilayers.

4. Discussion

The interaction of plastocyanin with its redox partners, namely cytochrome b_6/f complex and PS I, has been suggested to occur as a two-step event [4,9,11]. First, docking of Pc onto its redox partner would ensure complex formation through long-range electrostatic interactions. In a second step, the structural rearrangement of the proteins would define an interface stabilized by both electrostatic and short-range interactions, leading to a protein complex competent in electron transfer. It is also well known that light induced electron flow in chloroplasts gives rise to proton pumping which causes a drop in the pH of the intrathylakoid compartment of around 2 units. Therefore, it is reasonable to propose that plastocyanin conformation could be modulated by factors such as electrostatic interactions and pH.

Our results show that at neutral pH the electrostatic interaction between Pc and oppositely charged lipid bilayers results in an overall destabilization of the protein structure. This effect is indicated by a decreased thermal stability of the membrane-bound form and by the experimentally observed surface potential dependent destabilization of the protein structure. Both observations have also been made for cytochrome *c*, as well as for protein complexes such as cytochrome *c*–cytochrome *c* peroxidase [15,16]. These data suggest that electrostatic interactions (between oppositely charged molecules) could act as the driving force that modulates the conformation of the interacting proteins. Interestingly, this could, in turn, result in conformational changes around the metal ligand that might be involved in redox potential changes. In this context, it is particularly important to note that Pc binding to lipid bilayers containing the positively charged surfactant $DODAC^+$ shows an enhanced reactivity [17]. Our observations complement well these functional studies, as they demonstrate that electrostatic interactions affect the overall protein structure and not only the immediate environment of the copper ligand. Furthermore, since the comparison between the amide I band of free and membrane-bound Pc indicates that membrane association does not significantly modify the secondary structure of the protein, the decreased thermal stability should be a consequence of alterations in the tertiary structure of Pc.

The comparison between Pc and cytochrome *c*, regarding the regulation of their conformations by charged interfaces, shows how proteins belonging to different structural classes (mainly β and α, respectively) respond likely to similar stimuli. In spite of their different secondary structures, both proteins segregate charged residues in well-defined patches at their surface. As seen here, and in related studies with cytochrome *c*, the interaction of these patches with oppositely charged bilayers induce a similar destabilization of both proteins, although more pronounced for cytochrome *c* [16]. This conformational effect, associated to changes in the reactivity of both proteins, is most likely the expression of secondary effects that are transmitted through covalent and hydrogen bonds into the core of the protein, and results in an overall destabilization of their tertiary structures.

The second interesting finding of this study is related to the different membrane-bound conformations of reduced and oxidized Pc, observed at pH 4.8. The rate of reaction of reduced Pc with oxidized $P700^+$, in the core of PS I, increases as the environmental pH decreases [35,36], in spite of the higher redox potential and the reduction of the electron transport capabilities of Pc at low pH [37]. This apparent discrepancy has been rationalized by considering that the enhanced rate of association between Pc and PS I overcompensates the reduced activity of Pc. The interaction between these redox partners is believed to be stabilized by hydrophobic contacts between the flat hydrophobic surface of Pc and PS I, that allows electron transfer from the copper, most likely via His87, to $P700^+$ [4]. Furthermore, as seen

by NMR and molecular dynamic studies at pH 6.0, the interaction of Pc with cytochrome *f* leads to a single-orientation, specific complex whose interface is stabilized by both electrostatic and hydrophobic interactions. The structure of this complex also favors electron transfer from cytochrome *f* to Pc, via His87. It follows from these data that the same region of the Pc molecule, the hydrophobic 'northern' face, might be used to interact with both redox partners, and that a redox-dependent conformational change of the protein could help Pc to specifically recognize cytochrome *f* and $P700^+$.

Our results indicate that reduced, but not oxidized, Pc is able to undergo a structural change in the presence of lipid bilayers that leads to a protein conformation which most likely exposes hydrophobic residues. The conformational change does not depend on initial electrostatic interactions, since it is also observed upon Pc binding to neutral bilayers. This observation is of particular interest, since the isoelectric point of the inner surface of the thylakoid membrane is around pH 4.5 [38]. The spectroscopic indication of such exposure of hydrophobic patches at the protein surface is the appearance in the IR spectrum of membrane-bound, reduced Pc of a well-defined component band at 1626 cm^{-1}. This band has been attributed to intermolecular interactions, stabilized through hydrophobic contacts, between partially folded protein molecules at the membrane surface. The disappearance of this signal upon pH neutralization indicates that this membrane-bound conformational state is not a dead-end product, but can reversibly adopt a native-like conformation. The fact that membrane-bound, reduced Pc cannot be reoxidized at acidic pH suggests that the flat hydrophobic surface at the 'northern' end of the protein molecule tightly interacts with the lipid bilayer. This would account for the observed restricted access of ferricyanide, which is known to interact with this region of the protein [39], to the protein redox center.

The biological relevance of our observations requires to extrapolate the experimentally observed effects on Pc conformation to the interaction between Pc and its natural redox partners. In this context, it is important to note that the surface charges of the thylakoid membrane are predominantly due to the exposed segments of integral membrane proteins, the majority of its structural lipids being electroneutral [38]. Therefore, the electrostatic interaction studied in this work attempts to mimic the putative effect of the formation of the initial electrostatic complex on Pc conformation. Considerations of the electrical charges at pH 4.8 will be closer to the physiological conditions, since proton pumping could lead to a neutralization of surface charges on the inner thylakoid membrane. Interestingly, under these experimental conditions both redox states of the protein behave differently, the reduced form showing a considerable higher increase in hydrophobicity which leads to intermolecular interactions at the membrane surface. This remarkable difference between oxidized and reduced Pc might reflect a redox-dependent conformational change that could mediate specific recognition of cytochrome *f* and PS I by Pc.

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