A Thermal Unfolding Study of Plastocyanin from the Thermophilic Cyanobacterium *Phormidium laminosum*†

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ABSTRACT: The thermal unfolding of the plastocyanin from *Phormidium laminosum*, a thermophilic cyanobacterium, is herein described. The main objective of this work is to identify structural factors responsible for the higher stability observed in proteins from thermophilic organisms. With the aid of fluorescence spectroscopy, EPR, and NMR, the factors influencing the unfolding process of the protein were investigated, and procedures for its study have been standardized. The different spectroscopic techniques used provided consistent results showing that the thermal unfolding of plastocyanin is irreversible under all the conditions investigated and that this irreversibility does not appear to be related to the presence of oxygen. The oxidized plastocyanin species has proven to be more stable than the reduced one, with respect to both the required temperature for protein unfolding (up to a 9 °C difference between the two forms) and the kinetics of the process. The behavior of this plastocyanin contrasts with that of other cupredoxins whose unfolding had previously been studied. The unfolding pH dependence and kinetic studies indicate a process with a tight control around the physiological pH in which plastocyanin plays its redox role and the protein's isoelectric point (5.2), suggesting a close compromise between function and stability.

Thermophilic organisms thrive at temperatures where proteins from mesophilic organisms are often completely unfolded and nonfunctional. Remarkably, mesophilic and thermophilic protein homologues usually share quite similar core structures (*1*), a fact that allied with the irreversibility of some of the unfolding processes impairs analysis of enhanced stability. The molecular determinants of protein thermostability are extremely complex and not fully understood (*2*), although an increase in thermostability is generally caused by the additive effect of multiple and subtle changes such as a slightly higher number of electrostatic interactions (salt bridges, hydrogen bonds), an increase in the fractional polar surface, a decrease in the number of loops and turns, or the stabilization of α-helices (*3*, *4*). Thermostable proteins are important tools in biochemistry and essential for studies of protein folding and stability, but the understanding of their thermal resistance is also critical for designing thermostable proteins for a variety of biotechnological applications (*5*).

For this study of protein unfolding we have chosen as a model plastocyanin (Pc)[1] from *Phormidium laminosum* (hereinafter *P. laminosum*), a filamentous thermophilic cyanobacterium that grows in hot springs at temperatures up to 60 °C (*6*). Pc is a small (ca. 10.5 kDa) soluble blue copper protein which functions as an electron shuttle between the cytochrome b_6f complex and photosystem I (PS I) in oxygenic photosynthesis and whose structure and function are very well described (*7*−*11*). The 97−105 amino acids that form this cupredoxin are arranged in a β-barrel formed by eight β-strands and a small α-helix. Pc contains a single type I copper site that is coordinated by two histidines (His 39 and His 92 in *P. laminosum*), one methionine (Met 97), and one cysteine (Cys 89) in a distorted tetrahedral geometry (*9*). This copper site is responsible for the protein typical spectral properties that result in its intense blue color in the oxidized state, with an absorbance maximum at 598 nm and small hyperfine EPR splitting (*12*, *13*). The copper center geometry is also responsible for the high midpoint reduction potential exhibited by Pc (around +378 mV at pH 7.0) (*14*, *15*).

Earlier thermal stability studies on Pc have focused on the protein isolated from spinach (*16*−*18*). Initial results

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[1] Abbreviations: Pc, plastocyanin; PS I, photosystem I; T_M, midpoint temperature of the protein thermal transition; 1-D ^{1}H NMR, one-dimensional proton nuclear magnetic resonance.

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showed that, under aerobic conditions, the reduced protein was more thermostable ($T_M = 71$ °C) than the oxidized one ($T_M = 61$ °C) (*16*). Upon cooling, the end state of neither of the forms resembled the native protein and, in the case of the reduced Pc, resulted in two distinct final states. Reduced Pc showed an increased thermostability under anaerobic conditions. Differential scanning calorimetry, UV/vis spectroscopy, and EPR spectroscopy were later used to confirm the irreversibility of thermal unfolding (*17*). Intermolecular aggregation and degrading covalent modifications at temperatures above T_M were suggested to account for the irreversibility, following the general Lumry−Eyring scheme:

$$N \leftrightarrows U \rightarrow D$$

where N is the native protein, U is the reversible unfolded protein, and D is the irreversibly denatured protein. Assuming that the slow kinetic nature of the irreversible step allows separation of the irreversible and reversible steps (*19*, *20*), the authors devised a method to extract thermodynamic parameters for the reversible step alone based on the dependence of the heat capacity changes of Pc upon the heating scanning rate (*17*). Recently, however, it has been demonstrated that the reduced spinach Pc under anaerobic conditions undergoes reversible thermal unfolding, thus allowing direct extraction of thermodynamic parameters (*18*). These results corroborated similar studies undertaken with a closely related cupredoxin, azurin (*21*), where removal of dissolved oxygen led to reversible thermal unfolding. On the basis of these findings, a general mechanism for the irreversible thermal denaturation of Pc (applicable to all cupredoxins) was proposed. This mechanism is based on the covalent modification that occurs as a result of the copper-catalyzed oxidation of the metal-ligating cysteine sulfur under aerobic conditions.

To the best of our knowledge, the only data available on the thermostability of Pc from thermophilic organisms was provided by a comparative study of the thermal unfolding of the Pc/PS I system from different cyanobacteria (*22*). This work revealed not only that Pc from *P. laminosum* is more thermostable than its counterparts from mesophilic cyanobacteria but also that Pc is the partner limiting the thermostability of the Pc/PS I couple. However, no data were provided on the nature of the thermal unfolding process.

Hence, in the present study, we have investigated in detail the thermal unfolding process of *P. laminosum* Pc (both reduced and oxidized) under aerobic and anaerobic conditions, which revealed some peculiar features compared with those of other plastocyanins described to date. The results obtained provide an insight into the study of protein thermal unfolding and a useful contribution toward the understanding of the structural features that govern thermal stability.

MATERIALS AND METHODS

Protein Expression and Purification. Recombinant *P. laminosum* Pc was overexpressed in *E. coli* BL21(DE3)-pLysS transformed with the plasmid pET11Pc containing the *petE*-coding region (courtesy of Dr. B. Schlarb, University of Cambridge, U.K.) according to the procedure described previously (*15*) with the following modification: cupric citrate was added to the LB medium in a final concentration of 1 mM at the same time as the starter culture to increase the yield of Pc expression (*23*) and reduce the formation of apoprotein as a result of basal expression of the plasmid.

Periplasmic extracts of harvested cells were loaded onto a DEAE-cellulose column preequilibrated with 1 mM phosphate buffer, pH 7.0. The protein was eluted by a 1−100 mM gradient of this buffer. Fractions containing Pc were concentrated, loaded onto a HiLoad 16/60 Sephadex-75 column (Pharmacia), preequilibrated with 10 mM phosphate buffer, pH 7.0, with 0.1 M NaCl, and coupled to an Akta-FPLC system (Amersham Pharmacia BioTech), and Pc was eluted with the same buffer. The purity of the resulting protein fractions was assessed through the A_{280}/A_{598} ratio, and a value of 3.0 was considered for the pure protein (*24*). The protein concentration was determined spectrophotometrically using an absorption coefficient at 598 nm of 4.3 mM^{-1} cm^{-1} for the oxidized form of Pc (*24*). Pure protein preparations were desalted, concentrated by ultrafiltration, and stored at −80 °C.

For experiments with oxidized Pc, samples were fully oxidized by addition of potassium ferricyanide (Sigma). Oxidant was eliminated by passing the sample through a PD10 Sephadex G-25M gel filtration column (Pharmacia). For experiments with reduced Pc, samples were treated with sodium ascorbate (Sigma) and the reducing agent was removed as described above. Experiments conducted with different protein concentrations showed that there was no concentration dependence of the unfolding experimental data.

Fluorescence Spectroscopy. Fluorescence spectra were recorded using a Perkin-Elmer LS-5 fluorescence spectrophotometer. Samples contained 25 μM Pc in 10 mM sodium citrate buffer adjusted to pH values ranging from 4.5 to 7.5. Reduced Pc samples were studied in the presence of equimolar concentrations of sodium ascorbate to avoid autoxidation of the protein throughout the experiment. Samples were excited at 275 nm, and light emission was recorded between 280 and 500 nm.

Temperature changes during thermal denaturation of Pc were controlled by a Polystat cc2 circulation bath (Huber), and the temperature was monitored by a Digitron 2008 thermocouple fitted to a flexible probe inserted directly into the 1 cm path length cell. A scan rate of 1 °C/min was used between 25 and 95 °C. The average emission of fluorescence between 345 and 355 nm was considered to reduce the background noise. Data were corrected for the sloping of the baselines for the folded and unfolded protein forms and, whenever possible, normalized to calculate the fraction of folded protein. Curves were fitted to a two-state equilibrium mechanism for protein unfolding to estimate the values for T_M (*25*). The kinetic studies were conducted under the same conditions. The fluorescence spectrophotometer was supplied with an electronic stirrer (Variomag) coupled to the cell holder to allow rapid mixing of the protein upon injection into the thermally preequilibrated buffer, and fluorescence intensity measurements were carried out at regular intervals (minimum of 1 s). All experiments were conducted under both aerobic and anaerobic conditions.

NMR Measurements. NMR measurements were conducted with a sample of 1 mM reduced Pc in 10 mM citrate buffer, pH 6.0, 10% (v/v) D_2O, and 200 μM 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as an internal reference. The sample was deoxygenated and sealed in a restricted NMR tube. Single-scan ^{1}H NMR spectra were recorded on

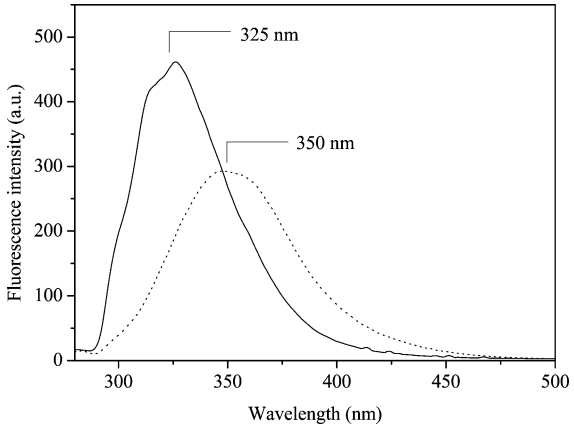

FIGURE 1: Fluorescence emission spectra of oxidized *P. laminosum* plastocyanin in the native (25 °C, continuous line) and thermally unfolded (90 °C, broken line) states. Samples contained 25 μM plastocyanin in 10 mM citrate buffer, pH 5.5, and were excited at 275 nm in a 1 cm path length cell.

an 800 MHz Varian spectrometer as the protein was heated from 30 to 90 °C and back down to 30 °C with 2 °C increments during the unfolding transition and a 5 min equilibration period after each temperature change. Temperature calibration was performed under identical conditions using 100% ethylene glycol (*26*). NMR data were processed using the MestRe-C 2.3a software package (http://www.mestrec.com). The chemical shifts of the reduced *P. laminosum* Pc had been previously assigned (*27*). The combined peak area of the resonances of the methyl group protons Ile 41 $H^{\gamma 1}$ and Lys 30 $H^{\gamma 1}$, as well as the His 39 $H^{\epsilon 2}$ peak, was normalized against the internal reference and plotted as a function of the temperature. Data were analyzed in Microcal Origin 6.0 to calculate the T_M of the transition.

EPR Measurements. EPR spectra were recorded at 20 K on a Bruker ESP 380 spectrometer equipped with an ESR 900 continuous-flow helium cryostat from Oxford Instruments. The spectra were acquired with 300 μM samples of oxidized Pc in 10 mM citrate buffer, pH 5.5 and 7.5, either in the native state or following a 5 min incubation at each given temperature and immediate freezing of the sample in liquid nitrogen. On the basis of the parameters obtained, the spectra were simulated and the fractions of folded and unfolded Pc were quantified to determine the T_M of the transition.

RESULTS AND DISCUSSION

Fluorescence Spectroscopy. Fluorescence spectroscopy is a useful technique for the study of protein unfolding due to the high sensitivity of the signal to the microenvironment of the aromatic residues that act as probes to the protein's structural changes (*28*). The fluorescence emission spectrum of the oxidized *P. laminosum* Pc at pH 5.5 is displayed in Figure 1. The protein has a high content in aromatic residues (six phenylalanines, four tyrosines, and one tryptophan), but given that all the residues are deeply buried within the protein core and that the quantum yields of both the phenylalanine and tyrosine residues are considerably low, the fluorescence emission spectrum is dominated by the features of the unique tryptophan.

At 25 °C, in the native state of the protein, the spectrum is mainly composed of a band with a maximum around 325 nm (Figure 1), typical of the $\pi-\pi^*$ transitions of the indole ring of the tryptophan when surrounded by the polar protein groups (*29*), although small contributions from the four tyrosine residues can also be observed. Upon thermal unfolding of Pc, the tryptophan residue becomes progressively more exposed to the aqueous environment, assuming a spectral form III characterized by a wide fluorescence band with a maximum around 350 nm (*30*). Moreover, the tryptophan fluorescence is quenched by water, which leads to the observed decrease in quantum yield upon denaturation (Figure 1). The fluorescence emission spectra of the native and unfolded states of the reduced protein are identical to the ones described above for the oxidized protein (data not shown).

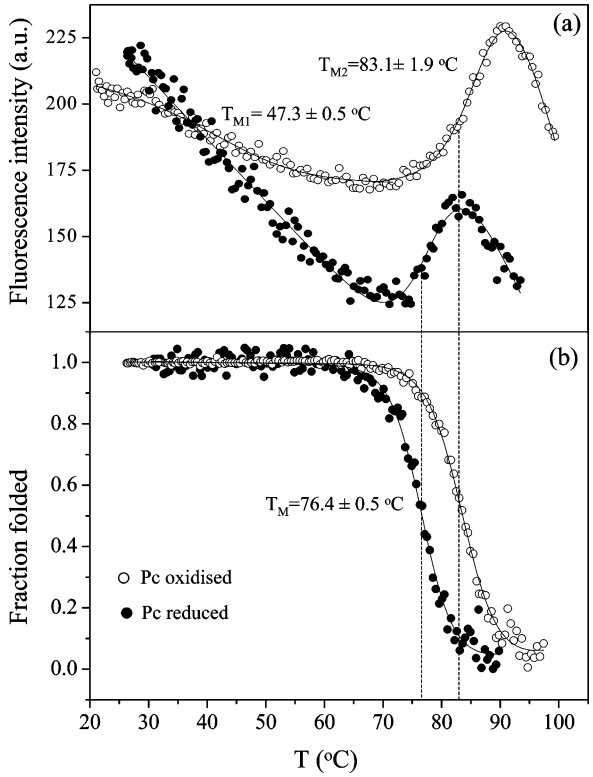

FIGURE 2: (a) Thermal unfolding of the oxidized and reduced *P. laminosum* plastocyanin followed by the fluorescence emission at 350 nm under aerobic conditions. Protein samples (25 μM) in 10 mM citrate buffer, pH 5.5, were subject to a temperature gradient of 1 °C/min. **(**b**)** Normalized curves for the unfolding of the protein. The curve for the oxidized form of plastocyanin represents an apparent fraction of folded protein, assuming that there is no intermediate formation in the unfolding pathway. See the text for more details.

Typical unfolding curves for both the oxidized and reduced forms of the protein while exposed to a temperature gradient of 1 °C/min under aerobic conditions can be seen in Figure 2. Following the fluorescence intensity of the emission maximum at 350 nm for the reduced Pc at pH 5.5, a characteristic unfolding transition can be observed with a T_M of 76.4 ± 0.5 °C (Figure 2a). The baseline slopes were used as floating fitting parameters in the data fitting process (*25*). Curves could be adequately adjusted to a two-state equilibrium mechanism for protein unfolding assuming that the irreversible step is much slower than the initial reversible process and takes place mainly at temperatures above T_M (*20*). This way, the data were normalized to describe the fraction of folded protein, as is shown in Figure 2b.

For the oxidized state of Pc at pH 5.5, thermal unfolding induced by the temperature gradient showed two unfolding transitions (Figure 2a, upper curve). The first one exhibits a T_M of 47.3 ± 0.5 °C, and the second (and much more noticeable) one has a T_M value of 83.1 ± 1.9 °C. The presence of these two transitions suggests the existence of an intermediate of unknown proportions; hence, the normalization of the data can only be undertaken by ignoring this intermediate and will be referred to as an apparent fraction of folded protein (Figure 2b). These results highlight an important difference in the mechanism of the unfolding process and thermal stability between the oxidized and reduced forms of Pc. It is worth mentioning that the transition observed at the lower temperature is reproducibly present for all samples at pH lower than 6 but is not observed for higher pH values.

Thermal unfolding of both oxidized and reduced Pc was also followed under anaerobic conditions. The difference in T_M values determined for both conditions was not considered significant (less than 1 °C). In addition, the unfolding of Pc has proven irreversible under all conditions tested, and regardless of the concentration used for the experiments, precipitation of the protein was always observed. This fact prevents the determination of the unfolding thermodynamic parameters and is not in agreement with previously published results on the thermal unfolding of some cupredoxins, namely, azurin (*21*) and spinach Pc (*18*) (see below).

All T_M values determined for *P. laminosum* Pc thermal transition in this study are higher than that previously published for the oxidized protein, 65.6 °C (*22*). The differences observed are due to the different methods used to impose the temperature gradient: a continuous temperature gradient (this work) against a step gradient followed by equilibration between increments (*22*), which considerably increases the length of time the sample is exposed to the high temperatures.

NMR. Protein fluorophore groups constitute internal probes that can reflect changes in the structural local environment that surrounds the residue more than the overall unfolding process. To validate the data obtained by fluorescence spectroscopy, *P. laminosum* Pc unfolding was also studied by NMR spectroscopy.

The 1-D ^{1}H NMR spectrum of reduced *P. laminosum* Pc was acquired in 10 mM citrate buffer, pH 6.0 (Figure 3a, lower trace), and comparison with previously obtained spectra (*27*) indicated that the assignment of the major resonances was not greatly affected by the buffer composition. Therefore, to investigate the thermal unfolding of the reduced Pc, 1-D ^{1}H NMR spectra were recorded within the temperature range of 30−90 °C under anaerobic conditions. Comparison of the 1-D spectra recorded at 30 °C, before and after heating, shows that the protein does not fully refold to a native structure (Figure 3a), and visual examination of the sample showed the presence of aggregates and precipitation. These results are in disagreement with those previously published for spinach Pc that under equivalent conditions showed a reversible unfolding process (*18*). Besides, it can be noted that the chemical shift of the copper ligand residue His 39 $H^{\epsilon 2}$ measured at 11.4 ppm does not return to the original position (Figure 3a), indicating that the type I copper site is not restored with the original coordination structure.

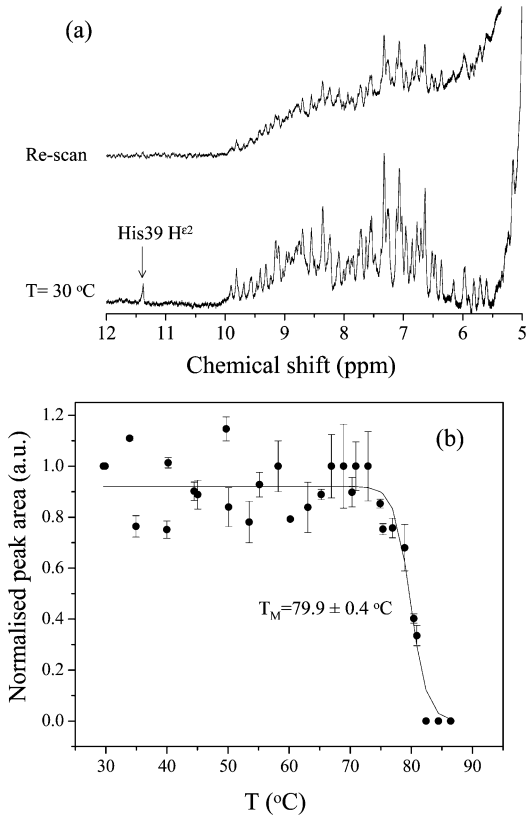

FIGURE 3: (a) ^{1}H NMR spectra of reduced plastocyanin (1 mM) in 10 mM citrate buffer, pH 6.0, both at 30 °C and after a cycle of heating the sample to 90 °C followed by slow cooling to the initial temperature (rescan). (b) Thermal unfolding of reduced *P. laminosum* plastocyanin followed by ^{1}H NMR. The normalized peak area corresponds to the average of the combined area of the peaks of His 39 $H^{\epsilon 2}$ (11.4 ppm), Ile 41 $H^{\gamma 1}$ (−0.41 ppm), and Lys 30 $H^{\gamma 1}$ (−0.46 ppm) taking the DSS peak as the normalizing factor (see the Materials and Methods). Error bars represent the standard deviation of two independent experiments, whereas the error reported for the calculated T_M value is that of the curve fitting procedure.

The resonances of the methyl group protons Ile 41 $H^{\gamma 1}$ and Lys 30 $H^{\gamma 1}$ (−0.41 and −0.46 ppm, respectively, not shown) as well as the His 39 $H^{\epsilon 2}$ (11.4 ppm) peak were well resolved throughout the unfolding transition. Analysis of their combined peak areas at each temperature resulted in a single unfolding transition (Figure 3b) with a T_M of 79.9 ± 0.4 °C. Identical transitions could be observed if the peaks were analyzed separately, indicating global unfolding as the three protons from three residues are evenly spread over the entire protein structure and their chemical shifts are sensitive to minute structural perturbations. Thus, no obvious intermediate was detected throughout the NMR experiments in agreement with the fluorescence profiles obtained for reduced Pc. The 1−2 °C difference observed for the T_M determined by NMR and fluorescence emission spectroscopy is not deemed significant, given the considerable difference in sample concentration and experimental procedure. As previously stated, a step gradient followed by equilibration between temperature increments usually results in lower T_M values, as compared to a continuous gradient; however, this is not the case when we compare the fluorescence and NMR results in this work. This apparent contradiction could be attributed to the unusual unfolding kinetic behavior exhibited by the protein (see Kinetic Studies for more details).

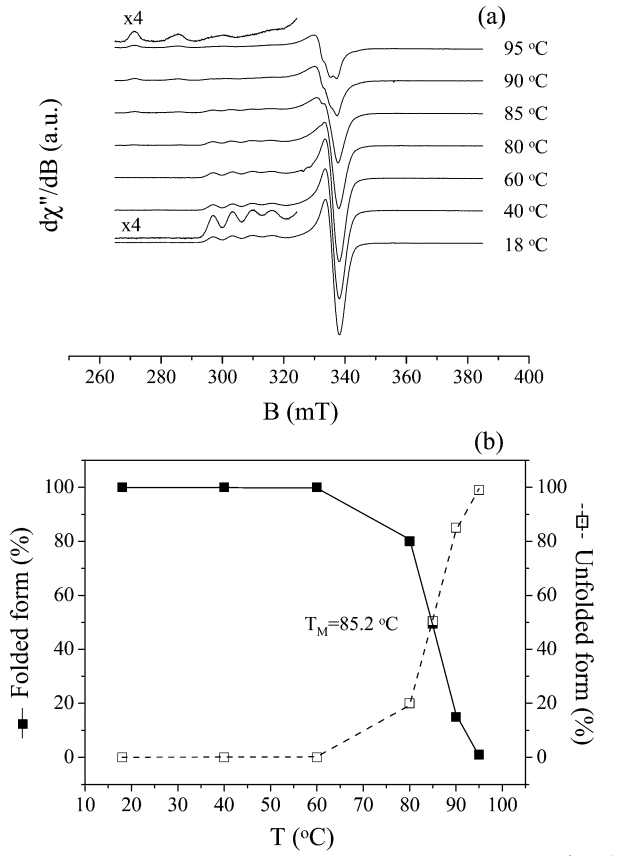

FIGURE 4: Thermal unfolding of oxidized *P. laminosum* plastocyanin followed by EPR. **(a)** EPR spectra as obtained for the different temperatures studied. Experimental conditions: 300 μM protein in 10 mM citrate buffer, pH 5.5, temperature 20 K, modulation amplitude 0.9 mT, microwave power 2.4 mW, and microwave frequency 9.64 GHz. Insets show the hyperfine splitting area magnified 4-fold. **(b)** Proportion of folded and unfolded plastocyanin as a function of temperature.

Electron Paramagnetic Resonance. Whereas fluorescence and NMR signals can be assigned to changes in the protein backbone throughout the protein unfolding process, EPR spectroscopy specifically monitors the integrity of the copper redox center, thus providing information about the changes occurring in the copper site during the protein unfolding. The copper ligand atoms are located in different portions of the β-sheets; hence, any significant changes in the secondary or tertiary structure would be expected to alter the electronic properties of the redox center.

Spectral features of the native oxidized *P. laminosum* Pc are typical of a type I copper center in a distorted tetrahedral coordination symmetry (Figure 4a). The calculated parameters $g_z = 2.247$, $g_y = 2.057$, $g_x = 2.038$, and the low $A_{||} = 68.2 \times 10^{-4}$ cm^{-1} are in good agreement with previously published data for this type of copper coordination (*31*).

The thermal unfolding of Pc was followed at pH 5.5 by EPR after incubation of the sample at increasing discrete temperatures for 5 min periods. After thermal transition to the denatured state, the low-temperature spectra of Pc resemble the features of a type II copper complex in a square planar coordination (Figure 4a). In fact, the high $A_{||}$ values observed after protein unfolding are characteristic of this kind of geometry (*32*, *33*). However, this geometry cannot be easily distinguished from a highly distorted octahedral geometry, which is normally assumed by a free copper ion in solution. After unfolding, the precipitated protein was removed by centrifugation and the EPR spectrum of the supernatant recorded: the spectrum obtained was identical to that of the fully denatured sample.

All spectra collected above 60 °C (Figure 4a) show a contribution of two unfolded species present in a 1:1 ratio (denominated 1 and 2) with relatively similar parameters: $g_{z1} = 2.373$, $g_{y1} = 2.080$, $g_{x1} = 2.064$, and $A_{||} = 144 \times 10^{-4}$ cm^{-1} for one of the species and $g_{z2} = 2.350$, $g_{y2} = 2.085$, $g_{x2} = 2.064$, and $A_{||} = 159 \times 10^{-4}$ cm^{-1} for the other. The formation of two denatured species during the unfolding of plastocyanin has previously been reported for spinach Pc following thermal unfolding by circular dichroism (*16*), but no EPR data are available. It was not possible to confidently assign any of these species to either square planar copper bound to the protein or free copper released into the solution, contrary to what has been published in the literature as a clean outcome of similar studies (*17*). Control samples of $Cu(NO_3)_2$ with 150 mM $NaClO_4$ in water and in 10 mM citrate buffer, pH 5.5, were also tested (data not shown). The parameters obtained for the complex in solution do not differ significantly from those obtained for the copper center after thermal denaturation of Pc, an observation that is compatible with the tetragonal distortion from the regular octahedral geometry assigned to copper in solution due to the Jahn−Teller effect (*12*) but which prevents the assignment of either of the two species observed.

In addition, samples exposed to the exact same temperature gradient described above were used for analysis by MS in an attempt to ascertain whether the copper ion remained bound to the unfolded protein. The results obtained were consistent with the EPR data, and it became clear that, once Pc unfolds, the copper is either more susceptible to being liberated due to higher exposure to the solvent or more loosely bound to the protein residues. Nevertheless, due to the aggressive methods required for the sample preparation, the results obtained were not fully conclusive.

From the deconvolution of the EPR signals at different temperatures, the T_M value for the loss of the copper center native coordination can be roughly estimated as 85.2 °C (Figure 4b). This value agrees with that previously determined by fluorescence spectroscopy for the second, and predominant, unfolding transition of oxidized Pc at similar pH (see Figure 2a) and suggests that the loss of the copper coordination is a phenomenon that occurs simultaneously with the loss of the protein's tertiary/secondary structure. Nevertheless, the results clearly show that the first transition detected by fluorescence for the oxidized Pc is not observed by EPR.

From the above results, it is evident that the minor transition observed at low temperature by fluorescence spectroscopy at pH $\leq$ 6.0 was not observed by any of the other techniques used for the study. Therefore, this transition does not seem to affect either the overall unfolding of the protein or the structure of the copper center, and must be associated with a local change in the electronic configuration of tryptophan, probably due to the protonation/deprotonation equilibrium of a neighboring amino acid residue (*34*). Thus, the thermostability studies will focus on the parameters obtained for the transition observed at higher temperature.

pH Dependence. The existence of structural changes in the redox center of eukaryotic Pc's has been proposed to

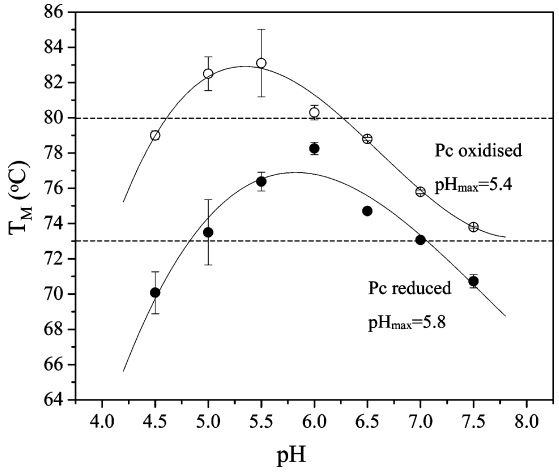

FIGURE 5: pH dependence of the midpoint unfolding temperature (T_M) for the oxidized and reduced forms of *P. laminosum* plastocyanin as determined by fluorescence emission spectroscopy under aerobic conditions. In all cases 25 μM Pc in 10 mM citrate buffer at the indicated pH value was used.

occur under protonation of the copper ligand His 87 (pK_a ca. 5.5), and the subsequent loss of the tetrahedral coordination geometry (*8*). Crystallographic studies of oxidized and reduced poplar Pc crystals at different pH values showed however that the overall fold of Pc remains the same despite changes in the oxidation state of the copper atom and in the pH (*35*). The structural changes related to the oxidation state and pH are therefore highly localized, a phenomenon that is consistent with optimization for biological effectiveness since efficient electron transfer will be assisted by a low reorganization energy.

To establish the effect of pH upon the thermal stability of Pc, a full dependence of the T_M of unfolding upon pH for both the oxidized and reduced Pc's was carried out by fluorescence spectroscopy (Figure 5). Experiments were conducted in both aerobic and anaerobic conditions, and the T_M values determined in both cases did not differ significantly. The results obtained for the range of pH values studied confirm the initial assessment of stability carried out at pH 5.5. The oxidized form of *P. laminosum* Pc is always more stable than the reduced form and exhibits a difference in midpoint transition temperature of up to 9 °C. In both cases, the curves exhibit a bell shape with an estimated maximum pH value of 5.4 and 5.8 for the oxidized and reduced proteins, respectively. EPR studies were also conducted at pH 7.5 to ascertain whether the pH effect observed by fluorescence spectroscopy also influenced the coordination of the copper center. The results obtained did not differ from those described above for the experiments ran at pH 5.5, and the T_M at neutral pH (83.6 °C) was slightly lower than that calculated for the unfolding at pH 5.5, a result which is consistent with the data obtained by fluorescence spectroscopy (Figure 5, and see below).

The pH inside the thylakoid of higher plants under illumination is reported to be around 5.5 (*36*), and a similar value is assumed for cyanobacteria. The fact that both oxidation states of the *P. laminosum* Pc have maximum thermostability around their operative pH and very close to its pI (5.2) (*37*) is of great significance in the context of the balance required between the functional properties of the protein as a soluble electron carrier in oxygenic photosynthesis and its thermodynamic and kinetic stability properties. These results are in conformity with a recent study on a large number of proteins that has revealed that the optimum pH of a protein is related to the pH of maximal protein stability, a relationship that is governed by the amino acid composition and the organization of the titratable groups within the 3D structure (*38*). However, this protein exhibits a behavior different from that of spinach Pc, where the reduced form has always been reported as more stable than the oxidized one (10 °C difference in T_M) (*16*, *18*). To justify the higher stability of reduced spinach Pc, both the role played by the copper center structure in the protein thermal stability and/or the effect of one extra charge buried in the protein have been suggested (*16*). A point to remark on is that the reported pK_a for His 92 in *P. laminosum* plastocyanin is 5.1 (*39*), also opening the possibility that the protonation state of the copper ligand may figure in the mechanism of unfolding. But, overall, in our case, the higher thermal stability of the oxidized Pc over its reduced form must be related to the structural differences between the Pc isolated from a thermophilic cyanobacterium and its eukaryotic homologues. Direct structural comparison between *P. laminosum* and spinach plastocyanins shows that, despite the relatively unchanged topology, the sequence alignment indicates a considerable replacement of conserved acid residues in the spinach protein by neutral or basic residues in its cyanobacterial counterpart with a concomitant change in the overall surface charge. Moreover, in *P. laminosum* Pc, additional residues at positions 52−55 and replacement of a group of three residues at position 62, although leaving the overall number of residues untouched, are associated with an additional turn of the α-helix and a substantial structural rearrangement in the fifth strand of the polypeptide (*9*). Further investigations are in progress to identify the specific factors responsible for the dissimilarities observed.

Kinetic Studies. To ascertain the kinetics of the thermal unfolding in *P. laminosum* Pc, the unfolding was followed by fluorescence spectroscopy as a function of time. To minimize the number of variables, all experiments were conducted at a fixed temperature, 80 °C for the oxidized Pc and 73 °C for the reduced Pc, which are the temperatures corresponding to the average of the T_M values determined at different pH values (Figure 5, dashed lines). Experiments were carried out under aerobic and anaerobic conditions, with similar results being obtained in both cases.

Typical unfolding curves are shown in Figure 6a. All curves obtained with the oxidized Pc could be fitted to an exponential function, and the values for k_{obs} could be determined. A strong dependence of this kinetic parameter on pH was observed, and an inverted bell-shaped curve was obtained by theoretical fitting to a Gaussian function (Figure 6b), from which a minimum pH value of 5.6 could be estimated. This pH value, which leads to the minimum k_{obs} and consequently is associated with the highest kinetic stability of the protein, is once again very close to the physiological pH in the thylakoid and the pI described for the protein. These results indicate that the *P. laminosum* Pc structure has been evolutionarily optimized at both the thermodynamic and kinetic levels to ensure its optimum effectiveness and stability under physiological conditions.

Under the same experimental conditions, the unfolding reaction of the reduced form of Pc revealed considerably

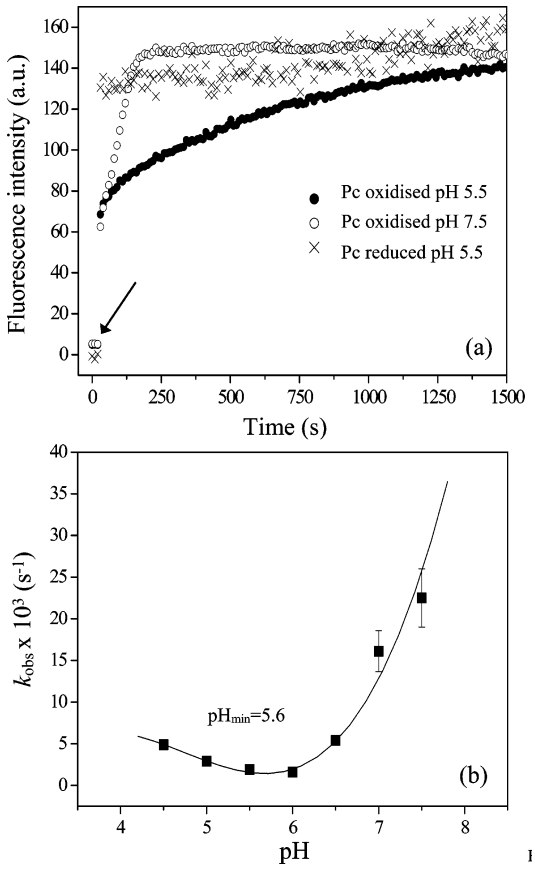

FIGURE 6: **(a)** Kinetic curves for oxidized and reduced *P. laminosum* plastocyanin unfolding under aerobic conditions. All experiments were conducted with 25 μM plastocyanin in 10 mM citrate buffer at the indicated pH values and followed by fluorescence emission spectroscopy. The arrow indicates the fluorescence increase due to protein injection into the cell. (b) Dependence of the observed rate constant (k_{obs}) on pH for the unfolding kinetics of oxidized plastocyanin.

different results. As can be seen in Figure 6a, at pH 5.5 the kinetics of unfolding of the reduced Pc are considerably faster than for the oxidized Pc (more than 10-fold), and the determination of k_{obs} is not possible given that the protein completely unfolds within the dead time of the technique. Changes in the pH of the reaction mixture rendered comparable results, and only addition of NaCl (0.10−0.40 M) to the buffer seemed to slow the unfolding reaction (not shown), though never enough for the kinetic parameters to be reliably calculated. The results indicate that reduced Pc is kinetically less stable than the oxidized form, evidence that is in tune with the lower thermodynamic thermostability observed for this reduced species.

FINAL REMARKS

The different spectroscopic techniques used provided consistent results regarding the thermal unfolding of Pc from the thermophilic organism *P. laminosum*. Thermal unfolding of this protein is irreversible under all the conditions investigated, and despite previous suggestions, this irreversibility does not appear to be related to the presence of oxygen. Even though the data accumulated did not clarify whether the irreversible unfolding of this protein is accompanied by the loss of the copper ion, the recurrent aggregation observed suggests that it is rather due to some spatial or chemical difficulty in the refolding of the amino acid chain. The main conclusion of this work is that the oxidized Pc species has proven to be more stable than the reduced Pc, with respect to both the required temperature for protein unfolding and the kinetics of the process, which is in disagreement with results previously published for spinach Pc. The unfolding pH dependence and kinetic studies on *P. laminosum* Pc indicate a process with a tight control around the physiological pH in which Pc plays its redox role, suggesting a close compromise between function and stability. In addition, the high T_M values observed for this thermophilic protein open an interesting field regarding comparison between this protein and its mesophilic counterparts. Further studies on wild-type and mutant proteins will elucidate these interesting questions.

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