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Thermal stability of wild type and disulfide bridge containing mutant of poplar plastocyanin

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Abstract

A comparative study of the thermal stability of wild type poplar plastocyanin and of a mutant form containing a disulfide bridge between residues 21 and 25 was performed using differential scanning calorimetry and optical spectroscopic techniques.

For wild type plastocyanin the transition temperature, determined from the calorimetric profiles, is 62.7 °C at the scan rate of 60 °C/h, whereas for the mutant it is reduced to 58.0 °C. In both cases, the endothermic peak is followed by an exothermic one at higher temperatures.

The unfolding process monitored by optical absorption at 596 nm also reveals a reduced thermal stability of the mutated plastocyanin compared to the wild type protein, with transition temperatures of 54.8 and 58.0 °C, respectively. For both proteins, the denaturation process was found to be irreversible and dependent on the scan rate preventing the thermodynamic analysis of the unfolding process.

In parallel, small conformational changes between wild type and mutant plastocyanin emerge from fluorescence spectroscopy measurements. Here, a difference in the interaction of the two proteins between the microenvironment surrounding the fluorophores and the solvent was proposed.

The destabilization observed in the disulfide containing mutant of plastocyanin suggests that the double mutation, Ile21Cys and Glu25Cys, introduces strain into the protein which offsets the stabilizing effect expected from the formation of a covalent crosslink.

Keywords: Plastocyanin; Thermal unfolding; Disulfide bridge insertion; Site-directed mutagenesis; DSC; Fluorescence

1. Introduction

Poplar plastocyanin (PCWT) is an all-beta protein of 99 residues arranged in eight β-strands forming a Greek key folding motif. It belongs to the cupredoxin family and binds a type-1 copper ion, Cu^{2+}, coordinated by two N^{δ} atoms from His-37 and His-87 and two S atoms from Cys-84 and Met-92 in a tetrahedral array. Plastocyanin is involved in the photosynthesis process in plants where it catalyses electron transfer from cytochrome-*f* of the membrane-associated cytochrome-b_6f complex to P700+ in photosystem I [1]. Recently, the functional property of electron transfer of blue copper proteins has become very attractive for their potential biotechnological application, such as in protein-based electronic devices [2,3]. An important requisite for this task is a stable immobilization of the redox metalloprotein on a metal substrate, favoured by the presence of exposed sulfur-containing residues and disulfide links. Within the cupredoxin family, azurin is the only protein containing a native disulfide bond close to the protein surface that has been used for an efficient anchoring on Au(111) metal [4–9]. With this aim, a surface disulfide bridge has been engineered in poplar plastocyanin, a protein which resembles the folding topology, function and spectroscopic features of azurin. The mutation, realized by site-

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directed mutagenesis of both Ile-21 and Glu-25 with cysteine residues, is localized in a region of the protein opposite to the active site. In previous papers [10,11], by using different spectroscopic techniques, it has been shown that the double mutation has not perturbed, to a significant extent, the global protein conformation, although X-ray data show some deviations from the wild type protein structure in localized regions of the protein. However, the active site is well preserved in the mutant form of the protein and the redox midpoint potential of the couple CuII/CuI was found very close to the value of the wild type protein [11]. On the other hand, molecular dynamics (MD) simulations focusing on a comparison between wild type and mutated plastocyanin have reported that the two proteins differ in their overall flexibility [12,13]. As a further investigation of the conformational properties of the mutant with respect to the wild type protein, fluorescence spectroscopy was applied. The results obtained show that the effect of the disulfide insertion is reflected on a protein region opposite to the mutation site, i.e., it seems to exhibit an increase in flexibility with the respect to the wild type protein, in agreement with the previous MD and crystallography studies.

A comprehensive characterization of a mutant protein requires a knowledge of the effect of the mutation on the energetics of the system. Despite the similarity of the structural features of the Ile21Cys/Glu25Cys plastocyanin (PCSS) compared to PCWT, the thermodynamic consequences of mutations are hard to predict because of the complexity of the protein fold. In fact, the cooperative nature of the protein fold may cause the transmission of the energetic effects of even a single mutation throughout the protein molecule [14]. To quantify the effects of a mutation on the stability of a protein, comparative thermal studies are then required.

The impact of disulfide bonds on protein stability and folding has been investigated by both experimental [15–20] and theoretical [21] approaches by considering the removal of natural –SS– as well as the insertion of novel –SS– bridges in a protein. From the results reported, it turns out that the removal of a natural –SS– bond is usually accompanied by a decrease of protein stability which has been attributed to the increase of the conformational entropy in the unfolded state [16,18,19,22]. In contrast to this, attempts to increase protein stability by insertion of novel disulfide bonds have met with limited success [15,17,20,23,24]. The contribution to stabilization due to the effect of the crosslink on the entropy of the unfolded state is, in some cases, offset by the strain associated with the formation of the disulfide bonds in the folded protein. The flexibility of the region of the insertion and the size of the loop between Cys residues participating in the disulfide bond are two aspects that can be important in determining the extent of the stabilization obtained [15,24].

Here we report the effects of the disulfide bridge insertion on the thermal stability of plastocyanin in comparison with the wild type protein. The results obtained with differential scanning calorimetry and optical absorption indicate a decrease in the stability of the mutated protein. This destabilization is explained in terms of strain energy associated with the formation of the disulfide bond in the folded state of plastocyanin.

2. Materials and methods

Wild type poplar plastocyanin and the mutant containing a disulfide bridge between the residues 21 and 25 have been obtained as previously reported [10,11]. The purity of the proteins has been checked by means of standard I.E.F and SDS-gel electrophoresis.

Differential scanning calorimetry (DSC) measurements have been performed on a VP-DSC microcalorimeter (MicroCal). Both protein solutions and reference have been extensively degassed before each scan using the ThermoVac unit provided by MicroCal. At least five buffer–buffer runs were carried out in order to obtain a reproducible baseline before each protein scan in the 20–90 °C temperature range. The equilibration time at the starting temperature was 15 min. The scan rates were 18, 30, 42 and 60 °C/h. Each sample was scanned twice in order to check the protein reversibility. No heat absorption was observed during the second scan. The concentration of the proteins, $2–3\times10^{-5}$ M in 20 mM of phosphate buffer solution at pH=6, has been spectroscopically determined using a ε_{280} of 4200 M^{-1} cm^{-1} [11]. The DSC data analysis was performed using the MicroCal Origin software package. The starting point was obtained by subtracting the corresponding buffer–buffer reference scan from the sample thermograms; the corrected data were normalized to protein concentration. Cubic baselines were generated and subtracted from the data.

Optical density (OD) experiments were performed with a JASCO 7850 spectrophotometer equipped with a Peltier-type thermostated cell holder, Model TPU-436 (precision±0.2 °C) and the EHC-441 temperature programmer. Quartz cuvettes with a 1-cm optical path were used throughout. The temperature of the samples was measured directly by a YSI thermistor dipped in the cuvette. The measurements were started after equilibration at the starting temperature of 20 °C and continued until the end of the thermal transition. The scan rates of these experiments were the same of the DSC ones as well as the proteins concentration. The transition temperature, T_t, was derived from the negative of the first derivative of the optical density at 596 nm as a function of the temperature.

Fluorescence spectra of 50 μM PCWT and PCSS in 20 mM sodium phosphate at pH 6.0 were recorded on a Spex FluoroMax™. All spectra were corrected for the Raman peak of water. The latter correction was necessary, since it contributed up to ~40% of the total emission at 310 nm. The excitation wavelength for the emission spectra was 278 nm with a slit width of 5 nm for the excitation and emission monochromator.

The denaturation of both proteins was carried out for 2 h in 8 M of urea at room temperature. The fluorescence quenching was induced by adding increasing amounts of a stock solution of 2.5 M of acrylamide.

3. Results and discussion

3.1. UV–Vis spectroscopy

Fig. 1 shows the UV–Vis spectra of PCWT (solid line) and PCSS (dashed line) in aqueous solution recorded at room temperature. As can be seen from the figure, the spectral features of the two proteins are similar over the whole wavelength range. The maximum absorption in the visible range at 596 nm is assigned to π–π* transition from a Cu–S bonding orbital to the π* antibonding orbital [25]. Also in the near-UV region the two proteins show the same fine structure, which is attributed to aromatic residues such as tyrosine and phenylalanine. The similarity of the spectra shows that the introduction of the –SS– bridge does not perturb the electronic structure of the active site of the protein.

Moreover, results previously obtained by electron paramagnetic resonance and resonance Raman scattering [11] indicate that the active site of the PCSS mutant retains the spectral properties of the wild type protein. The preservation of the Cu site properties is not surprising considering that the mutation site is ~20 Å away from the metal site. In addition, the X-ray structure at 1.6 Å resolution shows that the overall structure of PCSS matches closely the β-barrel folding of PCWT [10]. Nevertheless, the new structural constraint imposed by the presence of the disulfide bridge leads to a perceptible structural deviation of the loop bearing the two mutation sites. In fact, the root mean square deviation (rmsd) calculated for the C^{α} atoms in the loop of the wild type plastocyanin relative to the mutant is 1.10 Å compared to 0.49 Å for the rmsd of the C^{α} averaged over the whole polypeptide chain [10].

3.2. Fluorescence spectroscopy

Additional information on the protein conformation in the native state can be obtained by fluorescence measurements. Fig. 2 shows the room temperature emission spectra of PCWT (line a) and PCSS (line b) at pH 6.0, upon excitation at the tyrosine absorption peak at 278 nm (no tryptophan residue is present in plastocyanin). Both spectra display a maximum at 302 nm, while at longer wavelength they present two different emission maxima, at 314 nm for PCWT and at 338 nm for PCSS. These emission maxima, which are in the emission range of tryptophan in solution, were observed before in proteins which lack tryptophan residues in their sequence. They exhibit an emission band with a maximum in the 315–350 nm wavelength range at neutral pH, rather than at the expected wavelength of 305 nm. These red shift emissions cover the wavelength region that includes tyrosinate fluorescence, which in water has a maximum near 340 nm. On the basis of this similarity, the red shift emission in these proteins has been identified variously as due to hydrogen bonding of the phenol hydroxyl group or due to the tyrosinate formation in the excited state [26].

The same behaviour has been observed in the parsley plastocyanin [27,28]. In this case, the fluorescence emission maximum at 315 nm observed upon excitation at 275 nm was attributed to the emission of the phenolate anion in a low polarity environment.

However, to rule out definitively the presence of possible tryptophan contaminants as the origin of the red shift, the two proteins used in this study have been denaturated at room temperature using a high concentration of urea (8 M). In Fig. 2, the spectra for the denatured proteins (traces c and d) are displayed together with those of the native proteins

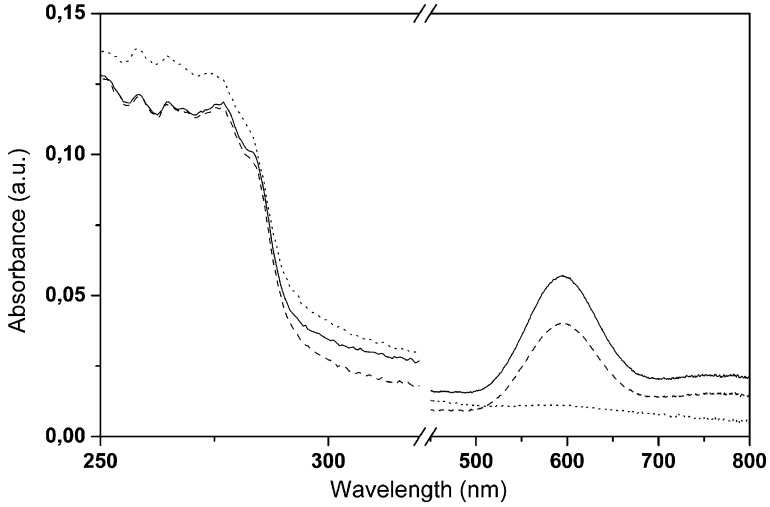

Fig. 1. Optical absorption spectra of PCWT (solid line) and PCSS (dashed line) in 20 mM PBS pH=6, recorded at room temperature in the native state. The dotted line denotes the UV–Vis spectrum of PCWT after the thermal denaturation.

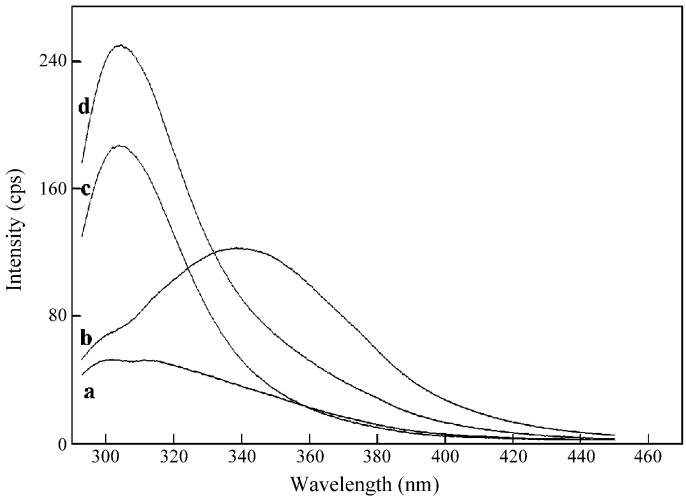

Fig. 2. Emission spectra of: native (a) and denaturated (c) PCWT, native (b) and denaturated (d) PCSS. The spectra are recorded at room temperature using an excitation wavelength of 278 nm.

(traces a and b). The emission spectra of the denatured proteins have a maximum at 304 nm which is compatible with the fluorescence emission typical of the tyrosine in solution [26]; clearly, in the unfolded proteins the tyrosine residues have lost their non-covalent interactions. Thus the differences, in the region at longer wavelength of the emission spectra, between the mutant and the wild type protein in the native state, reflect difference in the micro-environment of the tyrosine residues of the two proteins.

Poplar plastocyanin has two tyrosine residues in positions 80 and 83, which are differently accessible to the solvent. From the crystallographic data [29], it is known that Tyr-80, which is placed inside the protein, makes hydrogen bonds with Glu-59, while the Tyr-83, exposed at the surface of the protein close to the negatively charged patch, makes hydrogen bonds with Asn-76. To elucidate which tyrosine is responsible for the red shift in both fluorescence spectra, a small and highly diffusible quencher agent, i.e., acrylamide, was used. Acrylamide is an efficient quencher of tyrosine [30], which does not interact with proteins and is not influenced by nearby charged residues [31,32]. Fig. 3 shows the room temperature fluorescence emission spectra of the (A) PCWT and (B) PCSS in the presence of increasing amounts of acrylamide. As can be seen from this figure, PCSS undergoes a progressive quenching of the emission band at 338 nm, while a shoulder appears at 314 nm. On the other hand, no significant quenching effect is seen in the spectrum of the wild type protein. These results indicate that the peak at 338 nm in the emission spectrum of the PCSS can be traced back to Tyr-83.

Combining these observations, we tentatively conclude that the peaks at 314 nm in the PCWT and at 338 nm in the PCSS are due to different tyrosinate emissions. Thus, Tyr-83 in PCSS seems to have a different interaction with its microenvironment and consequently with the solvent molecules. This could explain the reduction of the emission of PCSS at 338 nm when acrylamide is added to the protein solution, since it could compete with water molecules in the collisional interaction with that region.

It can be concluded that while the native 3D structure of the PCSS protein is not affected in a significant way by the double mutation and the formation of the disulfide bridge, the solvent accessibility of Tyr-83 is affected.

3.3. Thermal stability

For a quantitative investigation of the thermal stability of PCSS as compared to PCWT protein, DSC and optical measurements were used. The DSC profile of the PCWT in 20 mM phosphate buffer solution, pH 6, recorded at a scan rate of 60 °C/h is shown in Fig. 4A. It shows an endothermic peak with a maximum heat absorption at T_m=62.7 °C followed by an exothermic one at 74.7 °C, indicating a complex unfolding pathway, which is not compatible with a two-state model. A similar result, but shifted towards higher temperature, has been previously found for spinach wild type plastocyanin [33,34]. The two proteins share the same folding topology and a high degree of homology (78%), with different amino acids in only 22 of the 99 positions. Of these 22 residues, 9 can be considered as conservative replacements, which means that the general properties of side chains of amino acids at corresponding positions are similar. Therefore, the difference observed in the thermostability may be an indication that it is a property depending on the sequence and not on topological factors as found also for the folding rates [35].

The overall endothermic enthalpy value, ΔH, could not directly be determined by integrating the experimental heat capacity curve, due to the presence of an exothermic peak in the denaturation curve. Moreover, the thermal unfolding was irreversible as judged by the absence of any endothermic peak on samples that were previously heated up to 90 °C, i.e. up to the end of the thermal transition, and then cooled down to 20 °C. The same result has been obtained if the first scan

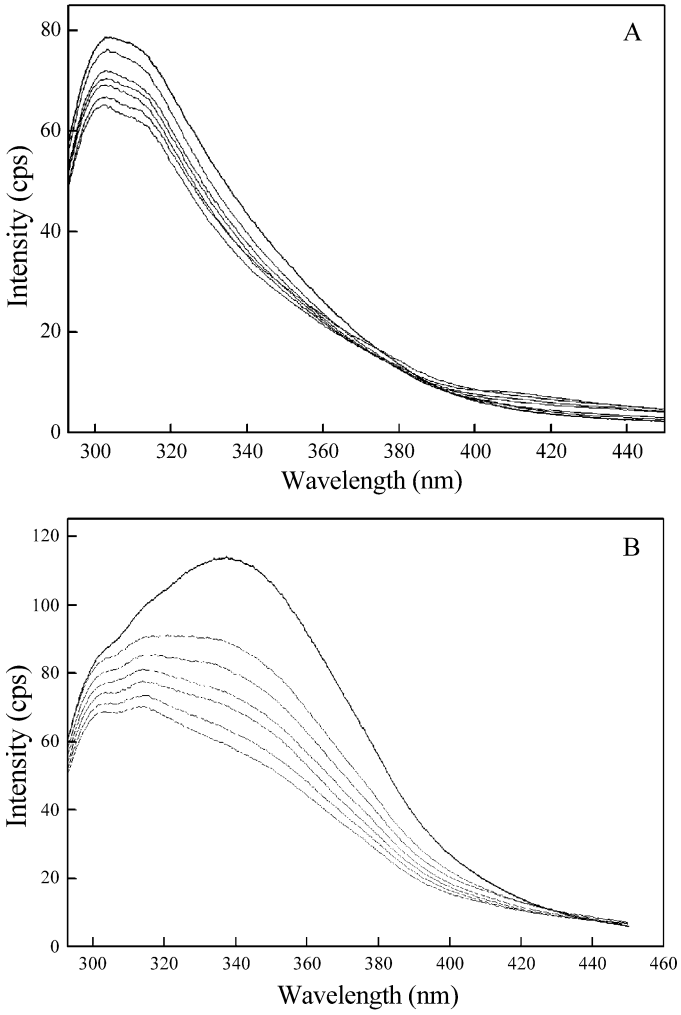

Fig. 3. Emission spectra of (A) PCWT and (B) PCSS in the presence of increasing amounts of acrylamide (from the top to the bottom). The spectra are recorded at room temperature using an excitation wavelength of 278 nm.

was stopped just below the T_m value in order to test the reversibility of the thermal transition [36,37]. The irreversibility of the thermal unfolding is commonly observed in other blue copper proteins belonging to the cupredoxin family like azurin [19,34,39–41] and amicyanin [42]. In a recent paper on spinach plastocyanin, Karlsson et al. [34] give an explanation of the origin of this irreversibility. They show that the presence of molecular oxygen associated with the oxidized state of copper are responsible of the thermal irreversibility. These arguments could apply also in our case, since the high level of homology between poplar and spinach plastocyanin. Moreover, we also have done a DSC experiments on reduced PCWT deoxygenated by N_2 gas bubbled through it for 30 min. However, we still have found thermal irreversibility probably because the risk of O_2 contamination during the filling of the cell of the calorimeter cannot be completely excluded in our experimental equipment.

Along with scanning calorimetry, which gives information on the conformational stability of the whole protein molecule, spectroscopic methods are most commonly used to study unfolding transitions. In copper proteins, the CT band in the Vis range is a suitable probe to monitor the local structural variations in the active site region induced by a change in temperature. In Fig. 4A, also the normalized OD at 596 nm is shown as a function of temperature of PCWT recorded under the same experimental condition as the DSC measurement. The curve follows a sigmoidal trend with a transition temperature, T_t, of 58.0 °C (Table 1). The comparison of the two thermograms shows that before T_t is reached there is a small heat absorption, so that the modification of the copper site precedes the global unfolding of the protein. After cooling down to 20 °C, the protein solution looses its characteristic blue colour and the intensity of the CT band is no longer recovered (Fig 1, dashed line). No precipitate was observed. The lower thermostability of the poplar plastocyanin compared to spinach plastocyanin [33] was confirmed also by the OD results.

Fig. 4B shows the DSC thermogram of PCSS obtained as for PCWT. A comparison of the DSC curves for the two proteins reveals a similar shape although the maximum heat absorption is downshifted to 58.0 °C. Again, an exothermic peak is present centered at 72.5 °C which prevents a correct

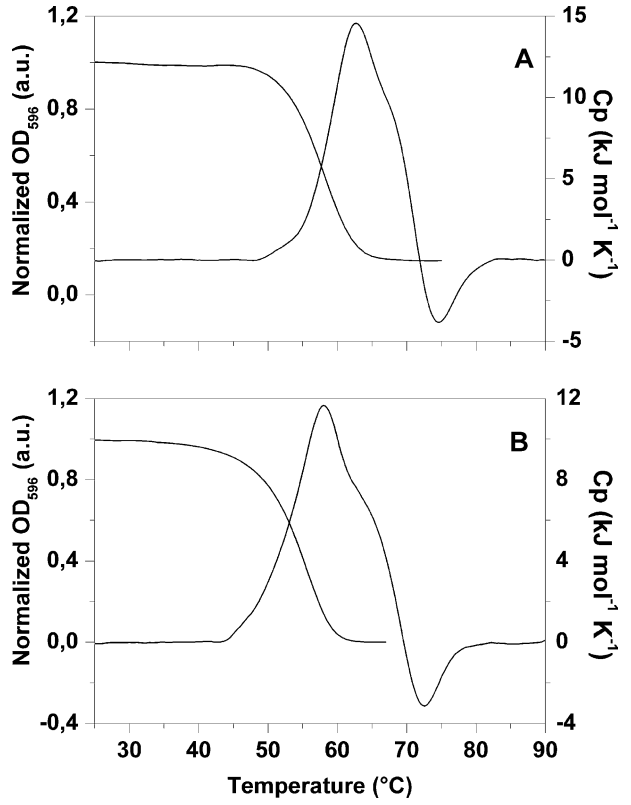

Fig. 4. DSC profile (right scale) and normalized OD_{596} vs. temperature (left scale) of (A) PCWT and (B) PCSS at 60 °C/h.

experimental determination of the ΔH. The reduction of the T_m value compared to that obtained for PCWT indicates that the introduction of the disulfide bridge at position 21–25, close to the protein surface, reduces the thermostability of plastocyanin. In the same figure the variation of the normalized OD at 596 nm is shown as obtained at a scan rate of 60 °C/h in the 20–70 °C temperature range. The T_t value is 54.8 °C, slight below the value obtained for PCWT (Table 1). The data in Table 1 show that although also for PCSS the disruption of the copper environment precedes the protein global unfolding, the difference between T_m and T_t is less than for the native plastocyanin, suggesting the occurrence of a conformational modification of the protein matrix surrounding the copper site induced by the new structural constraint.

3.4. Stability and dynamics

The addition of a disulfide bridge is one of the strategies pursued in an attempt to improve protein stability, although this approach is not always successful [15,20,24]. Several experimental results have shown that the mechanism by which crosslinks, such as disulfide bonds, stabilize native proteins is mainly related to entropy reduction in the unfolded state, which in turn depends on the length of the loop closed by the disulfide. Information on these aspects has mainly derived from thermodynamic studies on proteins in which the native disulfide was removed by mutagenesis. In general, deletion of an –SS– bridge closing a long loop in the protein main chain leads to greater destabilization than when shorter loops are involved as demonstrated by systematic studies on barnase [17] and lysozyme [38] with some exceptions [15,24]. However, only a few examples on the thermal effects due to –SS– addition are present in literature [15,20,23,24].

In our case, the overall results indicate a lower thermostability of the mutant relative to the wild type protein. Different factors can be invoked to explain this finding taking into consideration the very short loop closing off by the disulfide, only three residues. The introduction of the cysteine pairs could perturb stabilizing interactions, such as hydrophobic interactions, hydrogen bonds and van der Waals contacts that stabilize the wild type structure. This perturbation could propagate throughout the protein molecule due to the cooperative nature of the interaction that stabilize the protein structure [14]. Our findings support this interpretation.

Molecular dynamics simulations can be used to obtain structural and dynamical information about a protein and they can help in exploring the conformational changes in the native state of the mutant with respect to the wild type plastocyanin [12,13]. In particular, by analysing the root mean square fluctuation (rmsf) of the backbone atoms, which describes the flexibility around the mean structure, it has been found that the rmsf values for PCSS are, on average, higher than those observed for PCWT. The mutation has only modest effects on the mobility of the turn where the –SS– bond is located, whereas significant differences have been found for two turns close to the active site. This higher mobility is reflected in the H-bond pattern of this region where a number of interactions are lost.

This result is in agreement with the less pronounced variation between T_m and T_t values for PCSS (see Table 1) as well as with the overall destabilization of the PCSS molecule. Moreover, simulations for the two proteins in water show a reduction of the potential energy, E_{pot}, for PCWT ($E_{pot}=-172.3\pm0.5$ MJ) [12] with respect to PCSS ($E_{pot}=-159.3\pm0.5$ MJ) [13]. This result suggests that there is strain introduced in the folded protein structure with the formation of the disulfide bond as found also for some disulfide containing lysozyme mutants [15]. Indeed, a correlation between mutations that increase flexibility (and

Table 1
Transition temperatures, derived from calorimetric and optical absorption experiments for PCWT and PCSS at different scan rates

Scan rate (°C/h)	PCWT		PCSS	
	DSC T_m (°C)[a]	OD T_t (°C)[b]	DSC T_m (°C)[a]	OD T_t (°C)[b]
18	56.5	51.2	52.1	48.5
30	58.7	54.5	53.6	51.4
42	59.4	55.6	55.6	52.3
60	62.7	58.0	58.0	54.8

[a] Estimated error ±0.1 °C.
[b] Estimated error ±0.2 °C.

hydrophobicity) of the native state and destabilization has been reported for *Cucurbita maxima* trypsin inhibitor-V [24].

3.5. Irreversible unfolding

A common feature of PCWT and PCSS is the irreversibility of the thermal transition as followed by DSC and optical absorption, suggesting that the denaturation process cannot be described by a simple two-state model. Thermal irreversibility prevents a direct thermodynamic description of the protein unfolding process. However, in some cases, where the irreversible modification of the unfolded state occurs at higher temperatures than the reversible transition, the equilibrium transition models can be applied to the calorimetric data [36,37,43]. The applicability of this approach requires the analysis of the scan rate dependence of the DSC transitions and the kinetic distortions due to the irreversible processes must become negligible at sufficiently high scan rates, strictly speaking, at infinite scan rates [37]. In the commercial DSC instruments the scan rate range is limited and, very often, the highest scan rate available is not enough to eliminate the distortions due to the irreversible processes.

In Fig. 5A, the DSC curves obtained for PCWT at four different scan rates between 18 and 60 °C/h are shown. The curves show approximately the same shape with the exothermic peak always present and a shoulder after the maximum heat absorption which became less evident at the highest scan rate. Moreover, the T_m value increases with the scan rate (see Table 1). This dependence indicates that the kinetic distortion of the DSC profiles cannot be considered negligible and equilibrium thermodynamics analysis is not possible in this case.

The presence of the shoulder in the endothermic part of the DSC thermograms is not easily explained as plastocyanin is a monomeric protein. Since the OD profile (Fig. 4) shows a single transition, it is not necessarily related to a modification of the active site region, but it can also be associated with a rearrangement of the almost completely unfolded structure.

The dependence of the optical signal on the scan rate has also been investigated and the results are shown in Fig. 5B as the negative first derivative of the OD_{596} with respect to the temperature. The trend is similar to that observed in the DSC experiments with a upward shift of the transition temperature (see also Table 1). The scan rate dependence for the PCSS sample investigated by both DSC and OD techniques (data not shown) follows the same trend as the wild type protein with T_m values ranging from 52.1 °C at 18 °C/h to 54.8 °C at 60 °C/h as reported in Table 1. The T_t data derived by the OD profiles are also given.

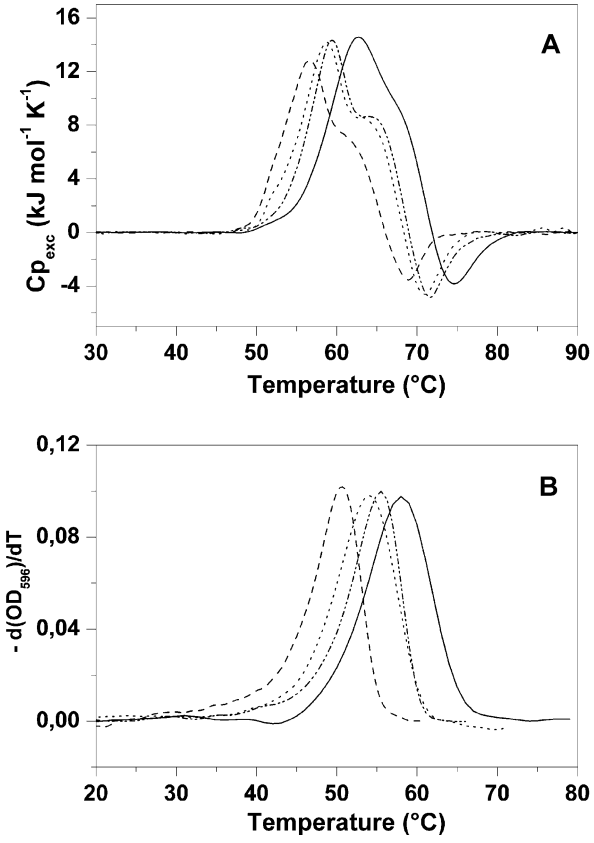

Fig. 5. Scan rate dependence of the (A) DSC curves and (B) negative of the first derivative of the optical density at 596 nm with respect to the temperature of wild type plastocyanin. The scan rates are as follows: (dashed line) 18; (dotted line) 30; (dash-dot-dot line) 42; (solid line) 60 °C/h.

4. Conclusions

The data presented in this paper have shown that the introduction of a disulfide bridge between residues 21 and 25 in poplar plastocyanin reduces the thermostability of the native protein. The global protein unfolding is preceded by conformational changes in the active site; this is less pronounced in the PCSS. The calorimetric profiles show a complex shape indicating a non-two state mechanism for the unfolding of plastocyanin. This behaviour does not depend on the presence of the cross link. Both proteins show a significant scan rate dependence of the thermal DSC and OD profiles in terms of a shift to higher temperatures at higher scan rates, which indicates the presence of kinetic factors within the unfolding pathway and prevents a thermodynamic analysis of the unfolding process.

Taking into account the results of molecular dynamics simulations performed on the two proteins, the reduced stability of PCSS can be explained in terms of an increased strain introduced at the site of the mutation which in turn affects the whole structure.

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