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Thermal unfolding of holo and apo pseudoazurin

A. Stirpe and R. Guzzi

Dipartimento di Fisica, Università della Calabria
Ponte P. Bucci, Cubo 31C, 87036 Rende (CS), Italy

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Summary. — The role of the copper ion in the thermal unfolding of pseudoazurin has been investigated by differential scanning calorimetry, optical density and fluorescence. In the presence of copper the denaturation of pseudoazurin (holo form) is irreversible and scan rate dependent. The melting temperature ranges between 60.0 and 67.3 °C, depending on the scan rate and the technique used. The DSC data analysis indicates that the denaturation pathway of the holo pseudoazurin is described by the classical Lumry-Eyring model, $N \Leftrightarrow U \Rightarrow F$. The simulation of the experimental DSC profiles according to this model has allowed the calculation of the thermodynamic and kinetic parameters related to the two steps. The destabilization of the copper active site and of the hydrophobic core precedes the global denaturation of the protein. The removal of the copper ion (apo from) significantly reduces the stability of the protein: the denaturation occurs at 41.8 °C. Moreover, the thermal unfolding of apo pseudoazurin is compatible with a two-state reversible process, $N \Leftrightarrow U$.

PACS 87.14.Ee – Proteins.
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1. – Introduction

For their peculiar spectroscopic and chemical properties, the blue copper proteins (cupredoxins) have been the object of intense investigations. Recently, the attention for such proteins has been also directed towards biomedical and bioelectronic applications [1, 2].

Our main interest lies in the thermal unfolding study of cupredoxins, proteins that share a high structural homology and a similar arrangement of the active site, despite of a low sequence homology [3]. There are minor structural differences within the proteins of this family mainly concerning the amount and the position of helical structure. The so-called "Greek-Key" topology of cupredoxins is one of the most common protein folds and is adopted by a variety of superfamilies (immunoglobulins, cadherins). Therefore, a better understanding of the folding process of this structural motif has implications for many different proteins. Previously thermal studies on azurin (Az) and plastocyanin

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(Pc) have shown that their unfolding follows the same pathway, which include an intermediate state [4, 5]. This would suggest that the folding topology plays an important role in modulating the folding mechanism. However, before that a general extension can be formulated more data on the folding of β-sandwich proteins are required. In this framework, we present a thermal unfolding investigation on pseudoazurin (Paz) from *Alcaligenes faecalis* S-6, a redox protein containing a single type-1 copper atom which, *in vivo*, functions as a direct electron carrier to copper-containing nitrite reductase (NiR) [6]. The copper ion is coordinated by two histidines (His 40 and His 81), one cysteine (Cys 78) and one methionine (Met 86) in a distorted tetrahedral configuration. The more evident difference in the Cu site of Paz with respect to that of Az and Pc, concerns the conformation of the Met side chain and the Cu-S (Met 86) bond length, which is significantly shorter (≈ 0.4 and 0.2 Å, respectively) [7].

An important feature in the thermal behaviour of many proteins, cupredoxins included, is the irreversibility of the transition, *i.e.* after thermal unfolding they are unable to recover their starting three-dimensional structure. This incapacity is commonly attributed to the formation of intermolecular aggregation and degradative covalent modifications occurring at high temperature [8, 9], to the aerobic conditions of the experiments as well as to the presence of native cofactors [10, 11].

A large amount of proteins (about 30%) in living cells requires metals or organic moieties to perform their biological function. However, the role of these cofactors in the folding stability and kinetics is poorly understood. In particular, for cupredoxins comparative thermodynamic and kinetics folding data on native and metal depleted form of protein are available only on Az [4, 12]. Copper removal in Paz produces the apo form, which adopts a folded structure very similar to that of the holo form. In fact, the root-mean-square displacement of the main chain atoms of the apo protein from the native Paz is 0.16 Å [13].

The unfolding process of Paz in the holo and apo form is here investigated by differential scanning calorimetry (DSC), optical density (OD) and fluorescence spectroscopy. The overall results show that the thermal denaturation pathway of Paz follows the classical Lumry-Eyring model, $N \Leftrightarrow U \Rightarrow F$, as for Az and Pc [4, 5]. The experimental calorimetric profiles have been simulated according to this model and the equilibrium and kinetic parameters characterizing the two steps have been determined.

The comparison of the calorimetric and spectroscopic results suggests that the disruption of the active site and the destabilization of hydrophobic core precede the protein global unfolding.

The unfolding model of Paz is closely related to the presence of copper. In fact, the removal of the metal ion (apo Paz) makes the unfolding pathway compatible with a two-state reversible model: $N \Leftrightarrow U$. In addition the stability of the protein in the apo form is dramatically reduced.

2. – Materials and methods

Protein purification and preparation. – Pseudoazurin from *Alcaligenes faecalis* S-6 was produced in *Escherichia coli* and was purified by following the procedure previously described [14]. The purity ratio, A_{277}/A_{595}, was 1.9. The copper depletion has been obtained as reported in ref. [15].

For the calorimetric and spectroscopic measurements the samples were prepared in 20 mM phosphate buffer solution, $p\text{H} = 7$ (PBS).

Differential Scanning Calorimetry. – DSC experiments were performed on a VP-DSC MicroCalorimeter (MicroCal, Inc.), with cell volumes of 0.52 mL. The temperature resolution is 0.1 °C. Protein samples were extensively degassed before measurements. The scan ran from 20 °C to 100 °C at different scan rates (0.3; 0.5; 1.0 and 1.5 °C/min), or from 10 to 80 °C at 1.0 °C/min (apo Paz). No scan rate dependence was observed for the apo protein. Protein concentration used in the experiments was 25 μM for holo Paz and 32 μM for the protein in apo form. To obtain a reproducible baseline, at least four buffer-*vs.*-buffer scans were performed. After the reference measurements, the sample cell was emptied, reloaded with the protein solution and equilibrated for 50 min at the starting temperature. The reversibility of the transition was checked by a second scan of a previously scanned sample. The Cp data were analysed by using the Origin software (MicroCal). The simulations were performed by means of an in-house program in MatLab environment.

Spectroscopy. – Optical thermal profiles were acquired for holo Paz with a JASCO 7850 spectrophotometer equipped with a Peltier thermostated cell, model TPU-436 (precision ±0.1 °C) and an EHC-441 temperature programmer. Quartz cuvettes with a 1 cm optical path were used throughout. The absorbance at a fixed wavelength (λ = 594 nm) was followed over a temperature range from 20 °C to 90 °C. The scan rates were 0.3, 0.5, 1.0 and 1.5 °C/min. Protein concentrations amounted to 20 μM. At the end of each experiment, the temperature was decreased to 20 °C and then an optical spectrum was recorded to check for refolding. In the protein apo form, the absence of copper has not allowed optical measurements.

Fluorescence curves were obtained with a Perkin-Elmer LS 50B spectrofluorimeter equipped with a Peltier Temperature Programmer PTP-1. The excitation wavelength was 280 nm. Temperature was scanned from 20 to 85 °C at 1 °C/min. The emission spectra were recorded at 400 nm/min. Protein concentration was 20 μM for holo Paz and 12 μM for apo protein.

3. – Results and discussion

3˙1. *Calorimetric analysis of the thermal unfolding of Paz.* – In fig. 1 (solid line) the calorimetric profile of Paz in PBS, recorded at 1 °C/min is shown in the 40–90 °C temperature range. One can observe an intense endothermic peak at $T_{\max} = 66.0$ °C and an exothermic one, located at the end of the thermal transition, at approximately 76.1 °C. The rescan of the sample did not show any peak. This indicates that the thermal unfolding of the protein is irreversible, as for other cupredoxins (Az, Pc and amicyanin). Thermal irreversibility in monomeric proteins is usually ascribed to degradation reactions occurring at temperatures higher than $T_{\max}$ [8, 9] and to the presence of dissolved molecular oxygen in the solution, which oxidizes the ligand cysteine [11, 16].

For an irreversible transition it is important to verify the scan rate dependence of the thermal profiles [17]. DSC runs on Paz at different scan rates, 0.3, 0.5, 1.0 and 1.5 °C/min, show a shift at higher temperature with the scan rate (data not shown). In table I the temperature of maximum heat absorption, $T_{\max}$, the temperature of the maximum heat release, $T_{\min}$, and the calorimetric enthalpy, ΔH_{cal}, values as a function of the heating rate are listed. As can be seen, $T_{\max}$ and ΔH_{cal} increase with the scan rate, in agreement with other results [4, 5, 18]. Moreover, it is interesting to note that the amplitude of the exothermic peak progressively reduces and at 1.5 °C/min only the endothermic peak is present, suggesting that the heating release is time dependent.

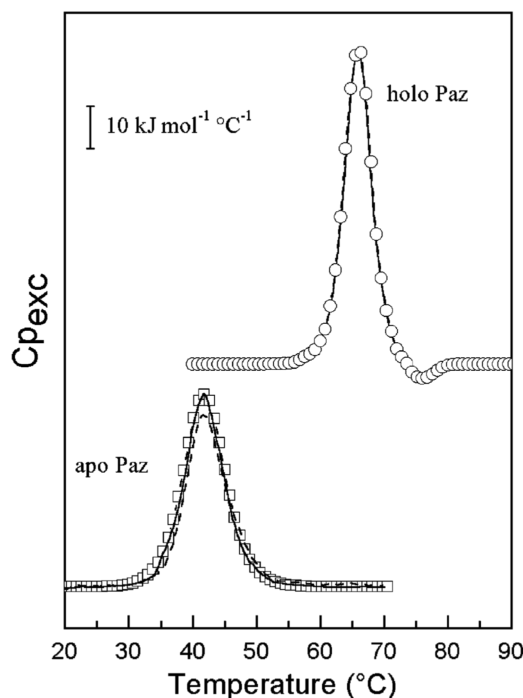


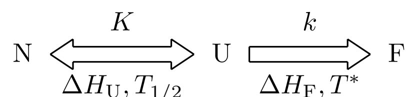
Fig. 1. – Experimental (solid line) and simulated (dashed + symbol) DSC thermograms of holo Paz at a scan rate of 1 °C/min. The parameters for the simulation are: $\Delta H_\mathrm{U} = 524\,\mathrm{kJ\,mol^{-1}}$, $T_{1/2} = 65.9\,^\circ\mathrm{C}$, $T^* = 76.9\,^\circ\mathrm{C}$, $E_\mathrm{a} = 447\,\mathrm{kJ\,mol^{-1}}$. Experimental (solid line) and simulated (square symbol) DSC profiles of apo Paz. The rescan is also shown (dashed line). The parameters for the simulation are: $\Delta H_\mathrm{U} = 380\,\mathrm{kJ\,mol^{-1}}$, $T_{1/2} = 41.8\,^\circ\mathrm{C}$.

TABLE I. – *Optical transition temperature, maximum heat absorption temperature, the maximum heat release temperature and the experimental unfolding enthalpy of pseudoazurin in PBS,* pH = 7 *as a function of the scan rate.*

Optical density		Differential scanning calorimetry		
Scan rate (°C/min)	T_t (°C)	T_max (°C)	T_min (°C)	ΔH_cal ($\mathrm{kJ\,mol^{-1}}$)
0.3	60.0	62.5	68.0	346 ± 27
0.5	61.5	64.0	62.5	382 ± 20
1.0	63.2	66.0	76.1	440 ± 18
1.5	65.5	67.3	/	520 ± 15

Estimated error on T in DSC measurements is $\pm 0.1\,^\circ\mathrm{C}$. Estimated error on T in OD measurements is $\pm 0.5\,^\circ\mathrm{C}$. ΔH_cal is expressed as mean ± standard deviation.

According to the experimental data obtained, the simplest scheme to describe the unfolding pathway of holo Paz is the Lumry-Eyring model, which has been already applied to azurin, wt [4] and C3A/C26A double mutant [19], and plastocyanin [5]. In this model a reversible and time-independent transition, containing thermodynamic information, is followed by an irreversible and rate-limiting (kinetically controlled) step:



where N, U and F are the native, unfolded and final states. ΔH_U and ΔH_F are the enthalpy variations associated with the two steps, $T_{1/2}$ and T^* are the temperatures at which the thermodynamic equilibrium constant, K, and the kinetic constant, k, approach the unity value, respectively.

The experimental calorimetric profiles of Paz, at the different scan rates, were simulated with the following equation derived from the above scheme [20]:

$$Cp_{\text{exc}} = \left[\frac{K\Delta H_U}{(K+1)^2}\left\{\frac{k}{v} + \frac{\Delta H_U}{RT^2}\right\} + \Delta H_F \frac{1}{v}\frac{kK}{K+1}\right] \cdot \exp\left[-\frac{1}{v}\int_{T_0}^{T} \frac{kK}{K+1}\mathrm{d}T\right], \tag{1}$$

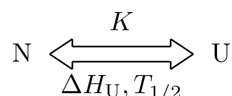
where R is the gas constant, K and k are given by

$$k = \exp\left[-\frac{E_a}{R} \cdot \left(\frac{1}{T} - \frac{1}{T^*}\right)\right], \tag{2}$$

$$K = \exp\left[-\frac{\Delta H_U}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right]. \tag{3}$$

The input parameters in the simulation of the experimental profiles were the activation energy, E_a, and T^* obtained as reported in ref. [21], whereas the unknown parameters $T_{1/2}$, ΔH_U and ΔH_F were obtained from the simulation procedure. In fig. 1 the simulated profile at 1.0 °C/min (dashed line + symbol) is compared with the corresponding experimental curve (solid line). The agreement is very good as also for the other scan rates (data not shown) and testifies the validity of the unfolding pathway proposed for Paz in the holo form.

To investigate the role of the copper ion in the stability of Paz, calorimetric studies on the apo form of the protein have been also performed. The DSC thermogram of apo Paz (fig. 1, solid line), recorded in the same experimental conditions of the holo form, shows a single and symmetric peak with a maximum heat absorption at $T_{\max} = 41.8\,^\circ\mathrm{C}$ and a ΔH_U of $380\,\mathrm{kJ\,mol^{-1}}$. This result indicates that the removal of the copper ion strongly reduces the stability of the protein. Moreover, the rescan of a previously scanned sample, until the end of the transition, shows about 88% reversibility (see dashed line in fig. 1) and no scan rate dependence has been observed. Therefore, the unfolding pathway proposed for the thermal denaturation of the apo Paz is the one-step reversible model:



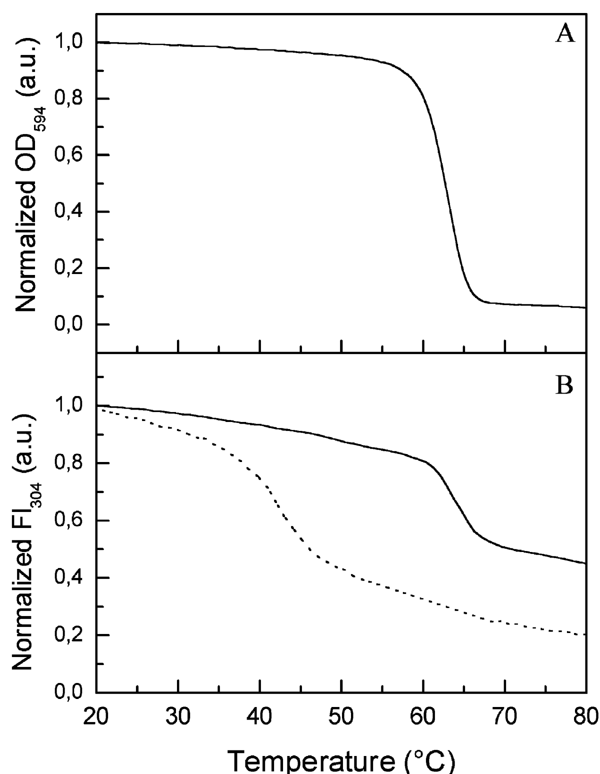


Fig. 2. – (A) Normalized OD_{594} *vs.* temperature of holo Paz, recorded at a scan rate of 1 °C/min, and (B) temperature dependence of the fluorescence intensity at 304 nm of holo (solid line) and apo (dashed line) Paz, $\lambda_{\mathrm{exc}} = 280\,\mathrm{nm}$.

According to this model the Cp_{exc} can be written as

$$Cp_{\mathrm{exc}} = \frac{\Delta H_{\mathrm{U}}^2}{R T_{1/2}^2} \frac{K}{(K+1)^2} \,. \tag{4}$$

The calorimetric profile simulated by using eq. (4), with input values of $\Delta H_{\mathrm{U}} = 380\,\mathrm{kJ\,mol^{-1}}$ and $T_{1/2} = 41.8\,°\mathrm{C}$ is also shown in fig. 1 (square symbol). Also in this case the agreement between the experimental and the simulated curves is very satisfying.

3·2. *Spectroscopic analysis of the thermal unfolding of Paz*

Optical density (holo Paz). – The thermal stability of Paz can also be studied by spectroscopic methods. In particular, the UV-Vis spectrum of holo Paz shows an intense absorption band at 594 nm assigned to the S(Cys 78) → Cu^{++} ligand-to-metal charge transfer transition [22]. The variation of the intensity of this band *vs.* temperature can be used to monitor the local conformational changes in the copper site region with the temperature. In fig. 2A is reported the normalized optical absorption variation at 594 nm, in the temperature range 20–80 °C, of Paz in PBS, recorded at a scan rate of 1 °C/min.

The transition temperature, T_t, derived from this thermal profile is 63.2 °C. Similarly to the DSC finding, when cooling the sample at room temperature, after the thermal denaturation, the characteristic blue colour is lost and the starting intensity is no more recovered as already observed in other cupredoxins [4, 5, 19, 23]. Since the copper centre lies in a hydrophobic region, formed by two loops [24], such an effect could be assigned to the conformational changes of the tertiary structure of Paz, that permanently alter the copper coordination sphere and, in particular, the Cu-S(Cys 78) bond length and angle.

The scan rate dependence of the OD_{594} in the same conditions as DSC experiments shows the same trend: T_t increases with the scan rate, but its values are lower compared to T_{max} at each scan rate (table I).

Fluorescence. – The fluorescence of Paz is mainly due to four tyrosine residues, two of them are partially exposed to the solvent. When the protein sample, in the holo and apo form, is excited at 280 nm the maximum emission is obtained at 304 nm indicating that the removal of the copper ion does not alter the microenvironment of the Tyr residues.

The variation of the fluorescence intensity at 304 nm as a function of temperature for holo (solid line) and apo (dashed line) Paz is reported in fig. 2B. The thermal profile shows the same trend in both proteins. In fact, by increasing the temperature a reduction of the fluorescence intensity at 304 nm is registered. The transition temperature, corresponding to the midpoint of the fluorescence variation at 304 nm, is 63.7 °C for holo Paz and 42 °C for apo Paz. These results are in agreement with those obtained by means of the other experimental techniques and confirm the reduction of stability of Paz after the removal of the copper. Moreover, since the fluorescence measurements as a function of temperature give information on the exposure to the solvent of the fluorophore residues, the comparison between the fluorescence and optical data reveals that in holo Paz the destabilization of the hydrophobic core occurs almost simultaneously with the disruption of the active site.

3˙3. *Thermodynamic analysis of the unfolding process*. – The stability of a protein is described by the temperature dependence of the Gibbs free energy:

$$\Delta G(T) = \Delta H_U \frac{T_{1/2} - T}{T_{1/2}} - \Delta Cp(T_{1/2} - T) + T \Delta Cp \ln \frac{T_{1/2}}{T} . \quad (5)$$

The irreversibility of the DSC transition prevents the experimental determination of $\Delta Cp = Cp_U - Cp_N$, because the Cp at the offset temperature is related to the final (F) state and not to the unfolded (U) one. In these cases, ΔCp can be calculated by means of theoretical models [25, 26]. The two different approaches used are both based on the amino acidic sequence of the protein but, in addition, the model proposed by Milardi *et al.* [26] also includes a temperature dependence of ΔCp. The ΔCp values obtained are very similar 8.5 ± 1.0 and $8.1 \pm 0.8\,\mathrm{kJ\,K^{-1}\,mol^{-1}}$, respectively.

The average of these two values have been used to calculate the temperature dependence of the ΔG function for the holo (solid line) and apo (dashed line) Paz (fig. 3). Moreover, the ΔH_U and $T_{1/2}$ values were those determined by the simulation (holo Paz) or by the experiment (apo Paz). By comparing the values of ΔG at 25 °C we found $39.2\,\mathrm{kJ\,mol^{-1}}$ for holo Paz and $16.5\,\mathrm{kJ\,mol^{-1}}$ for the apo form. This result suggests that the presence of copper in the protein significantly contributes to its stability ($\Delta\Delta G = 22.7\,\mathrm{kJ\,mol^{-1}}$). Another cupredoxin for which thermodynamic values for the holo and apo forms are available is Az. In this case, the $\Delta\Delta G$ value is $23\,\mathrm{kJ\,mol^{-1}}$ [12].

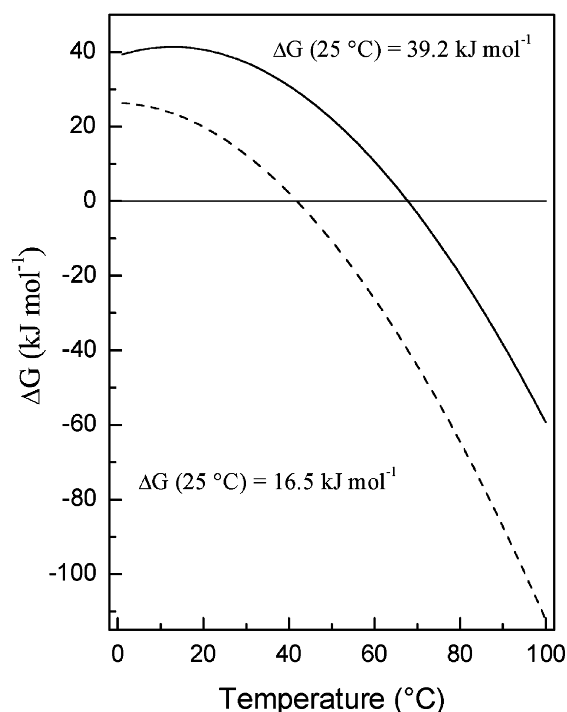


Fig. 3. – Gibbs free energy for Paz in holo (solid line) and apo (dashed line) form calculated using eq. (5). The parameters are: $\Delta H_{\mathrm{U}} = 498\,\mathrm{kJ{\cdot}mol^{-1}}$, $\Delta Cp = 8.3\,\mathrm{kJ{\cdot}{}^{\circ}C^{-1}{\cdot}mol^{-1}}$ and $T_{1/2} = 67.6\,{}^{\circ}\mathrm{C}$ for holo Paz and: $\Delta H_{\mathrm{U}} = 380\,\mathrm{kJ{\cdot}mol^{-1}}$, $\Delta Cp = 8.3\,\mathrm{kJ{\cdot}{}^{\circ}C^{-1}{\cdot}mol^{-1}}$ and $T_{1/2} = 41.8\,{}^{\circ}\mathrm{C}$ for apo Paz.

The similarity of the two values could be a coincidence or could be related to the contribution of the copper ion to the stability of blue copper proteins. However, to validate this hypothesis, more thermodynamic data are required.

Finally, comparing the thermodynamic data of the three cupredoxins we found that Paz shows a lower stability of Az (ΔG is $59.7\,\mathrm{kJ\,mol^{-1}}$ [4]), but higher compared to that of Pc ($\Delta G = 16\,\mathrm{kJ\,mol^{-1}}$ [5]). All of three proteins contain a copper ion in the active site. Az and Paz have a similar size (123 residues in Paz against 128 in Az), whereas Pc is more reduced (99 residues). In addition, Az has a disulfide bond which strongly contributes to the protein stability [19, 27].

The lowest ΔG value of Pc could be related, in first approximation, to its size.

4. – Conclusion

In the present work, the thermal denaturation of Paz has been followed by DSC, fluorescence and OD techniques. The denaturation of the holo form of the protein can be described by the sum of two contributions: one endothermic, related to the reversible unfolding of the protein; the second is exothermic and associated with the irreversibility of the whole process.

The analysis of the calorimetric and spectroscopic data indicates that the disruption

of the active site and the destabilization of the hydrophobic core precede the protein global unfolding.

The removal of the copper ion affects the protein stability, as put into evidence by the reduction of ΔG, and makes the unfolding process reversible.

By comparing the thermodynamic data of Paz with other blue copper proteins (azurin, plastocyanin), it seems that a correlation between the folding topology of the protein and the unfolding pathway exists. The structural differences among these proteins do not affect the denaturation model, but the stability extent.

* * *

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