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THERMODYNAMICS OF AZURIN FOLDING
The role of copper ion

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The role played by the metal ion in thermodynamics of azurin folding was addressed by studying the thermal denaturation of the apo-form by differential scanning calorimetry (DSC), and by comparing the results with data concerning the holo protein. The thermal unfolding experiments showed that at 25°C the presence of metal ion increases the thermodynamic stability of azurin by 24 kJ mol^{-1}. A comparison between the unfolding and the copper binding free energies allow us to assert that the unfolded polypeptide chain binds copper and subsequently folds into native holo azurin, being this the thermodynamically most favourable process in driving azurin folding.

Keywords: *apo-azurin, binding, DSC, folding Gibbs free energy, Lumry–Eyring models, stability*

Introduction

Studies of protein denaturations provide information about the driving force of the spontaneous folding of a protein into its native three-dimensional structure. In metallo-proteins, the understanding of the contribution of the metal ion to the thermodynamic stability of the protein structure is an additional requirement for elucidating all the features of the folding/unfolding process. A commonly adopted strategy for these investigations is the comparison of the thermodynamic stability of the apo protein with respect to the holo form [1–6]. In principle, if only two states, i.e. native (*N*) and unfolded (*U*) are involved in the unfolding process, the observed changes in protein stability can be ascribed to the effects of metal insertion/removal in the native and/or in the unfolded state of the protein. It is difficult to assess which of these two states are more affected by copper in stabilizing the protein because unfolding studies measure only relative free energy differences between the *N* and *U* states. Additional information on the metal contribute to the protein stability can be derived by metal binding experiments. In the present paper, this approach was applied to azurin.

Azurin, a well characterized small blue copper protein, shows a very intense absorption band in the visible spectrum at λ_{max}=630 nm with an $\varepsilon \cong 5000$ M^{-1} cm^{-1}, an unusually high redox potential (320 mV) and a characteristically narrow hyperfine splitting in the ESR spectra [7]. High-resolution X-ray diffraction studies have led to the determination of the three-dimensional structure of various forms of azurin [8–11]. Azurin is highly resistant to thermal denaturation: temperatures exceeding 70°C are necessary for irreversible denaturation. This unusual thermal resistance has been generally ascribed to a number of factors enhancing the structural stability including the presence of both a disulfide bridge and a copper atom [1, 2, 12–15].

Recently, a lot of studies concerning the folding dynamics of azurin has been published [16, 17]. In particular, it was evidenced that the azurin folding is much faster when the copper ion binds to the unfolded polypeptide chain than to the folded apo-azurin. The results obtained in the present paper demonstrate that this folding pathway is also the most thermodynamically favourable.

Experimental

Materials and methods

Chemicals

Holo azurin from *Pseudomonas Aeruginosa* was obtained as previously described [18]. Apo-azurin was prepared by a cyanide treatment of the holo form according to the procedure described elsewhere [18]. After the cyanide treatment, the protein was dissolved in 10 mM phosphate buffer (PBS) at pH 7.0. The ionic strength was adjusted at 0.1 M by sodium chloride. Incubation of apo-azurin with a three-fold excess of Cu(II) resulted in a rapid reconstitution of the

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holo-form. The concentration of apo-azurin was determined by measuring the UV absorbance of the protein solution at 280 nm using 9800 M^{-1} cm^{-1} as molar extinction coefficient [19].

1,4,8,11-tetraazacyclotetradecane (Cyclam) and 2-hydroxy-1-(1-hydroxy-naphthylazo)-6-nitro-4-naphthalene-sulfonic acid or Eriochrome Black T (EBT) were obtained from Sigma Chemical Co.

Differential scanning calorimetry

DSC scans were carried out with a Setaram (Lyon, France) micro differential scanning calorimeter (microDSC III) with stainless steel 1 mL sample cells, interfaced with a BULL 200 Micral computer. The sampling rate was 1 point s^{-1} in all measuring ranges. The same solution without the protein was used in the reference cell. Both the sample and reference were heated with a precision of 0.08°C at the scanning rates of 0.3, 0.5 and 1°C min^{-1}. The protein concentration was 1.3 mg mL^{-1} in all the experiments.

In order to obtain the excess heat capacity ($C_{p_{exc}}$) curves, buffer–buffer base lines were recorded at the same scanning rate and then subtracted from sample curves as previously described [20]. Calibration in energy was previousy obtained by dissipating a well defined amount of power, electrically generated by an EJ2 Setaram Joule calibrator within the sample cell. All DSC measurements were performed in nitrogen atmosphere.

Circular dichroism

CD measurements in the far-UV region (200–330 nm) were performed with a JASCO 700 spectropolarimeter using quartz cuvettes of 0.1 cm optical path. Protein concentration was 0.58 mg mL^{-1} in all the experiments. Other experimental condition are the same chosen in DSC experiments.

UV-Visible spectroscopy

UV-Vis spectra were recorded on a UV-JASCO 530 spectrophotometer, by using quartz cells of a 1 cm path length. Wavelength scans were performed at 25°C from 200 to 850 nm with a 200 nm min^{-1}scan rate.

Results and discussion

DSC of apo-azurin

In Fig. 1a the experimental calorimetric profile of apo-azurin recorded at a heating rate of 0.5 °C min^{-1} in the temperature range 30–70°C is reported (curve A). One symmetric peak centered at about 64°C was detected. A partial reversibility of the thermally induced transition

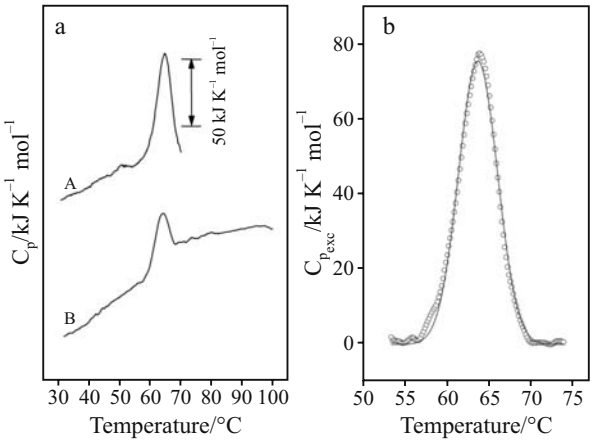

Fig. 1 a – DSC runs of apo azurin in 10 mM phosphate buffer pH 7.03 at ionic strength 0.1 M in NaCl, at the scan rate of 0.5°C min^{-1}: curve A – first scan up to 70°C; curve B – rescan of previous sample after rapid cooling. b – Curve fitting of the ○○○ – experimental $C_{p_{exc}}$ profile of apo-azurin at a heating rate of 0.5°C min^{-1} with —— – the simulated one by using Eq. (1)

was observed. In fact, when the protein solution was rapidly cooled and then re-heated up to 100°C, it exhibited an endothermic peak centered at the same temperature (Fig. 1a, line B). The area of this second peak is about 50% of the one obtained after the first heating. In order to establish to what extent kinetic factors can affect DSC traces, different calorimetric runs at increasing heating rates (0.3, 0.5 and 1.0°C min^{-1}) were carried out. The corresponding values of the temperature of maximum heat capacity (T_{max}), the experimental enthalpy values (ΔH) and the van't Hoff ratios ($\Delta H_{cal}/\Delta H_{vH}$) [20] as a function of the heating rates are listed in Table 1. It can be noted that T_{max} increases with the heating rate. Such an effect is indicative of the partial irreversible character of the thermal denaturation of apo azurin. Moreover, increasing values of the experimental denaturation enthalpy on increasing heating rates have been observed in agreement with DSC studies of other proteins that exhibit calorimetric irreversibility [22] and

Table 1 Scanning rate dependence of the maximum heat absorption temperature (T_{max}), of the experimental unfolding enthalpy (ΔH) and of the van't Hoff ratio ($\Delta H_{cal}/\Delta H_{vH}$) for apo-azurin obtained at pH=7, 10 mM PBS, ionic strength 0.1 M in NaCl

Scan rate/ °C min^{-1}	T_{max}/ °C[a]	ΔH/ kJ mol^{-1}[a]	$\Delta H_{cal}/\Delta H_{vH}$
0.3	63.4 ± 0.1	358 ± 10	0.43
0.5	64.0 ± 0.1	419 ± 11	0.59
1.0	64.6 ± 0.1	445 ± 11	0.72
∞*	65.2 ± 0.1	469 ± 18	0.95

*Extrapolated values at infinite heating rate
[a]Values expressed as mean ± standard deviation

with the theoretical predictions proposed in the literature [23]. In addition, the van't Hoff ratio also increases on increasing heating rates; this means that the irreversible component of the unfolding process is no more accessible when high heating rates are reached.

Our findings have been compared with the ones obtained in a previous study concerning the thermal unfolding on apo-azurin [1]. The results presented in that paper showed for apo-azurin two distinct melting peaks characterized by different amplitudes. The first peak was centered at 62°C whereas the second one at 86°C. No reversibility was observed for both peaks. The first peak of the calorimetric curves reported in that paper is very similar to the DSC peak found in our experiments, but we have not found evidence for a second calorimetric peak at high temperatures.

In general, the main problem concerning irreversible or partially reversible thermal transitions is that they cannot be analyzed in the light of classical thermodynamics. In these cases, the knowledge of the energetics of the system requires the use of theoretical models able to describe the unfolding pathway. In a previous paper, some of us applied to azurin a method to extract thermodynamic information from irreversible calorimetric data [20]. According to that procedure, the thermal denaturation of apo-azurin can be described as the sum of two steps: the first is reversible and contains thermodynamic information about the energetics of the protein unfolding; the second is irreversible and under kinetic control. The following scheme summarizes the proposed denaturation pathway:

$$N \underset{\Delta H_U, T_{1/2}}{\overset{K}{\Leftrightarrow}} U \underset{\Delta H_D, T^*}{\overset{k}{\Rightarrow}} D$$

where N, U and D are the native, unfolded and final (or denatured) states of apo-azurin, ΔH_U and ΔH_D are the enthalpies of the U and D states taking N and U respectively as reference state, $T_{1/2}$ and T^* are the temperatures at which the equilibrium constant K, and the kinetic constant k, approach unity, respectively. This model however can be applied to protein unfolding only if the molecularity of the irreversible step does not change, i.e. oligomerization of protein in the unfolded state and/or hydrolysis of the polypetide chain does not occur. DSC experiments carried out on apo-azurin at different concentrations ranging from 0.5 to 1.8 mg mL^{-1} have shown that the calorimetric curves are independent from protein concentration. This observation rules out the occurrence of any association/dissociation phenomenon during the thermally-induced transitions (data not shown for brevity).

The reversible component of the whole denaturation process can be separated from the irreversible one by using an extrapolation procedure of the $C_{p_{exc}}$ curve at infinite scanning rate as already described. Unfolding data, ΔH_U and $T_{1/2}$, related to the reversible component and calculated according to the above mentioned method [20–24] are reported in Table 2. In order to test the validity of the denaturation pathway proposed, the experimental DSC curves, obtained at different heating rates v, have been fitted with the following equation [25]:

$$C_{p_{exc}} = \left[\frac{K\Delta H_U}{(K+1)^2}\left\{\frac{k}{v}+\frac{\Delta H_U}{RT^2}\right\}+\Delta H_D \frac{1}{v}\frac{kK}{K+1}\right]\cdot \exp\left\{-\frac{1}{v}\int_{T_0}^{T}\frac{kK}{K+1}dT\right\} \quad (1)$$

where the previously experimental determined quantities unfolding enthalpy (ΔH_U) and temperature ($T_{1/2}$) have been used as fixed parameters, R is the gas constant, ΔH_D is the enthalpy of irreversible component, T_0 is the starting temperature and:

$$K = \exp\left\{-\frac{\Delta H_U}{R}\left(\frac{1}{T}-\frac{1}{T_{1/2}}\right)\right\} \quad (2)$$

$$k = \exp\left\{-\frac{E}{R}\left(\frac{1}{T}-\frac{1}{T^*}\right)\right\} \quad (3)$$

where E is the activation energy and T^* the peak of the irreversible step. It is noteworthy to remark that Eq. (1) has been derived from the hypothesized pathway $N \Leftrightarrow U \Rightarrow D$, and that it is not compatible with any other denaturation model [23]. Equation (1) by setting $v=\infty$, will became the equation describing the two state model ($N \Leftrightarrow U$) [26] in agreement with the observation that at very high scan rates kinetically-controlled processes do not occur:

$$C_{p_{exc}} = \frac{\Delta H_U^2}{RT^2}\frac{K}{(K+1)^2} \quad (4)$$

Table 2 Kinetic parameters obtained by fitting of DSC curves carried out at different scan rates. Minimum increases in the minimization procedure were 1 kJ mol^{-1} for enthalpies; 0.1°C for the temperatures. All the fitting parameters are freely floating

Scan rate/ °C min^{-1}	ΔH_U/ kJ mol^{-1}	$T_{1/2}$/ °C	E/ kJ mol^{-1}	T^*/ °C	δ[a]
0.3	469	65.2	36.2	15.91	2.40
0.5	471	64.9	35.4	16.12	2.66
1.0	469	65.0	38.0	17.13	3.16

[a]δ is the standard deviation of the $C^i_{p_{theor}} - C^i_{p_{exp}}$ function calculation in the denaturation range, where $C^i_{p_{exp}}$ is the i^{th} value of the experimental C_p curve and $C^i_{p_{theor}}$ is the

In Fig. 1b the experimental excess heat capacity curve of apo-azurin obtained at 0.5°C min^{-1} (circles) is compared with the corresponding fitted one obtained by using Eq. (1) (solid line). The agreement between experimental and fitted curves suggests that the unfolding model proposed provides a reliable description of the thermal denaturation of apo azurin. The same curve fitting procedure has been successfully applied to the experimental curves obtained at different scan rates (0.3 and 1°C min^{-1}) confirming the validity of the denaturation model proposed and the reliability of the values of ΔH_U and $T_{1/2}$ independently from the heating rate adopted (Table 2).

The calculation of the Gibbs free energy relative to the unfolding process in the temperature range considered requires three parameters: ΔH_U, $T_{1/2}$, and ΔC_p. Unfortunately, the partial irreversibility of the thermal transition of apo-azurin prevents the experimental determination of $\Delta C_p = C_{p_U} - C_{p_N}$, because the value of C_p at the offset unfolding temperature is ascribable to the denatured (*D*) state and not to the unfolded (*U*) one.

Thus ΔC_p can be estimated only by using thermodynamic data referring to the hydration of small model compounds [27, 28]. According to this procedure, the ΔC_p value depends only on the amino-acidic composition of the protein, and the value for apo azurin is approximately 8.5 kJ K^{-1} mol^{-1} [20].

The denaturational thermodynamic functions $\Delta G(T)$ have been calculated by using the equations [29]:

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

In Fig. 2 the $\Delta G(T)$ functions of holo- and apo-azurin are reported. It can be observed that the temperature of maximum stability is about 20°C in both cases. Moreover, the difference in the free energy of the proteins is approximately constant in all the temperature range investigated. This indicates that the presence of copper contributes significantly to the stability of the protein independently from temperature.

In particular the final value of the unfolding free energy of apo-azurin at 25°C is $\Delta G_{(N\rightarrow U,\ apo)} = 35\pm5$ kJ mol^{-1}. When compared to the value previously obtained for holo azurin, 59±6 kJ mol^{-1} [19] a reduction of 24 kJ mol^{-1} is observed as a consequence of the copper removal. The error into ΔG calculation was determined by using a procedure reported from other authors [30]. In a previous paper [31], azurin unfolding was induced by chemical denaturation (GuHCl). Despite of the different approaches adopted in the two studies the agreement between the results confirms the applicability of both strategies in determining metal-induced effects in the thermodynamic stability of proteins.

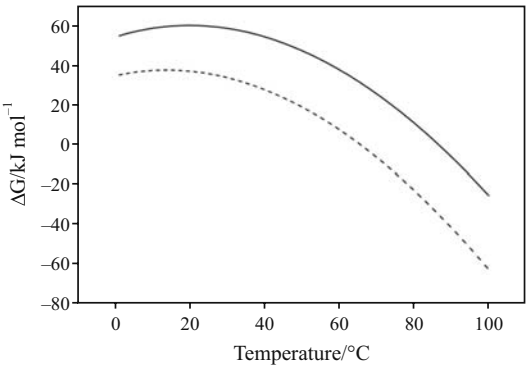

Fig. 2 Comparison of the thermodynamic stability ($\Delta G(T)$ functions) of —— – thermal unfolding of holo- and · · · – apo-azurin

Copper–azurin binding experiments

The binding free energy of a metal to a ligand can be determined from equilibrium experiments by means of the equation:

$$\Delta G = -RT \ln K_{binding} \quad (6)$$

In principle, independently from the experimental technique adopted the equilibrium binding constant ($K_{binding}$) of a metal with a ligand can be measured by using two different approaches: a) by direct titration of the ligand with the metal solution; b) or by competition experiments between a competitor ligand with known affinity for the metal and the metal-loaded complex. The last approach is particularly useful if the metal affinity for the ligand is very high, as in the case of copper proteins. This method requires the metal affinity of the chosen competitor ligand to be known. In the case of metal proteins this approach is particularly useful because it would rule out any undesired metal binding at different moieties than the native active site. In the present work metal binding experiments have been spectrophotometrically carried out either on native or denatured holo-azurin with the aim of measuring the equilibrium constant ($K_{diss}=1/K_{binding}$) of the following equilibrium:

$$CuAz \xleftarrow{K_{diss}} ApoAz + Cu^{++} \quad (7)$$

where CuAz is the holo-protein and ApoAz is the apo-protein.

The reaction (7) can be decomposed in the following partial reactions

$$ApoAz + CuL \xleftarrow{K_{app}} CuAz + L \quad (8)$$

$$Cu^{++} + L \xleftarrow{K_L} CuL \quad (9)$$

where K_L is the binding constant of Cu^{++} to ligand L. Competition experiments with a strong copper chelating agent (*L*) can be used to measure the apparent equilibrium constant K_{app} of the reaction (10). If the

chelating agent is properly chosen for its spectroscopic and thermodynamic properties, K_{diss} (reaction (7)) can be calculated by solving the following equations system:

$$K_{app}=\frac{K_{diss}}{K_L}=\frac{[CuL][ApoAz]}{[CuAz][L]} \quad (10)$$

$$[L]=b-[CuL] \quad (11)$$

$$[CuL]=a-[CuAz] \quad (12)$$

$$[CuL]=[ApoAz] \quad (13)$$

$$A_\lambda=\varepsilon_{\lambda(L)}[L]+\varepsilon_{\lambda(CuAz)}[CuAz]+\varepsilon_{\lambda(ApoAz)}[ApoAz]+\varepsilon_{\lambda(CuL)}[CuL] \quad (14)$$

where [L] is the total molar concentration at equilibrium of free ligand, b is the aliquot of the free ligand added for the titrations, [CuL] the molar concentration at equilibrium of ligand-copper complex, a is the initial concentration of CuAz, [CuAz] and [ApoAz] are the molar concentrations at equilibrium of holo- and apo-azurin. A_λ is the optical absorbance of the solution determined at a fixed wavelength λ and after reaching equilibrium, $\varepsilon_{\lambda(L)}$, $\varepsilon_{\lambda(CuL)}$, $\varepsilon_{\lambda(CuAz)}$ and $\varepsilon_{\lambda(ApoAz)}$ are the molar extinction coefficients determined at 25°C at wavelength λ for the chelating agent, the copper complex, the copper protein and the copper-depleted protein respectively.

The binding affinity of Cu(II) to the native apo-azurin was measured by spectrophotometric titration of CuAz by using 1,4,8,11-tetraazacyclotetradecane (Cyclam) as the chelating agent. Cyclam has been chosen because its copper complex has a weak optical absorption at 628 nm and titrations can be followed by monitoring the decrease of the optical absorption intensity of the solution at this wavelength. The affinity constant of Cyclam/copper complex at pH=7, ionic strength=0.1 M and T=25°C is $\log K_L$=27.2 [32]. Stepwise titrations were performed by adding 6 aliquots (20 μL each) of a freshly prepared aqueous solution of Cyclam at a concentration of 4.8 mM to a 0.1 mM solution of native holo azurin in 10 mM phosphate buffer pH=7 and ionic strength 0.1 M in NaCl. The absorbance values were collected after the equilibrium was reached, typically after 20 min. Corrections were made for dilution. For the native state of the apo azurin the binding constant at T=25°C was $K_{binding}$=1.057±0.003 and the corresponding Gibbs free energy obtained by means Eq. (6) was $\Delta G_{(Cu/Apo,N)}$= –137.3±8 kJ mol^{-1}. The binding affinity of Cu(II) to the previously thermally denatured azurin was measured by spectrophotometric titration of the protein solution by using 2-hydroxy-1-(1-hydroxy-naphthylazo)-6-nitro-4-naphthalenesulfonic acid or Eriochrome Black T (EBT) as the chelating agent. EBT was chosen because its copper complex has an evident absorption at 540 nm and, as a consequence titrations can be followed by monitoring the absorption of the solution at this wavelength. The affinity constant of EBT/copper complex at pH=7, ionic strength=0.1 M and 25°C is $\log K_L$=22 [33]. Stepwise titrations were performed by adding 6 aliquots (20 μL each) of a freshly prepared aqueous solution of EBT at a concentration of 4.8 mM to a 0.1 mM solution of denatured copper protein in 10 mM phosphate buffer pH=7 and ionic strength 0.1 M in NaCl. The absorbance was taken after the equilibrium was reached i.e. when the absorbance not change, typically after 10 min. Corrections were made for dilution. For the denatured state of the apo-azurin the binding constant at T=25°C was $K_{binding}$=1.042±0.003 and the corresponding Gibbs free energy obtained by means Eq. (6) was $\Delta G_{(Cu/Apo,D)}$= –99.3±8 kJ mol^{-1} (Table 3). On the other hand, DSC experiments allowed to estimate the unfolding free energies of holo-azurin, $\Delta G_{(N\rightarrow U,holo)}$ and of the apo form, $\Delta G_{(N\rightarrow U,apo)}$. According to the cycle reported in Fig. 3 the following equation can be written:

Table 3 Competition experiments of holo-azurin in its native state with Cyclam and holo azurin in the unfolded state with ETB. Six different titrations were carried out for the determination of the $K_{binding}$. For the native azurin $K_{binding}$=1.057±0.03 and for the denatured azurin $K_{binding}$=1.042±0.03

Native holo-azurin/Cyclam				Unfolded holo-azurin/ETB			
Titration	A_{628}/nm	Molar ratio*	$K_{binding}$	Titration	A_{540}/nm	Molar ratio	$K_{binding}$
1	0.6262	0.1274	1.054	1	0.3259	0.1075	1.044
2	0.6149	0.2548	1.055	2	0.3911	0.1259	1.042
3	0.5910	0.3823	1.061	3	0.4563	0.1329	1.040
4	0.5811	0.5097	1.057	4	0.5215	0.1740	1.039
5	0.5686	0.6372	1.059	5	0.6519	0.2050	1.047
6	0.5591	0.7646	1.060	6	0.7822	0.2544	1.041

*Molar ratio it is the ratio between the molar concentrations of ligand respect to protein

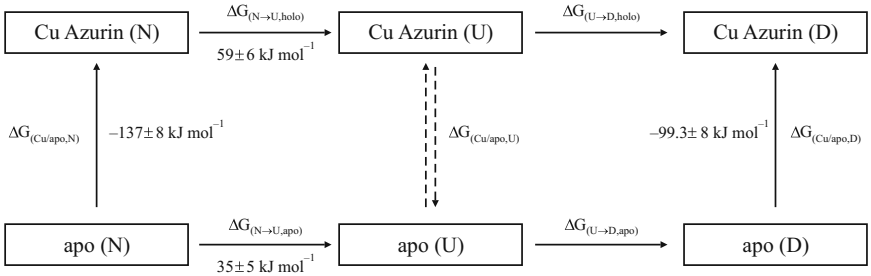

Fig. 3 Thermodynamic cycle showing the linkage between copper binding and the thermodynamic stability of holo- and apo-azurin. Solid arrows indicate directly accessible processes. Dotted arrows indicate not experimentally accessible processe

$$\text{apo}(N)\rightarrow\text{Cu azurin}(N)\rightarrow\text{Cu azurin}(U)\rightarrow\text{Cu azurin}(D)=$$
$$\text{apo}(N)\rightarrow\text{apo}(U)\rightarrow\text{apo}(D)\rightarrow\text{Cu azurin}(D)$$

Taking in account the calculated values of ΔG we obtain that by substituting the numerical values to symbols in the equation we obtain:

$$137\pm8\ \text{kJ mol}^{-1}-59\pm6\ \text{kJ mol}^{-1}+\Delta G_{(U\rightarrow D,holo)}=$$
$$35\pm5\ \text{kJ mol}^{-1}+\Delta G_{(U\rightarrow D,apo)}-99.3\pm8\ \text{kJ mol}^{-1}$$
$$\text{i.e. } \Delta G_{(U\rightarrow D,holo)}-\Delta G_{(U\rightarrow D,apo)}=14\ \text{kJ mol}^{-1}$$

This means that, within experimental uncertainties, the two terms $\Delta G_{(U\rightarrow D,holo)}$ and $\Delta G_{(U\rightarrow D,apo)}$ can be considered equal, and as a consequence $\Delta G_{(Cu/apo,\ U)}\cong\Delta G_{(Cu/apo,D)}$ (Fig. 3).

In principle azurin folding can be depicted according to two alternative pathways [16]: a) copper binds to the unfolded apo-form and then the protein folds into its native state; b) the unfolded apo-form folds into apo-folded state and then binds the copper ion, as schematically summarized in Fig. 4. Since the denaturated states obtained after thermal unfolding of both holo- and apo-azurin are very similar as demonstrated by the close similarity of the CD spectra (Fig. 5) it is reasonable to assume that the structure of the unfolded state of azurin is, as first approximation, independent from the presence of copper. Then, if we assume the unfolded state of apo-azurin as the reference state, we can note that the free energy gain of the first step in pathway a) (copper binding to unfolded apo azurin) if compared with the first step of pathway b) (folding of the apo-azurin without copper) is much higher: –99.3 kJ mol^{-1} *vs.* –35.3 kJ mol^{-1}. This finding suggests that folding pathway a) is thermodynamically favored with respect of pathway b) also confirming the conclusions recently obtained by other authors by means of kinetic investigations [16, 17]. Our findings are in agreement with these studies that propose the copper ion as a nucleation site for azurin folding.

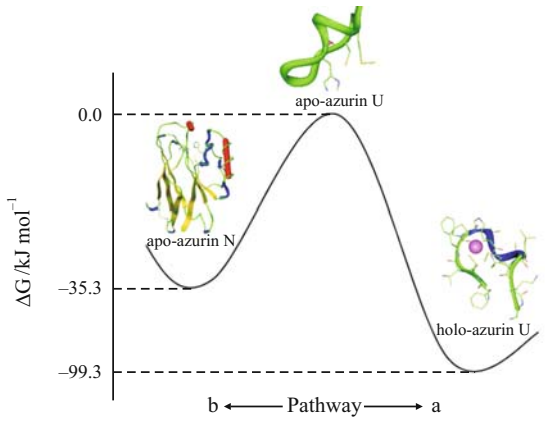

Fig. 4 Schematic drawing representing the two possible routes to the following. According to process a – the unfolded chain first binds the copper and then folds it into holo-azurin. Otherwise, in process b – the polypeptidic chain first folds and then binds the copper. Details about the energetics of of each step are reported in the text

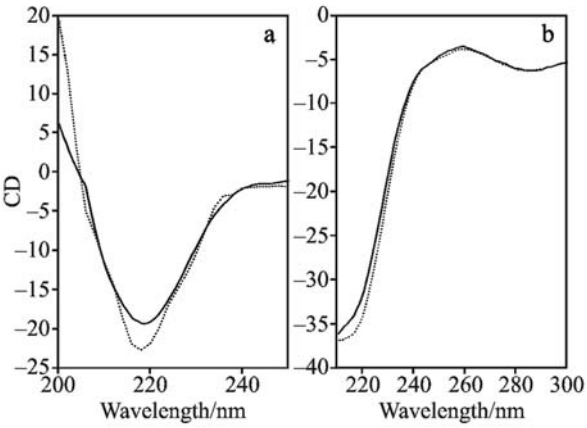

Fig. 5 a – CD spectra of —— – native apo azurin and · · · – holo-azurin obtained at 25°C; b – CD spectra of —— – denatured apo-azurin and · · · – holo-azurin obtained at 85°C. Experimental conditions are reported in the text

Abbreviations

DSC	differential scanning calorimetry
CD	circular dichroism
UV-Vis	UV-visible spettroscopy
CuAz	holo-azurin
apoAz	apo-azurin
Cyclam	1,4,8,11- tetraazacyclotetradecane
EBT	2-hydroxy-1-(1-hydroxy-naphthylazo)-6-nitro-4-naphthalenesulfonic acid or Eriochrome Black T

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