J Therm Anal Calorim (2009) 98:877–883
DOI 10.1007/s10973-009-0141-1

Reversible unfolding of poplar iso-plastocyanins

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Received: 26 March 2009 / Accepted: 26 May 2009 / Published online: 3 July 2009

Abstract We attempted to determine the experimental conditions under which poplar plastocyanin iso-forms PC*a* and PC*b* undergo reversible thermal unfolding studied by differential scanning calorimetry (DSC). Our results indicate that an exothermic unfolding process exists always in the presence of molecular oxygen. Reversible unfolding and almost perfect two-state transitions were exhibited in the presence of TCEP under anaerobic conditions. This suggests that the second endothermic peak is due to copper-site disulfide dimmers formed during thermal denaturation. The conformational thermal stability of reduced PC*b* (ΔG (25 °C) = 33.9 kJ mol^{-1}) has proven to be higher than that of reduced PC*a* (ΔG(25 °C) = 22.9 kJ mol^{-1}).

Keywords Plastocyanin · Iso-forms · Differential scanning calorimetry · Thermal stability

Introduction

Thermal analysis of protein stability by differential scanning calorimetry (DSC) is the most direct experimental method to study the thermodynamic conformational parameters. It enables thermal events and heat capacity changes in protein molecules to be measured sensitively and quantitatively. Ideally the proteins should be able to undergo complete and reversible cooperative thermal unfolding thus allowing thermodynamic equilibrium transition models to be applied to the calorimetric data [1]. However, reversibility of thermal transitions of proteins is comparatively rare because of some slow kinetic processes of aggregation at temperatures above T_m and degradative covalent reactions at high temperatures [2–5]. This is the case indicated for thermal stability studies on closely related blue copper proteins cupredoxins, plastocyanin and azurin. Recently, the role of copper ion on the thermodynamics of azurin thermal denaturation has been investigated [6].

Plastocyanin (PC) is a small (MW ≈ 10.5 kDa) monomeric blue copper protein which acts as an electron carrier between the cytochrome b_6f complex and photosystem I in oxygenic photosynthesis. Its structure and function have been very well described [7, 8]. Recently the presence of two plastocyanin iso-forms (PC*a* and PC*b*) in poplar was reported [9]. The dimorphism has been found in other higher plant plastocyanins [10–14]. It has been shown that *Arabidopsis* transgenic plants need both iso-forms to survive [15]. Deficiency of PC*a* or PC*b* has resulted in partial development of *A. thaliana* assuming their different role in plant development. This role requires specific biochemical characteristics important for interactions with the iso-form redox partners. The meaning for existence of two PC molecules could be associated either with some specific interactions with the redox partners or with some internal properties of the iso-forms. Some of our comparative biochemical studies on poplar PC*a* and PC*b* resulted in differences much more considerable than could be due to low amino acid difference of only about 10% [2, 16, 17].

Recently, we showed that the thermal unfolding of oxidized PC*a* and PC*b* under aerobic conditions was an irreversible process with two endothermic and one exothermic calorimetric peaks whose positions on the DSC curves were scan rate dependent [2]. Similar thermal unfolding results have been reported for poplar

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plastocyanin supposing one protein fraction only [18]. In general, the irreversible thermal denaturation of PC (and other cupredoxins) is attributed to the covalent modification that occurs as a result of copper-catalyzed oxidation of metal-ligating cysteine to sulfinic acid (RSO_2H) and disulfide (RSSR'). Reversible unfolding of spinach plastocyanin has been reported only in anaerobically denatured species of reduced PC form [19]. The interpretation of these results is that copper-site disulfide-linked dimmers are formed in anaerobic oxidized samples which exhibit full reversibility and melt at a higher temperature. Reversible thermal unfolding has been obtained for azurin in two investigations: in the first study by removal of both disulfide bonds in its structure [20] and in the second study by ligation to the protein nonreducible metal ions. Both Cu^{1+}– and Zn^{2+}– coordinating wild-type azurin unfold reversibly in anaerobic solutions [21]. The thermal unfolding study of plastocyanin from the thermophilic cyanobaterium *Phormidium laminosum* has shown irreversibility under all conditions [22].

This study is an attempt for further evaluation of the thermal behavior of poplar PC*a* and PC*b* under aerobic and anaerobic conditions. The Cu^{2+} ion in the proteins was reduced to Cu^{1+} either by ascorbate or by TCEP [Tris (2-carboxyethyl) phosphine hydrochloride]. It is well known that TCEP reduces disulfide bonds to thiols rapidly and qualitatively since it does not react with other functional groups of proteins. DSC scans of deaerated solutions of both proteins in the presence of this reducing reagent exhibited reversibility. Thus, thermodynamic equilibrium two-state models can be applied to data analysis. They assign a higher stability of reduced PC*b* compared to reduced PC*a*. The presence of oxygen always gave rise to an exothermal component in the thermal curves. Under anaerobic conditions the thermal unfolding curves for both reduced by ascobate and oxidized PC*a* and PC*b* consisted of two endothermic components.

Materials and methods

Protein sample preparation

Plastocyanin *a* and *b* iso-forms (PC*a* and PC*b*) were isolated and purified from poplar (*Populus nigra* var. *italica*) as described [9]. Samples were fully oxidized by addition of potassium ferricyanide which was removed by ultrafiltration using an Amicon concentration cell with YM-5 membrane. The proteins were extensively dialyzed at 4 °C against 20 mM sodium phosphate buffer, pH 7.0. The ratio $A_{278\ nm}/A_{597\ nm}$ was ≈ 1.0 for both iso-plastocyanins. Protein concentration was determined spectrophotometrically using milimolar extinction coefficients of 4.65 and 5.32 $mM^{-1}\ cm^{-1}$ at 597 nm for PC*a* and PC*b*, respectively [16]. The protein concentrations were between 0.9 and 1.15 mg mL^{-1}. For experiments with reduced PC, samples were treated with Na-ascorbate and the reduced agent was removed as described above. Anaerobic samples were degassed under vacuum for 30 min with gentle stirring prior to being loaded into the calorimetric cell. They were transferred to the calorimeter through the septum using gastight syringes and steady flow of N_2 gas. When TCEP was used, the solutions were deoxygenated with concomitant reduction of Cu(II) to Cu(I). Ten micro liters of TCEP stock solution were added to both sample and reference solutions up to a final concentration of 20 mM.

Differential scanning calorimetry

DSC measurements were performed on a DASM4 differential scanning calorimeter (NPO "Biopribor", Pushchino, Russia) with cell volumes of 0.5 mL, at temperature ranging 20–105 °C and heating rate of 1.0 °C min^{-1}. In order to check reversibility of the heat-induced denaturation reactions the first scan was completed at the end of the back slope of calorimetric maximum. Then the samples were cooled down to 15 °C for 40 min and rescanned under the same experimental conditions. Before each run three blank measurements were performed with buffer in both compartments. An average of the last two runs was used as the instrumental baseline. Each experiment was performed at least three times. Raw calorimetric data were converted into heat capacity by subtracting the instrumental baseline and after normalizing the data for protein concentration. Thereafter, the sample baseline was subtracted from the original results in order to calculate excess heat capacities. Step interpolation of pre- and post-transition baselines was used as sample line. The data obtained were subsequently analyzed with the Windows-based software package (Origin) supplied by Micro Cal.

The values of the calorimetric enthalpy ΔH_{cal} were determined from the area under the excess heat capacity profiles. The values of van't Hoff Enthalpy (ΔH_{vH}) of plastocyanin iso-form thermal unfolding was calculated by DSC curves by Strutevant's equation:

$$\Delta H_{vH} = \frac{4RT_m^2 Cp_{max}}{\Delta H_{cal}} \quad (1)$$

where Cp_{max} is the height of the heat capacity maximum at denaturation temperature T_m (or melting point), R is gas constant.

One of the forms of van't Hoff equation suggests a linear plot of $\ln K$ vs. $1/T$ to obtain ΔH_{vH}:

$$\ln K = -\frac{\Delta H_{\mathrm{vH}}}{\mathrm{RT}} + \frac{\Delta S}{\mathrm{R}} \quad (2)$$

where K is equilibrium constant, ΔS is entropy of the thermal process.

Results and discussion

DSC measurements

Our previous study on the stability of poplar plastocyanin iso-forms was concentrated on the thermal unfolding of oxidized forms of PC*a* and PC*b* under aerobic conditions investigated by differential scanning calorimetry [2]. The thermal unfolding process of both iso-forms appeared to be irreversible under those conditions and passed through two endothermic and one exothermic phases. Because of irreversibility DSC data were analyzed by available kinetic methods to determine some thermodynamic parameters associated with the thermal unfolding of the poplar plastocyanin iso-forms. The temperature of maximum heat capacity of the main thermal peaks (T_{m}) differs for both PC*a* and PC*b*. Moreover the difference was independent of calorimetric scan rate and salt concentration. PC*a* and PC*b* calorimetric enthalpy values at high salt concentration, obtained by extrapolation of the heat capacity data at infinite scan rate indicated also that PC*b* seemed to be more stable than PC*a*.

The calorimetric profiles of the Na-ascorbate reduced and TCEP reduced PC*a* under aerobic conditions are shown in Fig. 1. Comparison of the two curves shows two main differences. The first one is that the best fitting gives three independent transitions in the presence of Na-ascorbate and two transitions for TCEP reduced proteins. The second difference concerns the shift of the peaks of both DSC profiles. As in the case of aerobically oxidized PC isoforms both thermal processes in Fig. 1 were found to be irreversible since upon a second heating of the protein solutions no thermal effect was observed.

In Fig. 2 the calorimetric curves of oxidized and Na-ascorbate reduced PC*a* recorded under anaerobic conditions are compared. These results are in disagreement with those previously published for spinach plastocyanin under same conditions [19]. Anaerobic thermal transition of reduced PC*a* did not exhibit a single heat absorption peak. As seen in Fig. 2 both curves could be fitted to two endothermic transitions. The melting point for the first transition of reduced PCa was shifted to a temperature higher by about 4 °C than that for oxidized. The second transition appeared at the same temperature for oxidized and reduced plastocyanin ($T_{\mathrm{m}} = 74.82$ °C). Second

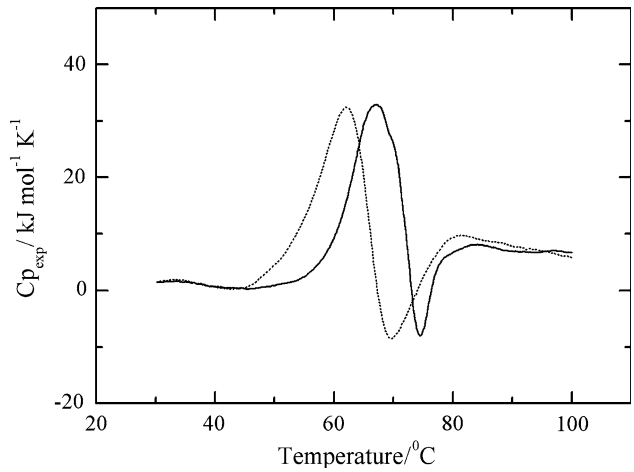

Fig. 1 DSC curves of reduced PC*a*. PC*a* reduced by 2 mM Na-ascorbate (*solid line*) and by 2 mM TCEP (*dotted line*) in aerobic conditions. Total protein concentration about 1 mg mL^{-1} in 20 mM Na-phosphate at pH 7.0

heating of the oxidized and reduced samples gave a single endotherm, centered at 74.8 °C.

Evidently these data show that: (i) The thermal unfolding of poplar plastoicyanins in the presence of dissolved molecular oxygen always involves an exothermic oxidation reaction which causes irreversibility [19, 21]; (ii) The melting point values of the main endothermic peaks are increased by oxygen removing and sample reducing as has been previously suggested for spinach plastocyanin [21, 23]. It means that with respect to the required temperature for protein unfolding reduced PC species have proven to be more stable than the oxidized PC and that regardless of the redox state a removal of dissolved oxygen causes some increasing of thermal stability; (iii) The

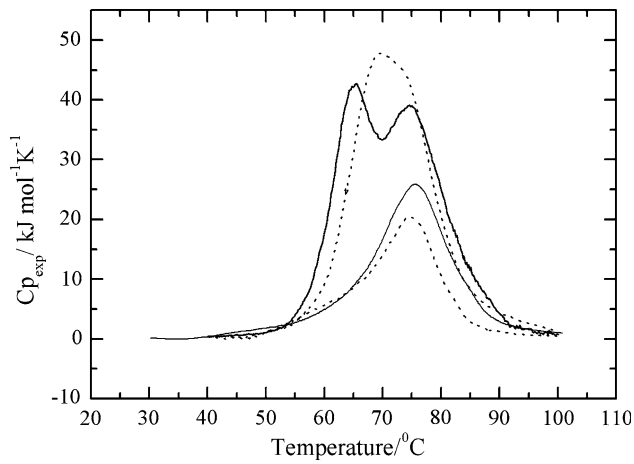

Fig. 2 DSC curves of oxidized PC*a* (*solid lines*) and reduced by 2 mM Na-ascorbate PC*a* (*dotted lines*) in anaerobic conditions. Scans and rescans from 20 to 100 °C. Protein concentration about 1 mg mL^{-1}, in 20 mM Na-phosphate buffer pH 7.0

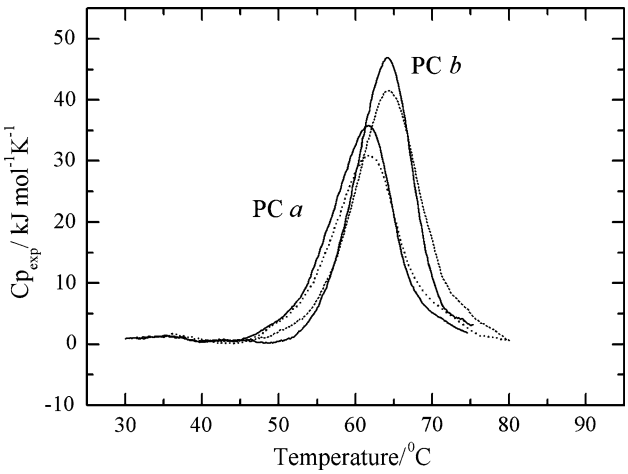

Fig. 3 DSC curves of reduced by 2 mM TCEP PC*a* and PC*b* under anaerobic condition. First scans (*solid lines*) was from 20 to 75 °C, rescans (*dotted lines*) from 20 to 100 °C. Protein concentration about 1 mg mL^{-1} in 20 mM Na-phosphate buffer at pH 7.0

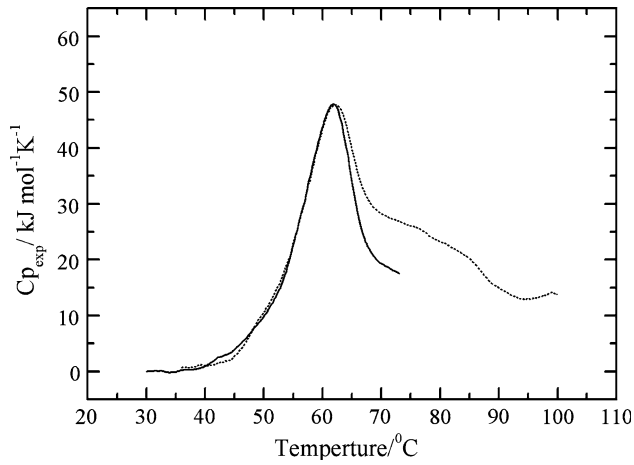

Fig. 4 DSC curves of reduced by 2 mM TCEP PC*a*, scan up to 75 °C (*solid line*) and rescan (*dotted line*) after subtraction of instrumental baseline only

second endotherm could be attributed to cooper-site disulfide dimmers [21, 23] formed during the thermal unfolding and in principle refoldable under both reduced and oxidized conditions. DSC profiles of the thermal unfolding of PC*b* under above conditions were not shown for simplicity. They are qualitatively the same as those of PC*a* with a typical for PC*b* shift of the thermal peaks to higher temperatures.

To prevent intermolecular disulfide bond formation by oxidation we performed measurements under reducing conditions provided by the presence of TCEP and under strictly oxygen-free conditions. In this way the disulfide reducing agent keeps reduced the only cysteine (Cys 84) in plastocynin molecule that appears to be one of the four copper ligands. DSC scans of PC*a* and PC*b* in the presence of TCEP, as shown in Fig. 3, exhibit single unfolding transitions with high reversibility which is the equilibrium criterion for a thermal process [1]. Thus, the thermodynamic parameters ΔH_{cal}, T_m and ΔH_{vH} for both plastocyanins could be calculated directly from the DSC data in Fig. 3. Figure 4 illustrates the results of this experiment for PC*a* scanned to 75 °C and rescanned to 100 °C without subtraction of sample baseline. It can be seen that thermal degradation of protein during prolonged exposure at high temperature prevents an accurate determination of the post-transition value of heat capacity.

Thermodynamic analysis of unfolding process

The thermal denaturation parameters of the reversible thermal unfolding of PC*a* and PC*b* are summarized in Table 1. Near unit values of the ratio $\Delta H_{cal}/\Delta H_{vH}$ indicate that denaturation is thought to obey a two-state cooperative unfolding transition [1]. This equation excludes all possibilities for significantly populated intermediates, irreversible processes or uncertainty in the baseline determination. On the other hand a coincidence of the values for ΔH_{vH} obtained by Eq. 1 and Eq. 2 provides a proof of accuracy in the determination of the protein concentration and purity of the sample. It is important to note that all thermodynamic values of PC*b* unfolding process exceed those of the PC*a*'s.

It is known that under equilibrium conditions the stability of a protein is identified by Gibbs free energy of stabilization [24]. Gibbs free energy is completely specified by enthalpy (ΔH_{cal}), the unfolding temperature T_m and the heat capacity differences (ΔC_p) between the native and the unfolded state of the protein [25, 26]. The evaluation of (ΔC_p) was accompanied by considerable uncertainty applying the alternative experimental methods and theoretical models discussed in literature [27, 28]. In principle, ΔC_p can be determined directly from the individual DSC measurements. However, in our experiments comparing different calorimetric measurements the directly measured heat capacity differences were scattered significantly by inaccuracies in the baseline of denatured state. One the other hand, the details about structural features of PC*b* are not published yet. This situation makes the methods using the exposure of the polar and the nonpolar water accessible surface areas inapplicable to describe the heat capacity changes upon unfolding [29–33]. Because of the high linear correlation coefficients for van't Hoff plots in Fig. 5, it was accepted as a reasonable approximation that ΔC_p is constant and does not vary with temperature. The variation, if any, is usually evaluated as small [33].

According to Murphy and Gill model [29] applied in thermodynamic analysis of plastocyanin and azurin molecules, ΔC_p can be evaluated by the set of equations:

Table 1 Parameters of thermodynamic reversible unfolding of PC isomers

	T_m (°C)	ΔH_{cal} (kJ mole^{-1})	ΔH^a_{vH} (kJ mole^{-1})	ΔH^b_{vH} (kJ mole^{-1})	$\Delta H_{cal}/\Delta H_{vH}$	ΔG(25 °C) (kJ mole^{-1})
PC*a*	61.7 ± 0.1	353.6 ± 28	323.3 ± 19	338.1 ± 16	1.09[a];1.05[b]	22.9 ± 3.0
PC*b*	64.3 ± 0.1	428.5 ± 23	425.9 ± 17	410.6 ± 19	1.00[a]; 1.04[b]	33.9 ± 3.0

[a] According to Eq. 1

[b] According to Eq. 2

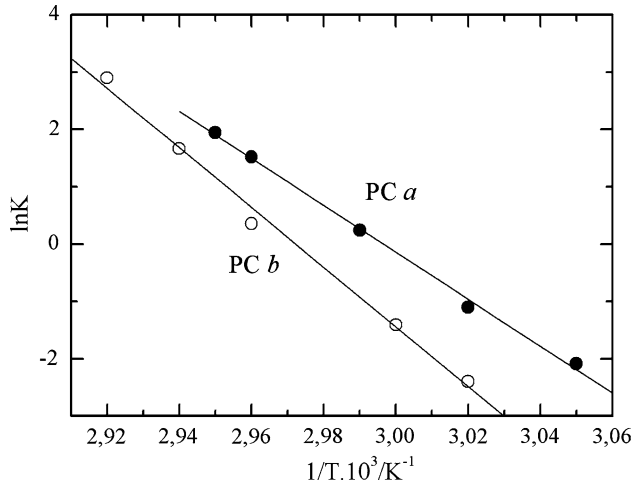

Fig. 5 Dependence of the logarithm of equilibrium unfolding constants of PC*a* and PC*b* on the reciprocal value of the absolute temperature determined from calorimetric curves in Fig. 3. Correlation coefficients for linear fits higher than 0.99. The slopes of the lines give ($-H_{vH}$/R)

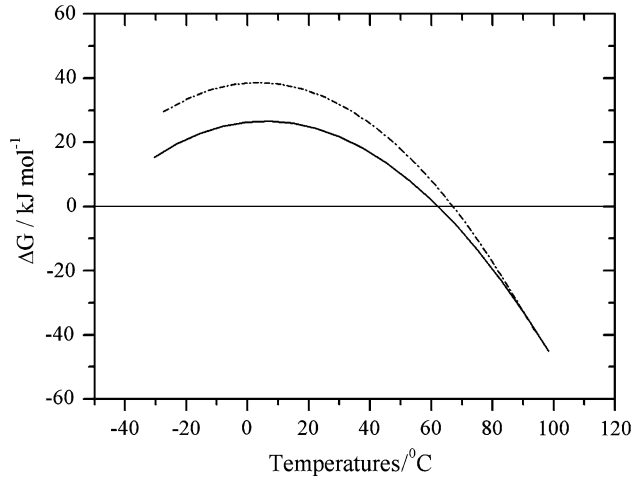

Fig. 6 Temperature dependence of the unfolding Gibbs energy (ΔG) of PC*a* (solid line) and PC*b* (*dash dotted line*)

$$\begin{aligned}\Delta Cp &= \Delta Cp_{ap} + \Delta Cp_{pol}, \Delta Cp_{ap} \\ &= f_{ap} \times N_{CH} \times (\Delta C^0)p_{-CH-} f_{ap} \\ &= 0.574 + 0.000702 \times N_{res} \Delta C_{pol} \\ &= 0.73 \times N_{res} \times (\Delta C^0)p_{-CONH-}, (\Delta C^0)p_{-CONH-} \\ &= -60 \pm 6\,\text{J mol}^{-1}\ \text{K}^{-1} (\Delta C^0)p_{-CH-} \\ &= 28 \pm 1\,\text{J mol}^{-1}\ \text{K}^{-1}\end{aligned}$$

where ΔCp_{ap} is the denaturational heat capacity change ascribable to apolar groups, ΔCp_{pol} is the heat capacity change ascribable to polar groups, f_{ap} is the fraction of apolar buried surface area, N_{res} is the number of the residues in the protein, N_{CH} is the number of apolar hydrogen atoms (i.e., the hydrogen atoms directly bound to a carbon atom), $(\Delta C^0)p_{-CH-}$ is the specific contribution of one mole of apolar hydrogens to the overall denaturational heat capacity change and $(\Delta C^0)p_{-CONH-}$ is the specific contribution ascribable to one mole of polar residues [29]. As there are 558 apolar hydrogen atoms in PC*a*, 565 apolar hydrogen atoms in PC*b* and 99 residues in both PC, ΔC_p is 5.72 kJ mol^{-1} K^{-1} for PC*a* and 5.90 kJ mol^{-1} K^{-1} for PC*b*.

Using the data in Table 1 and the above values of ΔC_p it is possible to calculate ΔG, ΔH and ΔS as a function of the temperature [24–26] using the following equations:

$$\begin{aligned}\Delta H(T) &= \Delta H_U - \Delta Cp(T_{1/2} - T), \Delta S(T) \\ &= \frac{\Delta H_U}{T_{1/2}} - \Delta Cp \ln \frac{T_{1/2}}{T}, \Delta G(T) \\ &= \Delta H_U \frac{T_{1/2} - T}{T} - \Delta Cp(T_{1/2} - T) \\ &\quad + T\Delta Cp \ln \frac{T_{1/2}}{T},\end{aligned}$$

The results for ΔG are shown in Fig. 6. By comparing the two ΔG functions it can be seen that the temperatures of maximum stability differ about 12 kJ mol^{-1} at 25 °C. Furthermore, it can be seen that $\Delta\Delta G$ decreases with the temperature and disappears in the denaturation temperature range of PC*a* and PC*b*.

Thus, in this work we report for the first time DSC thermal unfolding studies on reduced state of poplar plastocyanin. The thermal unfolding process of both plastocyanin iso-forms under equivalent conditions revealed considerably different features compared to those of other plastocyanins described up to now [19, 23, 34]. The results indicated that the dissolved molecular oxygen always gave rise to an irreversible exothermic process. In contrast, the

formation of disulfide dimmers during thermal denaturation did not appear to be related to the presence of oxygen and despite of previous suggestions [21] occurs also in the case of reduced anaerobic plastocyanins. In anaerobic solutions both PC*a* and PC*b* unfolded reversibly by inclusion of TCEP to prevent cysteine autooxidation and formation of dimmers. At present, the mechanism of dimmer formation in reduced species can not be explained because copper-catalyzed disulfide dimerization needs Cu^{2+} [19, 21]. The estimated thermodynamical parameters of reduced PC*a* and PC*b* species led to the idea that along with the twelve amino acid substitutions other reasons for the difference in the thermostability must be discovered. Four of substitutions are of PC*a* buried hydrogen-bonded polar residues to PC*b* nonpolar residues. According to general conclusions in [28, 31] they have normally very small effect on the enthalpy of protein unfolding. The remaining ones are nonpolar to nonpolar substitutions and one polar to polar substitution. This means that no significant difference between enthalpies of both PC iso-forms could be expect which disagrees with the reported estimates of the enthalpy (Table 1). Based on these results we can conclude that the increase of PC*b* enthalpy does appear to be result of a considerable structural perturbation.

Structural differences in the parts of the plastocyanin molecules were demonstrated in our crystallographycal studies [35] and the numerical calculation on these molecules [2, 16]. On the other hand PC*a* and PC*b* have subtle but crucial differences in their electrostatic behavior according to our experimental data and numerical calculations [2, 16]. Concerning the physiological role of PC iso-forms it is not out of place to notice that the conformational stability is a factor in biomolecular recognition because the energetics of the complexes depend on the energetics of complex partners [36, 37].

Acknowledgements This work was supported by grants of Fund "Scientific researches" of the Bulgarian Ministry of Education and Sciences (contract No. MY-Ь-1511/2005 and No. Ь-1519/05/2005).

References

1. Privalov P, Potekhin S. Scanning microcalorimetry in studying temperature-induced changes in proteins. Methods Enzymol. 1986;131:4–51.
2. Shosheva A, Donchev A, Dimitrov M, Kostov G, Toromanov G, Getov V, et al. Comperative study of the stability of poplar plastocyanin isoforms. Biochim Biophys Acta. 2005;1748(1):116–27.
3. Sanchez-Ruiz JM, Lopez-Lacomba JL, Cortijo M, Mateo PL. Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. Biochemistry. 1988;27(5):1648–52.
4. Sanchez-Ruiz JM. Theoretical analysis of Lumry-Eyring models in differential scanning calorimetry. Biophys J. 1992;61(4):921–35.
5. Milardi D, La Rosa C, Grasso D. Extended theoretical analysis of irreversible thermal protein unfolding. Biophys Chem. 1994;52(3):183–9.
6. Pappalardo M, Sciacca MFM, Milardi D, Grasso DM, La Rosa C. Thermodynamics of azurin folding. The role of copper ion. J Therm Anal Calorim. 2008;93(2):575–81.
7. Sykes A. Structure and electron-transfer reactivity of the blue copper protein plastocyanin. Chem Soc Rev. 1985;14:283–315.
8. Onodera J, Sugimura Y, Yoshizaki F. Novel plastocyanin containing phenyl-alanine-83 from the gymnosperm *Ginkgo biloba*. Protein Pept Lett. 2006;13(1):15–9.
9. Dimitrov M, Donchev A, Egorov C, Atanasov B. Complete amino acid sequence of poplar plastocyanin. FEBS Lett. 1987;226:17–22.
10. Dimitrov M, Donchev A, Egorov T. Microheterogeneity of Parsley plastocyanin. FEBS Lett. 1990;265:141–5.
11. Dimitrov M, Donchev A, Egorov TA. Twin plastocyanin dimorphism in Tobacco. Biochim Biophys Acta. 1993;1203(2):184–90.
12. Burkey K, Gizelice Z, Carter T. Genetic variation in soybean phosphosynthetic electron transport capacity is related to plastocyanin concentration in the chloroplast. Photosynth Res. 1996;49:1411–90.
13. Pesaresi P, Scharfenberg M, Weigel M, Granlund I, Schröder WP, Finazzi G, et al. Mutants, overexpressors, and interactors of *Arabidopsis* plastocyanin isoforms: revised roles of plastocyanin in photosynthetic electron flow and thylakoid redox state. Mol Plant 2009;2(2):236–48.
14. Salah EA-G. Contribution of plastocyanin isoforms to photosynthesis and copper homeostasis in *Arabidopsis thaliana* grown at different copper regimes. Planta. 2009;229:767–79.
15. Weigel M, Varotto C, Pesaresi P, Finazzi G, Rappoport F, Salmini F, et al. Plastocyanin is indispensable for photosynthetic electron flow in *Arabidopsis thaliana*. J Biol Chem. 2003;278(33):31286–9.
16. Shosheva A, Donchev A, Dimitrov M, Zlatanov I, Toromanov G, Getov V, et al. Experimental and numerical study of the polar plastocyanin isoforms using Tyr as a probe for electrostatic similarity and dissimilarity. Biochim Biophys Acta. 2004;1698(1):67–75.
17. Dobrikova AG, Dimitrov MI, Taneva SG, Petkanchin IB. Protein-coated β-ferric hydrous oxide particles: an electrokinetic and electrooptic study. Colloid Surf B: Biointerface. 2007;56(1–2):114–20.
18. Guzzi RC, Andolfi L, Cannistraro S, Verbeet MP, Canters GW, Sporelli L. Thermal stability of wild type and disulfide dridge containing mutant of poplar plastocyanin. Biophys Chem. 2004;112(1):35–43.
19. Sanberg A, Haroson D, Göran Karlsson B. Thermal denaturation of spinach plastocyanin: effect of copper site oxidation state and molecular oxygen. Biochemistry. 2003;42(34):10301–10.
20. Guzzi R, Sportelli L, La Rosa C, Milardi D, Grasso D, Verbeet MPh, et al. A spectroscopic and calorimetric investigation on the thermal stability of the Cys3Ala/Cys26Ala azurin mutant. Biophys J. 1999;77(2):1052–63.
21. Sanberg A, Leckner J, Ying S, Schwarz F, Göran Karlsson B. Effects of metal ligation and oxygen on the reversibility of the thermal denaturation of *Pseudomonas aeruginosa* azurin. Biochemistry. 2002;41(3):1060–9.
22. Feio M, Navarro J, Teixeira M, Harrison D, Göran Karlsson B, De la Rosa M. A thermal unfolding study of plastocyanin from the thermophilic cyanobacterium *Phormidium laminosum*. Biochemistry. 2004;43(46):14784–91.
23. Gross E, Draheim J, Curtiss A, Crombie B, Scheffer A, Pan B, et al. Thermal denaturation of plastocyanin: the effect of oxidation state, reductants and anaerobicity. Arch Biochem Biophys. 1992;298:413–19.

24. Privalov PL. Small globular proteins. Adv Protein Chem. 1979;33:167–241.
25. Privalov PL, Khechinashvili NN. A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J Mol Biol. 1974;86(3):665–84.
26. Privalov PL. Thermodynamic bases of the stability of protein structure. Thermochim Acta. 1990;163:33–46.
27. Pace NC, Grimsley GR, Thomas ST, Makhatadze GI. Heat capacity change for ribonuclease A folding. Protein Sci. 1999; 8(7):1500–4.
28. Loladze VV, Ermolenko DN, Makhatadze GI. Heat capacity changes upon burial of polar and nonpolar groups in proteins. Protein Sci. 2001;10(7):1343–52.
29. Murphy KP, Gill SJ. Solid model compounds and the thermodynamics of protein unfolding. J Mol Biol. 1991;22(3):2699–709.
30. Murphy KP, Freire E. Thermodynamics of structural stability and cooperative folding behavior in proteins. Adv Protein Chem. 1992;43:313–61.
31. Makhatadze GI, Privalov PL. Energetics of protein structure. Adv Protein Chem. 1995;47:307–425.
32. Makhatadze GI, Lopez MM, Richardson JM III, Thomas ST. Anion binding to the ubiquitin molecule. Protein Sci. 1998;7(3): 689–97.
33. Spolar RS, Livingstone JR, Record MT Jr. Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. Biochemistry. 1992; 31(16):3947–55.
34. Milardi D, La Rosa C, Grasso D, Guzzi R, Sporelli L, Fini C. Thermodynamics and kinetics of the thermal unfolding of plastocyanin. Eur Biophys J. 1998;27(3):273–82.
35. Kachalova GS, Bourenkov GP, Bartunik HD, Dimitrov MI, Donchev AA, Shosheva ACh. Crystal structure of poplar plastocyanin b. Acta Crystallogr. 2002;A58(Supplement):C303.
36. Sturtevant J. Biochemical applications of differential scanning calorimetry. Ann Rev Phys Chem. 1987;38:463–512.
37. Jelesarov I, Bosshard HR. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. J Mol Recognit. 1999;12(1):3–18.