Some Physicochemical Peculiarities of Poplar Plastocyanins *a* and *b*

Petya K. Christova[a], Anthony A. Donchev[b], Alexandra C. Shosheva[b], Vladimir I. Getov[b], and Mitko I. Dimitrov[b,*]

[a] Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia-1113, Bulgaria
[b] Institute of Biophysics, Bulgarian Academy of Sciences, Sofia-1113, Bulgaria.
Fax: +3 59 29 71 24 93. E-mail: mitkod@bio21.bas.bg

* Author for correspondence and reprint requests

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The redox potentials of poplar plastocyanins *a* and *b* (PC*a*, PC*b*) were determined by spectrophotometric titrations of their reduced forms with $[Fe(CN)_6]^{3-}$. It was found that the two isoforms have the following millimolar extinction coefficients ε_{597}, equilibrium constants K_{eq} of one-electron exchange with $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$, and standard electron potentials $E^{0'}$:

PC*a*: ε_{597} = (4.72 ± 0.08) mM^{-1} cm^{-1}, K_{eq} = 0.133 ± 0.009, $E^{0'}$ = (354 ± 11) mV;
PC*b*: ε_{597} = (5.23 ± 0.16) mM^{-1} cm^{-1}, K_{eq} = 0.175 ± 0.010, $E^{0'}$ = (363 ± 12) mV.

The pH dependence of the redox potential of PC*b* was studied too. It was found, that the value of $E^{0'}$ for PC*b* is constant in the pH range 6.5–9.5, but decreases in the range 4.8–6.5. On the whole, the dependence resembles that of PC from some well-known plant species, including poplar PC*a*. The changes of $E^{0'}$ in the pH-dependent region for poplar PC*b*, however, are smaller and are 13 mV per pH unit, whereas in the other well-known plant species the changes are about 50–60 mV per pH unit. It has been assumed that the weaker pH dependence of $E^{0'}$ of PC*b* accounts for some structural differences between PC*a* and PC*b*.

Key words: Plastocyanin, Dimorphism, Photosynthesis

Introduction

In the schemes of the photosynthetic chain plastocyanin (PC) is still presented as a homogeneous protein with one polypeptide chain (Allen, 2002). However, in 1987 our studies revealed that poplar (*Populus nigra* var. *italica*) PC represents an 1:1 mixture of two isoplastocyanins; we have designated them as PC*a* and PC*b* (Dimitrov *et al*., 1987). Primary structure analysis revealed that PC*a* represents the well-known biopolymer up to now studied in detail. PC*b* has proved to be a new isoform with different primary and three-dimensional structures compared to PC*a.* A similar PC dimorphism was further established in other higher plant species, such as parsley (Dimitrov *et al*., 1990), tobacco (Dimitrov *et al*., 1993), soybean (Burkey *et al*., 1996), *Arabidopsis thaliana* (Kieselbach *et al*., 2000), rice and moss (Pesaresi *et al*., 2009, and references therein). The primary structures of parsley and tobacco PC*a* and PC*b* have successfully been determined (Dimitrov *et al.,* 1990, 1993). The registered twin PC dimorphism in tobacco (Dimitrov *et al*., 1993) reflects its amphidiploid pattern (Gray *et al*., 1974; Last and Gray, 1990). This result supports the wide spreading of PC dimorphism in higher plants. In many cyanobacteria and algae, cytochrome c_6 transfers electrons between the cytochrome *bf* complex and photosystem I, replacing plastocyanin when copper is deficient (Hervás *et al.*, 2003).

Additional experimental and numerical studies revealed that poplar PC*a* and PC*b* possess some different physicochemical characteristics (Taneva *et al*., 1999, 2000; Shosheva *et al*., 2004, 2005; Toromanov *et al*., 2006; Dobrikova *et al*., 2007) including different crystal shapes and three-dimensional structures (Dimitrov *et al*., 2000, 2002; Kachalova *et al*., 2002). All these analyses could be considered as attempts to elucidate the nature of PC dimorphism. In some of those experiments the redox midpoint potentials of PC*a* and PC*b* have been potentiometrically estimated (Taneva *et al.*, 1999). The pH dependence of the poplar PC*a* redox potential has been analyzed (Freeman and Guss, 2001). However, such data about PC*b* is still absent in the literature.

The ultimate goal of the present work was to analyze and compare the redox potentials of PC*a* and PC*b* by the equilibrium constant of an one-electron exchange with $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ as

well as to study the pH dependence of the PC*b* redox potential and the extinction coefficients of both isoplastocyanins because of their functional significance.

Material and Methods

Preparation of PCa and PCb

PC*a* and PC*b* were isolated and purified from poplar (*Populus nigra* var. *italica*) as described elsewhere (Dimitrov *et al.*, 1987).

Determination of the redox potentials of PCa and PCb

The redox potentials of PC*a* and PC*b* were determined by spectrophotometric titrations of their reduced forms with $[Fe(CN)_6]^{3-}$. The two isoproteins were fully oxidized in an excess of $K_3[Fe(CN)_6]$. Their transition into a reduced state was carried out by an excess of Na-ascorbate. The excess amounts of the reductant or oxidant were removed by gel-filtration on a Sephadex G-25 column. The column was equilibrated with 5 mM tris(hydroxymethyl)aminomethane (Tris)/KH_2PO_4 buffer, pH 6.8. The protein concentration was determined by the absorption of protein oxidized with $K_3[Fe(CN)_6]$ at 597 nm.

The oxidant $K_3[Fe(CN)_6]$ and reductant $K_4[Fe(CN)_6]$ (Jamssen Chimica, Belgium) were dissolved in deionized water and stored in small flasks, protected from the light. The titrating agent was added to the protein solution in a photometric cuvette in aliquots of 4 μl from three different concentrations of the titrating reagent stoke solutions: 0.5 mM – for titration in the initial range of the curve, 1 mM – for the middle range, and 10 mM – for the final range. After gentle stirring a spectrum in the range of 360–650 nm was registered. At the working PC concentration of $(1.5–2.0) \cdot 10^{-5}$ M such aliquots of the oxidant ensured a minimal increase of the sample volume.

Spectra were recorded on a double beam spectrophotometer Specord UV-Vis, as the cuvette chamber was thermostated and supplied with a magnetic stirrer.

For the determination of the pH dependence of the redox potential, two buffer solutions were used: (a) 5 mM Tris/KH_2PO_4 in the pH range 5.5–9.5 and (b) Na-acetate for the pH range 4.5–5.5. The reduced PC could undergo 45% oxidation by addition of a mixture of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ in a suitable ratio (Pettigrew *et al.*, 1983). In this case, the reductant and the oxidant were in the ratio 7:1. The pH dependence was completed from three different curves of the oxidation process, corresponding to the pH intervals 7.0–9.5, 5.5–7.0 and 4.5–5. The pH value of the solutions was adjusted by 0.1 M KOH, 0.1 M H_3PO_3 and 0.1 M CH_3COOH, respectively.

Calculation of the millimolar extinction coefficients (ε_{597}) at 597 nm, the equilibrium electron exchange constants (K_{eq}), and the standard redox potentials ($E^{0'}$) of PC*a* and PC*b*.

For this purpose, the approach of Goldberg and Pecht (1976) and Nernst's equation (Dawes, 1956) were used.

Results and Discussion

The redox potentials of PC*a* and PC*b* were determined by means of the equilibrium constant of the one-electron exchange of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$. The hexacyanoferrate is an appropriate redox indicator (Dawes, 1956), because:

(i) The value of the redox potential of the ferro-/ferricyanide system is close to that of PC (418 mV).

(ii) The spectral (Cohen and Plane, 1957) and the thermodynamic (Hanania *et al.*, 1967; O'Reilly, 1973) properties of this redox pair are well studied.

The reaction between PC and $K_3[Fe(CN)_6]$ might be presented as follows:

$$PC(Cu^+) + Fe^{3+} \leftrightarrow PC(Cu^{2+}) + Fe^{2+}, \qquad (1)$$

where $PC(Cu^+)$ and Fe^{2+} are the reduced forms of PC and of Fe in cyanoferrate, whereas $PC(Cu^{2+})$ and Fe^{3+} are the oxidized forms.

The equilibrium constant is described as

$$K_{eq} = \frac{[PC(Cu^+)][Fe^{3+}]}{[PC(Cu^{2+})][Fe^{2+}]}. \qquad (2)$$

K_{eq} was determined by the approach of Goldberg and Pecht (1976). The method allows the simultaneous calculation of K_{eq} and the absolute value of the extinction coefficient by photometric titration of $PC(Cu^+)$ with the oxidant $K_3[Fe(CN)_6]$. For that purpose, (2) is transformed to

$$\frac{K_{eq}[PC(Cu^{2+})]}{[PC(Cu^+)]}[Fe^{2+}] = [Fe^{3+}] \qquad (3)$$

and

$$\frac{K_{eq}\,[PC(Cu^{2+})]}{[PC(Cu^{+})]}\,[Fe^{2+}] + [Fe^{2+}] = [Fe]_t, \quad (3a)$$

where $[Fe]_t$ is the total concentration of the Fe ions in the solution which in this case is equal to the added amount of $K_3[Fe(CN)_6]$. The concentrations of both PC redox forms, oxidized $[PC(Cu^{2+})]$ and reduced $[PC(Cu^{+})]$, are determined by the absorption at λ = 597 nm:

$$[PC(Cu^{2+})] = A_{597}/\varepsilon_{597},$$

$$[PC(Cu^{+})] = (A^0_{597} - A_{597})/\varepsilon_{597},$$

where A^0_{597} is the absorption of the fully oxidized protein and ε_{597} is the millimolar extinction coefficient at 597 nm. At one-electron exchange from Cu^+ to Fe^{3+} the equilibrium concentrations of the reaction products are equal, *i.e.* $[PC(Cu^{2+})] = [Fe^{2+}]$. The respective replacements in (3a) result in

$$\frac{K_{eq}}{\varepsilon_{597}} \cdot \left[\frac{A_{597}}{A^0_{597} - A_{597}}\right] + \frac{1}{\varepsilon_{597}} = \frac{[Fe]_t}{A_{597}}. \quad (4)$$

The values of $[Fe]_t$, A^0_{597} and A_{597} are experimentally measurable quantities. Fig. 1 shows the course of the spectral curves obtained by photometric titration of PC*b*(Cu^+) with $K_3[Fe(CN)_6]$. The spectra at 597 nm are recorded using the transmittance mode in the scale 80–100%. The absorption intensity A_{597} is proportional to the concentration of the product PC*b*(Cu^{2+}) (spectra 1–10). A^0_{597} is the absorption at saturated concentrations of $K_3[Fe(CN)_6]$ (spectra 11 and 12), corresponding to 100% oxidized PC. The band at 422 nm corresponds to free $K_3[Fe(CN)_6]$. The reduced form of the cyanoferrate, $[Fe(CN)_6]^{4-}$, does not absorb in the visible region of the spectrum (Cohen and Plane, 1957).

Fig. 2 shows the dependence of $[Fe]_t/A_{597}$ on $A_{597}/(A^0_{597} - A_{597})$ according to (4). The dependence represents a straight line with a slope, equal to K_{eq}/ε_{597}, and an intercept with the ordinate equal to $1/\varepsilon_{597}$. The values of ε_{597} and K_{eq} were determined by linear regression analysis of the data obtained for PC*a* and PC*b* from three independent experiments of photometric titration:

PC*a*: ε_{597} = (4.72 ± 0.08) mM^{-1} cm^{-1},
K_{eq} = 0.133 ± 0.009;

PC*b*: ε_{597} = (5.23 ± 0.16) mM^{-1} cm^{-1},
K_{eq} = 0.175 ± 0.010.

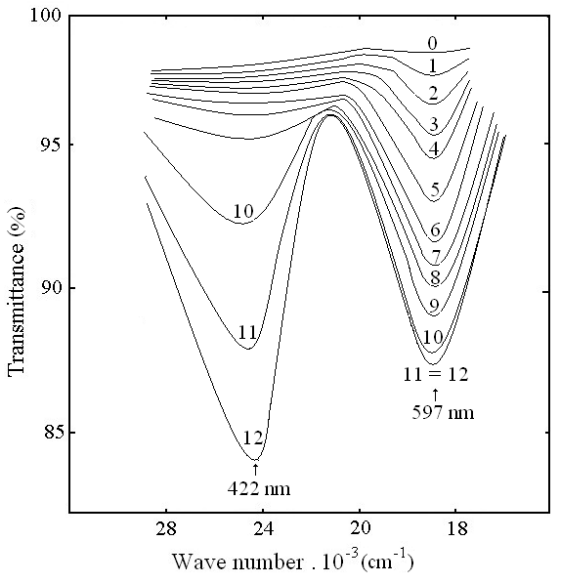

Fig. 1. Spectrophotometric titration curves of reduced PC*b* with $K_3[Fe(CN)_6]$. Protein concentration, 1.4 · 10^{-5} M; concentrations of the oxidant, 10^{-6} M to 45 · 10^{-6} M (spectra 1–12); buffer, 5 mM Tris/KH_2PO_4, pH 7.0, at 80–100% transmittant mode of operation; temperature, 20 °C.

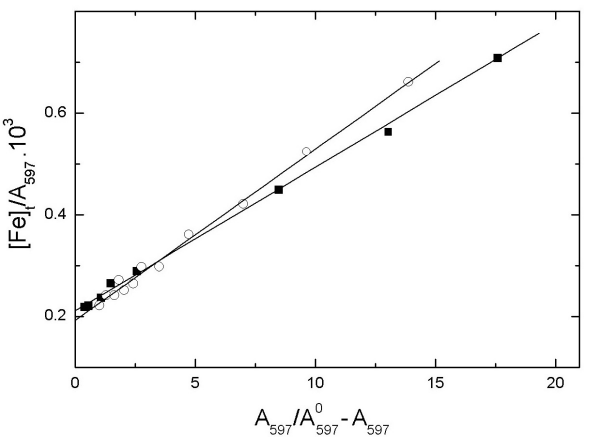

Fig. 2. Determination of the equilibrium constants of PC*a*/PC*b*-$[Fe(CN)_6]^{4-/3-}$ systems and the PC*a* (–■–■–) and PC*b* (–○–○–) extinction coefficients at 597 nm. Data from three different titrations of reduced PC*a* and PC*b* [(1.5–2.0) · 10^{-5} M] with ferricyanide at 20 °C are plotted according to (4).

The extinction coefficient of PC*a* determined above is in agreement with literature data for ε_{597} in the range 4.5–4.9 $mM^{-1}\,cm^{-1}$ for plastocyanins from different sources (Scawen *et al.*, 1975; Sykes, 1985; Gross *et al.*, 1985). The value of the extinction coefficient of PC*b* is close to that of PC*a*, taking in account the measured error.

The redox potential is connected with the electron exchange equilibrium constant by Nernst's equation (Dawes, 1956):

$$E = E^{0'} + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]}, \qquad (5)$$

where $E^{0'}$ is the standard electron potential of the redox pair at equal concentrations of the oxidized and reduced forms at pH 7.0, R = 8.314 J $M^{-1}\,K^{-1}$ is the gas constant, T is the temperature in kelvin, $F = 9.65 \cdot 10^4$ C/mol is Faraday's constant, and n is the number of the exchanged electrons.

Nernst's equation written for each of the redox pairs in the system PC-hexacyanoferrate at one-electron exchange results in

$$E = E^{0'}_{Fe} + 0.06 \log \frac{[Fe^{3+}]}{[Fe^{2+}]}, \qquad (6a)$$

$$E = E^{0'}_{Pc} + 0.06 \log \frac{[PC(Cu^{2+})]}{[PC(Cu^{+})]}. \qquad (6b)$$

If the system is in an equilibrium state, (6a) and (6b) give

$$E^{0'}_{Pc} + 0.06 \log \frac{[PC(Cu^{2+})]}{[PC(Cu^{+})]} = E^{0'}_{Fe} + 0.06 \log \frac{[Fe^{3+}]}{[Fe^{2+}]} \qquad (7)$$

or

$$E^{0'}_{Pc} = E^{0'}_{Fe} + 0.06 \log \frac{[Fe^{3+}][PC(Cu^{+})]}{[Fe^{2+}][PC(Cu^{2+})]}, \qquad (8)$$

i.e.

$$E^{0'}_{Pc} = E^{0'}_{Fe} + 0.06\, K_{eq}. \qquad (9)$$

The redox potential of PC can be calculated from (9) by the well-known value of $E^{0'}_{Fe}$ for the ferro-/ferricyanide pair and the value of K_{eq}, experimentally determined by (4) and Fig. 2. $E^{0'}_{Fe}$ strongly depends on the ionic strength of the medium, the nature of the buffer, and the pH value (O'Reilly, 1973). At 5 mM Tris/KH_2PO_4 buffer, pH 7.0, we take into account for = 408 mV. Thus, the calculated values for both PCs are as follows:

PC*a*: $E^{0'}$ = (354 ± 11) mV,

PC*b*: $E^{0'}$ = (363 ± 12) mV.

Fig. 3 shows the pH dependence of the redox potential of PC*b*. The values of K_{eq} are determined in the pH interval 4.8–9.5 at a constant concentration of $[Fe]_t = 4.4 \cdot 10^{-4}$ M, a $[Fe^{3+}]/[Fe^{2+}]$ ratio of 7:1, and a PC concentration of $2 \cdot 10^{-4}$ M. Under these conditions $[PC(Cu^{2+})]/[PC(Cu^{+})]$ = 0.45 at pH 7.0. The hexacyanoferrate excess functions as a redox buffer (Pettigrew *et al.*, 1983).

It is evident from Fig. 3 that the value of $E^{0'}$ for PC*b* is constant in the pH range 6.5–9.5 but decreases from pH 4.8 to pH 6.5. The common course of the dependence resembles that of PC from higher plants: spinach (Katoh *et al.*, 1962), *Cucurbita pepo* (Scawen *et al.*, 1975), parsley (Sykes, 1985), and poplar PC*a* (Freeman and Guss, 2001), as well as that from the alga *S. obliquus* (Freeman and Guss, 2001) and the cyanobacterium *A. variabilis* (Niles McLeod *et al.*, 1996). The changes of $E^{0'}$ in the pH-dependent region for poplar PC*b*, however, are smaller and are 13 mV per pH unit, whereas for the above-mentioned plastocyanins they are in the order of 50–60 mV per pH unit.

The changes of the redox potential of PC at low pH values could be due to the protonation of a ligand and its dissociation from the Cu atom in the active site (Sykes, 1985; Brill *et al.*, 1964). The difference between the redox potentials of the reagents appears to be a parameter, influencing

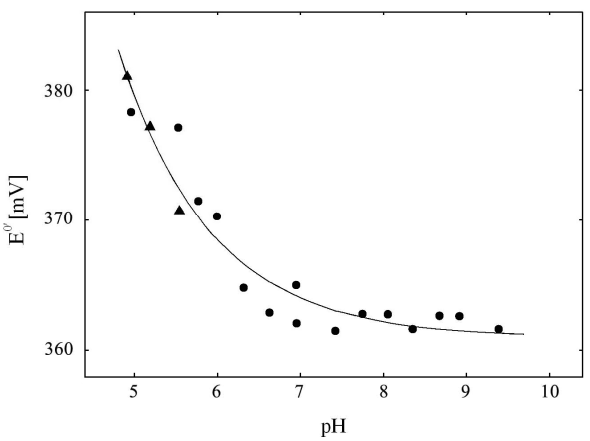

Fig. 3. pH dependence of the redox potential $E^{0'}$ of poplar PC*b* in 5 mM Tris/KH_2PO_4 buffer (–●–●–) and Na-acetate buffer (–▲–▲–).

the rate of the electron transfer. In this connection, the changes of the values of $E^{0'}$ concerning the PCs at decreasing pH value could be an original mechanism for the regulation of the electron transfer in the thylakoids (Guss *et al.*, 1986; Freeman and Guss, 2001). The comparative studies of the redox properties of PC*a* and PC*b* could undoubtedly shed light on their different functions as electron carriers.

Recently our X-ray analysis has proved that the geometry of the Cu site of poplar PC*b* is very close to that of poplar PC*a* in the pH range 6–8 (Dimitrov *et al.*, 2000, 2002; Kachalova *et al.*, 2002). That is why the small differences in the redox potentials measured by potentiometric titration (Taneva *et al.*, 1999) and by the ferro-/ferricyanide system here are reasonable. At pH 4.0, however, some differences occur in the Cu ligand geometry in the reduced state. In PC*b*, one sulfate anion was found to make a hydrogen bond to Nε_2-His 87 thus preventing the imidazole ring from swinging away as it does in PC*a* (Kachalova *et al.*, 2002). This structural difference could explain the smaller pH dependence of the redox potential of PC*b*.

In contrast to the conservatism of the PC*a*/PC*b* Cu site geometry, the 12th amino acid residue differences in their sequences (Dimitrov *et al.*, 1987) result in considerable conformational distinction. The structural models of the two isoproteins exhibit root mean square deviations of the structure exceeding by an order of magnitude those between the oxidized and reduced structures of PC*a* (Kachalova *et al.*, 2002). The most evident differences between the structures of PC*a* and PC*b* are obtained in the segment of amino acid residues 34 to 65. In this area the two functionally important acidic clusters (Asp 42–Glu 43–Asp 44 and Glu 59–Glu 60–Asp 61) with supposed different positions of their side chains between the two isoproteins are located (Shosheva *et al.*, 2004).

The 12th amino acid residue differences between PC*a* and PC*b* concern more radically the structural backbone of their globules than the geometry of the Cu site. This suggests specificities of the protein-protein interactions with their natural redox partners; that is an important part of the PC function.

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