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CRYSTAL FORMS OF POPLAR PLASTOCYANINS *a* AND *b*

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Summary: For the first time, the crystal form of oxidized poplar plastocyanin *b* (PC*b*) was defined. PC*b* crystals grew always as needles, forming druses. At protein concentration of 1%, pH 6.0 (0.1 M Na-phosphate buffer), 63% $(NH_4)_2SO_4$ and temperature of crystallization 4°C, the needle-shaped crystals were bigger in size and grew for 10-30 days. PC*b* crystals, obtained by the method of seeding, kept their forms but enlarged their size, allowing X-ray analysis of PC*b* with a resolution below 1.8 Å. The best plastocyanin *a* (PC*a*) crystals were obtained at 4°C during 10-14 days incubation time in 63% $(NH_4)_2SO_4$ and 1.5% protein concentration in 0.1 M Na-phosphate buffer (pH 6.0). The intensively colored blue mono-crystal prisms were ortho-rhombic - $P2_12_12_1$ space group with the following parameters of the elementary cell: a = 29.61 Å, b = 46.86 Å and c = 57.60 Å. The size and quality of these crystals permitted an X-ray analysis at a very high resolution below 1 Å. The different habits of PC*a* and PC*b* crystals represent an evident sign for differences in the three-dimensional structures of the two iso-PC's.

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Abbreviations: PC*a* – plastocyanin *a*; PC*b* – plastocyanin *b.*

INTRODUCTION

During the last three decades of the 20th century a series of investigations of the structure, function and genesis of plastocyanin (PC) have been carried out. They have demonstrated that this protein is a very important and crucial element of the photosynthetic chain. At present PC is one of the most investigated "blue" protein in structural and functional aspects. It is classified as "blue" or "Type-1" copper protein according to its UV/Vis and EPR spectral characteristics (Malkin and Malström, 1970) or as "cupredoxin" because of the availability of Cu in its molecule and its function as a redox component in the photosynthetic chain (Adman, 1985). PC is identified in some cyanobacteria (Aitken, 1975; Varley et al., 1995; Briggs et al., 1990; Suzuki et al., 1999; Onodera et al., 2006), many green alga species (Katoh, 1960; Simpson et al., 1986; Merchant et al.,

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1990; Yoshizaki et al., 1989; Onodera et al., 2006) and many higher plants (Katoh et al., 1961; Plesničar and Bendall, 1970; Rother et al., 1986; Nielsen and Gausing, 1987; Ramshaw and Felton, 1982; Kohzuma et al., 1999; Onodera et al., 2006).

The primary structure of PC has been comprehensively studied. Fifty primary structures of PC in one gymnospermous species (Ginkgo biloba), 11 cyanobacteria, 7 green alga, one moss, one fern and 24 higher plant species have been analyzed (Onodera et al., 2006). These analyses provided a three-dimensional structure of a globular type with a form of a flattened cylinder that is peculiar for the well-known monomeric cupredoxins. As a typical cupredoxin, PC also exhibits its conservative "sandwich" structure of β-type (Guss and Freeman, 1983; Baker, 1988; Chothia and Lesk, 1982), formed by seven parallel and anti-parallel polypeptide chains, one variable α-helical region in the "southern" end of the globule and a Cu site in the "northern" end on the top of one β-structure (Gough and Chothia, 2004). The two β-structures (universal for all cupredoxins) are situated vis-a-vis: the first one with domens G, F and D and the second one with domens A, C and E.

In the scheme of the photosynthetic chain PC is still present as a homogeneous protein of one polypeptide chain containing about 99 amino acid residues (MW ≈ 10500 Da).

In 1987, however, we found out that poplar PC represents a mixture of two iso-PC's - PC*a* and PC*b* (Dimitrov et al., 1987). Primary structure analysis revealed that PC*a* appeared to be the well known in the literature biopolymer with established structure and function. PC*b* was found to be new, yet unknown in science and structurally different from PC*a* iso-PC (Dimitrov et al., 1987). Similar PC dimorphism was established in parsley (Dimitrov et al., 1990), tobacco (Dimitrov et al., 1993), soybean (Burkey et al.,1996), *A. thaliana* (Kieselbach et al., 2000), lady fern – A.filix-femina (Toromanov et al., 2005) and the moss *Physcomitrella patens* (Rensing et al., 2008). The twin PC dimorphism of the amphi-diploide tobacco is based on a hypothesis for wide-spreading of this phenomenon in higher plants (Dimitrov et al., 1993). The data of Kieselbach et al., (2000) showed the genetic origin of the two PC isoforms.

The evidence for the wide-spreading of PC dimorphism in higher plants naturally brings up the question about the self dependent role of PC*a* and PC*b* in the photosynthetic electron transport chain. A number of comparative physico-chemical characteristics of PC*a* and PC*b* have been determined (Taneva et al., 1999, 2000; Shosheva et al., 2004, 2005; Dobrikova A et al., 2007, Dimitrov et al., 2008; 2011, Getov et al., 2009, Christova et al., 2009a,b). They additionally contribute to the knowledge about the existence of two simultaneously acting electron-transfer proteins PC*a* and PC*b*. In order to clarify the structural basis of the differences in the physico-chemical characteristics of PC*a* and PC*b*, with regard to their functions, comparative crystal structure analyses of the two iso-proteins was carried out.

Firstly, the structure-function investigations of the proteins need a good knowledge of the specific three-dimensional organization of their

polypeptide chains. In higher plants, the first protein crystals, suitable for X-ray analysis, have been successfully prepared in case of PC from poplar (*Populus nigra* var. *Italica*) (Chapman et al., 1977). The consecutive X-ray analysis has allowed determination of its three-dimensional structure with a resolution of 2.7 Å (Colman et al., 1978) and later up to 1.33 Å (Guss et al., 1992). Regarding the PC dimorphism, however, a question arises whether the above mentioned crystals and the three-dimensional structure determined by them belong to PC*a* or toPC*b*.

In the present work, we have shown for the first time the crystal form of poplar PC*b***.** We also analyzed in comparative aspect the crystal forms of PC*a* and PC*b* which allowed a X-ray analysis of a high resolution.

MATHERIALS AND METHODS

Preparation of PC*a* and PC*b*

PC*a* and PC*b* were prepared from poplar (*Populus nigra*, var. *italica*) as described in Dimitrov et al. (1987, 2010).

Crystallization of oxidized PC*a* and PC*b*

PC*a* и PC*b* crystals were prepared by the "vapour diffusion" method of the "hanging drop", using $(NH_4)_2SO_4$ as a precipitating reagent. PC*a* crystal forms were prepared in conditions described elsewhere (Chapman et al., 1977). For PC*b* crystallization different pH and temperature conditions were applied. The method of "seeding" of a single needle-shaped PC*b* crystal in saturated protein drops resulted in the growth of more bulky crystals.

RESULTS AND DISCUSSION

The first PC crystallized in a form suitable for X-ray analysis is that of poplar (*Populus nigra* var. *italica*) (Chapman et al., 1977). The crystals were prepared by the vapour-diffusion method of the "sitting drop" with $(NH_4)_2SO_4$ as a precipitating reagent: 5-10 mg ml^{-1} protein in 0.1 M Na-phosphate buffer, pH 6.0 and gradually increasing $(NH_4)_2SO_4$ concentrations from 1.6 to 2.6 M. Dark-blue crystals in the form of rhombic prism grew. Their elementary cell was ortho-rhombic, with parameters a = 29.6 Å, b = 46.9 Å, c = 57.6 Å, V = 80 030 $Å^3$. The crystals contained 36% (v/v) solvent. The asymmetrical cell included one protein molecule plus a solvent. The crystals were considerably robust and able to grow up to 2 mm thus allowing a large number of crystal-chemical and crystal-physical experiments.

The other PC's with published crystal structures (Freeman and Guss, 2001) were crystallized by the "vapour-diffusion" method of the "hanging drop" using $(NH_4)_2SO_4$ and PEG-4000/6000 as precipitating reagents and Na-phosphate, Na-acetate, glycine or HEPES as buffers. "Seeding" (additional crystal growth) and additives as $MgCl_2$, $CaCl_2$ and $CoCl_2$ as well as mutation of one or more surface amino acid residues (Xue et al., 1998; Romero et al., 1998) were used in some crystallizations.

Both the "sitting drop" and "hanging drop" methods followed the principle of diffusion saturation of the protein solution by the vapors of the precipitating reagent with an appropriate concentration. As a result, some concentration of the protein solution took place thus creating

conditions for crystallization (Chapman et al., 1977). The precipitating solution was situated on the bottom of the boxes (Linbro or "on the banches"). The drop ("sitting" or "hanging") consisted of 5 or 10 µl protein solution and the same volume of precipitating solution. Following the crystallization conditions and some period of time later (from 24 h to a month) the precipitating reagent diffusion resulted in equilibrium concentrations in the well-closed space. Along with that, the protein solution turned into a meta-stable phase of the protein/reagent molecules ratio thus increasing the probability for creating crystal nuclei. This process is multi-phase and depends on protein and reagent concentrations as well as on the temperature, pH and the purity of the solutions. The optimal conditions for crystallization of every protein can be found by multiple combinations of these parameters. The determination of these conditions was the main purpose of the experimental attempts for the preparation of PC*a* and PC*b* crystals.

Two experimental attempts were carried out for preparation of PC*a* and PC*b* crystals using the crystallizing reagent $(NH_4)_2SO_4$ (63%). Firstly, the protein concentration was below 1% in the drop. 0.1 M Na-phosphate buffer (for pH 5.5) and 0.1 M Tris.HCl (for pH 7.0, 7.5, 8.0 and 8.5) at 18°C were used. At these conditions the crystals grew for about 24-48 h. It was found that PC*a* and PC*b* exhibited rather different crystal shapes. PC*a* crystals grew as ortho-rhombic prism whereas those of PC*b* were very thin needles combined in druses. These two crystal types, however, are qualitatively unsuitable for X-ray analysis. The former were crystal conglomerates (Fig. 1) while the small sizes of the needle-shaped PC*b* crystals (Fig. 2) did not allow X-ray analysis with good resolution.

The second experimental attempt included the use of different concentrations of both $(NH_4)_2SO_4$ (63%, 50% and 35%) and proteins (up to 1.5% in a drop) as well as different pH values (from 5.0 to 7.5) and temperatures (18°C and 4°C).

Figure 1. Crystal conglomerate of oxidized PC*a* from poplar (*P. nigra* var. *italica*). Crystallization conditions: precipitating reagent $(NH_4)_2SO_4$ (63%); protein concentration in the drop below 1%; pH 5.5; temperature 18°C; incubation time 48 h.

Figure 2. Crystal druses of oxidized PC*b* from poplar (*P. nigra* var. *italica*). Crystallization conditions: precipitating reagent $(NH_4)_2SO_4$ (63%); protein concentration in the drop below 1%; pH 5.5; temperature 18°C; incubation time 48 h.

The best PC*a* crystals were obtained at 4°C after 10-14 days incubation time with concentrations of $(NH_4)_2SO_4$ and protein being 63% and 1.5%, respectively in 0.1 M Na-phosphate buffer (pH 6.0). The intensively colored blue mono-crystal prisms were ortho-rhombic - $P2_12_12_1$ space group with the following parameters of the elementary cell: a = 29.61 Å, b = 46.86 Å and c = 57.60 Å (Fig. 3). The size of these crystals permitted an X-ray analysis with a very high resolution below 1 Å. PC*b* crystals, however, always grew as needles, forming druses. At a protein

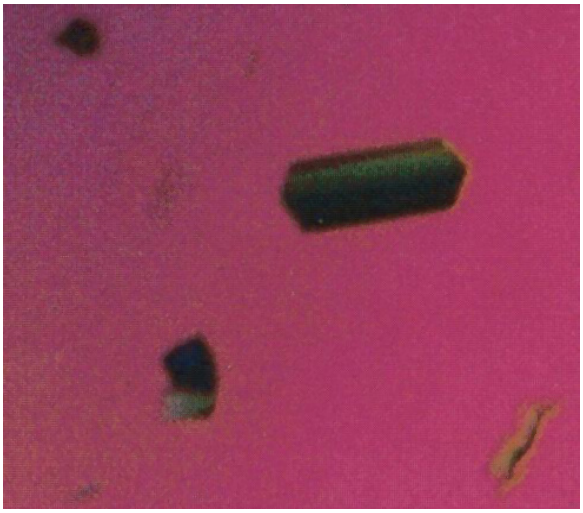

Figure 3. Crystal forms of oxidized PC*a* from poplar (*P. nigra* var. *italica*). Crystallization conditions: precipitating reagent $(NH_4)_2SO_4$ (63%); protein concentration in the drop 1.5%; pH 6.0; temperature 4°C; incubation time 10-14 days.

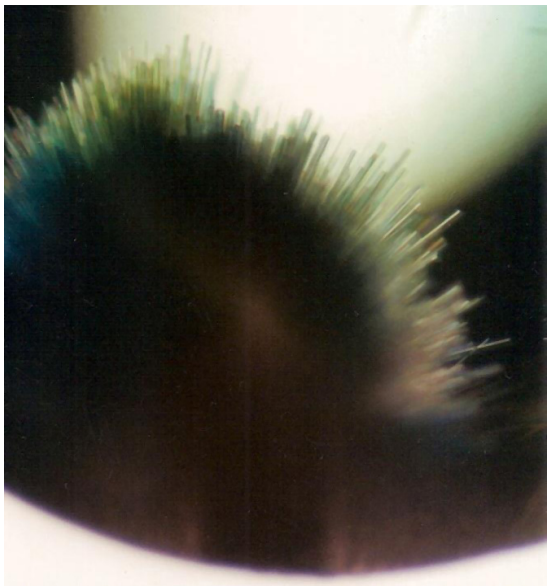

Figure 4. Crystal druses of oxidized PC*b* from poplar (*P. nigra* var. *italica*). Crystallization conditions: precipitating reagent $(NH_4)_2SO_4$ (63%); protein concentration in the drop 1%; pH 6.0; temperature 4°C; incubation time 10-30 days.

concentration of 1%, pH 6.0 (0.1 M Na-phosphate buffer), $(NH_4)_2SO_4$ – 63% and temperature of crystallization 4°C, the needle-shaped crystals were bigger in size and grew for 10-30 days (Fig. 4).

Additional attempts for preparation of PC*b* crystals with possibly bigger sizes were carried out. For that purpose, "seeding" of small crystals was used. The obtained crystals kept their forms but the

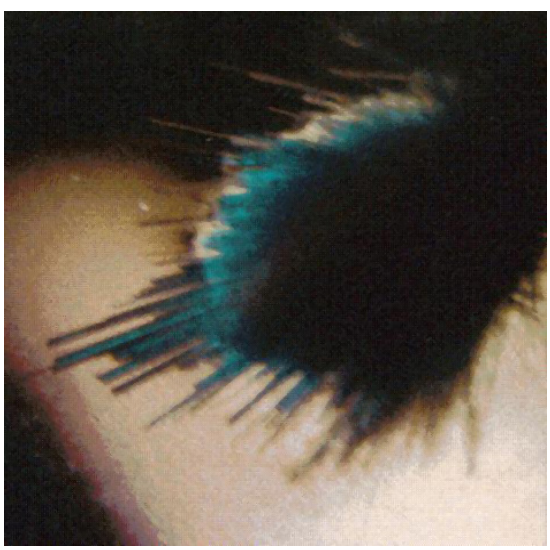

Fig.5. Enlarged crystals in the druses of oxidized PC*b* from poplar (*P. nigra* var. *italica*). Crystallization conditions "seeding": precipitating reagent 2.4 M $(NH_4)_2SO_4$; protein concentration in the drop 1.5%; pH 6.0; temperature 4°C; incubation time 10-30 days.

enlarged size allowed X-ray analysis of PC*b* with a resolution below 1.8 Å (Fig. 5).

The different habits of PC*a* and PC*b* crystals represent an evident sign for differences in the three-dimensional structures of the two iso-PC's. On the other hand, both the ortho-rhombic habit and the elementary cell parameters of PC*a* characterize the poplar PC crystals, which were studied earlier (Chapman et al., 1977; Colman et al., 1978; Guss et al., 1992). Obviously, that previously investigated crystal form concerned PC*a.*

Nowadays, poplar PC*b* is still considered the first and the only one PC *b*-isoform with prepared crystals suitable for X-ray studies. This fact gives a unique possibility for PC*b* three-dimensional structural analysis by X-ray. The knowledge of the PC*b* folding is a crucial stage in the way to clarification of the PC*b* physiological significance.

It is known that the discovery of PC was a surprise for its discoverer (Katoh, 1960). Besides, the investigation of this cupredoxin followed an intricate path of contradictory opinions and results (Katoh, 1995, 2003). The discovery and the comprehensive study of PC*b* is a possible continuation of this path.

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