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Functional characterization of the evolutionarily divergent fern plastocyanin

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Plastocyanin (Pc) is a soluble copper protein that transfers electrons from cytochrome b_6f to photosystem I (PSI), two protein complexes that are localized in the thylakoid membranes in chloroplasts. The surface electrostatic potential distribution of Pc plays a key role in complex formation with the membrane-bound partners. It is practically identical for Pcs from plants and green algae, but is quite different for Pc from ferns. Here we report on a laser flash kinetic analysis of PSI reduction by Pc from various eukaryotic and prokaryotic organisms. The reaction of fern Pc with fern PSI fits a two-step kinetic model, consisting of complex formation and electron transfer, whereas other plant systems exhibit a mechanism that requires an additional intracomplex rearrangement step. The fern Pc interacts inefficiently with spinach PSI, showing no detectable complex formation. This can be explained by assuming that the unusual surface charge distribution of fern Pc impairs the interaction. Fern PSI behaves in a similar way as spinach PSI in reaction with other Pcs. The reactivity of fern Pc towards several soluble *c*-type cytochromes, including cytochrome *f*, has been analysed by flavin-photosensitized laser flash photolysis, demonstrating that the specific surface motifs for the interaction with cytochrome *f* are conserved in fern Pc.

Keywords: *Dryopteris*; fern; *Nephrolepsis*; photosystem I; plastocyanin.

Plastocyanin (Pc) is a small copper-containing redox protein (molecular mass $\approx$ 10.5 kDa) that functions as a mobile electron carrier between the two membrane-embedded complexes cytochrome (Cyt) b_6f and photosystem I (PSI) in oxygenic photosynthesis (see [1,2] for reviews). Pc can be acidic in higher plants and green algae (pI $\approx$ 4), almost neutral in cyanobacteria such as *Synechocystis* or *Phormidium* (pI $\approx$ 6) or basic in other cyanobacteria such as *Anabaena* (pI $\approx$ 9) [3]. Acidic Pc exhibits two negatively charged surface regions formed by amino acids at positions 42–44 and 59–61, which are highly conserved. In neutral and basic Pc, however, such acidic residues are replaced by either neutral or positively charged amino acids [4,5].

At present, the high-resolution structures of Pcs from many different organisms are available [4,6–8]. The comparison of all these crystal and solution structures indicates that all Pcs possess an almost identical global fold, with the polypeptide chain forming eight β strands connected by seven loops along with a small α-helix [7,9]. The 'east side', comprising charged residues 42–44 and 59–61, has been proposed to be involved in electrostatic interactions with both PSI and Cyt *f*, but the solvent-exposed edge of His87, located in the hydrophobic patch at the so-called 'northern side' of the protein, constitutes the electron transfer pathway to PSI and from Cyt *f* [1,10,11].

The crystal structure of a novel Pc from the fern *Dryopteris crassirhizoma* has been solved [12]. This protein presents an acidic patch extended towards the amino-terminal end as well as other changes in the 42–45 positions, resulting in very distinct electrostatic properties as compared to typical eukaryotic Pcs, while maintaining the same global structure. Thus, in *Dryopteris* Pc the acidic region is relocated and surrounds the hydrophobic patch, the large dipole moment protruding through the north side surface [12].

Previous studies have shown that fern Pc shows similar reactivity towards metal complexes and similar electron self-exchange reaction rates to those of other plant Pcs [12,13], and a study on electron transfer between zinc-substituted Cyt *c* and fern Pc has shown that the reactivity towards this nonphysiological redox partner differs significantly from other plant Pcs [14]. However, no functional analysis has been reported up to now on the reaction of fern Pc with its physiological redox partners, Cyt b_6f and PSI complexes.

The reaction mechanism of PSI reduction has been analysed extensively in a wide variety of organisms from an evolutionary point of view, thereby yielding a hierarchy of kinetic models with a significant increase in efficiency

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Abbreviations: Cyt, cytochrome; dRf, dRfH, deazariboflavin; k_2, second order rate constant; K_A, equilibrium constant for complex formation; k_{et}, electron transfer rate constant; k_{obs}, observed pseudo-first order rate constant; k_{sat}, first-order rate constant at saturating donor concentration; Pc, plastocyanin; PSI, photosystem I; β-DM, β-dodecyl maltoside.

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[1,3,15]. PSI reduction by the donor proteins Cyt c_6 and Pc, isolated from different sources, can thus follow either an oriented collisional mechanism (type I), a mechanism requiring complex formation (type II), or complex formation with rearrangement of the interface to properly orient the redox centres to allow an efficient, fast electron transfer (type III).

The type I and II models are found in some cyanobacteria, whereas the type III model is observed mostly in eukaryotic organisms, in which the intermediate complex is first formed by electrostatic attractions and the further reorientation mainly involves hydrophobic interactions. The kinetics of PSI reduction are typically monophasic for the type I and II reaction mechanisms, but biphasic for the type III model. The proposal has been made that the appearance in evolution of a fast kinetic phase in the Pc/PSI system of higher plants would have involved structural modifications in both the donor protein and PSI [5,15]. Ferns are a division of the seedless vascular plants that are among the oldest terrestrial plant organisms known, and taking into account the peculiar structure of its Pc, ferns are an interesting case study to complete the evolutionary analysis of the electron donation to PSI.

In this work, we have analysed the reaction mechanism of electron transfer from Pc to PSI in ferns. The reactivity of fern Pc with PSI isolated from different eukaryotic and cyanobacterial sources has also been investigated in order to extend the evolutionary insights into the process. In addition, we have analysed the reduction of fern Pc by different *c*-type Cyts – including eukaryotic turnip Cyt *f* – as a complementary way to explore the electron transfer features and surface electrostatics properties of such an unusual Pc.

Experimental procedures

Proteins isolation and purification

Pc from *Dryopteris* was obtained by heterologous expression in *Escherichia coli*. The construction of the synthetic gene, expression conditions, purification protocol and characterization of the recombinant protein will be published elsewhere. Mass and NMR of the purified recombinant Pc indicated that it was indistinguishable from the native protein.

Nephrolepsis exaltata Pc was purified as follows: 200 g fern were homogenized in 1 L 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris/HCl pH 8 supplemented with a cocktail of protease inhibitors in a Waring blender for 5 min at medium speed. The resulting extract was filtered through four layers of cheesecloth. The thylakoidal membranes were sonicated in the presence of 0.1 M NaCl to remove bound Pc and then centrifuged at 12 000 ***g*** for 15 min. Solid ammonium sulphate was added to the supernatant to 60% saturation. Thereafter, the procedure was as described for the purification of *Synechocystis* Cyt c_{549} [16], except that fern Pc was eluted from the DEAE-cellulose column with a 0–0.3 M NaCl gradient and the protein was eluted directly with the pH gradient in the chromatofocusing step, avoiding the last salt gradient. About 3 mg of pure Pc with an absorbance ratio A_{275}/A_{590} of 1.27 were obtained. The protein concentration was determined spectrophotometrically using an absorption coefficient of 4.7 $mM^{-1}{\cdot}cm^{-1}$ at 590 nm for oxidized Pc [12]. Purification of other Pcs was carried out as described elsewhere [17–19].

Paracoccus versutus Cyt c_{550} was expressed using *Paracoccus denitrificans* as a host [20]. Cultures were grown on brain–heart infusion broth containing streptomycin (50 $\mu g{\cdot}mL^{-1}$) and spectinomycin (50 $\mu g{\cdot}mL^{-1}$) in a 5 L fermentor under vigorous agitation at 30 °C for 20 h followed by 4 h at 37 °C. The protein was purified according to Diederix *et al.* [21]. Turnip Cyt *f* and horse heart Cyt *c* were purchased from Sigma and used without further purification.

For reduction experiments, proteins were oxidized by potassium ferricyanide and then washed by several filtration–dilution cycles in an AMICON pressure cell.

Nephrolepsis PSI was prepared as follows: 200 g of fern were homogenized in 1 L 10 mM NaCl, 5 mM $MgCl_2$, 100 mM sodium ascorbate, 20 mM Tricine/KOH pH 7.5 buffer supplemented with a cocktail of protease inhibitors as stated before. The resulting extract was filtered through four layers of cheesecloth and the filtrate was centrifuged at 3000 ***g*** 1 min to remove debris. Thylakoid membranes were collected by centrifugation at 25 000 ***g*** 20 min, resuspended at 2 mg chlorophyll per mL in the homogenization buffer without ascorbate plus 20% (v/v) glycerol, and frozen. PSI particles from *Nephrolepsis* were obtained by β-dodecyl maltoside (β-DM) solubilization as follows. Thylakoidal membranes were diluted to 1 mg chlorophyll per mL with buffer D (20 mM Mes pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M D-mannitol, 20% glycerol) and solubilized for 30 min with 1.5% β-DM. The solution was centrifuged 5 min at 20 000 ***g***, and the supernatant centrifuged 20 min at 120 000 ***g*** to remove unsolubilized material. The resulting supernatant was diluted two in three with buffer A (20 mM Mes pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$) and applied to a discontinuous sucrose gradient (15, 20, 25, 40%) prepared on buffer B (buffer A + 0.5 M mannitol). After centrifugation for 20 h at 150 000 ***g***, the lower half of the only green band was collected, washed with 20 mM Tricine/KOH buffer pH 7.5, with 0.03% β-DM, and concentrated in an AMICON pressure cell. This fraction was applied to a continuous sucrose gradient (17–30%) and centrifuged as before. The lower half of the only green band was collected, washed and concentrated as before, and stored at −80 °C. The P700 content of PSI samples was calculated from the photoinduced absorbance changes at 820 nm using the absorption coefficient of 6.5 $mM^{-1}{\cdot}cm^{-1}$ determined by Mathis and Sétif [22]. Chlorophyll concentration was determined according to Arnon [23]. The chlorophyll/P700 ratio of the resulting PSI preparation was 280 : 1. Spinach, *Synechocystis* and *Anabaena* PSI were purified as previously described [15].

Amino acid sequence determination of *Nephrolepsis* Pc

Sequencing of *Nephrolepsis* Pc (Fig. 1) was performed with a Hewlett-Packard G1006A protein Sequencer system, connected on-line to a Hewlett-Packard Model 1100 HPLC system. As the amino terminus was unblocked, first, the intact protein was sequenced as far as possible. Protein sequencing of BrCN-generated peptides, and of peptides obtained by digestion with

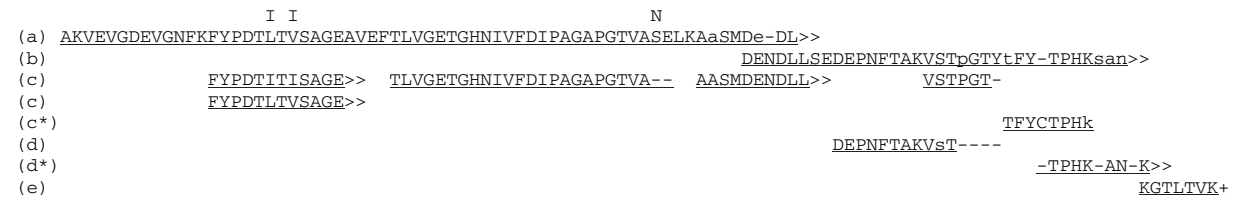

Fig. 1. Outline of sequence determination of *Nephrolepsis Pc*. Sequence results for (a) the intact protein, (b) the BrCN peptide starting after M-60, (c) selected endo-Lys-C peptides, (c*) the Cys containing endo-Lys-C peptide, after reduction and alkylation, (d) selected endo-Asp-N peptides; (d*) the peptide starting at C-87; and (e): the C-terminal BrCN-peptide. See also Experimental procedures.

endoproteinases Lys-C and Asp-N followed standard procedures (see also [24]).

Blocking of amino groups with acetic anhydride and the reduction and alkylation of cysteine residues were performed according to Amons [25,26]. The sequence starting at C-87 was obtained by treating the intact protein as follows: (1) digestion with endo-Asp-N; (2) acetylation with acetic anhydride; (3) oxidation with performic acid [27]; (4) redigestion with endo-Asp-N, now cleaving at the cysteic acid residue formed in step 3.

To isolate the small C-terminal BrCN peptide, the intact protein was cleaved by BrCN, and the mixture applied to a 2 × 10-mm reversed-phase C18 column. The small peptide was eluted at low acetonitrile concentration, and then coupled to aminoaryl-poly(vinylidene) difluoride (Millipore Inc) with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbonyl-imide hydrochloride (EDC) according to the manufacturer's instructions.

Laser flash spectroscopy

Kinetics of flash-induced absorbance changes in PSI were followed at 820 nm as described by Hervás *et al.* [28]. Unless otherwise stated, the standard reaction mixture contained, in a final volume of 0.2 mL, 20 mM buffer (Tricine/KOH pH 7.5 or Mes pH 5), 10 mM $MgCl_2$, 0.03% β-DM, an amount of PSI-enriched particles equivalent to 0.75 mg of chlorophyll per mL (0.35 mg·mL^{-1} in case of cyanobacterial PSI), 0.1 mM methyl viologen, 2 mM sodium ascorbate and Pc at the indicated concentration. All experiments were performed at 22 °C in a 1 mm path-length cuvette.

The optical set-up for kinetic experiments of intermolecular redox reactions between Cyts and Pcs has been described previously [29]. The standard reaction mixture contained, in a final volume of 1.2 mL, 5 mM potassium phosphate pH 7.0, 2 mM EDTA, 100 μM 5-dRf, and the different proteins at the indicated concentrations. Laser flash experiments were performed anaerobically at room temperature in a 1 cm path-length cuvette. Laser flash photolysis of the 5-dRf/EDTA system generated 5-dRfH, which in its turn reduced oxidized Cyt to yield the reduced species [30]. Further exponential absorbance decreases were concomitantly observed at 550 nm (554 nm for Cyt *f*) and 600 nm, which correspond to Pc reduction by Cyt. All of the kinetic experiments were performed under pseudo first-order conditions, in which the concentration of protein acceptor, either in the direct reduction by 5-dRfH, or in the interprotein electron transfer, was in large excess over the amount of dRfH or the donor protein, respectively. Deazariboflavin was a gift from G. Tollin (University of Arizona, Tucson, USA).

In all cases, kinetic data collection was as described previously [15]. Oscilloscope traces were treated as sums of several exponential components; exponential analyses were performed using the Marquardt method with the software devised by P. Sétif (CEA, Saclay, France). The estimated error in rate constants determination was 10%.

Results and Discussion

Fern Pc presents very distinct electrostatic properties as compared to other eukaryotic Pcs, with the acidic area extending into the hydrophobic patch (Fig. 2). Electrostatic forces play an important role in protein interactions and it is thus of interest to analyse the functional behaviour of fern Pc as compared to other plant Pcs. In this study, Pcs from the fern genera *Nephrolepsis* and *Dryopteris* were used. The amino acid sequence of *N. exaltata* Pc was determined and is shown in Fig. 3. A microheterogeneity was found in the sequence for three positions, 19 (Leu/Ile), 21 (Val/Ile) and 53 (Ser/Asn). At least the 19 and 21 positions are coupled, so proteins contain either Leu19 and Val21 or Ile19 and Ile21. Fern Pc sequences seem to be extremely well conserved, as illustrated by the alignment of the Pc sequences from *N. exaltata*, *D. crassirhizoma* [12] and *Polystichum longifrons* (Y. Nagai and F. Yoshizaki, Toho University, Japan, unpublished results), shown in Fig. 3. The functional analysis done in this work has shown that *Nephrolepsis* and *Dryopteris* Pcs behave in an indistinguishable manner, thus from this point fern Pc will refer to both proteins indistinctively.

The reaction mechanism of electron transfer from fern Pc to fern PSI has been analysed by laser-flash absorption spectroscopy and compared with spinach Pc. The kinetic traces of fern PSI reduction by fern Pc at pH 7.5 correspond to monophasic kinetics, whereas those with spinach Pc are better fitted to biphasic curves (Fig. 4). The amplitude of the fast phase of the latter represented up to 40% of the total amplitude, with a rate constant (k_{obs}) independent of Pc concentration (data not shown). From the k_{obs} values of this fast phase, a first-order electron transfer rate constant (k_{et}) of $\approx 3 \times 10^4$ s^{-1} can be directly estimated for the interaction between spinach Pc and fern PSI. The k_{obs} values with fern Pc and those for the slower phase with spinach Pc exhibit saturation profiles at increasing donor protein concentrations (Fig. 5, upper panel), thereby suggesting that the two metalloproteins are able to form transient complexes with

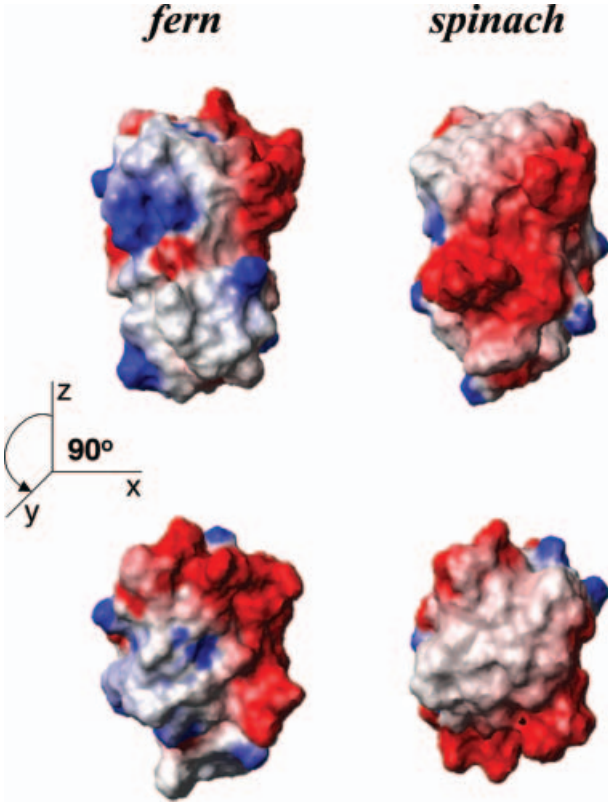

Fig. 2. Surface electrostatic potential distribution of fern (PDB entry 1KDI) and spinach (PDB entry 1AG6) Pc. The molecules are similarly oriented, with the lateral view showing the typical charged east patch of eukaryotic Pcs (upper) and the top view, obtained by rotating 90° around the horizontal x-axis as indicated, showing the standard hydrophobic patch (lower). Negatively and positively charged regions are shown in red and blue, respectively. The picture was generated with the program MOLMOL [36] at an ionic strength of 50 mM.

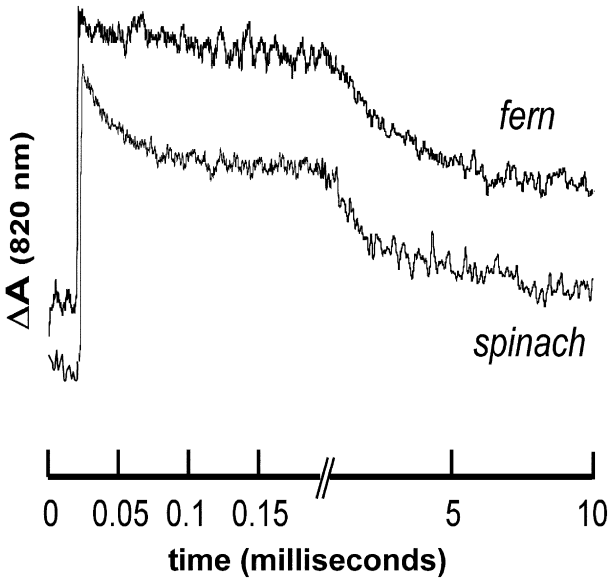

Fig. 4. Kinetic traces showing fern PSI reduction by fern and spinach Pc. Absorbance changes were recorded at 820 nm with 100 μM Pc. The kinetics were fitted to either biphasic (spinach) or monophasic (fern) curves. Other conditions were as described in Experimental procedures.

PSI. From the plots shown in Fig. 5, and applying the formalism previously developed [31], it is possible (Table 1) to estimate both K_A (equilibrium constant for complex formation) and k_{sat} (first-order rate constant at saturating Pc concentration). The fern Pc behaviour can be explained as following the two-step type II mechanism with its own PSI, involving complex formation and with k_{sat} corresponding to the further intracomplex electron transfer rate [15]. Spinach Pc, however, in which the k_{obs} values for the first initial fast phase of electron transfer does not match the values of k_{sat}, follows with fern PSI the classical three-step type III mechanism observed in other eukaryotic systems, with an additional rearrangement of redox partners within the intermediate complex prior to electron transfer [1,3,15]. From the data presented in Table 1, it seems clear that although fern PSI binds spinach and fern Pc with similar efficiency, as shown by the similar K_A values, the electron transfer step is one order of magnitude higher with spinach Pc (Table 1). Thus, whereas the kinetic constants presented here for the spinach Pc/fern PSI system are of the same order of magnitude as those observed for the spinach Pc/PSI system [15], the distance and/or orientation of the redox centers in the fern Pc/PSI complex seems to be nonoptimal for electron transfer. When checking fern PSI reactivity towards cyanobacterial Pcs, linear plots against Pc concentration were observed (data not shown), indicating the occurrence of a collisional type I mechanism with very low second-order rate constant (k_2) values for PSI reduction

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                          I I                                   N
Nephrolepsis  AKVEVGDEVGNFKFYPDTLTVSAGEAVEFTLVGETGHNIVFDIPAGAPGTVASELKAASM  60
              ***************** o*o********************************o******* 
Dryopteris    AKVEVGDEVGNFKFYPDSITVSAGEAVEFTLVGETGHNIVFDIPAGAPGTVASELKAASM  60
              ******* ********** ****** ********************** **********
Polystichum   AKVEVGDDVGNFKFYPDSLTVSAGETVEFTLVGETGHNIVFDIPAGAPGPVASELKAASM  60
              * *   *   * *    *  **   *       ** ***      * *       **
Spinacea      VEVLLGGGDGSLAFLPGDFSVASGEEIVFKNNAGFPHNVVFDEDEIPSGVDAAKI---SM  57

Nephrolepsis  DENDLLSEDEPNFTAKVSTPGTYTFYCTPHKSANMKGTLTVK  102
              ********** * ***************************
Dryopteris    DENDLLSEDEPSFKAKVSTPGTYTFYCTPHKSANMKGTLTVK  102
              ******************************************
Polystichum   DENDLLSEDEPSFKAKVSTPGTYTFYCTPHKSANMKGTLTVK  102
               * ***       *      *** *** **  * * *  **
Spinacea      SEEDLLNAPGETYKVTLTEKGTYKFYCSPHQGAGMVGKVTVN   99
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Fig. 3. Amino acid sequence alignment of *Nephrolepsis*, *Dryopteris*, *Polystichum* and *Spinacea* Pc. The alignment was made with CLUSTALW. Identical residues between the sequences are marked with an asterisk. The microheterogeneity found in *Nephrolepsis* Pc is indicated by the residues above the main sequence at positions 19, 21 and 53. Conservation of these residues is indicated with 'o'.

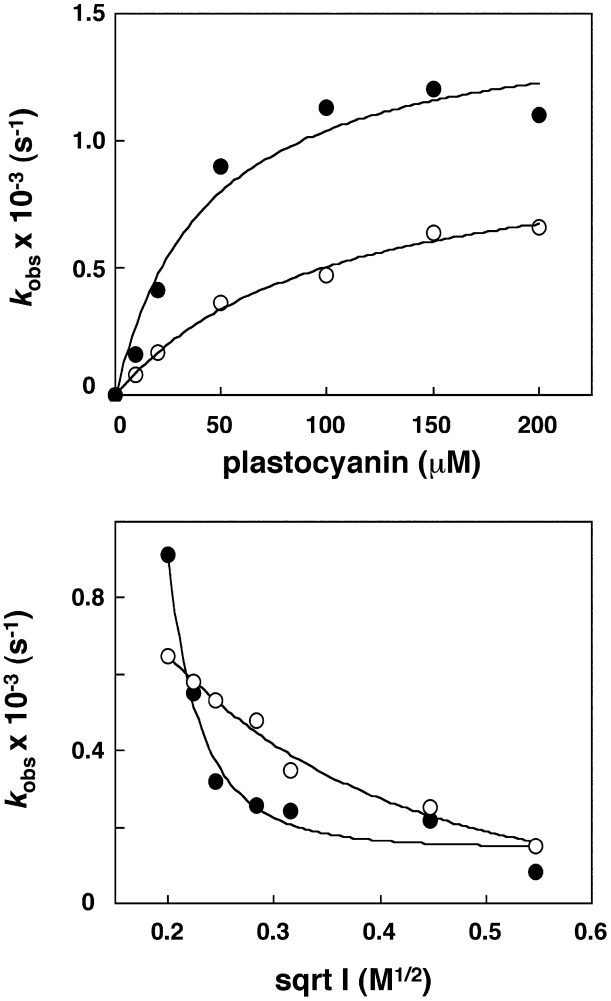

Fig. 5. Dependence of the observed rate constant (k_{obs}) on donor protein concentration (upper) and ionic strength (lower) for fern PSI reduction by fern (○) and spinach (●) Pc. In case of spinach Pc, the k_{obs} values correspond to the slow phase. Lines represent theoretical fits as described in the text (upper) and according to the formalism developed by Watkins *et al.* [37] (lower).

Table 1. Type of mechanism and kinetic constants for the reduction of fern PSI by Pc from different organisms.

Pc	pH	Type	K_A (M^{-1})	k_{sat} (s^{-1})	k_{et} (s^{-1})	k_2 ($M^{-1}·s^{-1}$)
Fern	7.5	II	1.0×10^4	1.0×10^3	1.0×10^3[a]	–
Fern	5.0	I	–	–	–	2.8×10^7
Spinach	7.5	III	2.5×10^4	1.5×10^3	2.7×10^4	–
Spinach	5.0	III	2.8×10^4	6.0×10^3	3.9×10^4	–
Synechocystis	7.5	I	–	–	–	2.6×10^6
Anabaena	7.5	I	–	–	–	1.3×10^6

[a] For fern Pc at pH 7.5, the value for k_{et} is inferred from that for k_{sat}.

(Table 1). This behaviour is very similar to that observed for spinach PSI when reacting with cyanobacterial Pcs [15]. Taken together, all of these data clearly demonstrate that fern PSI behaves as spinach PSI.

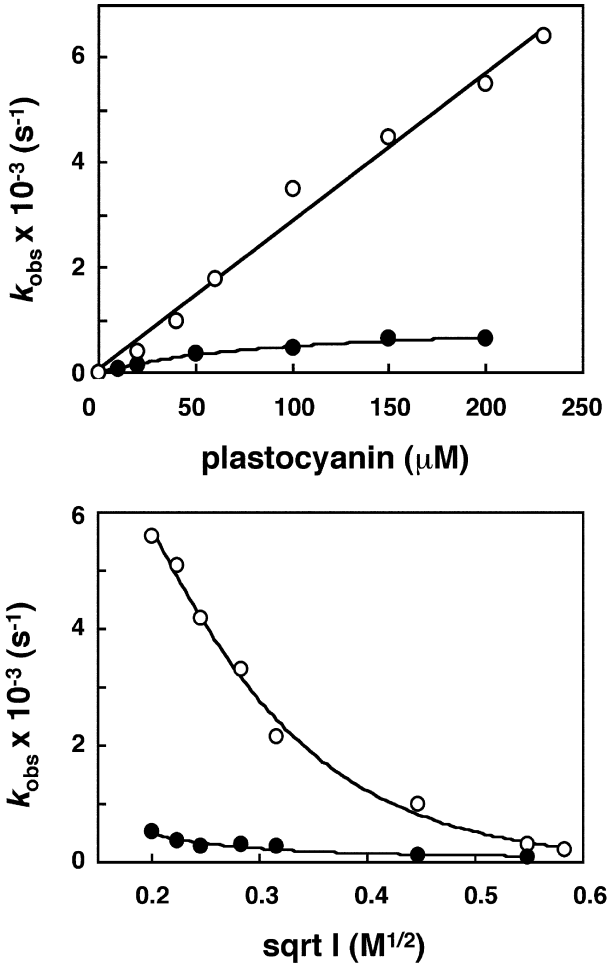

Fig. 6. Dependence of the observed rate constant (k_{obs}) on donor protein concentration (upper) and ionic strength (lower) for fern PSI reduction by fern Pc at pH 5.0 (○) and 7.5 (●). Lines represent theoretical fits as described in the legend of Fig. 5.

It has been reported previously that in fern Pc the redox potential exhibits a much less pronounced dependence on pH than other eukaryotic Pcs [32]. This has been ascribed to the absence of protonation of the His87 copper-ligand at low pH, a fact that strongly contrasts with pK_a values of ≈5.5 for this residue in other eukaryotic Pcs [12,32]. Consequently we checked the reactivity of fern PSI against fern and spinach Pc at pH 5, a value close to the physiological pH. As shown in Table 1, lowering pH only has quantitatively minor effects in the interaction between fern PSI and spinach Pc, as is the case for the spinach Pc/PSI system [15]. However, more drastic effects are observed in the fern Pc/PSI system, for which linear protein dependences are obtained (Fig. 6, upper), indicating the occurrence of a collisional type I mechanism. The k_2 value at low pH ($\approx 3\times10^7$ $M^{-1}·s^{-1}$) calculated from this linear plot cannot be directly compared with the kinetic values obtained at neutral pH, as different mechanisms (i.e. type II and type I) occur; however, the k_{obs} values obtained at pH 5 at high Pc concentration are about 10 times higher than those observed at pH 7

(Fig. 6, upper). Thus, it seems that at lower and more physiological pH values, fern Pc reactivity against its own PSI is significantly improved, thus approaching the efficiency attained by other eukaryotic systems. It is interesting to note that this pH effect is specific of the fern Pc/PSI system, as fern Pc reactivity with spinach PSI remains unchanged at low pH (data not shown).

The role of electrostatic interactions on fern PSI reduction by fern and spinach Pcs was investigated at varying NaCl concentrations (Fig. 5, lower panel). In plant Pc/PSI systems, it has been shown that salt initially stimulates PSI reduction and further slows down the reaction at high concentration, which has been explained in terms of rearrangement of Pc within the complex [15,33]. From the ionic strength profiles shown in Fig. 5, it is clear that fern PSI does not show the bell-shaped behaviour typical of other plant PSI, which is indeed observed in the fern Pc/spinach PSI system (data not shown). However, the ionic strength dependence of k_{obs} with fern PSI makes evident the electrostatic nature of the intermediate complexes with both Pcs, which are stabilized by means of attractive electrostatic forces. The ionic strength effect is more pronounced with spinach Pc than with fern Pc, mainly at low ionic strength (Fig. 5, lower panel), as expected from the differences in surface electrostatic potential between both proteins. However, at physiological ionic strength and pH, both proteins present similar reactivity (Fig. 6, lower panel).

Fern Pc reactivity against PSI obtained from organisms with negative (spinach), basic (*Anabaena*) or neutral (*Synechocystis*) Pcs was also checked. Significant electron transfer rates were only observed with spinach PSI, the cross-reaction showing monophasic kinetics of PSI reduction and linear plots against Pc concentration (data not shown), indicating the absence of any kinetically detectable Pc/PSI electron transfer complex. From these data, a second-order rate constant for spinach PSI reduction of 2.7×10^6 $M^{-1} \cdot s^{-1}$ was obtained under standard conditions. This value is significantly lower that those observed in homologous Pc/PSI systems from either eukaryotic or prokaryotic sources [15,34,35]. These findings indicate that the altered surface electrostatic potential of fern Pc drastically hinders its interaction with spinach PSI.

In order to extend the functional characterization of fern Pc, we have also compared the reactivity of this protein with that of other Pcs towards several soluble *c*-type Cyts (including Cyt *f*) by using the dRfH$^{\bullet}$ radical as a redox probe [30]. In all cases, the k_{obs} values for the electron transfer from Cyt to Pc depend linearly upon Pc concentration. As an example, Fig. 7 (upper panel) shows the linear protein concentration dependence observed for the electron transfer from either turnip Cyt *f* or horse Cyt *c* to fern Pc. From these plots, the k_2 values for the Cyt/Pc interaction can be estimated (Table 2). Efficient electron transfer is observed with any Cyt only when eukaryotic Pc is used as an acceptor, whereas no relevant reactivity is obtained with prokaryotic Pc (Table 2). This is in agreement with the electrostatic character of these proteins: positively charged in the case of Cyts, negatively charged in eukaryotic Pcs, and neutral or basic in prokaryotic Pcs [5,7].

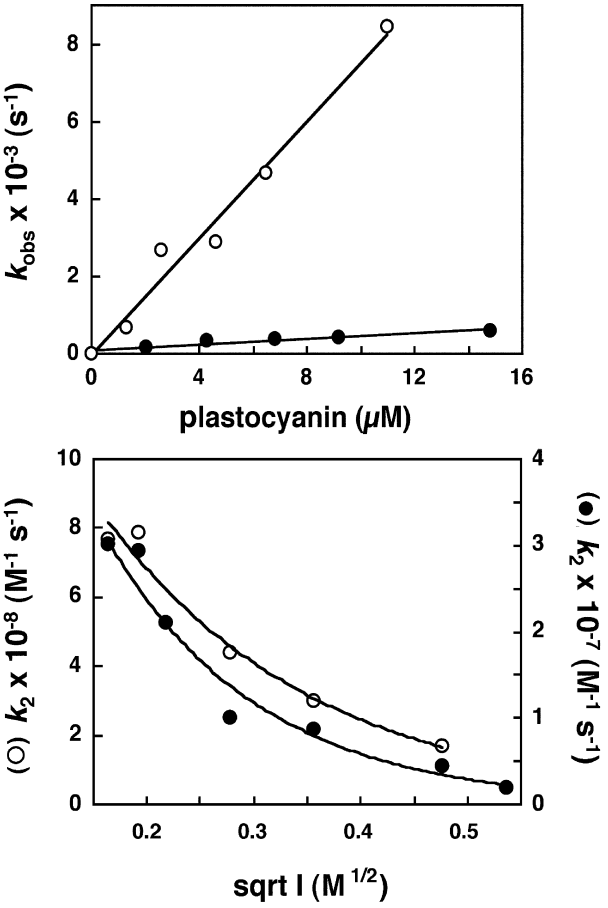

Fig. 7. Dependence of the observed rate constant (k_{obs}) on donor protein concentration (upper) and ionic strength (lower) for the reduction of fern Pc by turnip Cyt *f* (○) and horse Cyt *c* (●). Lines in the lower panel represent theoretical fits according to the formalism developed by Watkins *et al.* [37]. Other conditions were as described in Experimental procedures.

Table 2. Bimolecular rate constants (k_2, $M^{-1} \cdot s^{-1}$) for the overall reaction of reduction of different Pcs by *c*-type Cyts. n.d., Not determined.

	Plastocyanin			
Cytochrome	Fern	*Synechocystis*	*Anabaena*	Poplar
Cyt c_{550}	2.4×10^7	2.5×10^6	$< 10^4$	7.7×10^7
Horse Cyt *c*	4.1×10^7	$< 10^4$	$< 10^4$	4.6×10^7
Turnip Cyt *f*	7.5×10^8	n.d.	n.d.	6.0×10^8[a]

[a] Data from Meyer *et al.* [31].

The k_2 values presented in Table 2, obtained at ≈ 30 mM ionic strength, agree well with those previously reported for spinach Pc reduction by horse Cyt *c*, eukaryotic Cyt *f* or positively charged bacterial Cyts at similar ionic strength [31]. It is interesting to note that fern Pc reactivity against turnip Cyt *f* is one order of magnitude higher than with other Cyts (Fig. 7 and Table 2). Figure 7 (lower panel) shows that the rate constants for the electron transfer between any Cyt and fern Pc decrease as the ionic strength increases, indicating the existence of attractive

protein–protein electrostatic forces related to the complementarity in electrostatic charges between Pc and Cyt. Despite the peculiar surface charge distribution of fern Pc, this finding suggests that the specific interaction motifs between this copper protein and its natural electron donor, Cyt *f*, are conserved.

Concluding remarks

Fern Pc has electrostatic surface properties drastically different from those of other eukaryotic Pcs. The negatively charged area around positions 42–45 in the acidic patch is absent or strongly diminished, whereas new charged groups form an arc around the edge of the hydrophobic patch. From the data presented here we can conclude that fern Pc conserves the main electrostatic features of eukaryotic Pcs: its negatively charged character allows this protein to efficiently interact with plant Cyt *f* and PSI, but the unusual surface charge distribution in fern Pc seems to impede further rearrangement of the complex to attain an optimized electron transfer rate. In summary, fern Pc has followed a relatively independent evolutionary pathway since ferns diverged from other vascular plants, but keeping a charged area at the surface level that is crucial to drive the electrostatic attractive movements of the copper protein towards its membrane partners. Within the more general context of protein evolution, this finding reveals how important the surface electrostatic features of molecules are for their functional interactions within the living cells.

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References

1. Hope, A.B. (2000) Electron transfer amongst cytochrome *f*, plastocyanin and photosystem I: kinetics and mechanisms. *Biochim. Biophys. Acta* **1456**, 5–26.
2. Hervás, M., Navarro, J.A. & De la Rosa, M.A. (2003) Electron transfer between membrane complexes and soluble proteins in photosynthesis. *Accounts Chem. Res.* **36**, 798–805.
3. De la Rosa, M.A., Navarro, J.A., Díaz-Quintana, A., De la Cerda, B., Molina-Heredia, F.P., Balme, A., Murdoch, P.S., Díaz-Moreno, I., Durán, R.V. & Hervás, M. (2002) An evolutionary analysis of the reaction mechanisms of photosystem I reduction by cytochrome c_6 and plastocyanin. *Bioelectrochemistry* **55**, 41–45.
4. Gross, E.L. (1993) Plastocyanin: structure and function. *Photosynth. Res.* **37**, 103–116.
5. Navarro, J.A., Hervás, M. & De la Rosa, M.A. (1997) Co-evolution of cytochrome c_6 and plastocyanin, mobile proteins transferring electrons from cytochrome b_6f to photosystem I. *J. Biol. Inorg. Chem.* **2**, 11–22.
6. Guss, J.M., Harrowell, P.R., Murata, M., Norris, V.A. & Freeman, H.C. (1986) Crystal structure analyses of reduced (CuI) poplar plastocyanin at six pH values. *J. Mol. Biol.* **192**, 361–387.
7. Sigfridsson, K. (1998) Plastocyanin, an electron-transfer protein. *Photosynth. Res.* **57**, 1–28.
8. Romero, A., De la Cerda, B., Varela, P.F., Navarro, J.A., Hervás, M. & De la Rosa, M.A. (1998) The 2.15 Å crystal structure of a triple mutant plastocyanin from the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Mol. Biol.* **275**, 327–336.
9. Redinbo, M., Yeates, T.O. & Merchant, S. (1994) Plastocyanin: structural and functional analysis. *J. Bioenerg. Biomembr.* **26**, 49–66.
10. Ubbink, M., Ejdeback, M., Karlsson, B.G. & Bendall, D.S. (1998) The structure of the complex of plastocyanin and cytochrome *f*, determined by paramagnetic NMR and restrained rigid-body molecular dynamics. *Structure* **6**, 323–335.
11. Molina-Heredia, F.P., Hervás, M., Navarro, J.A. & De la Rosa, M.A. (2001) A single arginyl residue in plastocyanin and in cytochrome c_6 from the cyanobacterium *Anabaena* sp. PCC 7119 is required for efficient reduction of photosystem I. *J. Biol. Chem.* **276**, 601–605.
12. Kohzuma, T., Inoue, T., Yoshizaki, F., Sasakawa, Y., Onodera, K., Nagatomo, S., Kitagawa, T., Uzawa, S., Isobe, Y., Sugimura, Y., Gotowda, M. & Kai, Y. (1999) The structure and unusual pH dependence of plastocyanin from the fern *Dryopteris crassirhizoma*. The protonation of an active site histidine is hindered by π–π interactions. *J. Biol. Chem.* **274**, 11817–11823.
13. Sato, K., Kohzuma, T. & Dennison, C. (2003) Active-site structure and electron-transfer reactivity of plastocyanins. *J. Am. Chem. Soc.* **125**, 2101–2112.
14. Pletneva, E.V., Fulton, D.B., Kohzuma, T. & Kostic, N.M. (2000) Protein docking and gated electron-transfer reactions between zinc cytochrome *c* and the new plastocyanin from the fern *Dryopteris crassirhizoma*. Direct kinetic evidence for multiple binary complexes. *J. Am. Chem. Soc.* **122**, 1034–1046.
15. Hervás, M., Navarro, J.A., Díaz, A., Bottin, H. & De la Rosa, M.A. (1995) Laser-flash kinetic analysis of the fast electron transfer from plastocyanin and cytochrome c_6 to photosystem I. Experimental evidence on the evolution of the reaction mechanism. *Biochemistry* **34**, 11321–11326.
16. Navarro, J.A., Hervás, M., De la Cerda, B. & De la Rosa, M.A. (1995) Purification and physicochemical properties of the low-potential cytochrome c_{549} from the cyanobacterium *Synechocystis* sp. PCC 6803. *Arch. Biochem. Biophys.* **318**, 46–52.
17. Yocum, C.F. (1982) Purification of ferredoxin and plastocyanin. In *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R.B. & Chua, N.-H., eds), pp. 973–981. Elsevier Biomedical Press, Amsterdam.
18. Hervás, M., Navarro, F., Navarro, J.A., Chávez, S., Díaz, A., Florencio, F.J. & De la Rosa, M.A. (1993) *Synechocystis* 6803 plastocyanin isolated from both the cyanobacterium and *E.coli* transformed cells are identical. *FEBS Lett.* **319**, 257–260.
19. Molina-Heredia, F.P., Hervás, M., Navarro, J.A. & De la Rosa, M.A. (1998) Cloning and correct expression in *E. coli* of the *pet*E and *pet*J genes respectively encoding plastocyanin and cytochrome c_6 from the cyanobacterium *Anabaena* sp. PCC 7119. *Biochem. Biophys. Res. Commun.* **243**, 302–306.
20. Ubbink, M., Pfuhl, M., van der Oost, J., Berg, A. & Canters, G.W. (1996) NMR assignments and relaxation studies of *Thiobacillus versutus* ferrocytochrome *c*-550 indicate the presence of a highly mobile 13-residues long C-terminal tail. *Prot. Sci.* **5**, 2494–2505.
21. Diederix, R.E.M., Ubbink, M. & Canters, G.W. (2001) The peroxidase activity of cytochrome *c*-550 from *Paracoccus versutus*. *Eur. J. Biochem.* **268**, 4207–4216.

22. Mathis, P. & Sétif, P. (1981) Near infrared absorption spectra of the chlorophyll *a* cations and triplet state in vitro and in vivo. *Isr. J. Chem.* **21**, 316–320.
23. Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. *Plant Physiol.* **24**, 1–15.
24. Liang, P., Amons, R., MacRae, T.H. & Clegg, J.S. (1997) Purification, structure and in vitro molecular-chaperone activity of *Artemia* p26, a small heat-shock/alpha-crystallin protein. *Eur. J. Biochem.* **243**, 225–232.
25. Amons, R. (1996) Use of acetic anhydride in automatic sequencing. *Abstracts of the 11th International Conference on Methods in Protein Structure Analysis*, Annecy, France.
26. Amons, R. (1997) Determination of cysteine residues in protein sequence analysis at the picomole level. *Anal. Biochem.* **249**, 111–114.
27. Darbre, A., ed. (1986) *Practical Protein Chemistry – a Handbook*. John Wiley & Sons, NY, pp. 246–247.
28. Hervás, M., Myshkin, E., Vintonenko, N., De la Rosa, M.A., Bullerjahn, G.S. & Navarro, J.A. (2003) Mutagenesis of *Prochlorothrix* plastocyanin reveals additional features in Photosystem I interactions. *J. Biol. Chem.* **278**, 8179–8183.
29. Navarro, J.A., Hervás, M., Pueyo, J.J., Medina, M., Gómez-Moreno, C., De la Rosa, M.A. & Tollin, G. (1994) Laser flash-induced photoreduction of photosynthetic ferredoxins and flavodoxin by 5-deazariboflavin and by a viologen analogue. *Photochem. Photobiol.* **60**, 231–236.
30. Tollin, G. (1995) Use of flavin photochemistry to probe intra-protein and interprotein electron transfer mechanisms. *J. Bioenerg. Biomembr.* **27**, 303–309.
31. Meyer, T.E., Zhao, Z.G., Cusanovich, M.A. & Tollin, G. (1993) Transient kinetics of electron transfer from a variety of *c*-type cytochromes to plastocyanin. *Biochemistry* **32**, 4552–4559.
32. Dennison, C., Lawler, A.T. & Kohzuma, T. (2002) Unusual properties of plastocyanin from the fern *Dryopteris crassirhizoma*. *Biochemistry* **41**, 552–560.
33. Sigfridsson, K. (1997) Ionic strength and pH dependence of the reaction between plastocyanin and Photosystem 1. Evidence of a rate-limiting conformational change. *Photosynth. Res.* **54**, 143–153.
34. Bottin, H. & Mathis, P. (1985) Interaction of plastocyanin with the photosystem I reaction center: a kinetic study by flash absorption spectroscopy. *Biochemistry* **24**, 6453–6460.
35. Molina-Heredia, F.P., Wastl, J., Navarro, J.A., Bendall, D.S., Hervás, M., Howe, C.J. & De la Rosa, M.A. (2003) Photosynthesis: a new function for an old cytochrome? *Nature* **424**, 33–34.
36. Koradi, R., Billeter, M. & Wuthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14**, 51–55.
37. Watkins, J.A., Cusanovich, M.A., Meyer, T.E. & Tollin, G. (1994) A 'parallel plate' electrostatic model for bimolecular rate constants applied to electron transfer proteins. *Prot. Sci.* **3**, 2104–2114.