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The role of surface-exposed Tyr-83 of plastocyanin in electron transfer from cytochrome *c*

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The pathway of electron transfer from cytochrome *c* to the Cu atom of plastocyanin has been investigated using mutants of pea plastocyanin, in which the surface-exposed residue, Tyr-83, has been changed to Phe-83 or Leu-83 by site-directed mutagenesis. No significant differences in the reaction with the Tyr-83 and Phe-83 plastocyanins were observed. However, the Leu-83 mutant showed an 8-fold decrease in the rate of reduction, which was due entirely to a decrease in the intrinsic rate of electron transfer in the binary complex. This indicates that the major route for reduction of plastocyanin by cytochrome *c* involves Tyr-83, as has been shown previously for the natural donor, cytochrome *f*. The faster rates observed with cytochrome *f* are attributed to three factors. First, H-bonding between Tyr-83 and cytochrome *f*, but not cytochrome *c*; secondly, a slower dissociation of the reaction complex with cytochrome *f*; thirdly, a 20-fold faster intrinsic rate of electron transfer. Together, these factors indicate that most encounter complexes with cytochrome *f* are productive, whereas most with cytochrome *c* are unproductive.

Introduction

Plastocyanin (PC) is a small (M_r 10500), 'blue' copper protein which transfers electrons from cytochrome *f* to the primary electron donor of Photosystem I (P700) in the photosynthetic electron transport chain. It reacts rapidly with cytochrome *f* in vitro and also with several other cytochromes, although somewhat more slowly [1]. The crystal structures of both oxidized and reduced poplar plastocyanin have been determined and show that redox changes cause only small changes at the Cu site, leaving the structure of the rest of the molecule essentially unchanged [2,3]. The Cu atom and its ligands (His-37, Cys-84, His-87 and Met-92) are located in a hydrophobic pocket near one end of the molecule (the 'northern' end) such that only the imidazole ring of His-87 (the northern histidine) is accessible to solvent (Fig. 1). The negatively charged residues are concentrated on the 'eastern' face of the molecule, in an elongated acidic patch, composed of two distinctive clusters (residues 42–45 and 59–61) that are highly conserved in higher plant plastocyanins [2,4]. These solvent-exposed acidic residues are located on two prominent kinks in the polypeptide backbone. Between them the side-chain of Tyr-83, which is also highly conserved, is directed into the solvent.

The reaction of plastocyanin with cytochrome *f* slows down markedly with increased salt concentration, suggesting that the negatively charged residues of the acidic patch of plastocyanin are involved in binding to cytochrome *f* by electrostatic interaction with positively charged basic residues on the surface of cytochrome *f* [5]. This has been confirmed by chemical modification of acidic residues on plastocyanin [6] and by inhibition of electron transfer by small molecules which bind at the acidic patch [7]. Although cytochrome *f* is, overall, a neutral or weakly acidic protein its amino acid sequence shows several groups of basic residues that could form clusters that interact with the acidic patch of plastocyanin [8].

Two possible reaction sites on plastocyanin have been identified by studies with small molecules such as $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, one close to the copper ligand, His-87, in the northern hydrophobic patch, and the other close to the more remote Tyr-83, at the centre of the eastern acidic patch [4,7,9–12]. The distance from the surface of the molecule to the Cu atom is about 6 Å in the former case and 12 Å in the latter [2]. The fact that rates of electron transfer fall off exponentially with distance [13] tends to favour the former as the route for the electron. Indeed, Farver et

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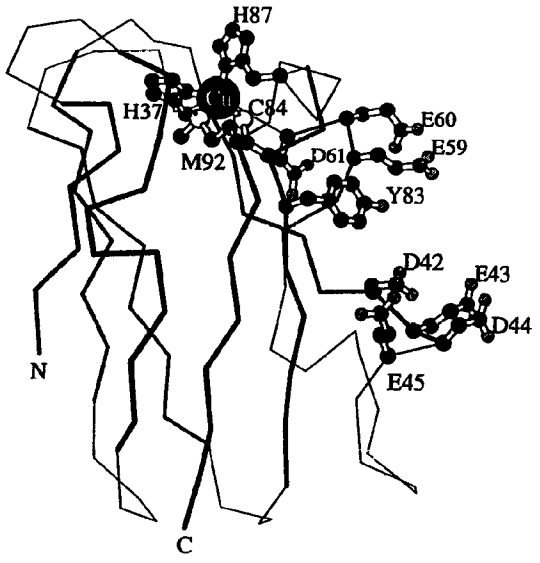

Fig. 1. Structure of plastocyanin. The programme MOLSCRIPT [36] was used to draw the α-carbon backbone, the Cu ligands and the side-chains of Tyr-83 and the residues of the eastern acidic patch with the coordinates of poplar plastocyanin from the Brookhaven Database, except for substitution of Glu-45 (as in pea plastocyanin) for Ser-45. β-Strands are shaded.

al. [14] suggested that His-87 is on the main route for electron transfer from cytochrome *f* on the grounds that labelling plastocyanin with Cr(III), in the region of the acidic residues Asp-42 to Glu-45, does not inhibit reduction of plastocyanin by chloroplasts. However, we have recently provided strong evidence, based on studies with plastocyanins modified by site-directed mutagenesis, that the main route of electron donation from cytochrome *f* involves Tyr-83 [15].

The reaction of plastocyanin with cytochrome *c* is also dominated by electrostatic effects similar to those found with cytochrome *f* [16]. Moreover, cytochrome *c* is more readily available than cytochrome *f* and its three-dimensional structure is well known [17], whereas that of cytochrome *f* has still to be determined. For these reasons, the u. physiological reaction of plastocyanin with cytochrome *c* has often been taken as a model for the reaction with cytochrome *f*. Since plastocyanin and cytochrome *c* are well-characterized proteins, both structurally and biochemically, and their electron-transfer reactions with each other [1] and with many other redox reagents [4,7,16] have been studied, their interaction offers excellent opportunities to explore the origins of specificity and efficiency in macromolecular recognition.

Evidence regarding the pathway of electron donation from cytochrome *c* is conflicting. A ring of basic groups is closely associated with the exposed edge of the haem ring [17], and it would be expected that these would interact with the eastern acidic residues of plastocyanin in such a way as to orient the haem ring towards the exposed Tyr-83 of plastocyanin. This has recently been confirmed by modelling based on the crystal structures of the two proteins and calculations of electrostatic potentials [18]. On the other hand, an analytical approach to the effect of ionic strength on bimolecular rate constants, taking into account dipole-dipole and dipole-monopole interactions, has led to the conclusion that cytochrome *c* donates predominantly through His-87 [19]. This would require reorientation within the initial precursor complex formed under the influence of the electrostatic forces. Evidence for relative mobility of the two proteins within the encounter complex has been provided by an NMR study of paramagnetic competition [20]. We have attempted to choose between these two possible routes by comparing the kinetics of electron transfer from cytochrome *c* to wild-type pea plastocyanin and to modified plastocyanins in which Tyr-83 has been changed to Phe or Leu by site-directed mutagenesis [15]. The conclusion reached is that, as with the analogous reduction by cytochrome *f* [15], the main route involves Tyr-83. The results are discussed in relation to the mechanisms of interaction between plastocyanin and each of the two cytochromes.

Materials and Methods

Site-directed mutagenesis and expression of the pea plastocyanin gene

Mutagenesis of a cDNA clone of pea plastocyanin in M13K8.2 [21], its expression in transgenic tobacco plants and purification of the mutant plastocyanins were carried out as previously described [15].

Kinetic and spectral measurements

The rate of electron transfer from reduced cytochrome *c* to oxidized plastocyanin was measured in a manner similar to that previously described for cytochrome *f* [15]. Oxidation of cytochrome *c* was monitored at 417 nm with an Applied Photophysics stopped-flow spectrophotometer (SF.17MV). For measurement of k_2, the concentrations of cytochrome *c* and plastocyanin were 0.8–0.9 μM and 8.0–9.0 μM, respectively, in a buffer containing 10 mM phosphate, 90 mM NaCl (pH 6.0) at 300 K. A standard pH of 6.0 was chosen for all experiments in this and the previous paper [15] because this was the pH at which ^{1}H-NMR assignments had been made. The observed pseudo-first-order rate constant was divided by the plastocyanin concentration to give k_2. Horse-heart cytochrome *c* (Type VI) was obtained from Sigma Chemicals and further purified by passage through columns of Sephadex G-100 and CM-cellulose [22]. Protein con-

centrations were determined from the following absorption coefficients: reduced horse-heart cytochrome c, $\epsilon_{550\,nm} = 2.76 \cdot 10^4\ M^{-1}\ cm^{-1}$; oxidised plastocyanin, $\epsilon_{597\,nm} = 4.7 \cdot 10^3\ M^{-1}\ cm^{-1}$.

The rate of binding of plastocyanin and cytochrome c was measured by following the increase in absorbance of oxidized cytochrome c at 410 nm in the stopped-flow spectrophotometer. Oxidized cytochrome c (10–12 μM) and oxidized plastocyanin (40–50 μM) were dissolved in 10 mM phosphate, 90 mM NaCl (pH 6.0).

Visible absorption spectra were recorded with a Perkin-Elmer Lambda 9 spectrophotometer.

Results

The overall bimolecular reaction between reduced cytochrome c and oxidized plastocyanin can be written as:

$$PC_{ox} + Cyt\ c_{red} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PC_{red} + Cyt\ c_{ox}$$

The simplest satisfactory kinetic model distinguishes between initial binding to form a complex $[PC_{ox} \ldots Cyt\ c_{red}]$, and the electron transfer process itself:

$$PC_{ox} + Cyt\ c_{red} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} [PC_{ox} \ldots Cyt\ c_{red}]$$

$$\overset{k_f}{\rightleftharpoons} [PC_{red} \ldots Cyt\ c_{ox}] \rightleftharpoons PC_{red} + Cyt\ c_{ox}$$

Application of the steady-state approximation to the concentration of the complex $[PC_{ox} \ldots Cyt\ c_{red}]$ under our conditions, in which $K_A[PC_{ox}] \ll 1$, gives the following equation [13]:

$$\frac{1}{k_2} = \frac{1}{k_a} + \frac{1}{K_A k_f}$$

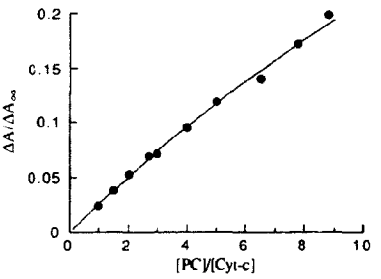

Fig. 2. Binding curve for the interaction of wild-type plastocyanin (PC) with cytochrome c (Cyt-c). The cytochrome c concentration was 20.1 μM. The increase in absorbance at 410 nm was measured when plastocyanin was present at concentrations between 0 and 176.7 μM. Proteins were dissolved in 10 mM phosphate, 90 mM NaCl (pH 6.0), at 300 K. The solid line is the best fit to the equation given in Ref. 15.

Thus, it was necessary to measure at least three of the four constants that appear in the above equation. The second-order rate constant (k_2) was measured by stopped-flow spectrophotometry, as described in Materials and Methods. K_A was measured by taking advantage of the increase in absorbance of the Soret band of oxidized cytochrome c at 410 nm that occurs on binding plastocyanin (Fig. 2), as previously described for cytochrome f [15]. A similar enhancement effect occurred when the reduced forms of the two proteins were mixed together and the same value for K_A was obtained. We have assumed that this value is a correct measure of K_A for the complex $[PC_{ox} \ldots Cyt\ c_{red}]$. Increased Soret band absorption has previously been observed on binding of cytochrome c to cytochrome c peroxidase [23] and cytochrome b_5 [24]. When the total concentration of the two proteins was held constant but the molar ratio varied, the maximum enhancement was observed at a molar ratio of 0.5. This indicated that plastocyanin and cytochrome c form a 1:1 complex. The rate constant for formation of the encounter complex (k_a) was measured in the stopped-flow spec-

TABLE I

Kinetic parameters for reduction of pea plastocyanin by cytochromes c and f

Values are given as mean ± SD.
Data for cytochrome c were obtained as described in the text. Data for cytochrome f are from Ref. 15.

Plastocyanin	$10^{-6} k_2$ ($M^{-1} s^{-1}$)	K_A (M^{-1})	$10^{-6} k_a$ ($M^{-1} s^{-1}$)	$10^{-3} k_{-a}$ (s^{-1})	$10^{-3} k_f$ (s^{-1})
Cytochrome c as donor					
wild type	3.26 ± 0.05	1253 ± 13	20.0 ± 0.4	16.0 ± 0.5	3.11 ± 0.22
Phe-83 mutant	3.28 ± 0.09	1295 ± 18	22.7 ± 0.5	17.5 ± 0.6	2.96 ± 0.27
Leu-83 mutant	0.421 ± 0.006	1260 ± 75	21.7 ± 0.7	17.2 ± 1.6	0.340 ± 0.047
Cytochrome f as donor					
wild type	40.6 ± 1.1	9890 ± 89	43.5 ± 1.2	4.40 ± 0.16	62 ± 35
Phe-83 mutant	5.43 ± 0.13	1270 ± 15	5.86 ± 0.12	4.61 ± 0.15	58 ± 27
Leu-83 mutant	0.955 ± 0.009	968 ± 13	1.27 ± 0.08	1.31 ± 0.10	4.0 ± 1.0

trophotometer as the rate of increase of A_{430} when oxidized cytochrome *c* was mixed with oxidized plastocyanin. Knowledge of these three constants allowed the value of the intrinsic rate constant for electron transfer (k_f) to be calculated.

The results obtained with wild-type pea-plastocyanin and with the two mutant forms containing Phe-83 and Leu-83, respectively, are shown in Table I. The data in the Table allow a comparison to be made with the earlier results obtained with cytochrome *f* [15]. The values for k_2 for reduction by cytochrome *c* were close to the activation-controlled limit ($k_f \ll k_{-a}$), unlike those with cytochrome *f*. In order to provide a simple mechanistic interpretation of the effects observed it is necessary to confirm that no major conformational change took place in the two mutant proteins. This has been demonstrated in a previous study of their reactivity with cytochrome *f* by measurement of circular dichroism spectra in the far ultraviolet and by 1 D ^{1}H-NMR [15].

No significant differences in the reaction of cytochrome *c* with the wild-type and Phe-83 mutant proteins were observed (Table I). This is in contrast with the observations with cytochrome *f* in which a seven-fold decrease in k_2 could be entirely explained by a decrease in binding to plastocyanin. However, the Leu-83 mutant showed an eight-fold drop in the rate of reduction by cytochrome *c* which, as in the case of cytochrome *f*, could be explained by a decrease in the intrinsic rate constant for electron transfer (k_f). This observation provides convincing evidence that the major route for reduction of wild-type plastocyanin by cytochrome *c* involves Tyr-83, just as we have previously shown for cytochrome *f* [15]. It should be noted that the previous study also demonstrated that these changes could not be explained by changes in redox potential of the mutant plastocyanins.

Discussion

The results reported above, and in our previous paper [15], demonstrate convincingly, for the first time, that Tyr-83 of plastocyanin is part of the main tunnelling pathway for the electron between the haem rings of both cytochrome *c* and cytochrome *f* and the Cu atom of plastocyanin. A leucine residue in this position is much less effective and it seems likely that the facilitation of electron transfer by tyrosine or phenylalanine is due to the aromatic nature of the ring, as has been proposed in other proteins [25]. The crystal structure of plastocyanin [2] reveals a fairly direct through-bond pathway from the solvent-exposed surface of the aromatic ring to the Cu atom; this includes the side chain of the tyrosine, the peptide bond between Tyr-83 and Cys-84, and the side chain of Cys-84, the sulphur atom of which is a Cu ligand. There is evidence that aromatic residues may not always enhance electron transfer [26], and this may apply to structures in which any advantage offered by the aromatic ring would be offset by one or more additional through-space steps, which are considered to be slower [27]. An alternative explanation for the slower rates with the Leu-83 mutant is that the leucine does not so effectively fill the space between the two proteins in the correctly oriented reaction complex. The residual intrinsic rates of electron transfer (k_f) may represent a slower rate of tunnelling through the leucine, or slow rates of transfer through His-87, or a mixture of the two.

A striking difference between the kinetics of reduction of plastocyanin by the two cytochromes is that the wild-type protein and the Phe-83 mutant behave identically towards cytochrome *c*, but not towards cytochrome *f*. In the latter case the rate constant for reduction of the mutant protein is about seven times smaller, a difference that can be ascribed entirely to weaker binding. We, therefore, predict that when the structure of cytochrome *f* becomes known it will reveal a surface residue in the region of the exposed haem edge which is capable of hydrogen bonding to the -OH of Tyr-83.

The second-order rate constant (k_2) and the affinity constant (K_A) for the reaction between plastocyanin and cytochrome *c* have not previously been measured together. The value of k_2 given in Table I for wild-type plastocyanin is similar to some other reported values [28,29], but significantly faster than others [1,16]. We have found that cytochrome *c* obtained from Sigma as Type III, which is prepared using trichloracetic acid, gives a second order rate constant identical to that originally reported by Wood [1], whereas the results in Table I were obtained with Sigma Type VI, which is prepared by a method avoiding the use of trichloracetic acid. King et al. [29] reported that in their work they used Type VI further purified by ion-exchange chromatography on CM-cellulose. Cytochrome *c* prepared by acid extraction is known to contain oligomeric forms and also to be prone to a variable loss of amide groups [30]. We found that Type III and Type VI cytochrome *c* gave virtually identical values for K_A, which were consistent with some reports [20,31] but approximately ten-fold lower than others [32,33], when the effects of ionic strength are taken into account.

An assumption inherent in our kinetic analysis is that the value of K_A measured by Soret band enhancement is appropriate for electron transfer. It seems likely that k_a, obtained by this method, does not measure strictly the formation of an encounter complex, but includes a term for the reorganisation that produces the relative configuration of the two proteins in which enhancement can occur. The enhancement is presumably caused by a slight increase in the rigidity of

the porphyrin ring or its immediate environment as plastocyanin binds, and this would be influenced by the same electrostatic forces that are involved in the electron transfer process. Moreover, reorganisation under the influence of electrostatic forces would be expected to occur on a sub-microsecond timescale [34].

The kinetic parameters reported here reveal three factors as contributing to the superiority of cytochrome *f* over cytochrome *c* as a donor to plastocyanin. First, there is the contribution of a hydrogen bond to Tyr-83 to the binding constant. Secondly, the significantly slower dissociation of the reaction complex (k_{-a}) with cytochrome *f* gives it a longer lifetime. The third, and most important factor, is the 20-fold increase in k_f. The last two, taken together, mean that with cytochrome *f* most of the encounter complexes are productive, whereas with cytochrome *c* most are unproductive. The difference in the intrinsic rate of electron transfer is likely to be attributable to a closer approach of the haem group of cytochrome *f* to the ring of Tyr-83 and to a more favourable relative orientation. A modelling study of the interaction with cytochrome *c* has recently been reported [18] and similar studies with cytochrome *f* must await the determination of the crystal structure [35].

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