Structural Dynamics in the Plastocyanin−Photosystem 1 Electron-Transfer Complex as Revealed by Mutant Studies†

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ABSTRACT: A series of plastocyanin mutants have been constructed by site-directed mutagenesis and expressed in *Escherichia coli* to elucidate the interaction between plastocyanin and photosystem 1 in the photosynthetic electron-transfer chain. Leu-12 has been replaced with alanine, asparagine, glutamate, and lysine, while Tyr-83 has been exchanged for histidine, phenylalanine, and leucine. Phe-35, Asp-42, and Gln-88 have been mutated to tyrosine, asparagine, and glutamate, respectively. The mutations that have been introduced do not seem to place any strain on the tertiary structure according to optical absorption and electron paramagnetic resonance (EPR) spectroscopic studies. However, there are changes in the reduction potential for the Leu-12 mutants that cannot be accounted for by electrostatic interactions alone. For some of the mutants, the p*I* shifts, in accordance with the changes in the number of titratable groups. Only the Leu-12 mutants show any major change in their photosystem 1 kinetics, while the mutants in the acidic patch show minor changes, suggesting that both the hydrophobic and acidic patches make contact with photosystem 1 but that the electron transfer occurs at the hydrophobic interface, most probably via the His-87 residue. The kinetics are best described with a model in which a rate-limiting conformational change occurs in the plastocyanin−photosystem 1 complex [Bottin, H., & Mathis, P. (1985) *Biochemistry 24*, 6453−6460; Sigfridsson, K., Hansson, Ö., Karlsson, B. G., Baltzer, L., Nordling, M., & Lundberg, L. G. (1995) *Biochim. Biophys. Acta 1228*, 28−36], where the changes observed are attributed to changes in the dynamics within the electron-transfer complex.

Plastocyanin (Pc)[1] is a small, blue copper protein which acts as an electron carrier between the cytochrome (cyt) $b_6 f$ and photosystem 1 (PS1) complexes in the photosynthetic electron-transfer (ET) chain. The photooxidized reaction-center chlorophyll P700 in PS1 is reduced by Pc, and the oxidized Pc is in turn reduced by cyt *f* [for reviews, see Haehnel (1986), Gross (1993), and Redinbo et al. (1994)].

Two areas on the surface of Pc were suggested to be important in the ET reactions already when the three-dimensional structure was first reported (Colman et al., 1978). One is the hydrophobic patch around the Cu ligand His-87, and the other is an acidic patch around Tyr-83. Support for this suggestion has come from studies of chemically modified Pc and of the reactivity between Pc and low-molecular weight complexes [see Sykes (1991) for a review].

In order to further elucidate the role of particular amino acid residues, site-specific mutations were recently introduced in the structural gene for Pc from spinach (Nordling et al., 1991; Haehnel et al., 1994) and pea (He et al., 1991). For one mutant, where Leu-12 in the hydrophobic patch is replaced with a Glu residue, the ET to PS1 was found to be much slower than for the wild type, suggesting an important role for this region in the interaction with PS1 (Nordling et al., 1991). This was further supported by the findings that replacement of Gly-10 and Ala-90 in the hydrophobic patch with Leu also results in a strong impairment of the reaction with PS1 (Haehnel et al., 1994). On the other hand, studies of Leu and Phe mutations of Tyr-83 suggest a crucial role for this residue in the reaction with cyt *f* (He et al., 1991).

A straightforward interpretation of the mutant studies would be that Pc employs different pathways in the reactions with its redox partners, Tyr-83 in the acidic patch when accepting electrons from cyt *f* and His-87 in the hydrophobic patch when donating electrons to PS1. The situation is, however, not entirely clear. For example, in the case of cyt *f*, a Leu12Asn Pc mutant showed a marked increase in binding constant, while the kinetics of reduction of an Asp42Asn mutant were not significantly different from those of wild type, despite the loss of a negative charge in the acidic patch (Modi et al., 1992). With regard to the reaction with PS1, a Tyr83Leu mutant from spinach displayed faster ET kinetics (Haehnel et al., 1994).

In the present paper, we report on extensive studies of a series of Pc mutants to further elucidate the interaction between Pc and PS1. Leu-12 has been replaced with residues ranging from small and neutral to large and positive, while Tyr-83 has been replaced with histidine, phenylalanine, and leucine. Phe-35, Asp-42, and Gln-88 have been mutated to tyrosine, asparagine, and glutamate, respectively. All the proteins have been characterized by optical absorption and electron paramagnetic resonance (EPR) spectroscopy, isoelectric focusing, and redox titrations, and the kinetics in their reaction with PS1 have been determined.

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[1] Abbreviations: cyt, cytochrome; ET, electron transfer; P, P700, reaction-center chlorophyll; Pc, plastocyanin; PS1, photosystem 1; WT, wild type.

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MATERIALS AND METHODS

Construction of Mutants. Recombinant wild-type plastocyanin [Pc(WT)] and the mutants were prepared using the previously described system for overexpression of Pc in *Escherichia coli* (Nordling et al., 1990) employing the expression vector pUG101t_r (Nordling et al., 1991). The mutant proteins were constructed using the polymerase chain reaction (PCR) according to the method of Landt et al. (1990) with the modifications described in Nordling et al. (1991). After mutagenesis, the entire gene of each mutant protein was sequenced and transformed into *E. coli* RV308 (ATCC 31608) (Maurer et al., 1980).

Expression and Purification of Pc. The transformed *E. coli* was grown overnight in a 20 L Chemap fermentor, where the Pc is expressed in the cell's periplasma. The periplasmic fraction was prepared and loaded onto a DE32 column after an acidic precipitation. The proteins were eluted with a salt gradient, and Pc-containing fractions were loaded onto a Pharmacia HiLoad 26/10 Q Sepharose High Performance column. Thereafter, the Pc fractions were dialyzed against dry polyethylene glycol and run on a Sephacryl S-100 HR gel filtration column. The amount of holo-Pc was determined spectrophotometrically under oxidizing conditions using an absorption coefficient of 4900 M^{-1} cm^{-1} at 597 nm (Katoh et al., 1962).

Isoelectric Focusing. The determination of the protein's p*I* was performed on a Pharmacia PhastSystem using PhastGel Dry IEF. The gel was rehydrated in a mixture of Pharmalyte 4−6.5 and 2.5−5 to give a pH range of roughly 3.5−6 and was then run according to the manufacturer's recommendations. The outside lanes of the gel contained p*I* markers from Pharmacia's Low p*I* Kit (pH 2.8−6.5). After Coomassie staining, the gel was scanned in Pharmacia's PhastImage using the marker lanes to create calibration curves. The reported p*I* values are mean values from at least six independent runs with a standard deviation of 0.02−0.03 for each Pc mutant. The theoretical values were determined with the computer program PCGENE from the Oxford Molecular Group.

Preparation of PS1. Digitonin-solubilized PS1 particles (D-144 particles) with 240 chlorophyll/P700 were prepared from spinach according to Boardman (1971). The particles were suspended in 20 mM Tris (pH 7.5) and stored at 77 K until use.

Optical Absorption and EPR Spectroscopy. Optical spectra were obtained at room temperature with a Cary 4 UV/vis spectrophotometer from Varian. EPR spectra were recorded at 77 K with a Bruker ER 200D-SRC spectrometer operating at 9.28 GHz. The modulation amplitude was 2 mT and the microwave power 2 mW.

Redox Titration of Pc. The reduction potential of Pc was determined in 20 mM Tris (pH 7.5 or 9.0) or in 20 mM Mes (pH 6.0) by monitoring the 597 nm absorbance as the ratio of (potassium) ferricyanide to ferrocyanide was varied. $MgCl_2$ was added (7 mM at pH 7.5 and 9 mM at pH 6.0 and 9.0) to obtain conditions similar to those for the kinetic studies (see below). The reported E^0 values are mean values from at least three independent experiments, and the uncertainty is estimated to within ±3 mV. The small amount of material precluded measurements at a constant ionic strength. Therefore, the absolute values reported are not as accurate as the relative shifts between different samples.

Table 1: Isoelectric Points (p*I*), Reduction Potentials (E^0), and Spectroscopic Parameters for Plastocyanin Mutants[a]

plastocyanin	p*I*[b]	λ_{max}(nm)	$g_{\|\|}$	$A_{\|\|}$(mT)	E^{0c} (mV)
wild-type	3.82	597	2.24	6.4	384
Leu12Ala	3.82	599	2.25	5.5	356[d]
Leu12Glu	3.81	597	2.24	5.9	354[e]
Leu12Lys	3.90	600	2.24	5.9	411
Leu12Asn	3.83	596	2.24	6.2	362
Phe35Tyr	3.80	597	2.24	6.3	385
Asp42Asn	3.87	597	2.24	6.4	387
Tyr83Phe	3.84	597	2.24	6.4	379
Tyr83His	3.88	597	2.24	6.4	419
Tyr83Leu	3.84	595	2.23	6.3	383

[a] λ_{max} values denote the position of maximal absorbance in the visible spectrum. $A_{\|\|}$ and $g_{\|\|}$ denote the hyperfine splitting and g value of the low-field EPR peak, respectively. Reduction potentials and spectra were obtained at pH 7.5; see Materials and Methods for other experimental conditions. [b] The uncertainty in the p*I* is estimated to ±0.03. [c] The uncertainty in the reduction potential is estimated to ±3 mV. [d] Values of 360 and 354 mV were obtained at pH 6.0 and 9.0, respectively. [e] Values of 368 and 354 mV were obtained at pH 6.0 and 9.0, respectively.

Kinetic Methods. The kinetics of the ET from Pc to PS1 were studied by monitoring the flash-induced absorption changes at 830 nm due to oxidized P700. Pc in 20 mM Tris (pH 7.5 or 9.0) or in 20 mM Mes (pH 6.0) was mixed with PS1 to a final chlorophyll concentration of 0.9 mg/mL. Sodium ascorbate (2 mM) and methyl viologen (0.1 mM) were added in order to reduce Pc and oxidize the PS1 acceptor side, respectively, between the flashes (spaced 20 s apart). $MgCl_2$ (7 mM at pH 7.5 and 9 mM at pH 6.0 and 9.0) was added to give a final ionic strength of 38 mM. The ET from Pc to PS1 is known to be facilitated by millimolar concentrations of $MgCl_2$ in this type of digitonin-solubilized PS1 particles (Lockau, 1979; Olsen & Cox, 1982). From studies at different $MgCl_2$ concentrations, it was found that the concentrations given above resulted in an optimal binding of Pc to PS1.

In pH titrations of the PS1 kinetics, a buffer mixture containing Hepes, Mes, and succinate (10 mM of each) was used. The pH was adjusted by addition of NaOH or HCl.

The experimental setup was essentially as described by Hoganson et al. (1991). Excitation of PS1 was obtained by short (10 ns) flashes (532 nm) from a Nd:YAG laser. Photooxidation and reduction of P700 were monitored at 830 nm with a continuous-wave diode laser using an Si photodiode (UDT PIN-10D) connected to a home-built amplifier (gain, 50; band width, 8 Hz to 30 MHz). The diode laser was driven with a Melles Griot 06DLD203 unit. Acquisition of the transient absorption signals and fitting of the signals to a sum of exponentials was made as previously described (Hoganson et al., 1991). Other experimental conditions are given in the figure legends.

RESULTS

Expression Yields. The expression level of the different proteins varies greatly. The mutations created in the hydrophobic patch do not seem to affect the expression level, while for the mutants in position 83, it can be as low as 5% of Pc(WT).

Isoelectric Focusing. The p*I* of the Pc(WT) and the mutant proteins was determined by isoelectric focusing chromatography, and the results are summarized in Table 1. Small but significant shifts from the Pc(WT) value of 3.82

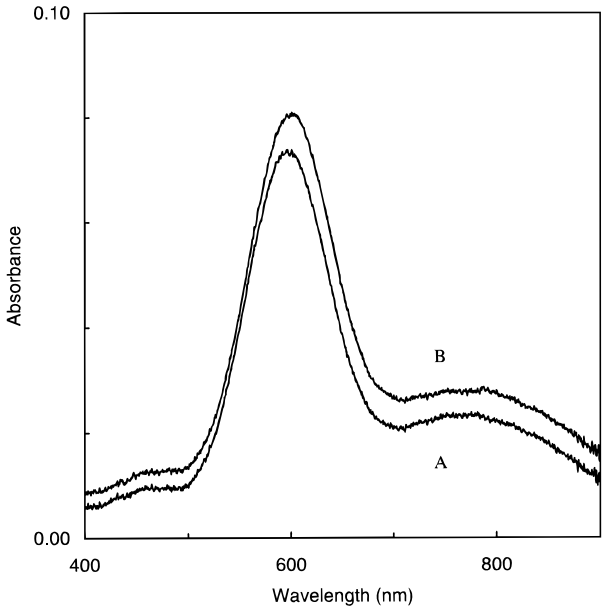

FIGURE 1: Optical absorption spectra at room temperature and pH 7.5 of oxidized wild-type (A) and Leu12Ala mutant (B) plastocyanin.

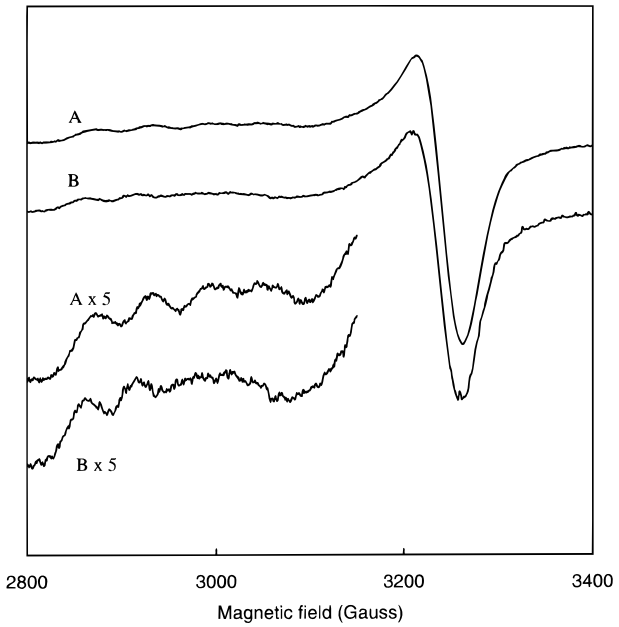

FIGURE 2: EPR spectra at 77 K of wild-type (A) and Leu12Ala mutant (B) plastocyanin. Samples containing 0.1 mM plastocyanin in 20 mM Tris buffer (pH 7.5) were oxidized by 0.2 mM potassium ferricyanide and frozen in liquid nitrogen. See Materials and Methods for spectrometer conditions.

are evident for some of the mutants, notably Pc(Leu12Lys), Pc(Asp42Asn), and Pc(Tyr83His).

Optical Absorption and EPR Spectroscopy. Figure 1 shows spectra in the visible region at pH 7.5 for oxidized Pc(WT), and Pc(Leu12Ala). The maximal absorbance occurs at $\lambda_{max} = 597$ nm for Pc(WT), while the mutant displays a small shift to a longer wavelength. The λ_{max} values for all proteins are listed in Table 1. Except for Pc(Leu12Glu), all mutations at position 12 cause small changes in the visible region. The λ_{max} for the Pc(Tyr83Leu) mutant is shifted to a shorter wavelength, as previously observed by Haehnel et al. (1994).

In the UV region, the spectra can be superimposed with that of Pc(WT), except for the Tyr-83 mutants and Pc(Phe35Tyr) [not shown, but see Nordling et al. (1991) and Haehnel et al. (1994)]. These mutations change the number of tyrosine residues in the protein [there are three in Pc(WT)], and this gives rise to changes in the 278 nm absorbance.

EPR spectra have been recorded in order to further investigate whether the mutations cause structural changes around the copper site. Figure 2 compares the EPR spectrum of oxidized Pc(Leu12Ala) with that of Pc(WT). There are small but significant changes in the parallel region of the mutant spectrum. The relevant EPR parameters are listed in Table 1 for all the proteins. Changes in $g_{||}$ or $A_{||}$ for Pc(Tyr83Leu) and the Leu-12 mutants coincide with shifts in λ_{max}, but there is no correlation between the direction of the shifts in the EPR and optical parameters.

Redox Titration of Pc. The reduction potential of the Pc mutants was determined by spectrophotometric titration, and the results are summarized in Table 1.

All Leu-12 mutants show a significant change in potential compared to Pc(WT). For the Pc(Leu12Ala), Pc(Leu12Asn), and Pc(Leu12Glu) mutants, there is a 25 mV decrease in E^0 at pH 7.5, and this difference persists at pH 9.0. However, at pH 6.0, the reduction potential of the Pc(Leu12Glu) mutant is increased toward the Pc(WT) value, which is unaffected in the range pH 6.0−9.0, while that of Pc(Leu12Ala) is only slightly elevated. In contrast, the reduction potential of the Pc(Leu12Lys) mutant is approximately 30 mV higher than the Pc(WT) value.

Of the other Pc mutants, only Pc(Tyr83His) shows a significantly increased E^0 value compared to Pc(WT) at pH 7.5, but at pH 8.9, the mutant is more similar to the Pc(WT) (390 and 388 mV, respectively) (Sigfridsson et al., 1995).

For some of the mutants (Leu12Asn, Leu12Glu, Asp42Asn, Tyr83His, and Tyr83Phe), E^0 values have also been calculated from stopped-flow kinetic data, and the values obtained by Kyritsis et al. (1993) agree with those reported here.

Kinetic Measurements. The Pc mutants' ET reactivity toward PS1 was investigated with time-resolved absorption measurements at 830 nm. Both $P700^{ox}$ and Pc^{ox} (ox = oxidized) absorb at this wavelength, but the absorption coefficients are different [5500 and 1000 M^{-1} cm^{-1}, respectively (Sigfridsson et al., 1995, and references therein)]. The reduced forms, $P700^{red}$ and Pc^{red}, do not absorb at 830 nm. As shown in Figure 3, flash excitation of a mixture of PS1 particles and Pc results in an instantaneous absorption increase at 830 nm (due to photooxidation of P700), followed by a multiphasic decrease (due to reduction of $P700^{ox}$ by Pc^{red}). Measurements were also done on time scales longer than that used in Figure 3. It was found that all kinetic traces eventually return to the preflash level with a 3 ms time constant for the slowest component (see below).

Overall, the Pc mutants can be divided into two groups, those that react with PS1 essentially as Pc(WT) (Table 2) and those that display a much slower kinetics (Table 3). The latter mutants all involve mutations of the Leu-12 residue, and representative kinetic traces for Pc(Leu12Glu) are shown in Figure 3B−D.

The absorption transients have been analyzed with a nonlinear least-squares curve-fitting program. At all Pc concentrations studied, good fits could only be obtained if the model function consisted of a sum of two decaying exponentials for the Leu-12 mutants and three for Pc(WT) and the remaining mutants. The amplitudes and rate

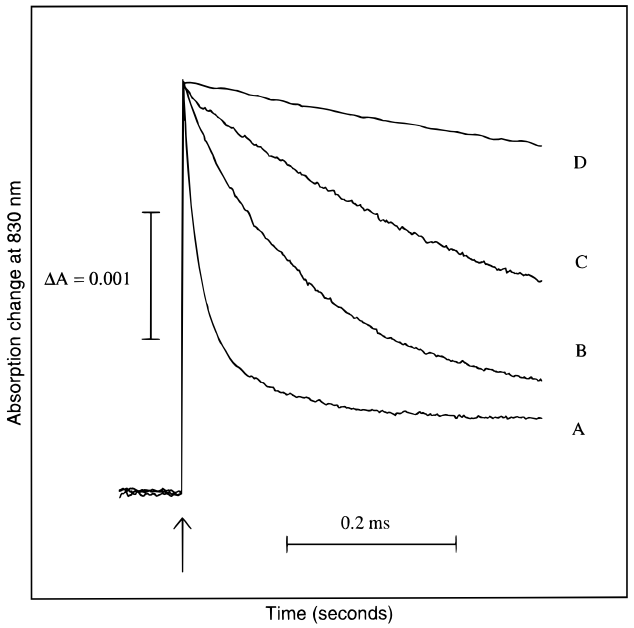

FIGURE 3: Flash-induced absorption transients at 830 nm of photosystem 1 particles with wild-type plastocyanin at pH 7.5 (A) and with Leu12Glu plastocyanin mutant at pH 6.0 (B), 7.5 (C), and 9.0 (D). Each sample contained photosystem 1 particles (0.9 mg of chlorophyll/mL), 2 mM sodium ascorbate, 0.1 mM methyl viologen, and 0.24 mM plastocyanin in 20 mM Tris (pH 7.5 or 9.0) or Mes (pH 6.0) buffer. $MgCl_2$ was added to a final ionic strength of 38 mM. The cuvette (thickness, 1 mm) was placed at 45° to the measuring beam. The arrow indicates the time of excitation. The traces shown are the averaged effect of 16 flashes spaced 20 s apart.

constants of the exponentials will be denoted by A_i and k_i, where $i = 1-3$ for the WT-like mutants and $i = 2-3$ for the Leu-12 mutants.

The analysis of the WT-like mutants was made as earlier described for Pc(WT) and Pc(Tyr83His) (Sigfridsson et al., 1995). Briefly, a two-step procedure is used. In a first, exploratory step, the transients are subjected to an analysis in which all A_i and k_i values are allowed to vary freely until a best fit is obtained. From this, we found k_1 and k_3 to be independent of the Pc concentration while the other parameters were concentration-dependent. The average value of k_1 is listed in Table 2. This phase essentially represents the intracomplex ET from Pc to P700, and of these mutants, only Pc(Tyr83His) is significantly slower than Pc(WT) (Sigfridsson et al., 1995).

An average value of 330 s^{-1} was obtained for k_3. This phase is ascribed to a slow rereduction of Pc^{ox} by ascorbate (Bottin & Mathis, 1985; Nordling et al., 1991). A second-order rate constant of 115 M^{-1} s^{-1} has been reported for this reaction (Wood, 1974), and under our conditions, this would result in an apparent first-order rate constant of 0.2 s^{-1}. However, the true rate constant of this reaction is obscured by the low-frequency cutoff of the AC amplifier (8 Hz).

In a second step of the analysis, k_1 and k_3 are fixed at the average values while the other parameters are varied until a best fit is obtained. From this, we found that both k_2 (see Figure 4) and the amplitude ratio $R = A_1/(A_1 + A_2)$, i.e. the relative contribution of the fast phase to the decaying kinetics, display a saturation at high Pc concentrations. The saturating values, k_{2max} and R_{max}, are different for the different mutants (Table 2), but the Pc concentration at which half the saturating value is obtained is similar for the WT-like mutants, approximately 30−50 μM (not shown). These parameters were obtained from fits of k_2 and R to hyperbolic functions of the Pc concentration.

For most of the mutants, the amplitude A_3 of the slowest component was found to be constant at about 20% of the total initial amplitude at Pc concentrations above 200 μM. This is in line with the assignment of this component to the rereduction of Pc^{ox}. However, for Pc(Asp42Asn) and Pc(Tyr83Phe), slightly larger values were obtained (36 and 47%, respectively). Also, all of these mutants, as well as Pc(WT), show larger relative values of A_3 below 200 μM Pc [not shown, but see Sigfridsson et al. (1995)].

Turning to the Leu-12 mutants, none of these displays a fast phase comparable to the 74 000 s^{-1} component (k_1) in Pc(WT), not even at high Pc concentrations [1.5 mM in the case of Pc(Leu12Glu) and Pc(Leu12Asn), 1.0 mM for Pc(Leu12Ala)]. While the slow, millisecond, phase is independent of Pc concentration (average values of 20% for the relative amplitude and 330 s^{-1} for the rate constant were obtained), we found a concentration dependence for the faster phase. The rate constant of this phase will be denoted by k_2 since it resembles k_2 of the WT-like mutants. There is a clear saturation in k_2 (see Figure 4), just as is the case for Pc(WT), but the saturating values, k_{2max}, are quite different for the different Leu-12 mutants and generally lower than for the WT-like mutants. Also, the Pc concentration C_{k2} at which half the saturating value is obtained is higher for the Leu-12 mutants (Table 3).

An apparent second-order rate constant for binding, k_{on}^{app}, can be obtained from the slope of the k_2 data at low Pc concentrations or from $k_{on}^{app} = k_{2max}/C_{k2}$. This rate constant is on the order of 0.3×10^9 M^{-1} s^{-1} for the WT-like mutants but thirty times less at pH 7.5 for the Leu-12 mutants in Table 3.

The pH dependence of the ET kinetics has been investigated, and representative absorption transients for the Pc(Leu12Glu) mutant are shown in Figure 3B−D. Figure 5B shows that the kinetics for this mutant become faster as the pH is decreased from 8.5 to 5.5 with an apparent pK_a of 6.6. The kinetics for the Pc(WT) and the other Leu-12 mutants are essentially independent of the pH in this range; only below pH 5.5 and above pH 8 is there a slight decrease in rate (Figure 5, Table 3).

The kinetics for the Pc(Leu12Lys) mutant was also investigated, but apparently, this mutant is unable to react with PS1. Preliminary experiments indicate that a Pc(Gln88Glu) mutant, in which an acidic residue has been introduced in the hydrophobic patch, exhibits PS1 kinetics that are similar to those of Pc(WT) (Sigfridsson and Young, unpublished observations).

DISCUSSION

Isoelectric Focusing. As part of an overall characterization of the Pc mutants, their p*I* was determined with isoelectric focusing. The low p*I* of Pc(WT) (3.82) is well-known and ascribed to a large excess of acidic residues, e.g. residues 42−45 and 59−61, which results in a total charge of −9 at neutral pH. The p*I* for the Pc(Leu12Lys), Pc(Asp42Asn), and Pc(Tyr83His) mutants is significantly shifted upward from the Pc(WT) value (Table 1). That the shifts are caused by major changes in secondary structure is considered

Table 2: Parameters Describing the Electron Transfer to Photosystem 1 at pH 7.5 for Plastocyanin Mutants[a]

plastocyanin	$k_1 \times 10^{-3}$ (s^{-1})	$k_{2max} \times 10^{-3}$ (s^{-1})	R_{max}	$k_{et} \times 10^{-3}$ (s^{-1})	$k_d \times 10^{-3}$ (s^{-1})	$k_c \times 10^{-3}$ (s^{-1})	K_{dc}[b]
wild-type	74	19	0.74	69	20	5.2	3.9
Phe35Tyr	82	13	0.64	76	14	6.2	2.3
Asp42Asn	66	6.9	0.56	61	7.4	4.9	1.5
Tyr83His	41	7.0	0.37	31	9.2	9.7	0.9
Tyr83Phe	70	12	0.67	65	13	5.0	2.6
Tyr83Leu	75	15	0.82	72	16	2.7	5.7

[a] k_1, k_{2max}, and R_{max} were obtained from analysis of flash-induced absorbance changes of P700 as described in the text. k_{et}, k_d, and k_c were calculated from eqs 1−3, assuming that the electron transfer can be modeled as in Scheme 2. [b] Calculated from the ratio k_d/k_c.

Table 3: Effect of pH on the Photosystem 1 Electron-Transfer Kinetics for Wild-Type and Leu-12 Plastocyanin Mutants[a]

plastocyanin	pH	$k_{2max} \times 10^{-3}$ (s^{-1})	C_{k2} (mM)	$k_{on}^{app} \times 10^{-9}$ (M^{-1} s^{-1})
wild-type	7.5	19	0.052	0.36
Leu12Glu	6.0	9.1	0.16	0.058
	7.5	6.4	0.51	0.013
	9.0	nd[b]	nd[b]	0.003[c]
Leu12Ala	6.0	nd[b]	nd[b]	0.012[c]
	7.5	1.5	0.15	0.010
	9.0	1.2	0.11	0.011
Leu12Asn	7.5	1.6	0.16	0.010

[a] k_{2max} and C_{k2} were obtained from analysis of flash-induced absorption changes of P700 at a range of plastocyanin concentrations. k_{2max} is the saturating value of the rate constant of the concentration-dependent phase. C_{k2} is the plastocyanin concentration at which half the saturating value is obtained. k_{on}^{app} was calculated from the ratio k_{2max}/C_{k2} or from the slope of the k_2 data at low plastocyanin concentrations (values indicated with[c]). [b] nd is not determined.

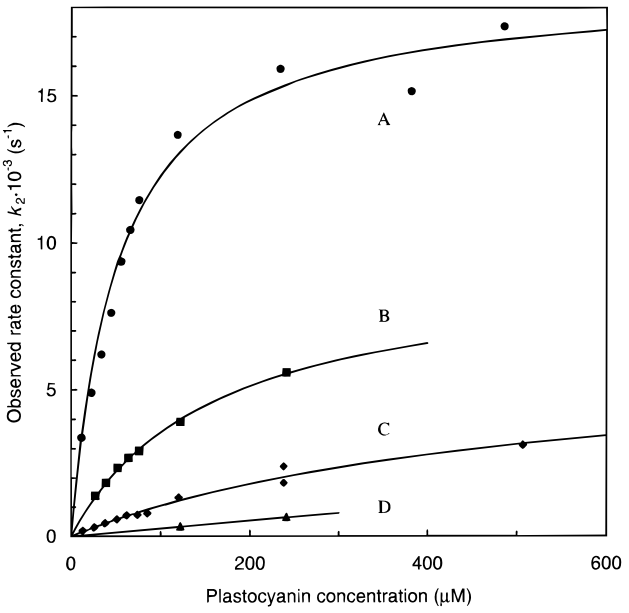

FIGURE 4: Effect of plastocyanin concentration on the observed rate constant k_2 in the decay kinetics of oxidized P700 for wild-type plastocyanin at pH 7.5 (A) and Leu12Glu plastocyanin mutant at pH 6.0 (B), 7.5 (C), and 9.0 (D). The experimental conditions were as in Figure 3. k_2 was obtained from a curve-fitting analysis as described in the text. The curves are hyperbolic functions that best fit the k_2 points. Characteristic parameters of the hyperbolae are given in Table 3.

unlikely, especially since the mutations are made at the surface. NMR studies of Pc(Asp42Asn) (Modi et al., 1992) and Pc(Tyr83His) (Kyritsis et al., 1993; Sigfridsson et al., 1995) show that there are no gross alterations in the structure. The changes in the optical absorption and EPR spectra that are reported here for some of the mutants are also too small

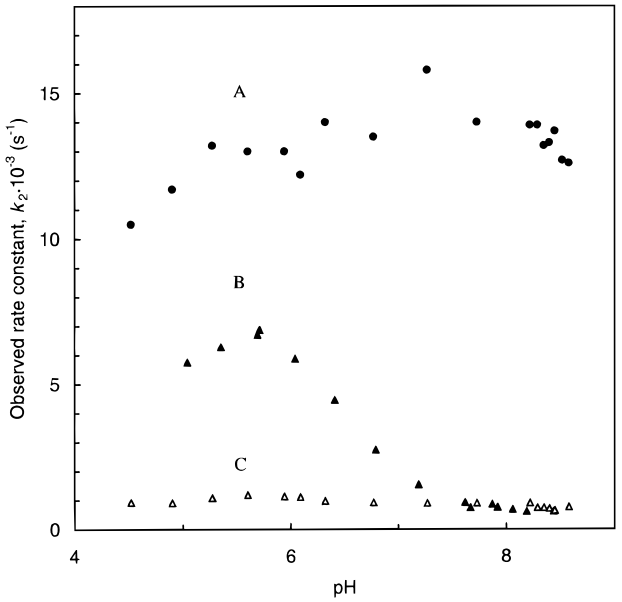

FIGURE 5: Effect of pH on the observed rate constant k_2 in the decay kinetics of oxidized P700 for wild-type (A) and Leu12Glu (B) and Leu12Asn mutant (C) plastocyanin. The experimental conditions were as in Figure 3, except that a buffer mixture containing Hepes, Mes, and succinate (10 mM of each) was used. k_2 was obtained from a curve-fitting analysis as described in the text.

to be caused by large differences in the overall structure (see below). In addition, the circular dichroism (CD) spectrum in the 195−260 nm region of the Pc(Tyr83His) mutant closely resembles that of Pc(WT) (Sigfridsson, to be published elsewhere).

A likely explanation for the upward p*I* shift is that a positive charge has been introduced by the Leu12Lys and Tyr83His mutations. Similarly, the exchange of an acidic residue for a neutral one in Pc(Asp42Asn) results in an overall increase of the net charge.

The p*I* values have been calculated with the program PCGENE. This program only takes into account the amino acids that titrate, i.e. arginine, aspartic acid, cysteine, glutamic acid, histidine, lysine, and tyrosine. This also means that it does not take into consideration where the residue is located in the protein's structure. Consequently, the p*I* values from PCGENE are higher than what has been determined from the gel (not shown). This can be explained by shielding of charges, bonding, and shifts in pK_a values caused by neighboring residues. Despite these restrictions, PCGENE predicts an upward shift in the p*I* values for Pc(Leu12Lys) of 0.13, Pc(Asp42Asn) of 0.10, and Pc(Tyr83His) of 0.13 compared to the wild type's p*I* (not shown). These increases are similar to what has been determined.

The mutations Leu12Ala, Leu12Asn, Phe35Tyr, Tyr83Leu, and Tyr83Phe do not change the overall charge, and as

expected, there are no significant shifts in the p*I* calculated by PCGENE (not shown) or determined experimentally (Table 1). The p*I* for Pc(Leu12Glu) is also close to the Pc(WT) value, despite the introduction of an acidic residue and PCGENE's prediction of a decrease in the p*I* by 0.05 p*I* unit (not shown). This can be explained by the high pK_a value of 6.6 for this residue [see below and Kyritsis et al. (1993)]; i.e. it is neutral around the p*I*.

Optical Absorption and EPR Spectroscopy. A number of the Pc mutants studied here display altered kinetics of ET to PS1. Since some of the mutated residues are close to the Cu ion, it is appropriate to ask if the mutations perturb the structure of the Cu site, altering its electron-donor capabilities. EPR and optical absorption spectroscopy in the visible are sensitive tools for studying the oxidized form of the Cu site. The unusual spectroscopic properties of Pc and other type 1 Cu proteins are attributed to the special coordination geometry around the Cu ion that is enforced by the protein [see Malmström (1994)]. Possible changes in the overall tertiary structure induced by a mutation are therefore expected to result in altered spectra.

Some of the Pc mutants in position 12 display a reduced value of the parallel hyperfine coupling constant, $A_{\|}$ (Table 1). In general, the small magnitude of $A_{\|}$ for the type 1 Cu site (<10 mT) is ascribed to a large degree of delocalization of the unpaired electron onto the ligands (Malmström & Vänngård, 1960), in particular the thiolate sulfur of the cysteine residue (Penfield et al., 1985). The degree of delocalization is sensitive to the detailed structure of the site (Gewirth & Solomon, 1985; Larsson et al., 1995). Evidently, changes of residue 12 can lead to slight perturbations of the Cu site geometry. Mutation of Leu-12 also leads to shifts in the NMR spectra for the His-N$_\epsilon$H resonance of the Cu ligand His-37 (Modi et al., 1992). Leu-12 is close to the other Cu ligand His-87, and it is possible that an interaction between these side chains gives rise to a structural change of the Cu site (Kyritsis et al., 1993).

The strong absorption band around 600 nm is another characteristic feature of oxidized type 1 Cu proteins. The major contribution to this band is a transition to an antibonding [Cu3dπ−S(Cys)3pπ] orbital, but due to the low symmetry of the site, other transitions also contribute (Gewirth & Solomon, 1985; Larsson et al., 1995). This can be seen from CD and low-temperature optical spectra (Gewirth & Solomon, 1985). In some of our Pc mutants (Leu12Ala, Leu12Asn, Leu12Lys, and Tyr83Leu), the position of the absorption maximum is shifted (Table 1). This may reflect small changes in the symmetry that affect the relative strength of nearby transitions which, in turn, leads to shifts in the absorption maximum. The blue shift of the Pc(Tyr83Leu) mutant was also noted by Haehnel et al. (1994). One may speculate that the smaller size of a leucine residue leads to a movement of residues 42−45 and 59−61 toward Leu-83. This could propagate to the Cu site and result in altered EPR and optical spectra.

Overall, it can be concluded that the spectral changes are very small and that the mutations do not induce any major structural changes. For comparison, a large number of azurin mutants have been spectroscopically characterized, and in several cases, their three-dimensional structure has been determined. Even if the mutations induce substantial spectral shifts, the structure is very similar to the WT protein (Romero et al., 1993; Tsai, 1995).

Redox Titration of Pc. A change in reactivity toward PS1 could, in principle, be due to a shift in reduction potential, since this determines the driving force for ET. The reduction potential for P700 is 0.49 V (Nordling et al., 1991, and references therein), resulting in a driving force of 0.11 V for Pc(WT). The Pc(Tyr83His) mutant and the Leu-12 mutants display altered reduction potentials (Table 1), and the possible cause for this will now be discussed.

The E^0 for the Pc(Tyr83His) mutant is increased by 35 mV compared to the Pc(WT) value at pH 7.5, but at higher pH, the difference vanishes due to a pH dependency in the Pc(Tyr83His) reduction potential. This was noted earlier and suggested to be due to an electrostatic influence from a protonated His-83 residue (Nordling et al., 1991). The pK_a for this residue has been found to be unusually high; an NMR titration yielded 8.44 (Sigfridsson et al., 1995), while stopped-flow kinetic studies suggested a value between 7.9 and 8.4 (Kyritsis et al., 1993), compared with typical values of 6−7. Thus, at pH 7.5, the protonated imidazole ring could stabilize the Cu(I) state by increasing the electrostatic potential at the metal ion. At a distance of 0.9−1.1 nm, estimated from the structure of poplar Pc (Guss et al., 1992), a 35 mV increase would result if the effective dielectric constant (ϵ_{eff}) of the intervening medium is 35−45. Similar high values were deduced from the influence of surface charges on the reduction potential of cyt *c* (Rees, 1980). This suggests that the His-83 residue is solvent-exposed, a conclusion which was also drawn from the high proton-exchange rate of this residue (Sigfridsson et al., 1995).

The effective dielectric constant used here is calculated from a simple application of Coulomb's law. Other, more elaborate theories of electrostatic interactions either treat ϵ inside the protein as a constant [e.g. 4 (Sharp & Honig, 1990) or 80 (Svensson & Jönsson, 1995)] or as a distance-dependent quantity (Mehler & Solmajer, 1991). Here, we simply interpret a high ϵ_{eff} as an indication of an interaction that involves solvent-exposed charges.

There is another possible explanation for the increase in E^0. The His-83 residue is surrounded by the acidic residues 42−45 and 59−61, and an attraction between these and the protonated imidazole could cause a conformational change that perturbs the Cu site. Consider, however, the Pc(Tyr83Leu) mutant whose reduction potential is similar to that of Pc(WT) (Table 1). The mutant displays shifts in its EPR and optical absorption spectra, and this perturbation is probably brought about by a conformational change similar to that considered above for Pc(Tyr83His) but for steric reasons (see above). Since the spectral properties of the Pc(Tyr83His) mutant are unchanged compared to those of Pc(WT), we consider a conformational change to be a less likely explanation for its increase in reduction potential.

The Pc(Tyr83Phe) and Pc(Phe35Tyr) mutants are very similar to Pc(WT), in terms of both reduction potential and spectral properties. This is also as expected from the similarities in size and charge between phenylalanine and tyrosine residues. The very small, if any, increase in E^0 for the Pc(Asp42Asn) mutant is somewhat unexpected since such a mutation should increase the electrostatic potential at the Cu ion in a way similar to that of the Pc(Tyr83His) mutant. The distance is, however, longer (1.6 nm), and it is also possible that ϵ_{eff} is larger.

Turning to the Leu-12 mutants, all of them display shifts in E^0 (Table 1). The increase for Pc(Leu12Lys) and the

decrease for Pc(Leu12Glu) compared to the Pc(WT) value suggest an electrostatic origin. At pH 7.5, the absolute values of both shifts are approximately 30 mV. The distance between the charged groups and the Cu ion is estimated to 0.6−0.9 nm, and from this, one can deduce an ϵ_{eff} of 50−70, reflecting the surface location of these side chains. The E^0 of Pc(Leu12Glu) is higher at pH 6.0 than at pH 7.5 by approximately 15 mV. Since the pK_a of the Glu-12 residue is 6.6 [see below and Kyritsis et al. (1993)], this can be explained by a partial neutralization at the lower pH.

Our results can be compared with those obtained for the Met44Lys and Met64Glu mutants of *Pseudomonas aeruginosa* azurin. These mutations also introduce charges close to the Cu ion in a hydrophobic region similar to the one in Pc. Compared to the WT value, shifts in E^0 of +60 and −28 mV were found for the Met44Lys (Van de Kamp et al., 1993) and Met64Glu (Van Pouderoyen et al., 1994) azurin mutants, respectively, at pH values where the side chains were ionized. The shifts were attributed to changes in electrostatic interactions and polarizability.

The reduction potentials of the Pc(Leu12Ala) and Pc(Leu12Asn) mutants are lower than the Pc(WT) value, but in these cases, electrostatic interactions can be excluded. However, both mutants display altered EPR and optical spectral properties. In addition, the NMR resonances of the N_ϵ protons of the Cu ligand His-37 were found to be shifted from the Pc(WT) value (Modi et al., 1992). Therefore, it is possible that the lower E^0 value for these two mutants is caused by a structural change of the Cu site.

In summary, the reduction potential does change for some of the mutants studied here. The shifts can be explained by electrostatic interactions or, in a few cases, by conformational rearrangements that lead to an altered electronic structure of the Cu ion.

Kinetic Measurements. The Leu-12 mutants display a reduced reactivity toward PS1 which is too drastic to be related to changes in the reduction potential or spectral properties. The curve-fitting analysis shows that these mutants lack the fast, 70 000 s^{-1}, component present in the $P700^{ox}$ decay kinetics of the WT-like mutants.

The slow kinetics for the Pc(Leu12Glu) mutant was noted already by Nordling et al. (1991) and suggested to be due to a negatively charged and more bulky Glu-12 residue which could reduce the affinity to PS1. A charge effect is supported by the pH study in Figure 5 which shows that the reactivity increases as the pH is lowered from 8.5 to 5.5. Pc(WT), Pc(Leu12Ala), or Pc(Leu12Asn) does not exhibit such a pH dependence, and we attribute it accordingly to the protonation of the Glu-12 residue. The Pc(Leu12Glu) mutant displays a similar pH dependence in the oxidation by ferricyanide (Kyritsis et al., 1993). The pK_a value obtained, 6.9, is similar to that obtained here for the PS1 reaction (6.6). Such a high value is unusual for a glutamate residue, compared with typical values of 4−5, but could be due to an interaction with the nearby Ser-11 or His-87 residues or to the hydrophobic environment (Kyritsis et al., 1993).

The reactivity of the Pc(Leu12Glu) mutant increases several fold when the pH is lowered, but there is still no fast phase in the PS1 kinetics. A neutral amino acid in this position is apparently not sufficient for full activity, as is also shown by the slow kinetics of the Pc(Leu12Ala) and Pc(Leu12Asn) mutants. In addition, the shape of residue 12 is crucial. A smaller side chain may lead to a local collapse of the structure and a worse fit to PS1.

The Gly-10 and Ala-90 residues, which also are located in the hydrophobic patch, have recently been replaced by a leucine residue (Haehnel et al., 1994). The mutations resulted in slow ET to PS1; notably, a fast phase was lacking in the kinetics. It was suggested that a leucine residue in these positions would protrude too much to allow the formation of a complex between Pc and PS1 (Haehnel et al., 1994). Thus, positions 10, 12, and 90 all seem to be part of a region around the Cu ligand His-87 whose conserved structure is very important for the binding to PS1. However, residues 35 and 88 are probably outside this critical region, since mutations of these residues do not cause any large changes in the PS1 kinetics (this work and Sigfridsson and Young, unpublished observations).

The Leu-12 mutants show a saturation in the observed rate constant, k_2, at high Pc concentrations. Therefore, the lack of a fast phase in the PS1 kinetics is intriguing. The simplest possible reaction scheme that gives rise to saturation kinetics can be formulated as

Scheme 1

$$Pc^{red} + P^{ox} \underset{k_{off}}{\overset{k_{on}[Pc]}{\rightleftharpoons}} Pc^{red}\cdot P^{ox} \xrightarrow{k_{et}} Pc^{ox}\cdot P^{red}$$

where k_{on} and k_{off} are rate constants for binding and debinding, respectively, k_{et} is the intracomplex ET rate constant, and P stands for P700. This scheme would result in a monoexponential decay of $P700^{ox}$ at high Pc concentrations but a biphasic decay at low concentrations (Sigfridsson et al., 1995), contrary to what is observed for the Leu-12 mutants.

Scheme 1 is also insufficient for explaining the kinetics of Pc(WT) (Bottin & Mathis, 1985) and the Pc(Tyr83His) mutant (Sigfridsson et al., 1995). Instead, the following reaction scheme, originally proposed by Bottin and Mathis (1985), can successfully account for the observed kinetics (Sigfridsson et al., 1995):

Scheme 2

$$PC^{red} + P^{ox} \underset{k_{off}}{\overset{k_{on}[Pc]}{\rightleftharpoons}} Pc^{red}\cdots P^{ox} \underset{k_c}{\overset{k_d}{\rightleftharpoons}} Pc^{red}\cdot P^{ox} \xrightarrow{k_{et}} Pc^{ox}\cdot P^{red}$$

In this model, a conformational change, determined by the rate constants k_d and k_c, constitutes a rate-limiting step that precedes the ET (d and c denote conformations where Pc is "distant" or "close" to P700). At high Pc concentrations, Scheme 2 results in two decay components in the $P700^{ox}$ kinetics with rate constants that are well-approximated by (Sigfridsson et al., 1995)

$$k_1 = k_c + k_{et} \quad (1)$$

$$k_{2max} = k_d k_{et}/k_1 \quad (2)$$

The fraction of the fast phase saturates at a value of

$$R_{max} = k_2/(k_c + k_d) \quad (3)$$

The experimental estimates, k_1, k_{2max}, and R_{max} can be used to solve eqs 1−3 for k_d, k_c, and k_{et}, and Table 2 lists the values obtained for the WT-like mutants. Examining the

k_{et} values first, only Pc(Tyr83His) deviates significantly from Pc(WT). This was noted by Sigfridsson et al. (1995) and attributed to the lower driving force for this mutant, since increasing the pH was found to result in a larger driving force and a k_{et} more similar to the Pc(WT) value.

The rate constants k_d and k_c determine the equilibrium constant K_{dc} ($=k_d/k_c$) for the conformational change. For most mutants in Table 2, K_{dc} is larger than 1, i.e. the close conformation is favored, but for some mutants there are deviations from the Pc(WT) value (see below). If, however, K_{dc} is smaller than 1, i.e. if $k_d < k_c$, formation of a close complex will be hampered and this will strongly influence the kinetics. Under such circumstances, the amplitude ratio R can be approximated by k_d/k_c, and if $R < 5\%$, the fast phase will pass undetected due to the limited signal-to-noise ratio of our experimental setup.

As seen from eqs 1 and 2, the saturating value k_{2max} is essentially governed by k_d. Thus, even if K_{dc} cannot be determined for the Leu-12 mutants, their low k_{2max} [in some cases almost 20 times lower than k_{2max} for Pc(WT), see Table 3] suggests that k_d is small for these mutants. If k_c is unaffected or raised, this means that the equilibrium is displaced toward the distant conformation. This would explain why the Leu-12 mutants lack a fast phase.

The results from our mutant studies and those from Haehnel et al. (1994) suggest that the hydrophobic area around the exposed His-87 residue is crucial for the interaction with PS1. Since His-87 is a Cu ligand, it is likely that the ET to P700 occurs through this residue. On the other hand, there is also support for an interaction between PS1 and the acidic residues 42−45 and 59−61. Detailed studies of the ionic strength and pH dependence indicate an attraction between Pc and a positively charged PS1 subunit (Ratajczak et al., 1988; Hope et al., 1994), and cross-linking studies have suggested this to be the PS1-F subunit (Wynn & Malkin, 1988; Hippler et al., 1989). Since modification of carboxyl groups on Pc inhibits cross-linking, the negative patch on Pc was proposed to be the binding site (Wynn & Malkin, 1988). In another study, chemical modification of positions 42−45 did not affect the ET to PS1 (Anderson et al., 1987), but it is uncertain whether the PS1-F subunit was present in the preparation used (Gross, 1993). More recent studies have questioned the involvement of the PS1-F subunit in the binding of Pc, at least in cyanobacteria (Xu et al., 1994).

An interaction between the acidic patch and PS1 is further supported by the results presented here. The equilibrium constant K_{dc} for the conformational change is approximately 4 for Pc(WT), but other values are obtained for some of the mutations in the acidic patch (Table 2). As also noted by Haehnel et al. (1994), the Pc(Tyr83Leu) mutant shows an increased overall reactivity, and this we can trace to an increase in K_{dc} to a value of approximately 6. Evidently, the smaller side chain of a leucine residue compared with a tyrosine favors formation of a close complex with PS1. Since the reactivity toward cyt *f* is much reduced for this mutant (He et al., 1991), it can, however, be understood why nature does not employ a leucine in this position. In contrast, K_{dc} is significantly lower for the Pc(Tyr83His) and Pc(Asp42Asn) mutants compared to the Pc(WT) value. The result for Pc(Tyr83His) was reported previously, and also reported was the fact that K_{dc} increases toward the Pc(WT) value when the pH is raised (Sigfridsson et al., 1995). This suggests that the protonation state of His-83 influences the equilibrium, with the protonated, positively charged, form favoring the distant conformation. The effect of the Asp42Asn mutation can be interpreted in a similar way. Also in this case, an increase in charge of the acidic patch results in less of the reactive close complex.

The conclusion is that both the hydrophobic and acidic patches make contact with PS1 but that the ET occurs at the hydrophobic interface, most probably via the His-87 residue. Electrostatic interactions between the negative patch and positive charges on PS1 may be important for achieving a proper orientation (Haehnel et al., 1994) and for the formation of ion pair bonds.

The kinetic data for our Pc mutants have been interpreted in terms of Scheme 2, which we consider to be a minimal model for the Pc−PS1 interaction. Further support for this reaction scheme comes from studies of the activity of cross-linked Pc-PS1 complexes (Wynn & Malkin, 1988; Hippler et al., 1989). Despite the fact that all PS1 centers can be cross-linked to Pc, photooxidation of bound Pc only occurs in a fraction of the PS1 centers [15% (Wynn & Malkin, 1988) to 55% (Hippler et al., 1989)]. This indicates a distribution of cross-linked products, with only some of them being in the right conformation for ET. In other words, a rearrangement between Pc and PS1 is necessary to bring the first encounter complex into a reactive one. Furthermore, the cross-linked product is unable to oxidize added Pc (Wynn & Malkin, 1988). This means that a proposed alternative to Scheme 2, which involves dual binding sites for Pc on PS1 (Bottin & Mathis, 1985, 1987), is unlikely. Finally, the well-known anomalous salt dependence of the Pc to PS1 ET rate, which shows an increase with ionic strength at low salt concentrations but a decrease at high salt concentrations [see Ratajczak et al. (1988) and references therein], has also been suggested to be due to a reorganization of the Pc−PS1 complex after the initial collision (Hervás et al., 1992).

A structural rearrangement in a biological ET complex is not unprecedented in the literature. Other donor−acceptor pairs where this has been suggested to occur include cyt *f*−Pc (Quin & Kostic, 1993), cyt c_2−bacterial reaction center [Tiede and Dutton (1993) but see Mathis (1994)], cyt *c*−cyt *c* peroxidase (Jeng et al. 1994, and references therein), and amicyanin−methylamine dehydrogenase (Brooks & Davidson, 1994). The picture that emerges is one where there is a substantial degree of flexibility in complexes of ET proteins.

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