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Flash-photolysis studies of the electron transfer from genetically modified spinach plastocyanin to photosystem I*

Margareta Nordling[1], Kalle Sigfridsson[1], Simon Young[1], Lennart G. Lundberg[1,2] and Örjan Hansson[1]

[1]*Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Goteborg, S-412 96 Goteborg (Sweden)* and [2]*Department of Cell Biology, Astra-Hassle AB, S-431 83 Molndal (Sweden)*

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Plastocyanin (Pc) has been modified by site-directed mutagenesis at two separate electron-transfer (ET) sites Leu-12-Glu at a hydrophobic patch, and Tyr-83-His at an acidic patch The reduction potential at pH 7 5 is decreased by 26 mV in Pc(Leu-12-Glu) and increased by 35 mV in Pc(Tyr-83-His) The latter mutant shows a 2-fold slower intracomplex ET to photosystem I (PSI) as expected from the decreased driving force The affinity for PSI is unaffected for this mutant but is drastically decreased for Pc(Leu-12-Glu) It is concluded that the hydrophobic patch is more important for the ET to PSI

Electron transfer, Flash photolysis, Plastocyanin, Photosynthesis, Photosystem I, Site-directed mutagenesis

1. INTRODUCTION

Plastocyanin (Pc) is a small, blue copper protein which acts as an electron carrier between the cytochrome b_6f and photosystem I (PSI) complexes in the photosynthetic electron transfer (ET) chain. The photooxidized reaction-center chlorophyll P700 in PSI is reduced by Pc and the oxidized Pc is in turn reduced by cytochrome *f* (see [1,2] for reviews).

Two areas on the Pc surface have been identified as important sites for ET [1,2] One is the hydrophobic patch around the Cu-ligand His-87, and the other is an acidic patch around Tyr-83 These regions are also referred to as the adjacent and remote sites, respectively [2] Based on studies of Cr-labeled Pc, it was suggested that the acidic patch is involved in the ET to PSI while the ET from cytochrome *f* involves the hydrophobic patch [3]. However, these assignments have been questioned [1,2]

In order to assess the importance of these sites we have introduced site-specific mutations in the structural gene for spinach Pc using our previously described system for overexpression of Pc in *E coli* [4] In one mutant, Pc(L12E), a Leu in the hydrophobic patch has been replaced with a Glu and in another, Pc(Y83H), the Tyr-83 has been replaced with a His The recombinant wild-type (Pc(WT)) and mutant proteins have been characterized by optical and EPR spectroscopy, and the reduction potential has been determined at pH 7 5 In addition, the kinetics of the ET reaction with PSI has been studied Both mutants show altered kinetics but the results suggest that the hydrophobic patch around the Cu-ligand His-87 is more important for the ET to PSI

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Abbreviations ET, electron transfer, P700, reaction-center chlorophyll, Pc, plastocyanin, PCR, polymerase chain reaction, PSI, photosystem I, Tris, 2-amino 2-hydroxymethylpropane-1, 3-diol

Correspondence address Ö Hansson, Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, S-412 96 Göteborg, Sweden. Fax (46) (31) 72 2813.

2 MATERIALS AND METHODS

2 1 *Construction of expression vector and mutant proteins*

The expression-vector used,pUG101t$_r$, was constructed as described in [4] with the exception of the addition of a termination sequence for stabilization of the mRNA In pUG101 the Pc gene is placed under control of the *lac* promotor in pUC18 The termination sequence originates from the expression vector pGK14 [5] which encodes the structural *Pseudomonas aeruginosa* azurin gene and the transcriptional termination sequence of this gene The vector pGK14 was cut with restriction enzymes *Kpn*I and *Ban*II This fragment, containing the azurin transcript termination sequence, was filled in using Klenow polymerase and dNTPs The blunt ended fragment was then ligated into pUG101 using a unique *Sph*I site at the stop codon of the Pc gene The new construction was named pUG101t,

The mutant proteins were constructed using PCR amplification in two steps The method of Landt et al [6] was used with the following modifications (i) The template was prepared by resuspending a colony of TG1 transformed with pUG101t, in 0 5 ml H_2O and 10 μl of this solution were used in each of the PCR steps (ii) For increased fidelity and thermostability the Vent polymerase (New England Biolabs) was used The reaction buffer was 20 mM Tris-HCl (pH 8 8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0 1% Triton X-100, 100 μg/ml BSA The dNTP concentration was 200 μM and 10 pmol of each primer was used. Oligonucleotides for mutagenesis were from MedProbe, Norway The reaction volume was 20 μl One PCR cycle consisted of

60 s at 93°C (denaturation), 60 s at 45°C (primer annealing) and 120 s at 72°C (primer extension) The PCR was run in 30 cycles (iii) The primers used in the first step were the pUC/M13 reverse primer together with the mutagenic primer (23 bases long) The PCR product was purified on agarose gel where the fragment was eluted from the gel slice by freezing it at −70°C for 10 min and afterwards crushing it The suspension obtained after adding 1/5 volume of H_2O to the crushed gel slice was shaken for 15 min and centrifuged (Eppendorf) for 5 min The supernatant was extracted once with phenol, twice with chloroform and precipitated with ethanol The yield of the purified PCR product was at least 50% (iv) In the second PCR step the entire fragment from above was used as 5'-primer The forward primer of pUC/M13 was used in the 3'-end A 500 basepair long fragment was amplified The conditions were the same as in the first step except that the temperature of the annealing reaction was raised to 55°C (v) The final PCR product was cut with restriction enzymes *Eco*RI and *Pst*I and ligated into pUC18 which had been cut with the same enzymes

2 2 *Bacterial strains and growth*

The strain used was TG1, a derivative of *E coli* K12 (*Δ(lac-pro), supE, thi, hsd*D5/F'*tra*D36, *pro*A⁺B⁺, *lac*I^q, *lac*Z*Δ*M15) [7] Growth media and conditions were as described in [4]

2 3 *Protein purification*

The Pc mutants were purified according to [4] with the exception that in the last step an ordinary Sephacryl S-100 column was used instead of the Superose 12 FPLC-column Apoprotein was present in small amounts but further purification was not attempted The amount of obtained mutant proteins was approx 1/3 of the wild-type yield (2 mg/l culture) Pc (Y83H) showed an increased tendency towards auto-reduction The concentration of holo-Pc was determined spectrophotometrically under oxidizing conditions using an absorption coefficient of 4900 M^{-1} cm^{-1} at 597 nm [8]

2 4 *Redox titration of Pc*

The reduction potential of Pc was determined in 20 mM Tris (pH 7 5) by monitoring the 597 nm absorbance as the ratio of (potassium) ferricyanide to ferrocyanide was varied 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 Pc mutants

2 5 *Preparation of PSI*

Digitonin-solubilized PSI particles (D-144 particles) with 240 chlorophyll/P700 were prepared from spinach according to [9] The presence of subunit III (PSI-F), which is thought to be important for the reduction of PSI by Pc [10], was confirmed by gel electrophoresis The reduction potential of P700 was determined to 0 49 V for this PSI preparation using the flash-photolysis technique described in [11]

2 6 *Spectroscopic and kinetic methods*

Optical and EPR spectra were obtained as in [4] Kinetic studies were made with the equipment described in [12] Briefly, excitation of PSI was by short (10 ns) flashes (532 nm) from a Nd YAG laser Photooxidation and reduction of P700 was monitored at 830 nm with a continuous-wave diode laser Acquisition of the transient absorption signals and fitting of the signals to a sum of exponentials were made as in [12] Other experimental conditions are given in the figure legends

3 RESULTS AND DISCUSSION

3.1. *Mutagenesis*

The mutagenesis method used here has several advantages over other PCR and ordinary mutagenesis methods [6] (i) The mutagenesis efficiency is 100%, (ii) Only one specific primer is needed, the other two primers are standard sequencing primers (iii) The final PCR product can be subcloned into different vectors using the different restriction enzyme sites in the pUC polylinker (iv) The mutated protein can be expressed using isopropyl-*β*-D-thiogalactopyranoside induction of the *lac* promotor in pUC (v) Double-stranded sequencing of the entire PCR amplified fragment can readily be done in the pUC-vectors (vi) The risk of introducing random mutations in the gene is reduced by using the Vent polymerase and it is also possible to raise the denaturation temperature to efficiently denature the DNA strands

When Vent polymerase is used for the amplification there is a risk for degradation of the primers due to the 3'→5' exonuclease activity. The exonuclease activity degrades primers that are not annealed to a template, leaving a product with a length of about 15 bases This problem was circumvented by using primers where the mutation is flanked by less than ten bases

3.2 *Spectroscopic studies and redox titrations*

Fig 1 compares the absorption spectra of the three plastocyanins The Pc mutants show charge-transfer bands at 597 nm and in the near infrared region with shapes similar to Pc(WT). The lower absorbance at wavelengths above 500 nm for the mutants is due to the presence of some apo-Pc Pc(Y83H) has a reduced absorption peak at 278 nm (Fig. 1, *insert*) consistent with the smaller content of Tyr in this mutant The EPR spectra are indistinguishable from each other (not shown)

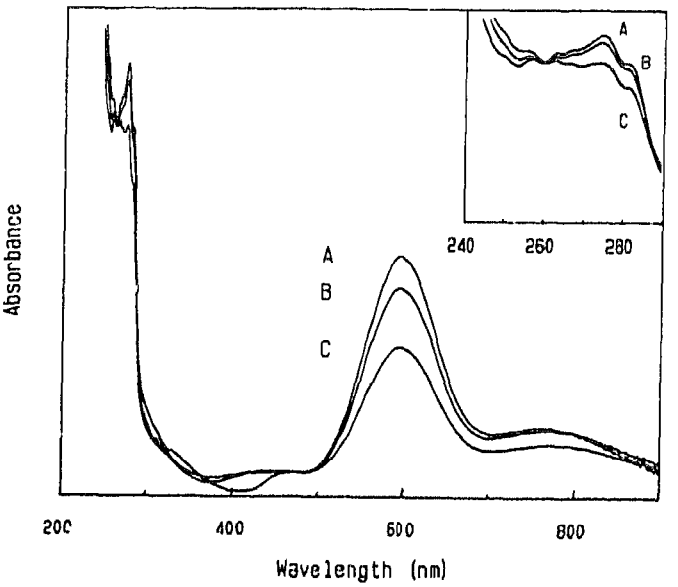

Fig 1 Absorption spectra of recombinant Pc(WT) (A), Pc(L12E) (B) and Pc(Y83H) (C) The *insert* is an enlargement of the UV region Potassium ferricyanide was added both to the sample (to oxidize Pc) and to the reference cuvettes (to suppress the contribution of ferricyanide to the absorption spectrum) The spectra have been normalized at 260 nm

Table I

Reduction potentials (E°) together with dissociation (K_{diss}) and rate (k_{on}, k_{et}) constants describing the electron transfer to photosystem I for different spinach plastocyanin mutants in 20 mM Tris (pH 7.5)

Plastocyanin	E° (mV)	K_{diss} (mM)	k_{on} (10^7 M^{-1} s^{-1})	k_{et} (10^4 s^{-1})
WT	384	0.17	3.7	3.8
Y83H	419	0.18	4.2	1.7
L12E	358	–	–	–

The reduction potentials are summarized in Table I. The potential of Pc(WT) is close to the value for Pc prepared from spinach [1,2,13]. The mutants display shifts in their potentials which probably in part are due to long-range electrostatic interactions since the spectroscopic data indicate that the Cu site is unperturbed. The 26 mV lower potential of Pc(L12E) is consistent with the introduction of a negative charge in the neighborhood of the Cu ion. The 35 mV higher potential of Pc(Y83H) is unexpected but could be explained by a high pK of His-83, rendering this residue protonated at pH 7.5. This idea obviously needs to be tested by measurements at other pH.

3.3. *Kinetic measurements*

Flash-excitation of a mixture of PSI particles and Pc results in an instantaneous absorption increase at 830 nm (due to photo-oxidation of P700) followed by a slower absorption decrease (due to reduction of $P700^{ox}$ by Pc) (Fig. 2). These experiments were made in the presence of ascorbate and methylviologen in order to reduce Pc and oxidize the PSI electron-acceptor side, respectively, between the flashes. $MgCl_2$ at millimolar concentrations is known to facilitate the ET from Pc to PSI in this type of digitonin-solubilized PSI particles [14,15] but was left out here in order to keep the ionic strength at a moderately low value (17 mM). The effect of higher ionic strength will be reported in a separate publication.

For Pc(WT) (Fig. 2A) three phases can be discerned:

Fast phase	A_1 = 24%	τ_1 = 22 μs
Intermediary phase	A_2 = 52%	τ_2 = 219 μs
Slow phase:	A_3 = 24%	τ_3 = 3 ms

The amplitudes (A_i) and decay lifetimes (τ_i) were obtained from a curve-fitting analysis of the data in Fig. 2A where the Pc concentration is high (36 Pc/PSI) and the ionic strength is moderately low (17 mM). At lower Pc concentration and/or higher ionic strength, A_2 and τ_2 increase while A_1 decreases and τ_1, A_3 and τ_3 remain the same. This behavior is consistent with earlier results that have been interpreted in terms of a formation of a Pc PSI complex (intermediary phase) followed by an intracomplex ET (fast phase) [16,17].

The analysis will be made in terms of the following simple model

$$Pc^{red} + P700^{ox} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Pc^{red} \cdot P700^{ox} \xrightarrow{k_{et}} Pc^{ox} \cdot P700^{red}$$

Here, k_{on} and k_{off} are rate constants for formation and dissociation, respectively, of a Pc PSI complex and k_{et} is the rate constant for intracomplex ET from Pc^{red} to $P700^{ox}$ ($^{red/ox}$= reduced/oxidized species). The absorption signal at 830 nm has contributions from both $P700^{ox}$ and Pc^{ox} (absorption coefficients, 5500 [18] and 1000 M^{-1} cm^{-1} (Fig. 1), respectively) and will according to this model show a biexponential decay to a constant level at 1000/5500 = 18% of the initial amplitude. In the present case Pc is in large excess over PSI and the decay lifetimes will be related to the rate constants through

$$\tau_1^{-1} + \tau_2^{-1} = k_{on}\,[Pc] + k_{off} + k_{et}$$
$$(\tau_1\,\tau_2)^{-1} = k_{et}\,k_{on}\,[Pc]$$

A third relation between the lifetimes, the rate constants and the amplitude ratio $A_1/(A_1+A_2)$ can also be found if it is assumed that the dissociation constant $K_{diss} = k_{off}/k_{on}$ is independent of the oxidation state of P700

$$\frac{A_1}{A_1 + A_2} = \frac{\tau_1^{-1} - k_{on} \cdot [Pc] - k_{off} - \dfrac{k_{et} \cdot k_{off}}{k_{on} \cdot [Pc] + k_{off}}}{\tau_1^{-1} - \tau_2^{-1}}$$

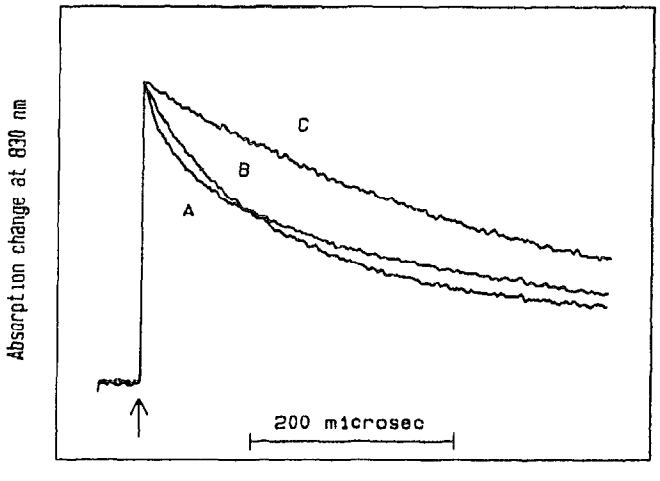

Fig. 2. Flash-induced absorption transients at 830 nm of PSI particles with Pc(WT) (A), Pc(Y83H) (B), and Pc(L12E) (C). Each sample contained PSI particles (0.9 mg chlorophyll/ml), 2 mM Na-ascorbate, 0.1 mM methyl viologen and Pc at concentrations of 0.15 mM (Pc(WT)), 0.24 mM (Pc(Y83H)) and 0.37 mM (Pc(L12E)) in 20 mM Tris buffer (pH 7.5). The cuvette (thickness, 1 mm) was placed at 45° to the measuring beam. The traces shown are the averaged effect of 16 flashes spaced 20 s apart.

Thus one can calculate all three rate constants from the amplitude ratio and the two shorter lifetimes obtained from the curve-fitting analysis. The experimentally observed third phase arises from the reduction of Pc^{ox} by ascorbate However, the observed lifetime (τ_3 = 3 ms) gives a lower limit for this reaction given by the low-frequency cut-off of the AC amplifier PSI particles unable to bind Pc but reduced by ascorbate may also contribute to the third phase

Analysis of the kinetic data in Fig 2A yields K_{diss} = 0 17 mM, $k_{on} = 3\ 7 \times 10^7$ M^{-1} s^{-1} and $k_{et} = 3\ 8 \times 10^4$ s^{-1} for Pc(WT) at pH 7.5 and 17 mM ionic strength (see Table I) These values differ slightly from the values obtained for Pc prepared from spinach. K_{diss} = (0.08–0 125) mM, $k_{on} = 1.9 \times 10^8$ M^{-1} s^{-1} and $k_{et} = 5\ 8 \times 10^4$ s^{-1} [17]. The difference may arise from the lack of $MgCl_2$ in the present experiments

The Pc mutants show altered kinetics For Pc(Y83H) the decay at early times is slower than for Pc(WT) while at longer times it becomes more rapid (Fig 2A,B) However, an analysis yields K_{diss} = 0 18 mM and $k_{on} = 4.2 \times 10^7$ M^{-1} s^{-1}, very similar to the Pc(WT) values (Table I). Thus the binding of Pc to PSI is not affected by this mutation The apparent acceleration of the intermediary phase is mostly due to the higher Pc concentration in this sample

The slowing down of the fast phase for Pc(Y83H) is reflected in a smaller intracomplex ET rate constant (k_{et}) The value obtained is only half the value obtained for Pc(WT) (Table I). A halving of the ET rate is also predicted from ET theory [19] if the 35 mV higher reduction potential of Pc(Y83H) is taken into account and if the electronic factor is assumed to be unaffected by the mutation This indicates that Tyr-83 is not in the ET path to PSI since a replacement of Tyr with His is expected to result in a smaller electronic factor and a slower ET rate than observed

The Pc(L12E) mutant (Fig 2C) shows no fast phase at all and therefore the above analysis cannot be applied An apparent second-order rate constant of 8 10^6 M^{-1} s^{-1} is obtained from the intermediary phase The lack of a fast phase indicates that K_{diss} has increased by a large amount for this mutant. Thus, introductio of a negatively charged, more bulky, amino acid in th hydrophobic patch significantly decreases the ability c Pc to bind to PSI and strongly suggests that this patc provides an important binding site for PSI

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REFERENCES

[1] Haehnel, W (1986) in Encyclopedia of Plant Physiology, vol 1' pp 547–559, Springer, Berlin
[2] Sykes, A G (1991) Structure and Bonding 75, 175–224
[3] Farver, O , Shahak, Y and Pecht, I (1982) Biochemistry 2 1885–1890
[4] Nordling, M , Olausson, T and Lundberg, L G (1990) FEE Lett 276, 98–102
[5] Karlsson, B G , Nordling, M , Pascher, T , Tsai, L -C , Sjolin, I and Lundberg, L G (1991) Prot Eng 4, 343–349
[6] Landt, O , Grunert, H -P and Hahn, U (1990) Gene 96, 125–12
[7] Carter, P (1986) Biochem J 237, 1–7
[8] Katoh, S , Shiratori, I and Takamiya, A (1962) J Biochem 5 32–40
[9] Boardman, N K (1971) Methods Enzymol 23, 268–276
[10] Bengis, C and Nelson, N (1977) J Biol Chem 252, 4564–456
[11] Setif, P and Mathis, P (1980) Arch Biochem Biophys 20 477–485
[12] Hoganson, C W , Casey, P C and Hansson, Ö (1991) Biochir Biophys Acta 1057, 399–406
[13] Takabe, T , Ishikawa, H , Niwa, S and Tanaka, Y (1984) Biochem 96, 385–393
[14] Lockau, W (1979) Eur J Biochem 94, 365–373
[15] Olsen, L F and Cox, R P (1982) Biochim Biophys Acta 67 436–443
[16] Haehnel, W , Propper, A and Krause, H (1980) Biochir Biophys Acta 593 384–399
[17] Bottin, H and Mathis, P (1985) Biochemistry 24, 6453–6460
[18] Mathis, P and Setif, P (1981) Isr J Chem 21, 316–320
[19] Marcus, R A and Sutin, N (1985) Biochim Biophys Acta 81 265–322