Interaction of Plastocyanin with the Photosystem I Reaction Center: A Kinetic Study by Flash Absorption Spectroscopy

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ABSTRACT: Interactions between plastocyanin and photosystem I have been studied by measuring the reduction kinetics of photooxidized P-700 with flash absorption spectroscopy. In pea chloroplasts, the reduction of $P\text{-}700^+$ shows two major phases. A fast phase of reduction has a $t_{1/2}$ = 12 μs, which is influenced by temperature (activation energy 4.9 kJ mol^{-1}) but is independent of the fraction of P-700 oxidized by the flash. Its amplitude titrates with E_m = 385 mV with no change in the half-time. In spinach photosystem I particles, this fast phase is observed upon addition of pure plastocyanin. This fast electron donation is attributed to reaction center bound plastocyanin (dissociation constant 8×10^{-5} M at 20 °C; binding enthalpy 8 kJ mol^{-1}). An intermediate phase indicates a bimolecular reaction between plastocyanin and photosystem I. In chloroplasts, its rate slows down when the viscosity increases and titrates with E_m = 390 mV. In photosystem I particles, its rate is proportional to plastocyanin concentration below 20 μM (second-order rate constant $k = 1.9 \times 10^8$ M^{-1} s^{-1}) but tends to saturate at higher concentration with a limiting $t_{1/2}$ of 110 μs. The saturation is attributed to the participation of a "distant" bound state of plastocyanin in the electron-transfer pathway from plastocyanin in solution to P-700. The results show that the kinetic behavior of $P\text{-}700^+$ in chloroplasts can be satisfactorily modeled in vitro, upon addition of pure plastocyanin. The interaction between plastocyanin and photosystem I can thus be interpreted, as for soluble cytochrome c_2 and reaction centers from the bacterium *Rhodopseudomonas sphaeroides* [Overfield, R. E., Wraight, C. A., & DeVault, D. (1979) *FEBS Lett. 105*, 137–142], in terms of two bound states in equilibrium with free plastocyanin.

In higher plant chloroplasts, most of the electron carriers are organized into three membranous complexes: the oxygen-evolving complex associated with the PS-II[1] reaction center, the cytochromes b_6/f complex, and the PS-I reaction center (Mathis, 1983). The functional organization of these proteins and the connections between the complexes have been studied by many approaches, but the problems of long-distance electron transport and particularly of electron donation to $P\text{-}700^+$ are still in debate. It is now known that this reduction involves cytochrome *f* and plastocyanin, but there is little information concerning the interactions between the molecules and molecular complexes, although the sequence Rieske iron–sulfur center → cytochrome *f* → plastocyanin → P-700 has been rather well established [for reviews, see Haehnel (1984) or Cramer & Crofts (1982)].

The isolated plastocyanin molecule, a blue copper protein of M_r 10500, has been studied in some detail. It contains one copper atom, and its structure has been recently established by X-ray crystallography at 1.6-Å resolution (Guss & Freeman, 1983; Garret et al., 1984). The oxidation–reduction potential of the purified protein is E_m = 370 mV for pH values above 5.4 (Katoh et al., 1962). A midpoint potential of 382 mV has recently been reported (Takabe et al., 1984). Studies using antibodies raised against plastocyanin, as well as other techniques, have evidenced that plastocyanin was localized exclusively inside the thylakoids (Haehnel et al., 1981). Chemical modifications of the protein and NMR studies have indicated the existence of at least two different sites for highly specific interactions with inorganic redox complexes (Lappin et al., 1979; Cookson et al., 1980; Handford et al., 1980; Farver et al., 1982). These sites are proposed to be electron-transfer pathways to and from the copper atom. The chemical modifications also brought informations on the ionic regulation of the interaction between plastocyanin and the PS-I reaction center (Burkey & Gross, 1981, 1982; Takabe et al., 1984).

Despite the structure and physicochemical properties of plastocyanin being well documented, the detailed mechanism of the reaction between P-700 and its immediate electron donor(s) is still controversial. Most work on this problem has been done by monitoring the absorption changes related to the photooxidation and the subsequent reduction of P-700 in chloroplasts or in isolated protein complexes. After a short flash, photooxidized P-700 returns to the reduced state following complex kinetics. In photosystem I subchloroplast particles, upon addition of reduced plastocyanin, the kinetics are influenced by numerous parameters, such as the pH, the ionic composition of the medium, or the chemical integrity of plastocyanin (Wood & Bendall, 1975; Lien & San Pietro, 1979; Haehnel et al., 1980a; Tamura et al., 1981; Burkey & Gross, 1981, 1982; Farver et al., 1982; Olsen & Cox, 1982; Takabe et al., 1983, 1984). These effects have been mostly interpreted within a bimolecular kinetic model, in terms of factors that control the interaction of plastocyanin with the reaction center, although the occurrence of a binding has not been established. In chloroplasts and algae, under nearly physiological conditions, the reduction of $P\text{-}700^+$ has been analyzed as the sum of at least three exponential components, the half-times of which are respectively 15 μs, 200 μs, and more than 5 ms (Haehnel et al., 1971, 1980b; van Best & Mathis, 1978). Haehnel et al. (1980b) first proposed that the 15- and

[1] Abbreviations: ΔA, absorbance change; Pc, plastocyanin; Pc(I) and Pc(II), reduced and oxidized plastocyanin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS-I, photosystem I; PS-II, photosystem II; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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200-μs phases are due to the reduction of P-700$^+$ by plastocyanin and that the fast decay is a first-order process, whereas the 200-μs phase behaves like a bimolecular reaction. On the contrary, Olsen et al. (1980, 1982), working at subzero temperatures in fluid organic media, proposed that the entire reduction of P-700$^+$ is a single bimolecular reaction. Bouges-Bocquet & Delosme (1978) interpreted their results as involving an unknown molecule, possibly located in subunit III of the reaction center, acting as the immediate fast donor to P-700$^+$.

In the present work we have reinvestigated the problem of electron transfer to photooxidized P-700, studying the influence of various physicochemical parameters on the rate of the reaction. The comparison of these properties in chloroplasts and photosystem I particles leads to a coherent scheme for the interaction of plastocyanin with the reaction center. We chose to study P-700$^+$, by means of its absorption peak around 820 nm [see Mathis & Sētif (1981)], instead of studying plastocyanin directly. In its oxidized form, this molecule has a broad absorption peak (maximum at 597 nm) (Katoh et al., 1962), but the absorption changes associated with changes in its redox state are overlapped, in the chloroplasts, by several other species that render the specific study of plastocyanin rather difficult, and perhaps uncertain. Problems associated with the weak absorption of oxidized plastocyanin at 820 nm will be discussed below.

Materials and Methods

Intact chloroplasts were isolated from leaves of 10–14-day-old pea plants as described by Nakatani & Barber (1977). The chloroplasts were osmotically shocked in the measuring cuvette as described by Telfer et al. (1983). The final composition of the suspension medium was 0.33 M sorbitol, 20 mM Tricine/NaOH, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 1 mM sodium ascorbate, 10 μM DCMU, 40 μM hydroxylamine, and 50 μM methylviologen, unless otherwise indicated. Spinach chloroplasts were prepared as described previously (Mathis & Haveman, 1977). The pellet was homogenized in a minimum volume of buffer (0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 50 mM Tricine/NaOH, pH 7.5) and kept on ice. Sodium ascorbate, DCMU, hydroxylamine, and methyl viologen were added in the cuvette at the same final concentrations as for pea thylakoids.

Spinach plastocyanin was isolated and purified according to Yocum (1982). The purification procedure was stopped after the second DEAE-cellulose (DE-52, Whatman) column chromatography and a 10-h dialysis against 10 mM potassium phosphate buffer, pH 7.0. Plastocyanin concentration was determined by absorption spectrophotometry with an extinction coefficient of 4900 M^{-1} cm^{-1} at 597 nm, as described by Davis & San Pietro (1979).

Digitonin-solubilized PS-I particles from spinach, named D-144 particles, were prepared according to Boardman (1971). The particles were suspended in 50 mM Tricine/NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM sodium ascorbate, and 100 μM methyl viologen. Triton-solubilized PS-I particles from pea (PSI-110) were prepared as described by Mullet et al. (1980) and suspended in the same medium as the digitonin particles. For oxidation–reduction titration experiments, the redox potential was continuously measured inside the cuvette, with saturated calomel and platinum-plate electrodes. The cuvette was continuously stirred. In these measurements, sodium ascorbate, methylviologen, and hydroxylamine were omitted, but 20 μM TMPD and 20 μM ferrocene were added. The oxidation–reduction potential of the medium was adjusted by adding appropriate amounts of potassium ferrocyanide and ferricyanide from stock solutions at 100 mM. After each titrant addition, the redox potential was allowed to equilibrate for 3 min before the flash absorption measurement.

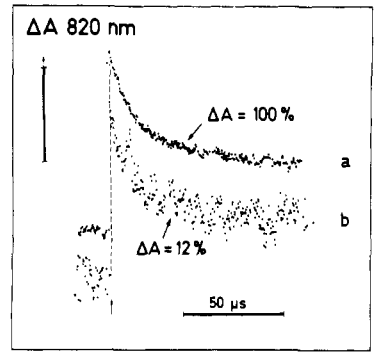


FIGURE 1: Kinetics of flash-induced absorption change, at 820 nm, in spinach chloroplasts at a concentration of 37 μg of chlorophyll/mL in 50 mM Tricine/NaOH, pH 7.5, 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 1 mM sodium ascorbate, 50 μM methylviologen, 10 μM DCMU, and 40 μM hydroxylamine. Cuvette was of 10-mm path, at 20 °C. (a) Signal induced by a nearly saturating laser flash (referred to here as 100% excitation); average of 16 experiments; vertical bar denotes $\Delta A = 2 \times 10^{-4}$. (b) Same conditions but the laser flash was 17-fold attenuated; average of 64 experiments; vertical bar denotes $\Delta A = 2.5 \times 10^{-5}$. The vertical arrow indicates the time of the laser flash.

Kinetics of flash-induced absorption changes at 820 nm were measured with an apparatus described previously (Van Best & Mathis, 1978). Excitation was provided by a Quantel ruby laser (λ = 694.3 nm; $t_{1/2}$ = 6 ns; E_{max} = 40 mJ). The laser flash was attenuated with calibrated neutral density filters and homogenized by a piece of ground glass. Absorption changes were recorded with a transient digitizer (Biomation, Model 1010) connected to a signal averager (Tracor Northern TN 1710). The kinetics from 4 to 64 separate experiments were added in the signal averager after a control of the reproducibility. The time between two excitations was 20 s. For the experiments with chloroplasts at 20 °C, we used a regular 10 × 10 mm square cuvette. The measurements with PS-I particles were done with a special lucite cuvette (0.8-mm thickness), which was oriented at 45° to the mutually perpendicular measuring and exciting beams. The same orientation was used to study the effect of temperature on the reactions. The cuvette was then inserted in a four-window cryostat that was cooled by flowing helium gas at a controlled temperature. In this case, the lucite cuvette was 2 mm thick and a chromel–constantan thermocouple was positioned in its internal volume. Analysis of the kinetics in terms of two or three monoexponential decays was performed on a computer, with the "simplex" method (Nelder & Mead, 1965) adapted by J. M. Thiery.

Results

Reduction of P-700$^+$ in Chloroplasts. Freshly prepared chloroplasts were excited by a short, just saturating flash. As shown in Figure 1a, the absorption at 820 nm rises practically instantaneously and then decays in a complex manner. The absorption increase is essentially due to the formation of P-700$^+$ in result of the primary reactions of photosystem I (Van Best & Mathis, 1978; Conjeaud et al., 1979). We confirmed that under our experimental conditions the contribution of P-680$^+$ to the absorption increase was negligible. This was conveniently controlled by adding 5 mM ferricyanide to the chloroplast suspension in order to chemically oxidize P-700 (neither ascorbate nor hydroxylamine was added in that case). Under such conditions, the remaining absorption increase at

820 nm is always smaller than 10% of the signal observed in the control; it decays with a half-time longer than 10 ms and does not show any significant component decaying in the microsecond range (not shown). The absence of any P-680$^+$ signal is presumably due to its faster (submicrosecond) reduction kinetics and also to the fact that the ruby laser, at 694 nm, excites photosystem I preferentially.

The multiphasic kinetics observed under the conditions of Figure 1a have been analyzed by using a sum of three monoexponential components, which typically are $t_{1/2}$ = 12 μs (50% of the initial ΔA amplitude), t = 200 μs (30%), and $t_{1/2}$ > 5 ms (20%). We also found that the respective proportions of each contribution and the half-time of the intermediate phase can be varied by changing the experimental conditions, for example, the salt or impermeant sugar concentration in the medium. Haehnel et al. (1980b) reported that in spinach chloroplasts addition of 3 mM $MgCl_2$, 20 mM KCl, and 200 mM sorbitol increases the fast phase to 70% of the total amplitude and accelerates the medium "200-μs" component to 140 μs. The authors concluded that the interaction between P-700 and plastocyanin is favored by a low internal volume of the thylakoids and a compensation of surface charges of the membrane. In our experiments, we used intermediate concentrations for salts and sugar (see Materials and Methods) in order to be able to measure all kinetic components under the same conditions.

Effect of Excitation Light Intensity. One simple way to investigate the mechanism and the order of a chemical reaction is to vary the concentrations of the reactants. In the case of P-700$^+$ reduction in thylakoids, this can be accomplished by changing the intensity of the excitation flash so as to induce different amounts of photooxidized P-700 without changing the amount of reduced electron donor or any other kinetic parameter. After attenuation of the laser flash intensity in order to obtain a just saturating excitation (maximum ΔA at 820 nm, referred to here as 100% photooxidized P-700 by one flash), the excitation light intensity was decreased by means of grey filters, and the effect of various nonsaturating excitations on the kinetics of P-700$^+$ reduction was measured. Extreme examples are shown in Figure 1. Curve a corresponds to the oxidation of 100% of the photoactive P-700, and curve b is the situation where 12% of the total P-700 is oxidized (by a 17-fold attenuated excitation flash). No significant difference can be detected between the two decay curves. This indicates that the reduction of P-700$^+$ under nearly physiological conditions is not a single bimolecular reaction as proposed by some authors (Olsen et al., 1980; Olsen & Cox, 1982). With such a reaction mechanism, the kinetics would indeed be strongly affected by a 10-fold decrease in the concentration of one of the reactants, which, at 100% oxidized P-700, are not far from being in equimolar concentrations (Plesnicar & Bendall, 1970; Katoh, 1979). The results of Figure 1, however, do not have a good enough signal to noise ratio to exclude that the slow phases of the decay correspond to a bimolecular reaction. Delosme et al. (1978) proposed that the fast kinetics of P-700$^+$ reduction may vary with flash energy; their experimental data, however, may have been influenced by double hitting occurring during a long flash.

Effect of Viscosity and Temperature. In order to confirm the kinetic analysis of P-700$^+$ reduction in chloroplasts, we have investigated the temperature dependence of this reaction. The addition of organic compounds such as glycerol or ethylene glycol is necessary for optical measurements at subzero temperatures. This addition provides a homogeneous glassy chloroplast suspension and prevents ice cracking even at very

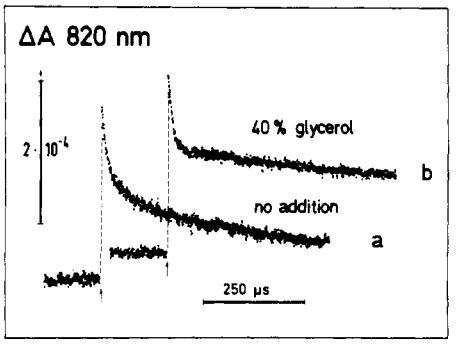


FIGURE 2: Time course of flash-induced ΔA, at 820 nm, in pea chloroplasts at the concentration of 40 μg of chlorophyll/mL. Cuvette was of 10-mm optical path, at 20 °C. Averaged effect of 16 flashes. (a) Pea chloroplasts were osmotically shocked in the cuvette in distilled water. The suspending medium was then adjusted to 20 mM Tricine/NaOH, pH 7.5, 0.33 M sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 1 mM sodium ascorbate, 10 μM DCMU, 50 μM methylviologen, and 40 μM hydroxylamine. (b) Same conditions as in (a), except the addition of 40% (v/v) glycerol.

low temperatures. We first examined the effect of such antifreeze media on the P-700$^+$ reduction at room temperature. Figure 2 shows that an increase in the glycerol proportion from 0% (Figure 2a) to 40% (v/v) (Figure 2b) slows down the overall reduction of P-700$^+$. The kinetic analysis clearly indicates, however, that the 12-μs fast phase remains absolutely unaffected in size and kinetics by the glycerol addition. By contrast, the half-time of the intermediate phase is progressively increased. Its values for 0, 10, 20, and 40% glycerol are respectively 210, 300, 410, and 1400 μs. No difference was detected between 40% and 60% glycerol. The effect of glycerol on the intermediate transient seems to indicate that this kinetic phase is a bimolecular reaction limited by the diffusion of the reactants. This particular point has been already investigated by Olsen & Pedersen (1983) and interpreted in terms of viscosity dependence of a second-order reaction. One can argue, however, that such large effects on reaction rates not only could be due to viscosity but also involve pH shifts and, more generally, changes in equilibrium constants and ionic interactions. However, the glycerol effect at least shows that the 12-μs kinetic phase does not originate from the same mechanism as the "200-μs" transient and is absolutely unaffected by addition of large quantities of glycerol. We obtained qualitatively similar results upon addition of ethylene glycol.

With these results at room temperature, it was then possible to undertake the analysis of the effect of temperature on the fast reduction phase of P-700$^+$ in chloroplasts. The kinetics were measured between room temperature and 160 K. The chloroplast suspension, with 60% glycerol, DCMU, and hydroxylamine, was illuminated with room light for about 10 s and then slowly cooled in complete darkness. The brief illumination was intended to block photosystem II in an inactive state; this was done because it could not be excluded that P-680 gives detectable transients at low temperature. For each temperature above −10 °C, we found that successive laser flashes given on one sample elicited identical signals. This was not true at lower temperature, presumably because the electron donor to P-700$^+$, being oxidized after one flash, has not the time to get rereduced before the next flash. For that reason, the chloroplast suspension was changed after every single laser flash in the low-temperature experiments.

Upon cooling, the general trend is a progressive slowing down of the fast phase, the relative amplitude of which also decreases. The slow phase soon becomes very slow, with kinetics not measurable with our instrument. Figure 3a is an

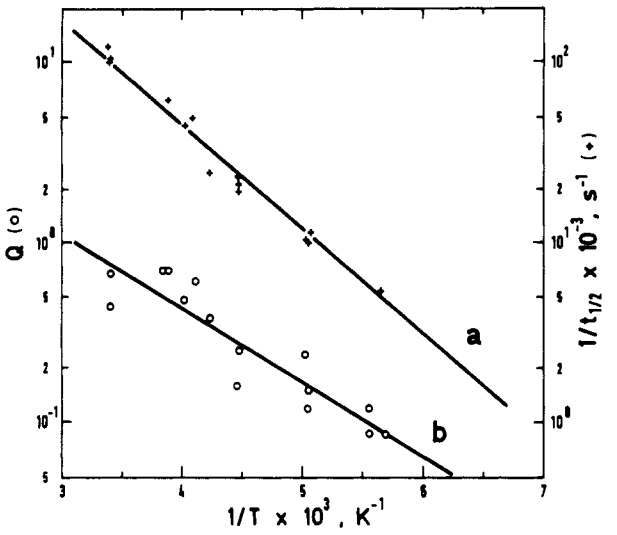


FIGURE 3: Temperature dependence of the fast P-700$^+$ reduction transient in pea chloroplasts. Same conditions as in Figure 2b, except for the cuvette (thickness 2 mm, position at 45° of the measuring beam) and the addition of 60% (v/v) glycerol. (a) Effect of temperature on the reciprocal half-time of the fast phase (right vertical scale). (b) Effect of temperature on the amplitude of the fast phase (left vertical scale). The experimental parameter Q is equal to $\Delta A_{fast}/(\Delta A_{max} - \Delta A_{fast})(2\Delta A_{max} - \Delta A_{fast})$, where ΔA_{max} and ΔA_{fast} are the total ΔA induced by a flash and the rapidly decaying ΔA, respectively. It can be assumed that Q is proportional to the equilibrium constant for the binding of plastocyanin to the PS-I reaction center.

Arrhenius plot of the P-700$^+$ fast reduction rate. It clearly indicates that this reaction is weakly depending on temperature ($t_{1/2}$ = 12 μs at 293 K; $t_{1/2}$ = 200 μs at 177 K) as quantified by the activation energy E_A = 4.9 kJ mol^{-1}. This low activation energy rules out the possibility that the fast phase could originate from a diffusion-controlled reduction of P-700$^+$.

The relative amplitude of the fast phase is also temperature dependent. It decreases from 50% at room temperature down to 13% at 170 K. We suppose that the fast phase originates from reaction centers where plastocyanin is bound, in equilibrium with free molecules: Pc + P-700 $\rightleftharpoons$ (Pc·P-700), with an equilibrium constant for binding K_b. If there are two plastocyanin molecules per P-700 [see Katoh (1979) or Graan & Ort (1984)], it immediately appears that K_b is proportional to the experimental parameter $Q = \Delta A_{fast}/(\Delta A_{max} - \Delta A_{fast})(2\Delta A_{max} - \Delta A_{fast})$, where ΔA_{max} and ΔA_{fast} are the total ΔA induced by flash (proportional to the total P-700 concentration) and the rapidly decaying ΔA (proportional to the concentration of bound plastocyanin), respectively. A plot of log Q vs. T^{-1} (Figure 3b) roughly gives a straight line, the slope of which is the enthalpy of the binding reaction: ΔH_b = 8 kJ mol^{-1}. The result is not appreciably different (ΔH_b = 9 kJ mol^{-1}) if we assume a stoichiometry of one plastocyanin per P-700. The entropy change cannot be calculated since we do not know the concentrations precisely enough.

Redox Titration of P-700$^+$ Reduction Kinetics. The redox titration of the P-700$^+$ reduction kinetics has been performed with pea chloroplasts. Experiments were conducted either in the reducing or in the oxidizing direction, and chloroplasts were changed after three individual measurements.

Figure 4 shows examples of P-700$^+$ reduction kinetics, for three different redox potentials. The major feature observed, when the potential is increased from 200 to 470 mV, is a slowing down of the overall P-700$^+$ reduction, which is due both to a decrease in the amplitude of the fast kinetic component and to an increase of the intermediate-phase half-time. The decay has been computer analyzed in terms of three

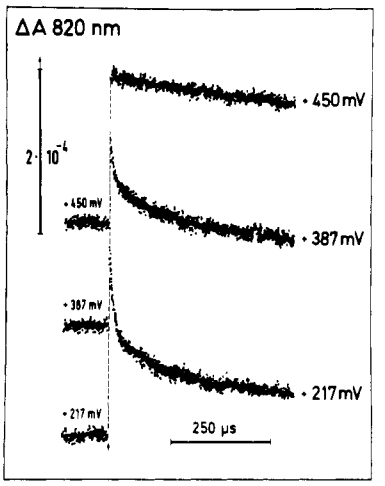


FIGURE 4: Time course of flash-induced ΔA at 820 nm in pea chloroplasts, for three values of the redox potential. Same conditions as in Figure 2a, except that ascorbate and hydroxylamine were omitted and that 20 μM TMPD, 20 μM ferrocene, and various amounts of ferro- and ferricyanide were added. Chlorophyll concentration was 50 μg/mL. Cuvette was of 10-mm path, at 20 °C. Each trace is the averaged effect of eight flashes. (Bottom trace) E_h = 217 mV; (middle trace) E_h = 387 mV; (top trace) E_h = 450 mV.

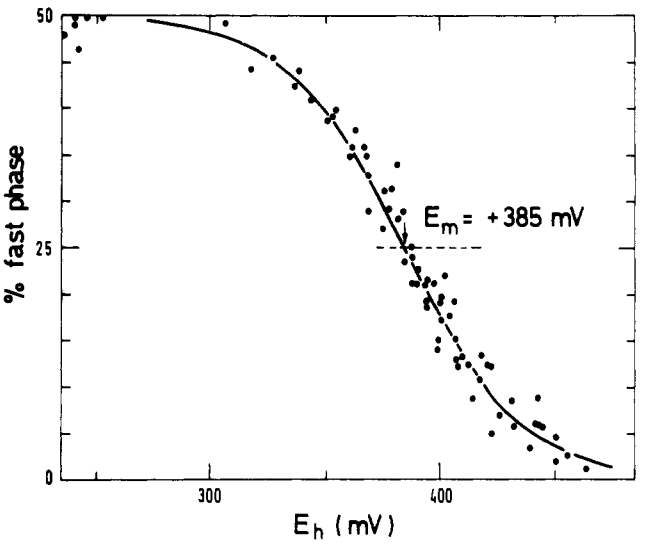


FIGURE 5: Redox titration of the amplitude of the fast phase of P-700$^+$ reduction in pea chloroplasts. Same conditions as in Figure 4. The solid line is the theoretical titration curve for a one-electron reaction according to the Nernst equation.

exponential phases. This analysis shows that a fast phase keeps an identical half-time (about 12 μs) at all the redox potentials where it can be measured. However, its amplitude varies, as shown by the titration in Figure 5. This curve shows that the "fast" donor to P-700$^+$ titrates with one electron (continuous line in Figure 5) and a midpoint potential E_m = 385 ± 5 mV (pH 7.5). This E_m is very close to the values reported for plastocyanin: 370 (Katoh et al., 1962) or 382 mV (Takabe et al., 1984).

The same titration experiments have also been performed with a lower time resolution in order to obtain a better evaluation of the medium-phase half-time. The results of a computer analysis are shown in Figure 6. Data points satisfactorily fit with the theoretical titration curve relating the reciprocal half-time of a second-order process to the oxidation–reduction potential of one of the partners. The best fit is obtained with the following parameters: E_m = 390 ± 10 mV (pH 7.5) and n = 1. This midpoint potential, although not very accurate, is similar to the one found for the amplitude

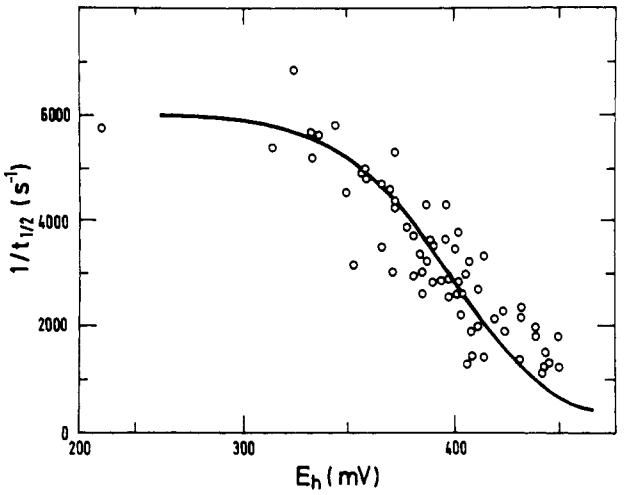


FIGURE 6: Redox titration of the reciprocal half-time of the intermediate kinetic phase of P-700$^+$ reduction in pea chloroplasts. Same conditions as in Figure 4. The solid line represents the theoretical redox titration curve for a one-electron reaction according to the Nernst equation and assuming that the intermediate kinetic phase originates from a simple second-order process.

of the fast transient. Thus, both fast and medium kinetic phases originate likely from the same electron donor but via two different mechanisms. It must be noted that the initial flash-induced absorption increase at 820 nm remains constant from 200 to 430 mV. It is only decreased by 20% at E_h = 460 mV. This is in agreement with the midpoint potential of P-700/P-700$^+$, E_m = 490 mV, measured by Sêtif & Mathis (1980).

Reaction between PS-I Particles and Purified Plastocyanin. Most biochemical studies on the interaction between plastocyanin and photosystem I have involved a kinetic analysis of the reduction of P-700, oxidized by light in various PS-I particles, under controlled experimental conditions [e.g., Wood & Bendall (1975), Lien & San Pietro (1979), Burkey & Gross (1981, 1982), Tamura et al., (1981) Olsen & Cox (1982), and Takabe et al. (1983, 1984)]. All published reports, however, were obtained with slow time-response methods. Since it appears that, in vivo, more than half of P-700$^+$ is reduced in a rapid reaction, we applied our flash spectrophotometric method to investigate the in vitro interaction between plastocyanin and PS-I particles. This approach is similar to that of Overfield et al. (1979) and Overfield & Wraight (1980a,b) in their study of the interaction between cytochrome c_2 and reaction centers from *Rhodopseudomonas sphaeroides*; the results also are very much comparable. We have used two types of PS-I particles: D-144 from spinach and PSI-110 from pea, with which we succeeded in partly mimicking the in vivo kinetics.

D-144 PS-I particles, prepared with digitonin, are devoid of plastocyanin and contain only little cytochrome *f* (one per 4.4 P-700; Boardman, 1971). When they were supplemented with 1 mM ascorbate, in order to keep P-700 reduced before illumination, and with 100 μM methylviologen to accept electrons from the bound iron–sulfur proteins, P-700 could be photooxidized by a flash and then very slowly rereduced, in hundreds of milliseconds, as shown previously by Ke (1972). Addition of pure reduced plastocyanin accelerates the reduction (Figure 7). When the plastocyanin concentration is below 20 μM, the absorption recovery at 820 nm includes a major exponential phase, the rate of which increases linearly with the plastocyanin concentration. This is consistent with a bimolecular reaction mechanism with a second-order rate constant k(D-144) = 1.9 × 10^8 M^{-1} s^{-1} (reduced plastocyanin

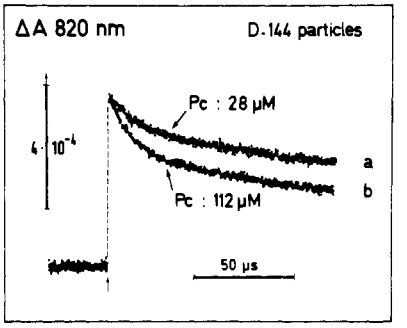


FIGURE 7: Time course of flash-induced ΔA at 820 nm, measured in D-144 particles (concentration 750 μg of chlorophyll/mL). Particles were suspended in 50 mM Tricine/NaOH, pH 7.5, 10 mM $MgCl_2$, 2 mM sodium ascorbate, 100 μM methylviologen, and 28 (curve a) or 112 (curve b) μM plastocyanin. Averaged effect of four flashes. The cuvette (thickness 0.8 mm) was placed at 45° of the measuring beam.

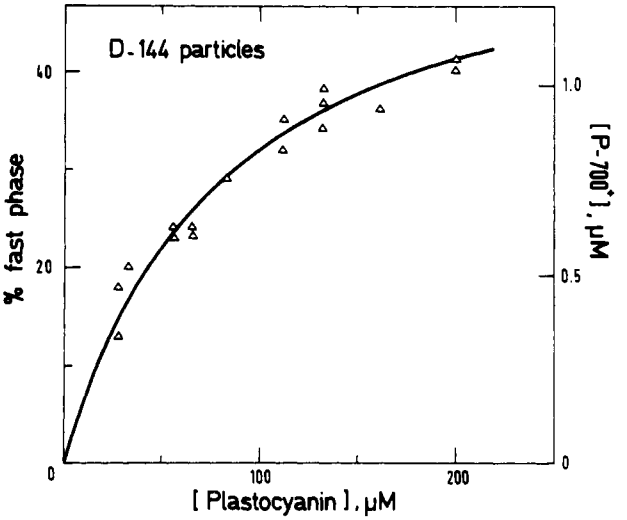


FIGURE 8: Effect of plastocyanin concentration on the relative amplitude of the fast ($t_{1/2}$ = 12 μs) reduction phase of P-700$^+$ in D-144 particles (left scale). Same conditions as in Figure 7. The solid line represents the calculated amount of plastocyanin bound to the reaction center assuming a simple binding equilibrium between free and bound plastocyanin and a dissociation constant K_d = 8 × 10^{-5} M (see Results). The right scale indicates the concentration of photooxidized P-700, which is reduced by the fast transient.

was always largely more abundant than P-700). About 15% of the absorption change recovered very slowly and could not be analyzed. This simple kinetic treatment does not fit when the plastocyanin concentration is above 20 μM. In that case, computer analysis of the signals, in terms of exponential phases, reveals two phases, in addition to the apparently nondecaying signal: a fast phase, the size of which increases in parallel with the plastocyanin concentration (Figure 8) but with a constant half-life $t_{1/2}$ = 12 μs (Figure 9, open symbols), and a slower phase with a concentration-dependent rate (Figure 9, closed symbols). This rate deviates rapidly from linearity with plastocyanin concentration and tends to saturate at high concentrations.

The properties of the fast (12-μs) reduction phase (constant half-time; increasing contribution at higher plastocyanin concentration) indicate the formation of a complex between plastocyanin and the PS-I center. Considering a simple binding equilibrium between PS-I and reduced plastocyanin, it has been possible to evaluate the dissociation constant of the complex. As the saturation of the binding apparently involves very high plastocyanin concentrations, it is difficult to estimate accurately which fraction of the reaction centers

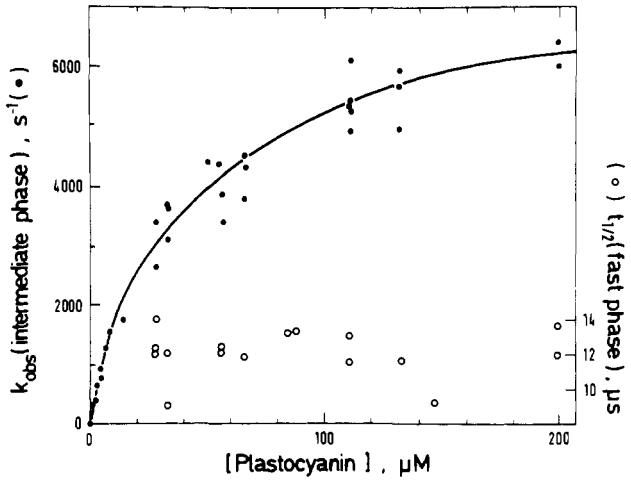


FIGURE 9: Apparent rate constant of the intermediate kinetic phase (closed symbols) and half-time of the fast phase (open symbols) in D-144 particles, as a function of plastocyanin concentration. Same conditions as in Figure 7.

is susceptible to bind plastocyanin. An acceptable theoretical fitting of the data, shown in Figure 8, is obtained when 50–70% of the reaction centers can bind plastocyanin and undergo a rapid reduction phase. From this, the corresponding dissociation constant K_d for the complex (P-700·Pc) is found to lie in the range (8–12.5) × 10^{-5} M.

The properties of the slower phase are in good agreement with a diffusive collisional mechanism for plastocyanin concentrations lower than 20 μM. Above this value, however, the nonlinearity of the rate constant with plastocyanin concentration (Figure 9) indicates that the simple second-order analysis can no longer fit the data. The saturation of the observed rate constant can be interpreted by the occurrence, in the electron-transfer pathway between reduced plastocyanin in solution and P-700$^+$, of another reaction step that limits the reaction for high plastocyanin concentrations. This is confirmed by a plot (not shown) of $t_{1/2}$ vs. $[Pc]^{-1}$, which gives a straight line (for high plastocyanin concentrations) intercepting the y axis at $t_{1/2}$ = 110 μs. This value represents the minimum half-time for this kinetic phase and is much larger than the theoretical value one can predict for a pure second-order process under the same conditions of reactant concentration. Following previous kinetic analysis for such rate constant limitation (Yoneda & Holwerda, 1978; Mauk et al., 1980), this can be described by the mechanism

$$\text{Pc(I)} + \text{P-700}^+ \underset{}{\overset{\text{diffusion}}{\rightleftharpoons}} \text{Pc(I)}\cdots\text{P-700}^+ \xrightarrow{110\ \mu s} \text{Pc(II)}\cdots\text{P-700}$$

where Pc···P-700 is a state of plastocyanin binding different from the state involved in the fast (12-μs) reduction phase.

The experiments reported in Figure 7 have been repeated in the presence of 60% glycerol (v/v). As in chloroplasts, it appears that this addition slows down drastically the intermediate kinetic phase but changes neither the size nor the kinetics of the 12-μs decay component.

Most of the experiments performed with D-144 particles have been repeated with PS-I particles from pea chloroplasts (PSI-110). All of the qualitative features observed with D-144 particles were also found with PSI-110. It appeared, however, that the dissociation constant associated with the 12-μs phase is larger [K_d = (2.5–3.0) × 10^{-4} M] so that, at the highest plastocyanin concentration tested (80 μM), only 20% of P-700$^+$ was reduced with rapid kinetics. The second-order rate constant characterizing the intermediate kinetic phase (k = 2 × 10^8 M^{-1} s^{-1}) is very close to that obtained with D-144 particles (1.9 × 10^8 M^{-1} s^{-1}).

DISCUSSION

A large part of the present work is devoted to the properties of the fast reduction of P-700$^+$. The constancy of its half-time, under all experimental conditions, except at low temperature, together with the variability of its amplitude, demonstrates that the immediate electron donor to P-700 is bound to the PS-I reaction center in chloroplasts. The reasonable reconstitution of that kinetic phase, upon readdition of pure plastocyanin to PS-I particles, indicates that this donor is plastocyanin. The redox titration (E_m = 385 mV) is also in good agreement with that attribution, although this result could also be attributed to cytochrome f (E_m = 360 mV; Bendall et al., 1971). Our kinetic data clearly eliminate the validity of a purely bimolecular collisional analysis of the electron donation to P-700 under physiological conditions. In this respect our results are complementary to those of Haehnel et al. (1980b), and our conclusions are identical. The binding of Pc to PS-I is rather weak: the results with PS-I particles lead to a dissociation constant K_d = 10^{-4} M. To calculate whether this constant can account for the fast phase in the chloroplast, we can suppose a thylakoid volume of 8 L/mol of chlorophyll (Rottenberg et al., 1972), a ratio of 500 chlorophylls per P-700, and two Pc's per P-700 (Graan & Ort, 1984). This yields a fraction of 70% of P-700 complexed with Pc, in good agreement with the maximum found by Haehnel et al. (1980b) and with our own results.

The absorption recovery after a flash also includes slow phases. The kinetic analysis in chloroplasts and the results obtained with PS-I particles upon readdition of plastocyanin clearly indicate a collisional, bimolecular reaction between Pc and the PS-I reaction center, in agreement with previous reports (Olsen et al., 1980; Olsen & Cox, 1982; Haehnel et al., 1980a,b; Tamura et al., 1981; Takabe et al., 1983). In this respect, our experimental approach is not ideal because the absorption changes at 820 nm also include a small contribution of oxidized Pc. Besides its characteristic absorption peak at 597 nm, oxidized Pc indeed has a weaker absorption band centered at 770 nm, which gives an extinction coefficient of 1200 M^{-1} cm^{-1} at 820 nm, i.e., 18% of that of P-700$^+$. Oxidized Pc produced in the fast electron donation to P-700$^+$ will contribute to the slower phases of absorption recovery. In vitro this should give (as indeed observed) a small very slowly decaying signal, since oxidized Pc is reduced very slowly by ascorbate (Katoh, 1979). In the chloroplast, oxidized Pc may be reduced with a $t_{1/2}$ = 240 μs (Selak & Whitmarsh, 1984); thus, it is possible that a small part of the 200-μs kinetics is due to the reduction of Pc(II) by the cytochrome b_6/f complex.

These reservations do not hold for our major observation that the bimolecular reaction between the PS-I center and plastocyanin in solution has a rate limit at high Pc concentration, corresponding to $t_{1/2}$ = 110 μs. This limit, which is close to the fastest intermediate reaction observed in chloroplasts (140 μs; Haehnel et al., 1980b), can be interpreted by the existence of another state of Pc binding to the PS-I reaction center, which we will name the "distant" bound state (denoted Pc···P) to distinguish it from the "close" bound state (Pc·P), which is responsible for the 12-μs electron transfer. We adopt the terminology of Dutton & Prince (1978), also used by Overfield et al. (1979), who studied the electron transfer from cytochrome c_2 to the reaction center of the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. They also analyzed their data in terms of a bimolecular process (k = 8 × 10^8 M^{-1} s^{-1}, in solution) and of two bound states corresponding

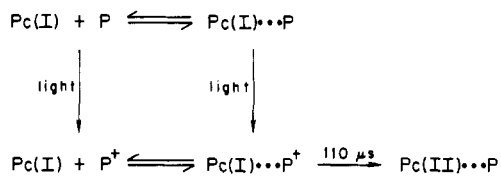
to electron-transfer half-times of 2 and 400 μs. The functional analogy between plastocyanin and cytochrome c_2 (or other soluble high-potential cytochromes) has often been stressed [see, e.g., Crofts & Wood (1978)]. It now appears that the analogy extends to the details of the kinetics. The binding of Pc, however, is significantly weaker since its dissociation constant is 10^{-4} M instead of 10^{-5} M for cytochrome c_2 (Overfield et al., 1979). However, the dissociation constant between cytochrome c_2 and the reaction center appears to be highly variable since Rosen et al. (1980) reported a value of 10^{-6} M, by a method of equilibrium dialysis, while Overfield et al. (1979) obtained values as high as 10^{-4} M under high-salt conditions.

The role of the two bound states cannot be fully decided on the basis of our experiments. Two schemes can be put forward, a "linear" one and a "parallel" one. The parallel scheme is written below. The linear scheme is simply obtained by assuming that the close bound state (Pc·P) is an obligatory intermediate in electron transfer from the distant bound state (Pc···P); i.e., the two bound states are in equilibrium (P stands for P-700):

fast reaction (close bound state)

$$\mathrm{Pc(I)} + \mathrm{P} \rightleftharpoons \mathrm{Pc(I)\cdot P} \xrightarrow{\text{light}} \mathrm{Pc(I)\cdot P^{+}} \xrightarrow{12\ \mu s} \mathrm{Pc(II)\cdot P}$$

slow reaction (distant bound state)



In their analysis of electron transfer from cytochrome c_2 to a bacterial reaction center, Overfield et al. (1979) have assumed that the close bound state was an obligatory intermediate in the reaction. Although there is no evidence for it, this model is quite reasonable and could work as well for the electron transfer from plastocyanin to the PS-I reaction center. The close and distant bound states are not to be understood in terms of well-defined distances but more in terms of two structures of a complex between plastocyanin and PS-I, leading to highly different rates of electron transfer. Our measurements distinguish only the interaction between reduced Pc and PS-I. The redox titration of the fast electron transfer, i.e., of the close state, gives an E_m of 385 mV, very close to the values reported for free plastocyanin (370 and 382 mV): this shows that Pc(I) and Pc(II) are nearly equally bound. If the difference between our measurement and those for isolated Pc is significant, this would mean that Pc(I) is slightly more tightly bound than Pc(II). The situation in this respect is more complex in the case of cytochrome c_2 and *R. sphaeroides* reaction centers, since the oxidized and reduced forms appear to bind equally (Rosen et al., 1980) to isolated reaction centers, but the E_m of the bound cytochrome is significantly lower in vivo than for the isolated cytochrome (Dutton & Prince, 1978).

Electron transfer from Pc(I) to P-700$^+$ in the close bound state becomes slower at low temperature. The activation energy (4.9 kJ mol^{-1}) is rather weak and would imply that the transfer remains fast at very low temperature. Under these conditions, however, photooxidation of plastocyanin has not been observed. Our results show that this is not due to kinetic reasons but to the large positive binding enthalpy of 8–9 kJ mol^{-1}, which results in a nearly complete absence of the close bound state at low temperature. This state thus appears to be equivalent to a transition state in classical reaction kinetics: it may be formed via the distant bound state with the requirement of an activation energy.

The conclusions presented in this work may be useful for future biochemical studies on the interaction between Pc and PS-I. A realistic approach would indeed require one to consider precisely the steps involved in electron transfer. The two bound states that we have studied are important in that respect, and it would be very useful to see how they correlate with the detailed structure of plastocyanin and of the PS-I reaction center.

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Pseudocatalase from *Lactobacillus plantarum*: Evidence for a Homopentameric Structure Containing Two Atoms of Manganese per Subunit†

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ABSTRACT: An improved procedure for the isolation of the pseudocatalase of *Lactobacillus plantarum* has been devised, and the quaternary structure and manganese content of this enzyme have been reexamined. Sedimentation equilibrium of the native enzyme at several salt concentrations gave a molecular weight of 172 000. The subunit weight, obtained by sedimentation equilibrium in 6.4 M guanidinium chloride, with or without prior reduction and carboxymethylation, was 34 kilodaltons. The amino acid composition indicated 150 Arg + Lys, and after exhaustive tryptic digestion, 32 peptides were resolved. These data suggest that the pseudocatalase is a homopentamer. Cross-linking with dimethyl suberimidate, followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, yielded five major bands, another indication of pentameric structure. The manganese content was found to be 1.8–2.4 per subunit. $s_{20,w}$ was found to be 9.6 S and f/f_0 = 1.2, suggesting a globular structure of Stokes radius 44 Å.

An azide-insensitive or "pseudo" catalase was first noted in certain species of bacteria which cannot synthesize heme (Delwiche, 1961; Johnson & Delwiche, 1964; Jones et al., 1965). We subsequently isolated this enzyme from *Lactobacillus plantarum* and found it to be an oligomeric manganienzyme (Kono & Fridovich, 1983a). Although the visible spectrum of the mangani-catalase resembled that of the mangani-superoxide dismutase, there was no overlap in the substrate specificities of these enzymes. The physiological function of this catalase was explored (Kono & Fridovich, 1983b), and studies of its structure and metal content suggested a hexamer containing one atom of manganese per

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