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Chlorophyll organization in P-700-enriched particles isolated from spinach chloroplasts. CD and absorption spectroscopy

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P-700-enriched particles having a Chl-*a*/P-700 ratio of 7–10 (Ikegami, I. and Katoh, S. (1975) Biochim. Biophys. Acta 376, 588–592) showed three derivative-shaped CD signals, i.e., CD694(−)/683(+), CD688(−)/678(+) and CD678(+)/663(−), where (+) and (−) indicate the signs of CD signal. The intensity of CD688(−)/678(+) was essentially constant under all redox conditions used here, whereas the intensities of the other two changed depending on the redox conditions. Maximum intensity of CD678(+)/663(−) and minimum intensity of CD694(−)/683(+) were observed on oxidation with ferricyanide. By subsequent reduction with ascorbate or ferrocyanide, the intensity of the CD678(+)/663(−) was decreased and that of the CD694(−)/683(+) was increased. A redox titration gave a midpoint potential of 400 mV for the CD678(+)/663(−) and 420 mV for the CD694(−)/683(+), each with a one-electron process. The reduced-minus-oxidized difference absorption spectra obtained during the redox titration of P-700 showed that the absorption change of a chlorophyll having a peak at 674 nm (Chl-*a*-674) overlapped with that of P-700. Chl-*a*-674 bleached on reduction, showing a midpoint potential of 390 mV, which was 25 mV lower than that of P-700. We concluded that the three derivative-shaped CD signals, CD694(−)/683(+), CD688(−)/678(+) and CD678(+)/663(−), originate from P-700, Chl-*a*-684 and Chl-*a*-674, respectively. A curve analysis of the absorption spectrum revealed the presence of three major chlorophyll species, Chl-*a*-684, Chl-*a*-674 and Chl-*a*-669 with a molar ratio of 2:2:2 to one P-700, which suggests two dimeric and two monomeric chlorophylls to one P-700 in the PS-I reaction center.

Introduction

The degenerate interaction among chromophores causes a splitting of the monomer absorption band, which results in a double CD signal that reverses sign close to the absorption maximum [1]. The chlorophyll dimer shows a very large, double CD, whereas the chlorophyll monomer shows only an extremely small CD [1,2]. Thus, the large CD signals observed in photosynthetic particles [1–6] gave strong evidence for chlorophyll–chlorophyll interaction among antenna pigments. A dimeric organization of the PS-I reaction center chlorophyll (P-700) has also been supported by a double CD characteristic in the light-induced CD spectrum of P700 [7–9]. The intensity of the CD signal of P-700 thus far reported [7–9] was, however, much smaller than that of antenna pigments, probably due to a large number of antenna chlorophyll participating in dimeric organization.

Abbreviations: PS, Photosystem; CD, circular dichroism; DCIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll.

Ikegami and Katoh previously found that 90–95% of the antenna chlorophyll is extractable from digitonin-treated PS-I particles with wet diethyl ether, while keeping most of P-700 in the photoactive state [10]. The P-700-enriched particles with Chl/P-700 ratios as low as 7–10 were obtained by this method [10]. Further enrichment of P-700 was not successful even by repeating the ether extraction. This suggests that the chlorophylls in the P-700-enriched particles, including P-700, might be in special environments, to which organic solvents such as ether, cannot readily be accessible. A part of these chlorophylls (2–3 molecules) was previously designated as Chl-*a*-684 [11]. This Chl-*a*-684 was suggested to be a constituent of the PS-I reaction center, since its fluorescence yield changes depending on the redox state of both P-700 and primary electron acceptors [11,12]. Thus, the P-700-enriched particle can be a good material to study the organization of the chlorophyll in (or near) PS-I reaction center.

CD and absorption spectroscopy of the P-700-enriched particles in the present studies revealed three dimer-like and two monomer chlorophyll *a* in (or near) PS-I reaction center. The dimer-like species were identified as P-700, Chl-*a*-684 and Chl-*a*-674. Chl-*a*-684 seems to be the emitter of the variable fluorescence from PS-I as identified previously [11]. Chl-*a*-674 bleached on reduction with ferrocyanide, showing a midpoint potential close to, but a little lower than, that of P-700. The two monomer chlorophylls which had an absorption maximum at 669 nm (Chl-*a*-669), were supposed to be the primary electron acceptor (A_0 and/or A_1) of PS-I.

Materials and Methods

P-700-enriched particles were prepared as described previously [10,11]. PS-I particles, prepared by digitonin treatment of spinach chloroplasts, were lyophilized and then extracted twice with diethyl ether containing water in 70–100% saturation, to yield P-700-enriched particles having a Chl/P-700 ratio of 7–10. The P-700-enriched particles obtained were solubilized with phosphate buffer (10 mM, pH 8) containing 0.1–0.2% Triton X-100 by incubation of 15 min at 0–4°C. Insoluble greyish-white materials were removed by centrifugation. The blue-green supernatant, which had a 7–11 Chl/P-700 ratio, was used for measurements (cf. Ref. 13). Prior to use, it was diluted about 20 times with the same phosphate buffer without Triton X-100 to attain a low detergent concentration. Temperature was kept at 7°C during all the following measurements to prevent the damage of samples.

CD spectrum was determined with a JASCO-J200B CD spectrometer equipped with a tungsten-iodine light source and with a cuvette of 1 cm path-length at a band-width of 2 nm. The signal was fed into a Hewlett-Packard computer (HP-9845B) and difference CD spectrum was calculated. The CD intensities were given in terms of ellipticity (m°), where 1 m° corresponds to $3 \cdot 10^{-5}$ of $(A_L - A_R)$, a difference in the absorption for left- and right-circular-polarized light.

Absorption and difference absorption spectra were determined with a Hitachi model 557 dual-wavelength spectrophotometer, as described previously [13]. Curve analysis of the absorption spectrum was carried out on a Hewlett-Packard computer (HP-9845B) with the program of Mimuro et al. [14]. P-700 was assayed from the ferricyanide (0.5 mM)-oxidized minus ascorbate (5 mM)-reduced difference spectrum, using the extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [15]. Chlorophyll concentration was measured by the method of Arnon [16].

Results and Discussion

CD spectrum of P-700-enriched particles

The solid lines in Fig. 1 show the absorption (A) and CD (B) spectra of P700-enriched particles in the absence of any redox reagents. Under this condition most of P-700 was in the oxidized state. The spectra were completely different from those of the original PS-I particles (dashed lines in Fig. 1A and B). It is noteworthy that the intensity of CD signal, when expressed on the chlorophyll basis, remained in the same range even after extraction of pigments. This indicates that both the CD-active and inactive antenna chlorophylls were extracted from PS-I particles by the ether treatment. Hence, the CD signals observed in P-700-enriched particles seem to arise from the chlorophylls locating in the vicinity of the PS-I reaction

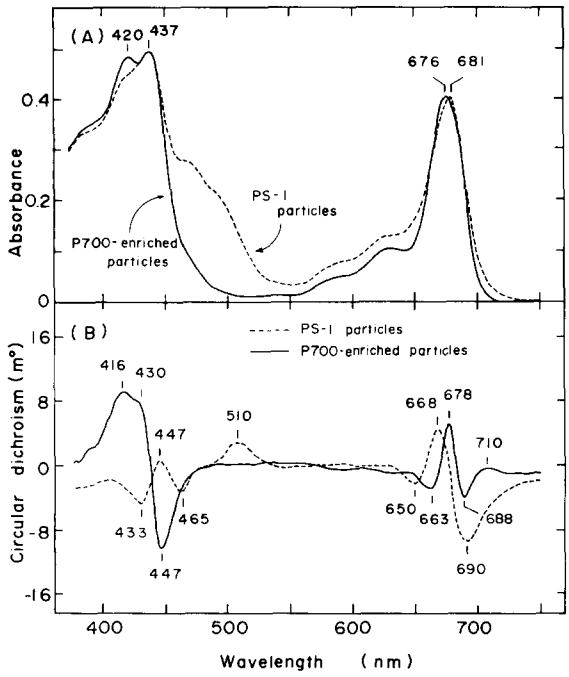


Fig. 1. Absorption (A) and CD (B) spectra of P-700-enriched particles (solid line) and PS-I particles (broken line). 6.7% of chlorophyll in the original PS-I particles remained unextractedly in the P-700-enriched particles. Chl/P-700 ratios were 142 and 8 in PS-I particles and P-700-enriched particles, respectively. The sample concentrations were adjusted to be 0.4 in absorbance at their α-band maxima. All spectra were measured without any redox reagents.

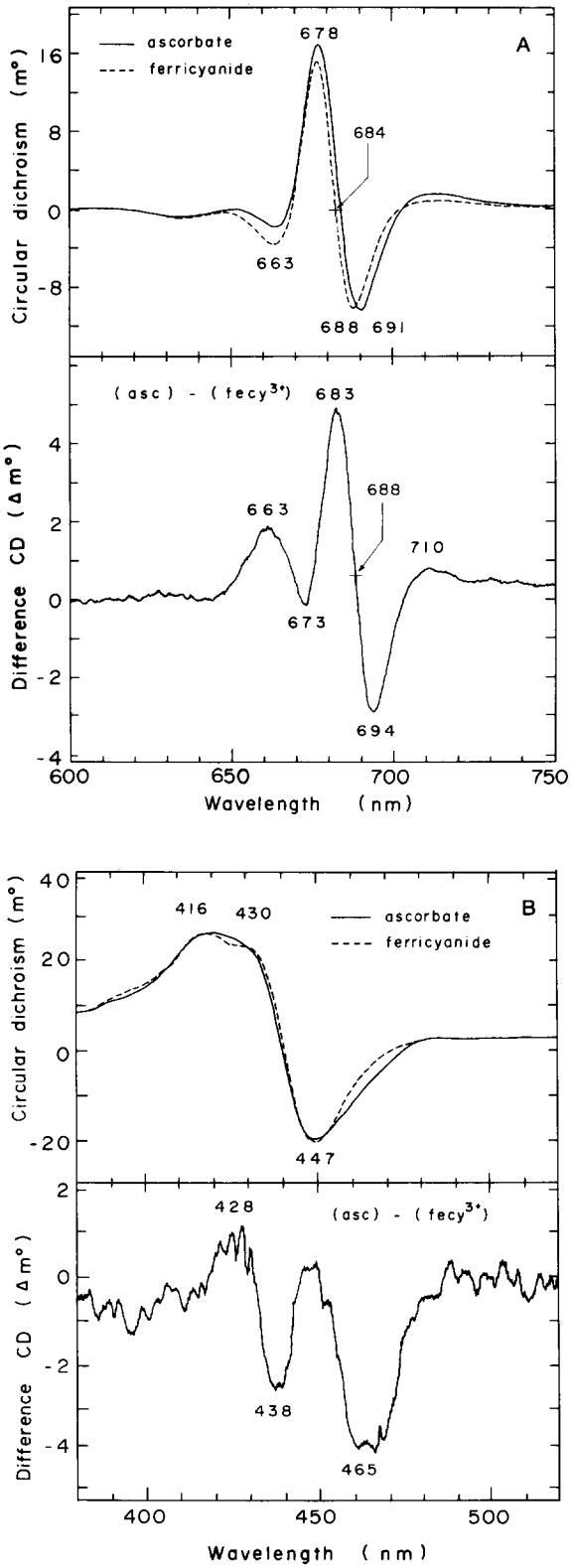
center. Three bands in the long-wavelength region are apparent with peaks at 688 nm (−), 678 nm (+) and 663 nm (−) in P-700-enriched particles, where (+) and (−) indicate the signs of the CD signal. The two major bands at 678 nm (+) and 688 nm (−) seem to be a degenerate component caused by a dimeric organization. This derivative-shaped CD crosses zero at about 684 nm (Fig. 2A) indicating that the chlorophyll responsible for this CD signal has an absorption peak at about 684 nm. The presence of Chl-*a*-684 in P-700-enriched particles has already been suggested from the excitation spectrum for variable fluorescence from PS I [11].

The negative CD peak at 663 nm was not recognizable until the Chl-*a*/P-700 ratio was lower than 10. It became prominent through further enrichment of P-700; the maximum development was obtained at the Chl-*a*/P-700 ratio of 7, where the CD peak at 663 nm was comparable to, or bigger than the 688 nm CD band. Therefore, the chlorophyll showing the 663 nm CD band may locate very close to P-700 and is resistant to ether extraction. This CD signal has a positive counterpart signal around 678 nm as will be seen in the following section (see Fig. 5B).

P-700-enriched particles have no CD bands at 650 nm (−), 510 nm (+) and 465 nm (−), which are present in the CD spectrum of PS-I particles. This is consistent with the fact that P-700-enriched particles have lost all the carotenoids and almost all the chlorophyll *b* originally present in PS-I particles (see Fig. 1A) [10,13].

Reduced-minus-oxidized difference CD spectrum

The addition of reducing agents such as ascorbate or dithionite to the oxidized P-700-enriched particles, induced obvious changes in the CD spectrum both in the α- and the Soret-band regions. In the α-band region (Fig. 2A), the trough at 663 nm became smaller and the positive peak at 678 nm became higher, while the negative peak at 688 nm shifted to 691 nm with small change in the signal intensity. The ascorbate-reduced minus ferricyanide-oxidized difference CD spectrum showed two major bands at 694 nm (−) and 683 nm (+), in addition to a small band at 663 nm (+) (the lower part in Fig. 2A). In the Soret band region (Fig. 2B), the difference CD spectrum shows a broad negative band at 465 nm and two sharp bands at 438 nm (−) and 428 nm (+). These profiles are fundamentally similar to the light-induced CD spectrum of P-700 determined previously by Shubin et al. [8], except the presence of a small but distinct peak at 663 nm in our difference CD spectrum. This peak became more prominent (sometimes its magnitude was comparable to the 683 nm CD band) with further improvement of Chl-*a*/P-700 ratio to about 7. This high 663 nm peak always accompanied a distinct negative peak around 675 nm in the difference CD spectrum, suggesing that these peaks be attributable to another dimeric chlorophyll *a* near P-700. Shubin et al. [8] and Karapetyan et al. [9] have also reported that the light-induced difference CD spectrum of P-700 sometimes accompanied a CD signal arising from antenna chlorophyll *a*.



Redox-potential-dependent change of the difference CD spectrum

Fig. 3 shows a few representive difference CD spectra obtained during a reductive titration with a ferri-ferrocyanide couple. The signal intensities increased gradually upon reduction. In addition, the spectral profile changed progressively. At the low ferro- to ferricyanide ratio, the band at 663 nm was hardly recognizable, so that the CD spectrum was composed of only the two peaks at 683 nm (+) and at 694 nm (−). With increasing a ferro- to ferricyanide ratio, the 663 nm band gradually developed accompanying a distinct trough around 673 nm. The full development of all these bands was attained on addition of ascorbate. Further addition of dithionite had no effect.

The midpoint potentials were determined at 663 nm peak and 694 nm trough. Each of the experimental data followed approximately a theoretical curve for one electron reaction with the midpoint potential of about 400 mV for the 663 nm band and about 420 mV for the 694 nm band (Fig. 4). The 683 nm peak seemed to change as the sum of a negative and a positive changes, each of which goes in parallel with the 663 nm and the 694 bands, respectively. The difference CD spectrum, thus, can be decomposed into two derivative-shaped species; the one with the CD peaks at 694 nm (−) and 683 nm (+) which have the higher midpoint potential and is ascribed to P-700, and the other with a minor peak at 663 nm (+) and a peak at 678 nm (−) (see Fig. 5B), which has the lower midpoint potential and may be ascribed to another dimeric chlorophyll locating near P-700.

A typical CD spectrum of P-700 was obtained at the low ferro- to ferricyanide ratio (Fig. 5A) (cf. Fig. 3). It has a characteristic derivative-shaped CD profile, but somewhat asymmetric on account of the larger 683 nm peak than the 694 nm trough. It crossed zero at 688 nm, which was a little

Fig. 2. CD spectra obtained in the presence of ferricyanide (1 mM) (broken line), or ascorbate (5 mM) plus DCIP (2 μM) (solid line) in α-band (A) and Soret-band (B) region. The lower part of the figure shows the reduced-minus-oxidized difference CD spectrum (solid line minus broken line). Chlorophyll concentration of P-700-enriched particles was 20 μg/ml (absorbance$_{676}$ = 1.16). Other experimental conditions are the same as in Fig. 1.

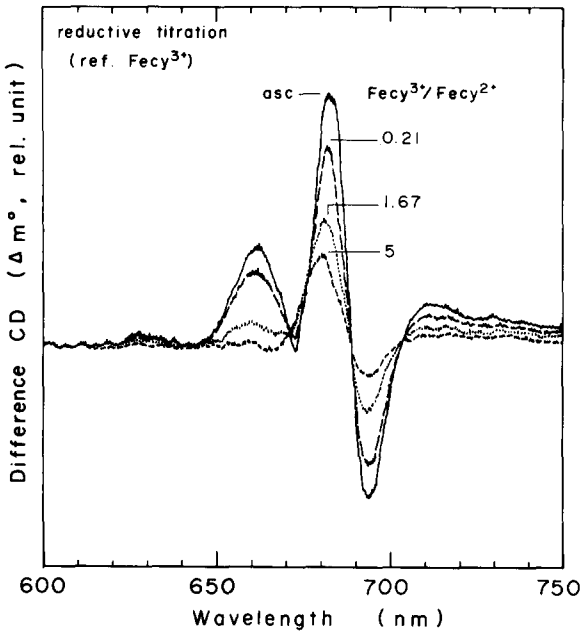


Fig. 3. Difference CD spectra obtained during a reductive titration of P-700-enriched particles. Increasing amount of ferrocyanide (0.2 mM–10 mM) was added to the sample already containing a certain amount of ferricyanide (0.5 mM or 1 mM). Ferri- to ferrocyanide ratios are 5 (dashed line), 1.67 (dotted line) and 0.21 (broken line), respectively. Solid line, ascorbate (5 mM) and DCIP (2 μM) were added. Other details are the same as in Fig. 2.

shorter than the maximum of P-700 difference absorption spectrum (695 nm in our preparation). These results can be explained as following that P-700 (reduced form) has an absorption maximum at about 688 nm showing a dimeric CD peaking at 694 nm (−) and 683 · nm (+), while P-700$^+$ (oxidized form) has an absorption maximum at about 683 nm (cf. Ref. 7) showing a relatively small CD peaking at 683 nm (+). Evidence supporting this has been provided previously by Schaffernicht and Junge [17], and Shubin et al. [8] who estimated, from curve analysis of the P-700 difference absorption spectrum, that the absorption peaks of P-700 (reduced form) and P-700$^+$ (oxidized form) were about 5 nm and about 10 nm, respectively, shorter than the P-700 difference absorption maximum.

The 663 nm CD component was spectrally isolated by subtracting the CD spectrum having no 663 nm band from that having the 663 nm band (cf. Fig. 3). In order to cancel the P-700 CD signal, each spectrum was normalized with the amplitude of the 694 nm band. The resultant spectrum (Fig. 5B) has a trough at 678 nm in addition to a 663 nm peak with a zero-crossing point of about 673 nm, suggesting that the chlorophyll responsible for this CD signal has also a dimer-like organization with an absorption peak at about 673 nm. The negative peak at 663 nm observed in the CD spectrum of the oxidized P-700-enriched particles (Fig. 1B) probably corresponds to the CD signal from the oxidized form of this chlorophyll, where the positive peak at 678 nm from this chlorophyll overlaps with the positive peak from Chl-*a*-684. On the other hand, the reduced form of this chlorophyll lost the CD signal (see Fig. 2A), because this chlorophyll loses its absorption on reduction, as will be shown in the following section.

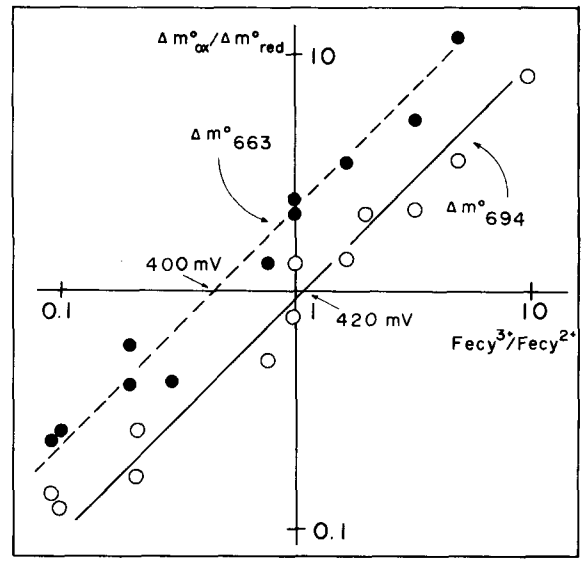


Fig. 4. Redox potential dependency of the 663 nm (solid circles) and the 694 nm (open circles) band in the difference CD spectrum of P-700-enriched particles. The extents for 0 and 100% yield were determined with samples incubated with ferricyanide (1 mM) and ascorbate (5 mM) plus DCIP (2 μM), respectively. A value of 419 mV was used for the normal redox potential of ferri-ferrocyanide couple [19]. The straight lines in the figure are theoretical curves for one electron titrations. Other details are the same as in Fig. 3.

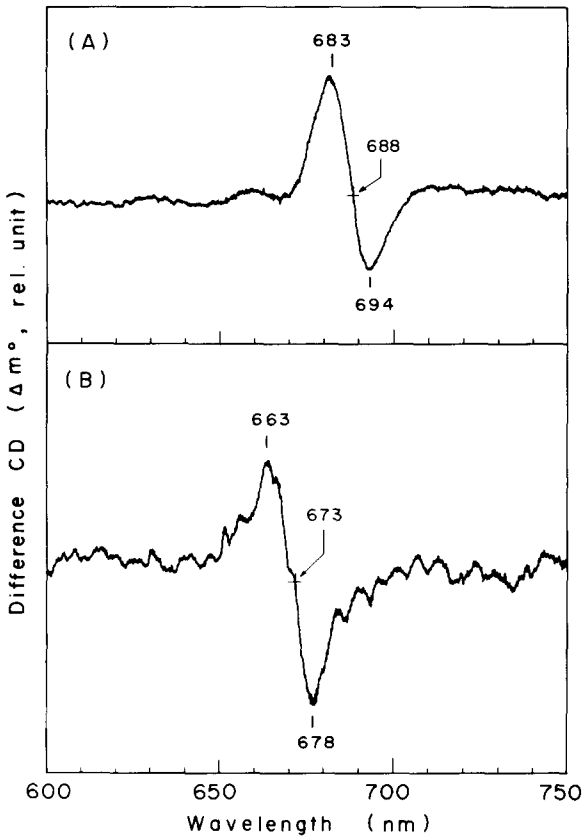


Fig. 5. Typical difference CD signals for major (A) and minor (B) component. Spectrum (A) was determined at a ferri- to ferrocyanide ratio of 2 during a reductive titration. Spectrum (B) was determined as a difference between the CD signals obtained with ascorbate (5 mM) plus DCIP (2 μM) and with ferrocyanide (0.2 mM) plus ferricyanide (1 mM), after normalization at the 694 nm CD intensity. Spectrum (B) was expanded by a factor of 4 relative to spectrum (A). Other details are the same as in Fig. 3.

In the Soret band region, all peaks had the same redox potential dependency with a midpoint potential of about 420 mV (data not shown). This suggests that these bands originate only from P-700, i.e., a double CD peaking at 428 nm (+) and 438 nm (−) is attributed to P-700 (reduced form) and a broad band at 465 nm (−) probably to $P\text{-}700^+$ (oxidized form). The latter band seems to relate to a small positive peak at 450 nm in the light minus dark difference absorption spectrum of P-700 [8,18].

Redox-potential-dependent change of the difference absorption spectrum

Fig. 6A shows difference absorption spectra obtained during a reductive titration with a ferri-ferrocyanide couple, where ferrocyanide solution was progressively added to the sample already containing a certain amount of ferricyanide. At a higher redox potential (ferri- to ferrocyanide ratio higher than 1), the spectrum practically consists of a main peak at 695 nm and a satellite peak at 675 nm. At the lower redox potential, the trough at 665 nm developed preferentially, resulting in the appearance of the third peak at 653 nm. The change in the spectral profile during the reductive titration was more clearly presented in Fig. 6B, where each difference spectrum was normalized at the main peak. Of particular interest is the finding that the satellite peak around 675 nm became smaller at the lower redox potential, which caused the development of a dip around 665 m and a peak around 653 nm. These results suggest that the 675 nm band is a mixture of a satellite peak due to P-700 and a certain component having an absorption around 675 nm; the lattter loses its absorption at the lower redox potential. Fig. 6C presents the change in the spectral profile during an oxidative titration, in which ferricyanide was progressively added to the sample already containing a certain amount of ferrocyanide. At the beginning of this titration, the satellite peak around 675 nm was quite low and the trough around 668 nm was marked so that it sometimes crossed over the base line. The satellite peak became higher at the higher redox potential, accompanied by shallowing a dip around 665 nm. The spectral profile obtained at the end of an oxidative titration somewhat resembled to that obtained at the end of a reductive titration (see Fig. 6B).

Fig. 7 shows the two typical difference spectra, each with the highest and the lowest satellite band obtained at the beginning of a reductive (Fig. 6B, the spectrum with ferri/ferrocyanide ratio of 4) (curve a) and an oxidative (Fig. 6C, the spectrum with ferri/ferrocyanide ratio of 0.125) (curve b) titration, respectively. Fig. 7 also shows the difference spectrum between the curves a and b. It has a chlorophyll-like absorption with a peak around 674 nm, so that we will hereafter designate this component as Chl-*a*-674. The results in Fig. 6

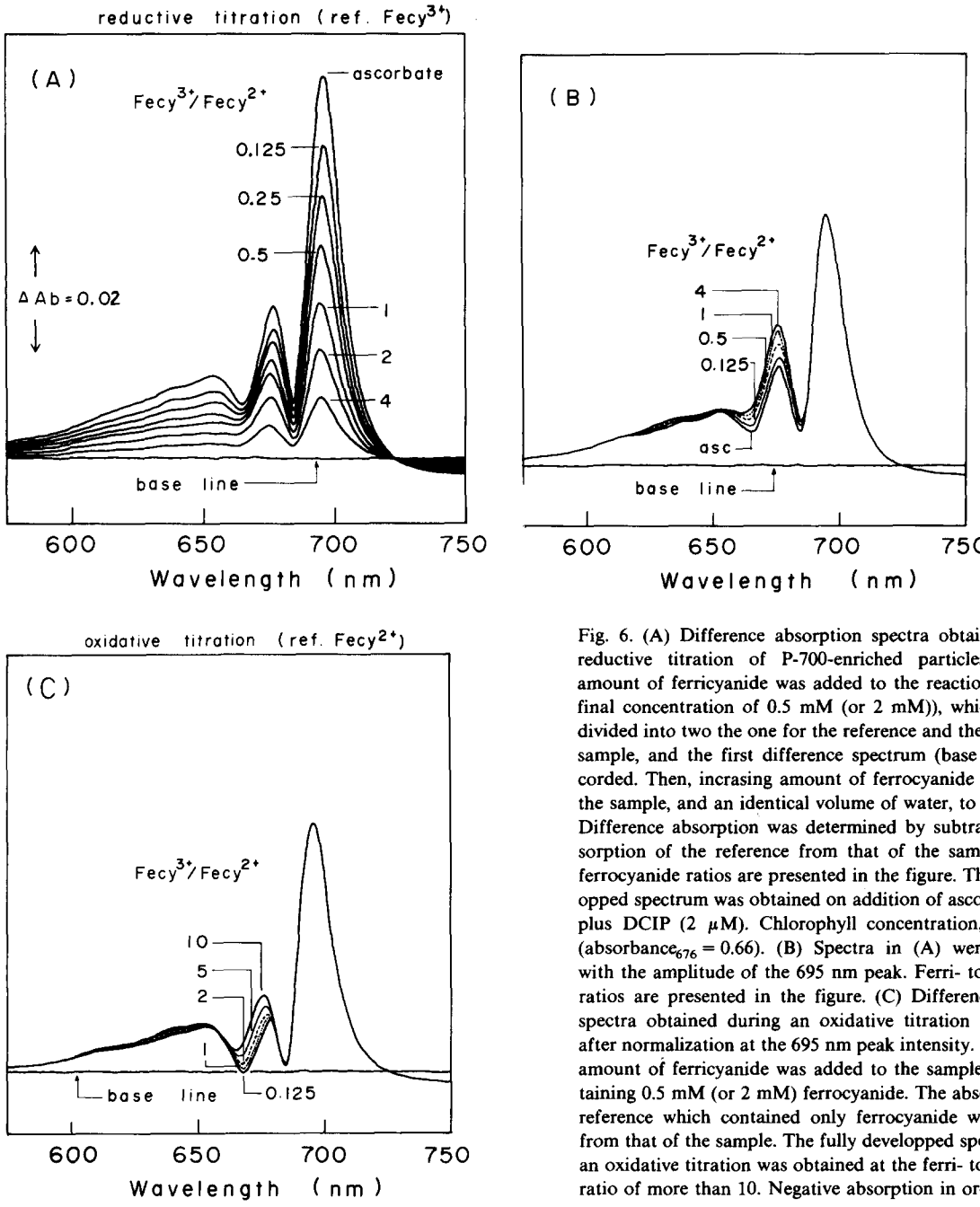


Fig. 6. (A) Difference absorption spectra obtained during a reductive titration of P-700-enriched particles. A certain amount of ferricyanide was added to the reaction mixture (at final concentration of 0.5 mM (or 2 mM)), which was, then, divided into two the one for the reference and the other for the sample, and the first difference spectrum (base line) was recorded. Then, incrasing amount of ferrocyanide was added to the sample, and an identical volume of water, to the reference. Difference absorption was determined by subtracting the absorption of the reference from that of the sample. Ferri- to ferrocyanide ratios are presented in the figure. The fully developped spectrum was obtained on addition of ascorbate (5 mM) plus DCIP (2 μM). Chlorophyll concentration, 11.5 μg/ml (absorbance$_{676}$ = 0.66). (B) Spectra in (A) were normalized with the amplitude of the 695 nm peak. Ferri- to ferrocyanide ratios are presented in the figure. (C) Difference absorption spectra obtained during an oxidative titration are presented after normalization at the 695 nm peak intensity. An increasing amount of ferricyanide was added to the sample already containing 0.5 mM (or 2 mM) ferrocyanide. The absorption of the reference which contained only ferrocyanide was subtracted from that of the sample. The fully developped spectrum during an oxidative titration was obtained at the ferri- to ferrocyanide ratio of more than 10. Negative absorption in ordinate.

suggest that Chl-*a*-674 has a redox midpoint potential a little different from that of P-700. In fact, the absorption change of Chl-*a*-674 obtained after subtraction of the contribution due to P-700 gave a midpoint potential of about 390 mV with one electron reaction (Fig. 8), while a midpoint potential of P-700 determined with the absorption change at 695 nm was about 25 mV higher than that of Chl-*a*-674. These values of midpoint potentials were consistent with those obtained by the

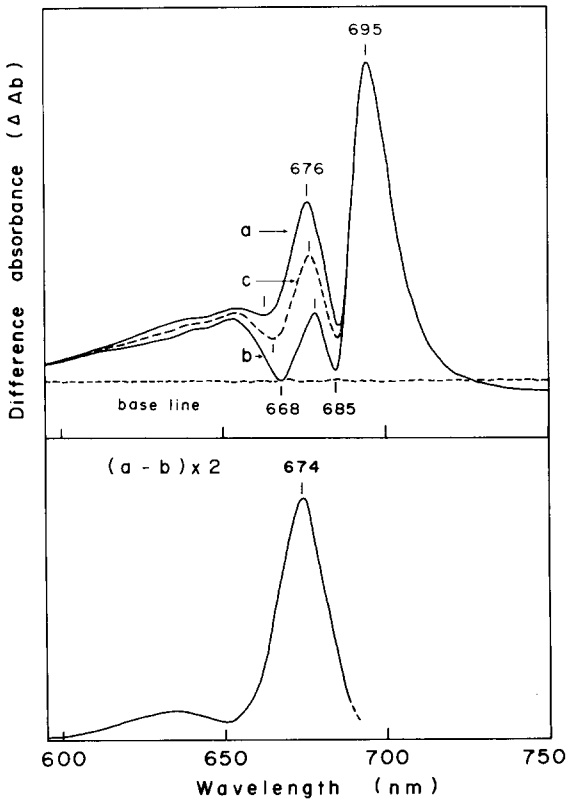


Fig. 7. Difference absorption spectrum for P-700 with the highest (a), the lowest (b), or the intermediate (c) satellite peak. They were obtained at the beginning of a reductive (curve with ferricyanide/ferrocyanide ratio of 4 in Fig. 6B) (a) and an oxidative (curve with ferricyanide/ferrocyanide ratio of 0.125 in Fig. 6C) (b) titration, and at the end of a reductive (curve with ascorbate added in Fig. 6B) (c) titration. The lower part of the figure shows the difference spectrum between curves a and b, expanded in ordinate by a factor of 2.

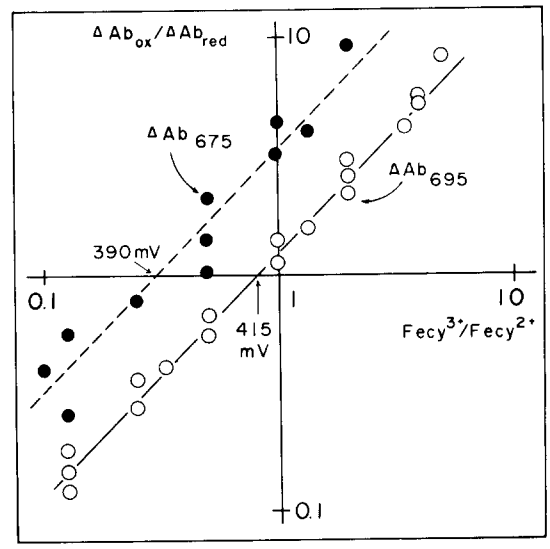


Fig. 8. Redox potential dependency of the 695 nm (open circles) and the 675 nm (solid circles) band in the difference absorption spectrum of P-700-enriched particles. Experimental data for the 695 nm band were taken from several reductive and oxidative titrations. Those for the 675 nm band were taken from reductive titrations as follows. The change in the 675 nm band during a reductive titration is the mixture of the positive development due to the satellite band of P-700 and the negative development due to Chl-*a*-674 bleaching. Based on the assumption that the spectrum obtained at the beginning of a reductive titration is only due to P-700, we estimated the signal change due to Chl-*a*-674 by a subtraction of the spectrum obtained at the beginning of a reductive titration from that at a certain redox potential after normalizing the former main peak with the amplitude of the latter one. The extent for 0 and 100% yield were determined with samples incubated with ferricyanide (1 mM) and ascorbate (5 mM) plus DCIP (2 μM), respectively. Other details are the same as in Fig. 4.

CD signal changes of the two CD components, CD694(−)/683(+) and CD678(−)/663(+), respectively, suggesting that the major CD component (CD694(−)/683(+)) originates from P-700 and that the manor one (CD678(−)/663(+)) originates from Chl-*a*-674.

The difference between the spectra obtained at the beginning of a reductive and an oxidative titration (Fig. 7, curves a and b) can be explained by the different contribution of Chl-*a*-674 in the absorption changes due to P-700. Judging from their midpoint potentials, the contribution of Chl-*a*-674 is minimum at the beginning of the reductive titration and maximum at the beginning of the oxidative titration. The difference absorption spectrum mainly due to P-700 thus obtained (curve a in Fig. 7) shows a high satellite band at 676 nm with a shallow dip around 662 nm. On the other hand, the maximum contribution of Chl-*a*-674 reflects a very low satellite band in the difference absorption spectrum (curve b in Fig. 7). These observations suggest that Chl-*a*-674 which is probably a neutral form in the oxidized state is reduced during the reductive titration giving the absorption decrease at 674 nm and is reoxidized during the oxidative titration. Both the oxidative and reductive titrations gave the same midpoint

potential to Chl-*a*-674 (cf. Fig. 8). At the end of the reductive and of the oxidative titration, P-700 and Chl-*a*-674 were both fully developed, so that the resultant spectra became similar to each other. They showed spectra approximately averaged the curves a and b in Fig. 7 (cf. curve c in Fig. 7).

There are some reports on a bleaching of bulk chlorophyll induced by chemical or photochemical oxidation, which had a midpoint potential of a little higher than that of P-700 [19,20] and always resulted in heightening a satellite peak in the difference absorption spectrum of P-700. On the other hand, no oxidation of bulk chlorophyll was observed during our measurements because of low temperature (5–7°C) and low detergent concentration (less than 0.01%) used here.

Curve analysis of the absorption spectrum of P-700-enriched particles

In order to confirm the presence of chlorophyll species corresponding to the CD and difference absorption components described above, we attempted to resolve the absorption spectrum into its components. Fig. 9 shows the results on a curve analysis of the absorption spectrum of P-700-enriched particles measured without any redox reagents. The best fit to the observed spectrum was obtained with three major Gaussians peaking at 669 nm, 675 nm and 684 nm, in addition to three minor gaussians peaking at 650 nm, 660 nm and 698 nm. The P-700-enriched particles used here contains only about eight antenna chlorophyll *a* per one P-700. Based on the results in Fig. 9, we can classify these eight antenna chlorophylls into four species, i.e., Chl-*a*-660, Chl-*a*-669, Chl-*a*-675 and Chl-*a*-684 with a quantitative ratio of less than about 1 : 2 : 2 : 2.

Chl-*a*-684 has already been identified as the emitter of the special fluorescence (F_{695}) of which yield changes according to the redox states of both P-700 and primary electron acceptors [11,12]. In addition, Chl-*a*-684 has a derivative-shaped CD signal with a positive peak at 678 nm and a negative peak at 688 nm (Fig. 1B), suggesting its dimeric organization. These observations, as well as the fact that the ratio of this chlorophyll to P-700 remains constant through ether extraction of pigments [11], suggest that it is a constituent of the PS I reaction center.

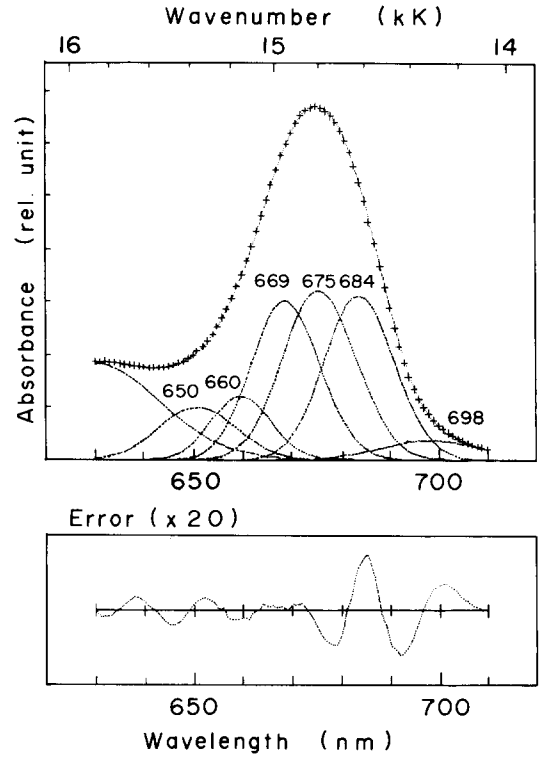


Fig. 9. The absorption spectrum of P-700-enriched particles fitted by the sum of Gaussian components. The observed data are plotted as cross marks while the line through them is the sum of the component curves of which characteristics are given in the following table. The error of fit at each point is shown below on a scale of 1 : 20. Chlorophyll content in P-700-enriched particles was 7% of that in PS-I particles and Chl/P-700 ratio was 8. The absorption spectrum was measured without any addition of redox reagents.

Peak locations:					
650.7	659.6	668.6	675.5	683.6	698.1
Relative peak heights:					
993	1190	3002	3200	3100	362
Band widths (1/e), cm^{-1}:					
523	395	435	459	428	603
Relative areas in %:					
7.4	6.7	18.6	21.0	18.9	3.1

Chl-*a*-674(5) seems corresponding to another species giving a derivative-shaped CD signal with a negative peak at 663 nm and a positive peak at 678 nm (Fig. 5B). This signal intensity was decreased on addition of reductants such as ascorbate or ferrocyanide (Fig. 2A), which enabled us to ascertain its derivative-shaped CD profile by an

oxidized minus reduced difference CD spectrum (Fig. 5B). Its absorption also decreased on addition of reductants, suggesting that a Chl-*a*-674 anion is formed by its reduction, with a midpoint potential of about 390 mV which was a little lower than that of P-700 (Figs. 4 and 8). This characteristics of Chl-*a*-674 apparently gave the spectral deformation of the difference CD and the difference absorption spectra of P-700 at shorter wavelength side of the main peaks. The observations that the CD signal arising from Chl-*a*-674 became more marked with more extraction of pigments suggests a close location of this chlorophyll to the PS-I reaction center. This CD signal, however, was a little broad and weak compared with those from P-700 and Chl-*a*-684 (Figs. 2 and 5), and, therefore, suggests a rather weak interaction between two chromophores in Chl-*a*-674.

The primary electron acceptor of PS-I (A_0 and/or A_1) was suggested to be a chlorophyll monomer and has an absorption maximum around 670 nm [13,21]. Therefore, a part of Chl-*a*-669 might be identified as A_0 (and/or A_1) like two molecules of bacteriopheophytin in a bacterial reaction center [31]. No fluorescence from this chlorophyll form has ever been detected in our particles [11,12], suggesting that it also act as an efficient antenna chlorophyll.

The presence of Chl-*a*-660 has already been reported by French et al. [22] who suggested that it is free chlorophyll *a* dissolved in lipid. The lower ratio of Chl-*a*-660 (less than 1 per one P-700) in our preparation shows that it may be possible to extract all of this chlorophyll by critical ether treatment.

A recent finding that P-700-enriched particles contain one or less than one chlorophyll *b* per one P-700 [13], is in good agreement with the presence of a minor band peaking at 650 nm in Fig. 9. This chlorophyll *b* may locate very close to the secondary electron acceptor $X(A_2)$ because its reversible reduction, possibly via $X(A_2)$, was observed at the redox level of $X(A_2)$ reduction [12,13].

Another characteristic of P-700-enriched particles is the absence of the far-red fluorescence band (F_{735}) [23] commonly present in PS-I particles with a high Chl-*a*/P-700 ratio [24–26]. This corresponds to the absence of a long-wavelength absorbing form of chlorophyll (Chl-*a*-700) in our particles [23]. Therefore, a minor band peaking at 698 nm in Fig. 9 may correspond not to the emitter of the far-red fluorescence, but to P-700 which remains partly in the reduced form without any redox reagents. The band due to the oxidized P-700, which probably has an absorption around 683 nm [cf. Refs. 8,17], may overlap the Gaussian peaking at 684 nm in Fig. 9. A curve analysis of the absorption spectrum in the presence of ascorbate gave the larger 698 nm component and a little smaller 684 nm component with no considerable change in the other components (data not shown). A deconvolution including Gaussians corresponding to the reduced and the oxidized P-700 is now in progress.

Conclusion

The results show the presence of three dimer-like chlorophyll *a* (P-700, Chl-*a*-684 and Chl-*a*-674 (5)) and two monomer chlorophyll *a* (Chl-*a*-669) (A_0 or/and A_1) in PS-I reaction center. Their strong resistance to the ether extraction suggests the close location of one with each other in a special site of the reaction center.

The physiological role of Chl-*a*-674 (5) is not clear now, but it may serve as an electron carrier around P-700. In our experiments, a part of Chl-*a*-674 (5) (i.e., a part of reaction center units) underwent this reaction, because both the CD signal change and the absorption change were only a part (about $\frac{1}{2}-\frac{1}{4}$) (cf. Figs. 2, 6, 7 and 9) of the total intensities. It suggests that the Chl-*a*-674 is not working as an electron carrier in the main path of normal electron transport in Photosystem I.

A large variety of the amplitude of the satellite band in the light-minus-dark difference absorption spectrum of P-700 has been reported in various membrane preparations [7,8,18,27–30]. These spectra have a good resemblance to those observed here at various redox potentials (Figs. 6 and 7). Therefore, the variation can be explained by assuming the occurrence of the light-induced redox change of Chl-*a*-674(5) with variable contribution to the reported spectra, probably due to the different procedures for measurement and to the different preparation.

Recently, Deisenhofer et al. have reported the detailed orientation of bacterichlorophylls from X-ray refraction analysis of the crystal of the reaction center of *Rhodopseudomonas viridis* [31]. The presence of three dimer-like and two monomer chlorophylls in our PS-I reaction center suggests the different arrangement of chlorophyll molecules between the bacterial and the PS-I reaction center.

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