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A 160-KILODALTON PHOTOSYSTEM-I REACTION-CENTER COMPLEX

LOW-TEMPERATURE ABSORPTION AND EPR SPECTROSCOPY OF THE EARLY ELECTRON ACCEPTORS *

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Treatment of a P700-enriched particle having a Chl/P700 = 7–12 (Ikegami, I. and Katoh, S. (1975) Biochim. Biophys. Acta 376, 588–592) with Triton X-100, followed by ammonium sulfate precipitation and sucrose-density-gradient centrifugation yielded a Photosystem-I reaction-center complex highly purified in both pigment and protein. The 160-kilodalton reaction-center complex contains 10–15 Chl:1 P700:12 atoms each of iron and sulfur, no carotenoids, and no (or little) cytochromes. Some complex can be prepared to contain equal or less 1 Chl *b* per P700, although it could also be completely removed by further extraction without appreciable change of the Chl *a*/P700 ratio. This PS-I reaction center, when appropriately redox-poised, is fully photochemically active at room and cryogenic temperatures. At room temperature, P700 could be oxidized by ferricyanide and re-reduced by excess ascorbate. In the complex containing dithionite at pH 10, intense illumination followed by freeze-trapping in the dark resulted in an absorption increase at 686 nm and some weak bleachings at 440 and 420 nm. These changes are attributed to possibly a contribution from the photoreduction of the secondary electron acceptor A_2 or X, as well as an electrochromic shift in the neighboring chlorophyll molecules. Extended illumination of the complex containing dithionite at approx. −40°C produced absorbance decreases at 670, 440 and 420 nm and an appearance of a 14 G-wide EPR signal at $g = 2.0$, indicating photoaccumulation of the reduced primary electron acceptor A_1^-. When this same sample was briefly thawed and then refrozen, the difference absorption spectrum and the 14 G-wide free-radical EPR signal attributed to A_1 reduction disappeared. Accompanying the reduction of A_2 (or X), additional absorbance decreases at 650 and 468 nm and an absorbance increase at 527 nm were observed, which could be accounted for by photoreduction of the chlorophyll-*b* molecule remaining in the complex.

Introduction

The concept of two photosystems in green-plant photosynthesis and the development of physical separation of thylakoid membrane fragments representing the two photosystems have amply been documented (e.g., Refs. 1–4). Although the membrane fragments obtained earlier were enriched in the reaction-center components, they still contained large complements of antenna chlorophylls and other extraneous components. Since the mid-1970's, various attempts have been made to isolate the 'reaction-center' particles [5–16]; most of these preparations were extensively freed of antenna

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chlorophylls, and some with reasonably well-defined polypeptide compositions [17].

Ikegami and Katoh [10] found previously that most of the antenna chlorophyll molecules in a digitonin-fractionated Photosystem-I (PS I) particle could be removed by extracting with ether containing suitable amount of water, without appreciable loss of P700 or its photochemical activity. The P700 content in the wet ether-extracted particles can be as high as 1 P700 in 6–9 chlorophyll molecules. The particles, because of their simple pigment composition, have been utilized initially to demonstrate effects of the redox state of the primary electron acceptors on the fluorescence yield in PS-I reaction centers [18,19].

As ether extraction only removes the bulk of the pigments and extraneous lipids, the P700-enriched particles still contained large amounts of proteins. We have recently treated these particles with the detergent Triton-X100 in order to remove the extraneous proteins, and obtained a solubilized and photochemically active PS-I preparation with a minimum molecular weight of 160 kDa. The chlorophyll/P700 ratio remains in the range of 10–15.

Difference absorption spectroscopy has been an extremely useful method for spectrally distinguishing the various functional electron carriers in photosynthesis [1]. For an electron carrier with a moderate midpoint potential, such as P700, it can be readily reduced or oxidized by chemical reagents. Thus, P700 may be recorded as an oxidized-minus-reduced difference spectrum. Very often, the difference spectra of certain electron carriers may also be obtained as a light-minus-dark spectrum, provided illumination causes a change in the redox state of the electron carrier of interest and an accompanying spectral change. One group of electron carriers, namely, the early electron acceptors of PS I, have characteristically highly negative redox potentials, making their chemical reduction rather difficult. Even when reduction is achieved, it is difficult to observe the spectrum changes because of their small amplitude or because of masking by such other components as the redox mediators.

Up to now, the absorption spectra of almost all the PS-I electron acceptors have been obtained from light-induced transient absorption changes. Depending on the relaxation kinetics of the species involved, the transients may be observed in the microsecond (for instance, P430 [20–22]) or picosecond (for instance, the chlorophyll acceptor of PS I [23–25]) domain. Certain species may be observed by using relatively long flashes in conjunction with lowering temperature so as to facilitate accumulation (for instance, the A_2 or X acceptor of PS I [23]). With the availability of the solubilized, highly enriched and photochemically active PS-I reaction-center complex reported here, we have attempted to use a combined chemical and photochemical treatments plus appropriate temperature lowering to effect selective reduction of PS-I electron acceptors for spectral measurements. The purpose of temperature lowering is either to retard the back reaction or to freeze-trap the desired reaction product.

This paper reports the difference spectra isolated for the various PS-I electron acceptors. Low-temperature EPR spectroscopy has been used to confirm the free-radical nature of the oxidized $P700^+$ and the reduced acceptor A_1^-. During this study, we have also discovered that chlorophyll *b*, which is approximately stoichiometric with P700 in this particular preparation, can undergo a reversible redox change at the redox level of acceptor A_2 (or X).

Experimental

Particles fractionated by digitonin treatment of spinach chloroplast and enriched in PS I, designated here as 'PS-I particle,' have a Chl *a*/*b* ratio above 7.0. These particles were washed twice with distilled water and, after lyophylization, extracted twice (at 4°C) with diethyl ether that was 80–100% saturated with water. Ether-extracted PS-I particles have a Chl/P700 ratio of 7–12, and hence designated at 'P700-enriched particles'. The preparation procedures and properties of the reaction-center particles will be described under Results.

Room- and low-temperature absorption and difference absorption spectra and the spectrophotometric assay of P700 were carried out in the Hitachi model 557 dual-wavelength spectrophotometer. P700 was assayed from the ferricyanide (0.5 mM) oxidized-minus-ascorbate (10 mM) re-

duced difference spectrum, using the extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [26]. Room-temperature photooxidation of P700 was monitored at 698 nm (vs. 730 nm as the reference wavelength) with ascorbate (10 mM) and DCIP (1 μM) present in the reaction mixture. EPR measurements were made in a Varian model 4500 spectrometer as described previously [31]. Chlorophyll concentration was determined according to Arnon [27]. Chlorophyll *b* was determined by the sensitive fluorometric method of Boardman and Thorne [28]. Protein was determined by the biuret method [29] using bovine serum albumin as the standard. Nonheme iron was determined by a modification of the method of Lovenberg et al. [30]. Inorganic sulfide was determined by the method of Golbeck and San Pietro [32].

Results and Discussion

The PS-I reaction-center complex – preparation and properties

The ether-extracted P700-enriched particles were not easily soluble in water, and their suspension showed strong light-scattering. However, these particles could be solubilized with Triton-X100 (0.1–0.3% w/w) by incubating at a Triton/Chl ratio of about 40 in 10 mM phosphate buffer (pH 8.0) at 0–4°C for 1 h. An insoluble greyish-white material was removed by centrifugation and discarded. Solid $(NH_4)_2SO_4$ was added to the green supernatant (at 0–4°C) with stirring. The supernatant became cloudy at about 0.1 g $(NH_4)_2SO_4$ per ml. Most of the chlorophyll and P700 were recovered as a blue-green precipitate by centrifugation. The precipitate was dissolved in a minimum volume of phosphate buffer (10 mM, pH 8.0) and the insoluble material was removed by centrifugation and discarded. Portions (0.5–1.0 ml) of the supernatant were layered on a discontinuous sucrose density gradient (5, 10, 20, 30 and 50%) in 10 mM phosphate buffer (pH 8.0)/0.05% Triton X-100, and centrifuged at 340000 × *g* for 5 h.

Two green bands appeared, one in the 20% sucrose layer and one at the interface between 30 and 50% sucrose layers. The ratio of Chl/P700 was 10–15 in the upper green band and 20–25 in the lower interface. The fraction present in the upper green band will be referred to as the 'PS-I reaction-center particles' and used in the work reported below. Table I presents an account of the pigment and protein contents of the particles at different stages of fractionation. It can be seen that extraction of the lyophylized (PS-I) particles resulted in about 50% loss of their weight, but only a small decrease in the protein content. On the other hand, more than 90% of protein of the P700-enriched particles was removed by Triton treatment and subsequent purification steps, but not much chlorophyll or P700 was lost. Thus, the reaction-center particle, which contains 160000 g protein per mol P700, still retained a low chlorophyll-to-P700 ratio.

The chlorophyll-*b* content in the PS-I reaction-center particle was determined by the sensitive fluorometric method [28]. A calibration curve was established first for our spectrofluorometer set-up

TABLE I

PIGMENT AND PROTEIN CONTENT IN PS-I PATICLES AT DIFFERENT STAGES OF FRACTIONATION AND PURIFICATION

Fractions	Dry weight (mg)	Protein (mg)	Chl (mg)	Chl *a*/*b* (w/w)	Chl/P700 (mol/mol)	Fe/P700 (atom/mol)	S/P700 (atom/mol)	Protein/P700 (g/mol)	P700 recovery (%)
Chloroplast	5000	3000	450	3.75	380	–	–	2600000	100
PS-I particles	450	270	62	8.0	158	–	–	572000	40
P700-enriched particles	240	200	3.1	15.5	9.1	11.0	12.6	512000	33
Reaction-center particles	–	15	0.92	16.5	13.0	11.9	12.3	160000	8

TABLE II

CHL-*b* CONTENT IN PS-I PARTICLES AND THE REACTION-CENTER PARTICLES IN RELATION TO THE EXTENT OF CHLOROPHYLL EXTRACTED BY DIETHYL ETHER AT DIFFERENT WATER SATURATION

	PS-I particles	Reaction-center particles					
Chlorophyll extracted (%)	0	94.3	94.4	94.6	95.7	97.3	97.5
Chl *a*/*b* (w/w)	8.0	17.5	16.5	16.5	24	48	58
Chl *a*/P700	158	14.0	13.0	13.0	12.0	12.0	16.0
CHl *b*/P700	20	0.8	0.8	0.79	0.5	0.25	0.27

[33]; this would provide an internal correction for the responses of the two monochromators (Jobin Yvon model H10) and the photomultiplier detector (Hamamatsu R928). A linear relationship was obtained for the plot of Chl-*a*/*b* ratio vs. the F666/F646 ratio for Chl-*a*/*b* ranging from 7 to 87. The Chl-*b* content in the digitonin-fractionated PS-1 particle and the Triton-fractionated reaction center particle is presented in Table II. As mentioned earlier, the PS-I particles prepared by digitonin fractionation had a Chl-*a*/*b* of 8 and a Chl/P700 ratio of 158. There was an excess of Chl *b* to P700 also (20 : 1). The Chl content in the P700-enriched particle decreases with increasing water saturation in diethyl ether used in extraction [10]. At 80% water saturation in diethyl ether, the resulting P700-enriched particles had only 5.6% Chl with a Chl-*a*/*b* ratio of 15.5. The corresponding reaction-center particle had a Chl-*a*/*b* ratio of 16.5, close to that in the original P700-enriched particles (see Table I). Increasing water saturation in diethyl ether led to a further loss of Chl and an even greater loss of Chl *b* in the resulting RC-particles (see Table II). These results indicate that about one Chl *b* (per P700) is located very close to the PS-I reaction center, although it seems unlikely to be a constituent of the reaction center (see below). All experiments reported in this work were carried out with reaction-center particles containing equal or less 1 Chl *b*/P700. The P700-enriched particles and the PS-I reaction-center particles contained 11.0 and 11.9 Fe atoms and 12.6 and 12.3 S atoms per P700, respectively, as determined from five separate measurements. These data indicate that the PS-I reaction center in both particles still retains all the iron-sulfur-center acceptors and that the membrane-bound iron-sulfur centers were unaffected by ether extraction or detergent treatment during the various preparation steps.

The reaction-center particles give a clear transparent solution. Its absorption spectrum (Fig. 1) has a broad red band with a maximum at 673–677 nm and a small shoulder around 480 nm, most of which could be estimated to be due to Chl *b* remaining in the reaction-center particles. The transparency of the reaction-center particle suspension thus makes it a better experimental material for more accurate P700 determination and other studies than the P700-enriched particles. P700 was initially present in the oxidized state in the reaction-center particles as prepared, as addition of a reducing agent such as ascorbate or dithionite led to an increase in absorbance around 697, 675 and 430 nm (Fig. 1, dashed spectrum). In contrast to the P700-enriched particles, there was no absorbance change around 560 nm, indicating that the particles contain no cytochromes.

P700 in the reaction-center particles was photochemically active. When P700 was reduced with ascorbate (5 mM) and DCIP (1 μM), almost all the P700 could undergo reversible photobleaching upon illumination with continuous light (not shown). This suggests that the electron-acceptor complex remained intact and functional in these particles. The time-course of the onset of photooxidation was somewhat slow, probably due to the diminished antenna size in these particles.

The photochemical activity of the reaction-center particles monitored at cryogenic temperatures by both EPR and absorption spectroscopy is shown in Fig. 2. Curve a, 1 in Fig. 2 is the EPR spectrum at 95 K of the maximum amount of $P700^+$ formed in the reaction-center particle in the dark after adding 0.5 mM ferricyanide to the sample at room temperature and then frozen. Adding excess reducing agent (10 mM ascorbate and 50 μM DCIP) to the thawed and oxidized sample in the dark converted all $P700^+$ back to P700, and

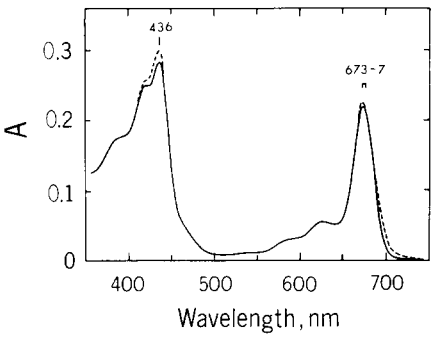

Fig. 1. Room-temperature absorption spectra of the PS-I reaction-center particle of 160-kilodalton molecular weight. Solid line: no addition (sample as prepared); broken line: sample containing dithionite.

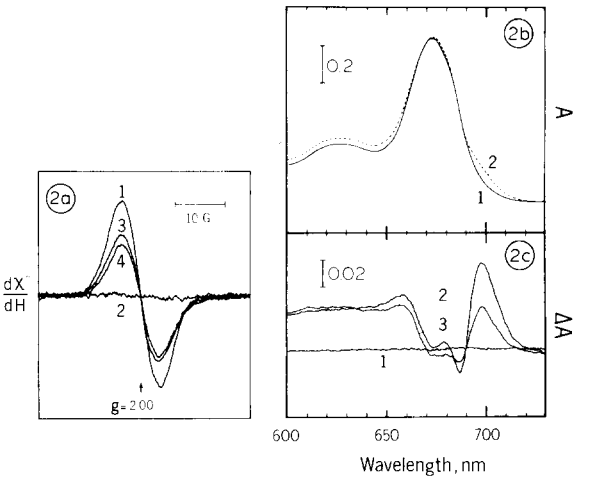

Fig. 2. (a) EPR spectra at 95 K of the PS-I reaction-center particles in the presence of 0.5 mM ferricyanide (spectrum 1) or the same sample with additional 10 mM ascorbate and 50 μM DCIP added (spectrum 2), both in the dark. Sample containing excess Asc/DCIP under illumination at 95 K (spectrum 3) and in the dark after illumination (spectrum 4). Sample was suspended in 0.1 M glycine-NaOH (pH 10)/60% glycerol and under anaerobic conditions. EPR conditions were: frequency, 9.1175 GHz; microwave power, 200 μW; modulation amplitude, 0.9 G; scanning speed, 100 G/12 min; instrument gain, $2 \cdot 10^3$; Chl concentration, 87.5 μg/ml. (b) Absorption spectra of the PS-I reaction-center particles at 77 K; sample containing 0.5 mM ferricyanide (spectrum 1) or the same sample with additional 15 mM ascorbate and 50 μM DCIP added (spectrum 2). (c) The difference spectra were obtained by manipulating the various spectra in (b): Spectrum 1 (baseline) = b, 1 − b, 1 (i.e., two identical samples, both containing 0.5 mM ferricyanide); spectrum 2 = b, 2 (dark) − b, 1; spectrum 3 = b, 2 (dark after light) − b, 1.

the free-radical spectrum also disappeared completely (spectrum a, 2). When the reduced sample was illuminated at 95 K, the free-radical $P700^+$ spectrum reappeared, the amplitude of which is approximately 70% that of the chemically oxidized $P700^+$ (spectrum a, 3). Rescanning the spectrum in the dark after illumination, approx. 17% of the light-induced signal decayed (spectrum a, 4). The extent of this decay is consistent with the previous finding on charge recombination in PS I at low temperatures by a temperature-dependent electron-tunneling mechanism [34].

Fig. 2b and c show the absorption and difference absorption spectra at 77 K of the same sample under various redox conditions similar to those used in measuring the EPR spectra. The low-temperature absorption spectrum of the reaction-center particles in the red region is similar to the room-temperature ones (spectrum b, 1 for sample containing 0.5 mM ferricyanide; spectrum b, 2 for the same sample with excess reducing agents added — 15 mM ascorbate and 50 μM DCIP). The difference between the two spectra, of course, reflects that between P700 and $P700^+$. Spectrum c, 1 was obtained from two identical reaction-center-particle samples, both containing 0.5 mM ferricyanide. This illustrates the measurement precision and the straight baseline as well as the great extent to which of the sample conditions were identical (i.e., maximum P700 oxidation in both samples). Spectrum c, 2 is the absorption difference between the reaction-center particle containing 15 mM ascorbate and 50 μM DCIP and the oxidized sample. Spectrum c, 3 was taken with the same sample pair used in spectrum c, 2, except after the ascorbate-reduced sample was illumination at 77 K. The difference between the amplitude of P700 photooxidation versus that caused by chemical oxidation, compared with the difference between the corresponding EPR spectra a, 1 and a, 3 (or a, 4), can mostly be accounted for by the difference in temperature at which the sample was illuminated (cf. Ref. 34).

Low-temperature difference absorption spectra of PS-I electron acceptors

We will first describe the various chemical and photochemical manipulations on the reaction-center particles at appropriate temperatures that provide selective and sequential reduction of the PS-I electron acceptors and, for the sake of briefness and simplicity, assign them alphabetical designations and, if necessary, additional notations for minor variations in conditions.

NA (no addition) – reaction-center particle as prepared;

A – sample containing ferricyanide (0.5–1.0 mM), maintained in the dark or illuminated (light);

B – sample with 10 mM dithionite, dark;

C – sample containing 10 mM dithionite, illuminated near 0°C for 2 min, then frozen in liquid nitrogen in the dark;

D – sample containing 10 mM dithionite, illuminated near −40°C for periods up to 20 min, then frozen immediately in liquid nitrogen;

D′ – the reduced and frozen sample in D above, briefly thawed in the cuvette and refrozen in liquid nitrogen.

For these experiments, the Hitachi cryogenic cuvette-and-dewar attachment was used. The reaction-center particle was suspended in 0.1 M glycine-NaOH (pH 10.0)/60% glycerol. Since a clear glass could not always be obtained reproducibly upon freezing the particle suspension, the sample was deliberately frozen (by repeated freeze and thaw) to yield maximum turbidity. The cuvette-and-dewar assembly was placed as close as possible to the photomultiplier cathode surface and the absorption spectra were monitored in the 'scattered transmission' mode. An identically frozen glycerol/buffer mixture containing no reaction-center particle was used as the reference. Only absorption spectra for sample NA (spectrum NA) and sample B (spectrum B) were shown in Fig. 3, top as illustrations.

In the red region of the difference spectrum for B-minus-NA in Fig. 3, the difference clearly represents that of (P700–P700$^+$), with peaks at 697, 687, 672 and 658 nm. In the Soret region, the difference is smaller than that expected for the P700 difference alone. This is most likely attributable to a counter absorbance decrease in this spectral region associated with reduction of iron-sulfur center A (and possibly center B also) in reduced sample B, making the net difference smaller. Note that spectrum (B − NA) is very similar to a ferricyanide-minus-ascorbate difference spectrum at 77 K reported by Lozier and Butler [35] for a PS-I subchloroplast particle. In their 77 K light-minus-dark difference spectrum, the amplitude of change in the blue region was negligible [35].

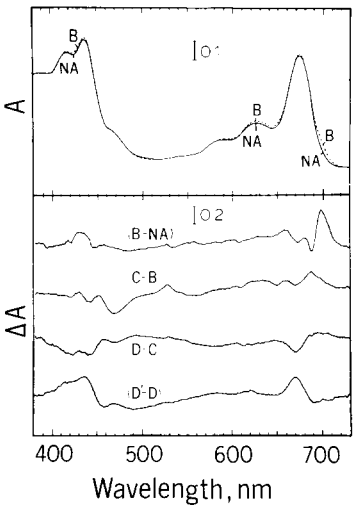

Fig. 3. The absorption (top) and difference absorption (bottom) spectra at 77 K of the PS-I reaction-center particle under various redox conditions: Spectrum NA (top) for sample without any addition (condition NA) but briefly illuminated beforehand near 0°C; spectrum B for sample containing 15 mM dithionite in the dark (condition B). Difference spectra (bottom) between samples under different redox conditions: (B-NA), (C − B), (D − C) and (D′ − D). See text for designations of the varous redox-poising conditions. Other experimental conditions the same as in Fig. 2b.

Under condition C, the acceptor A_2 or X is expected to be reduced. Thus, the (C − B) difference spectrum would be expected to reveal the difference (A_2^- − A_2). Previous measurements of A_2 reduction were all obtained from light-induced transient absorption changes [23,36–38]. The transient difference spectrum measured at 5 K appeared to be a composite of (P700$^+$ − P700) + (A_2^- − A_2) [23]. At 4°C, it was possible to photoaccumulate A_2^- in the Triton PS-I particle poised at −625 mV [23], yielding a net difference spec-

trum of $(A_2^- - A_2)$. The spectrum consisted of a broad bleaching between 420 and 520 nm, which was attributed to the reduction of the iron-sulfur center A_2, a broad but weak bleaching near 710 nm, an electrochromic shift near 450 nm, and development of bands at 672 and 688 nm (Refs. 23 and 36; cf. Ref. 38). The electrochromic shifts were explained as due to spectral changes in chlorophyll molecules induced by the electric field of nearby reduced acceptor A_2^- [23,36]. The light-induced transient difference spectrum measured by Koike and Katoh [37] showed only bleachings at 420 and 440 nm but lacked the electrochromic shifts of chlorophyll. Spectrum (C – B) in Fig. 3 indeed shows relatively weak bleachings at 420, 440 as well as 670 nm, plus an increase at 686 nm, which represent probably a combination of A_2 changes and chlorophyll electrochromic shifts. Furthermore, it shows rather prominent additional absorbance changes at 468 and 650 nm (decreases) and 527 nm (increase). As will be discussed below, the latter changes may be originated from the reduction of chlorophyll *b*.

As will be shown below from EPR-spectrum changes, condition D apparently can produce a maximum amount of A_1^-. Thus, spectrum (D – C) would represent the net difference of $(A_1^- - A_1)$. It shows bleaching at 670, 420 and 440 nm, some absorbance increase near 700 and 455 nm. Spectrum (D – C) indeed bears a close resemblance to that reported recently for $(A_1^- - A_1)$ by Swarthoff et al. [38], but differs with that reported by Shuvalov et al. [23–25] in the red spectral region. The $(A_1^- - A_1)$ difference spectrum reported by Swarthoff et al. [38] shows a bleaching at 670 nm and an absorbance increase beyond 710 nm; the bleaching in the Soret region is at 420 nm (with a shoulder at 440 nm), plus a large absorbance increase near 460 nm.

Spectrum (D′ – D) is the difference between sample D after a brief thawing and refreezing (D′) and the original sample D. Spectrum (D′ – D) appears exactly as a mirror image of spectrum (D – C). This result indicates that a brief thawing of sample D causes re-oxidation of the reduced acceptor A_1^-, but has no effect on the reduced acceptor A_2^-. In other words, the redox state of sample D′ as a result of the brief thawing, is almost identical to that of sample C.

EPR spectroscopy of the reaction-center particle at 95 K

EPR spectra were measured (at 95 K in the dark) for the reaction-center particles treated under some reductive conditions (see Fig. 4) and used as a confirmatory measure for the species assignment made in the absorption spectral data. Spectrum A in Fig. 4, top shows the $g = 2.0$ free-radical signal of $P700^+$ formed under condition A (dark), with a typical ΔHpp of 7–8 G. A straight baseline was obtained for the sample under condition B (data not shown), indicating that all $P700^+$ were reduced. No new development of EPR signal occurred under condition C either (spectrum C). Spectrum D was measured for sample under condition D, where the free-radical species would be A_1^-, as its ΔHpp is 14 G. Upon brief thawing and refreezing, the reduced A_1^- is expected to be re-oxidized and the redox state of the sample should be reverted to that of sample C; indeed the 14-G

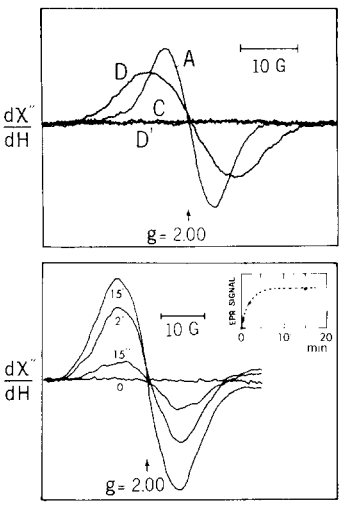

Fig. 4. Top: EPR spectra at 95 K of the PS-I reaction-center particles under different redox conditions A, C, D and D′. See text for the designations of the various redox conditions. EPR conditions were the same as in Fig. 2 except that the scanning speed was 100 G/3 min and instrument gain was $1 \cdot 10^3$. Other experimental conditions were the same as in Fig. 2a. Bottom: Development of the 14-G EPR signal due to freeze-trapping A_1^- before illumination and after illumination for 15 s and 2 and 15 min. EPR conditions were the same as in Fig. 2 except the scanning speed was 100 G/24 min and instrument gain was $5 \cdot 10^3$. Inset shows the amplitude of EPR-signal vs. illumination time.

free-radical signal disappeared, and only a straight baseline resulted in spectrum D'.

The A_1/A_1^- couple is expected to have a very negative redox potential (actual value not yet determined). This is shown by the fact that brief thawing (condition D') leads to its complete reoxidation. Ordinarily A_1^- is freeze-trapped by continuous and intense illumination [39,40]. We have found that illumination of the sample at a lower temperature (in our case, approx. $-40°C$) but for a longer duration can also effect accumulation of A_1^-. Trapping of A_1^- is accomplished presumably as a result of a favorable competition between electron donation by dithionite to $P700^+$ and the fast back-reaction between $P700^+$ and A_1^- at lower temperatures (cf. Ref. 41). The amount of A_1^- formed increases exponentially with time (Fig. 4, bottom). About 50% of the maximum amount of A_1^- is formed in about 60 s, and the maximum level is reached in about 7–8 min. To insure complete reduction of A_1, we have elected to illuminate for 15–20 min for condition D. The lower temperature used here for photo-trapping presumably is also beneficial in avoiding photobleaching of antenna chlorophyll molecules that might otherwise occur by intense illumination at higher temperatures.

A light-induced reversible redox change in chlorophyll b

As shown in Fig. 3, spectrum (C − B), there is ample evidence that certain spectral changes in the supposedly $(A_2^- - A_2)$ difference spectrum may consist of some contribution by absorption changes associated with (photo)reduction of chlorophyll *b*. Reduction of Chl *b* in vivo is expected to cause bleaching of the two major absorption bands at 468 and 650 nm, as seen in Fig. 3. In addition, an absorption increase occurred at 527 nm. Reduction of Chl *b* appears to occur at the same redox level of A_2 reduction. It is not certain whether Chl *b* is directly photoreduced or reduced by an intermediate such as A_2^-. Separate examination (results not shown) showed that both the onset kinetics in the light and the decay kinetics in the dark were relatively slow.

Concluding remarks

The PS-I RC-proteins reported in recent literature [5–16] are mostly highly depleted of antenna chlorophylls, with Chl/P700 ratio ranging from 6–9 [10] to 40 [7,9]. In general, all reaction-center preparations represent an aggregate complex consisting of 6 [9] to 13 [16] subunits. The polypeptide composition and enzymatic activity of some reaction-center preparations have been characterized in detail [9,16]. The 160-kDa PS-I reaction-center particle reported here, produced by first removing as much as possible the antenna chlorophylls and other lipoid materials from a crudely fractionated PS-I particle [10] followed by Triton treatment to remove the extraneous proteins probably represents the simplest aggregate complex available for the PS-I reaction center. Preliminary polypeptide analysis (work in progress; results will be reported elsewhere) indicate the aggregate complex to consist of one large P700-containing polypeptide, two smaller polypeptides presumably containing the iron-sulfur centers, plus 2 or 3 other small subunits. Thus, the 160-kilodalton PS-I reaction-center particle is structurally a rather complete assemblage of proteins (including all the early electron acceptors A_1, A_2 (X), and the iron-sulfur centers B and A) situated in a concerted interactive state and functionally capable of photochemical charge separation and charge stabilization.

The oxidative and reductive treatments of the PS-I reaction-center particles coupled with photochemical manipulations allowed us to reduce the electron acceptors sequentially with increasingly negative redox potentials. Because of the high concentration of the electron carriers in the PS-I reaction-center particle, it is perhaps for the first time that the difference absorption spectra of the various electron acceptors can be directly measured in the freeze-quenched reaction-center particles in the dark, instead of resorting to measuring the transient absorption changes induced by light pulses. In the case of ΔA(B − NA) or ΔA(P700 − $P700^+$), its close resemblance (except for slight spectral shifts of the red absorption bands toward shorter wavelength) to that of the more native particles indicates no major alteration of structure or conformation in the highly-pure and -enriched particles.

In the first two reductive stages, one would expect to see absorbance decreases in the Soret region due to chemical and/or photochemical reduction of the iron-sulfur proteins. The small amplitude of absorbance change in the Soret region in case (B − NA) was attributed earlier in the Results section to a counter absorbance change. In ΔA(C − B), an additional absorbance decrease is expected as a result of reduction of A_2, the iron-sulfur center X, with P700 change now remaining constant. This appears to be reflected in the Soret region by an overall greater absorbance decrease. In the red region, there should be no change originated from P700; the small spectral change most likely originated from electrochromic shifts in the neighboring chlorophyll molecules, as has previously been observed under comparable conditions [23,36].

In ΔA(D − C), all absorbance changes due to A_2 (X) and the iron-sulfur centers B and A cancel each other out in samples D and C, so the difference spectrum should reflect $\Delta A(A_1^- - A_1)$. Indeed, the Soret region shows only absorbance decreases at 420 and 440 nm and one major absorbance decrease at 670 nm. Interestingly, this difference spectrum agrees very well with the recently reported diffrence spectrum attributed to $(A_1^- - A_1)$ by Swarthof et al. [38]. It is, however, at variance with the $(A_1^- - A_1)$ difference spectrum obtained earlier from picosecond pulse-induced transient absorbance changes [24,25]. These latter changes presumably include a large component arising from the triplet state of P700 (Ref. 42; cf. Refs. 43 and 44). The difference absorbance attributed to A_1^- here is consistent with the notion that A_1 is a chlorophyll-*a* molecule. The assignment of the absorption difference spectrum, ΔA(D − C) to a chlorophyll acceptor is also corroborated by the 14 G radical EPR signal obtained under the same redox conditions (see Fig. 4). It is also worth noting that extended illumination of the same sample in the presence of dithionite and at approx. −40°C appears to be a very satisfactory procedure for trapping A_1^-.

The difference spectrum ΔA(D′ − D) is almost an exactly inverted image of ΔA(D − C). As sample D′ was obtained from sample D by briefly thawing it in the cuvette and refreezing, it means that such a brief thawing readily reoxidizes A_1^- back to A_1 without affecting the redox state of A_2^- or X^-.

Accompanying the reduction of A_2 or X in stage C, there was an apparent absorbance change that could be attributed to the reduction of chlorophyll *b*, namely, the absorbance decrease at 468 and 650 nm and an absorbance increase at 527 nm. The appearance of an absorption band at 522 nm accompanying photoreduction of chlorophyll *a* in solution was reported previously [45]. On the other hand, we have not yet detected any EPR signal that can be attributed to an anion radical of chlorophyll *b* under these reducing conditions (cf. Fig. 4). The reason for this anomaly is yet unknown. No similar chlorophyll-*b* reduction has been reported by other workers in other PS-I particles, presumably the disposition of chlorophyll *b* may have been altered in some way by the harsh detergent treatment during the preparation of the particles. Furthermore, its onset kinetics is exceedingly slow, making its detection difficult by more transient perturbation techniques. Reports on possible involvement of chlorophyll *b* in green-plant electron-transport processes appeared in the mid-1960's [46,47]. However, their significance has not been confirmed and its relationship to our present observation is unclear.

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