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Oxidation-Reduction Potentials of Bound Iron–Sulfur Proteins of Photosystem I

(photosynthesis/primary electron acceptor/P700/P430/electron paramagnetic resonance)

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ABSTRACT Digitonin-fractionated photosystem-I subchloroplasts were titrated potentiometrically between −450 and −610 mV at pH 10. Examination of the titrated subchloroplasts by low-temperature (13°K) electron paramagnetic resonance spectroscopy revealed resonances centered at values of 2.05, 1.94, 1.92, 1.89, and 1.86 on the g-factor scale. The peak heights depended on the potentials at which the chloroplasts were poised. The resonances of at least three iron–sulfur centers can be recognized: one with lines at g = 2.05 and 1.94; one with lines at g = 2.05, 1.92, and 1.89; and one for which only a line at g = 1.86 has been resolved. The midpoint potentials of the iron–sulfur species fall into two distinctly separate regions: the titration profile of the g = 1.94 signal, the first segment of the g = 2.05 plot, and the rise phase of the g = 1.86 signal had a value of −530 ± 5 mV; the upper segment of the g = 2.05 plot, the decrease phase of the g = 1.86 signal, and the g = 1.89 profile had a midpoint potential estimated to be ≦−580 mV. The oxidation-reduction reaction of each of the bound iron–sulfur species, as represented by the changes of the electron paramagnetic resonance spectra, was reversible and apparently involved a two-electron change.

Titration at pH 9 could only be carried to −560 mV, and essentially only the first half of the titration behavior as found at pH 10 was seen. At any given potential more positive than −560 mV, the part of the iron–sulfur protein that was not reduced electrochemically could be reduced photochemically, but only to the maximum extent reduced electrochemically at −560 mV. Whereas, chloroplasts illuminated at room temperature and then frozen while still being illuminated developed a signal similar to that produced by electrochemical reduction at −610 mV, illumination at 77°K did not bring about photoreduction beyond that accomplished electrochemically at about −560 mV.

Dithionite alone in the dark and under anaerobic conditions brought about a partial reduction to the extent of the first electrochemical reduction step. Dithionite plus illumination at room temperature or dithionite plus methyl viologen in the dark produced the maximum signal. Electron paramagnetic resonance spectra due to either light or electrochemically reduced iron–sulfur proteins showed no detectable decay for at least 3 days when samples were stored in the dark at 77°K.

In 1971 Malkin and Bearden (1) observed an electron paramagnetic resonance (EPR) signal characteristic of a plant-type ferredoxin when spinach chloroplasts were illuminated at 77°K and the spectrum was recorded at 25°K. Since detection of the EPR signal was independent of the presence of soluble chloroplast ferredoxin, and since reduction of ferredoxin could take place by a simple photon capture at 77°K, the authors attributed the EPR signal to a membrane-bound ferredoxin and suggested that "it may serve as a primary low-potential electron acceptor in chloroplast photosynthesis." It was soon found that the light-induced ferredoxin-like EPR signal was associated with photosystem I (2). These findings were subsequently confirmed by others (3–5). Leigh and Dutton (3) also found that chloroplasts poised at redox potentials from +350 to −250 mV were still capable of undergoing photoreactions, giving rise to the ferredoxin-type EPR spectrum. More recently, Bearden and Malkin (6) reported that the stoichiometric relationship between the bound ferredoxin and P700 is unity, which lends further support to the idea that membrane-bound ferredoxin is the primary electron acceptor.

Simultaneous with these developments a light-absorbing species was characterized by Hiyama and Ke (7) that exhibited kinetic behavior expected of the primary electron-acceptor of photosystem I. This assignment was mainly based on a kinetic correlation between the reduction of various artificial secondary electron acceptors and the decay of the newly found spectral species. A light-induced difference spectrum for this species showed a maximum absorption decrease at 430 nm and minor bands at 460 and 405 nm. The species was designated as "P430" (7, 8). The difference spectrum bears some resemblance to that of soluble spinach ferredoxin, but the difference extinction was greater (7–9). Subsequent measurements showed that the quantum efficiency for the formation of $P700^+$ and $P430^-$ in the primary photochemical charge separation was unity for the quanta absorbed at 710 nm (8), and the risetime for formation of both species was less than 100 nsec (9). Redox titrations of photosystem-I particles, correlated with attenuation of light-induced absorption changes, yielded a midpoint potential of −470 mV for P430 (9).

From these findings, which have uncovered components of the chloroplast system that could qualify as primary acceptor of photosystem I, it seemed of great interest to correlate the behavior of these components, namely, P430 and bound ferredoxin, in kinetic and redox-titration experiments. Indirect evidence for EPR spectroscopy that P430 of photosystem I is a membrane-bound iron–sulfur protein has recently been reported by use of a system in which $P700^+$ accumulates (10). We report here a redox titration with digitonin-fractionated photosystem-I subchloroplasts monitored by low-temperature (13°K) EPR spectroscopy.

Abbreviation: EPR, electron paramagnetic resonance.

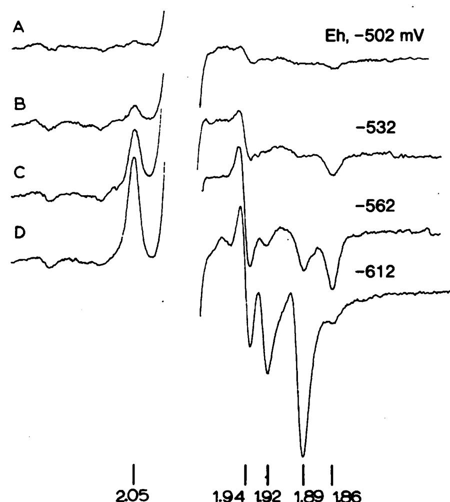

FIG. 1. EPR spectra of photosystem-I subchloroplasts at the initial (spectrum *A*), two intermediate (*B* and *C*), and the final (*D*) stages of a redox titration at pH 10. The poised redox potential (expressed relative to the hydrogen electrode) is indicated for each spectrum. The inner diameter of the tubes was 4 mm. All spectra were obtained from one single scan. The ordinate represents $d\chi'/dH$ in arbitrary units.

EXPERIMENTAL

Digitonin-fractionated photosystem-I subchloroplast particles (NADP-reduction activity about 1500 μmol/mg of chlorophyll per hr) were used in all experiments at a final concentration of 1.0 mg of chlorophyll per ml. A titration at pH 10 in 0.2 M glycine–NaOH buffer was extended to −610 mV. At pH 9, with the same buffer, only a partial titration up to −560 mV was possible. The following redox mediators (all at 0.1 mM) were used to cover the potential range from 0 to −610 mV (all potentials expressed against hydrogen electrode): indigo-tetrasulfonate (E_0' = −46 mV), indigo-disulfonate (−125 mV), anthraquinone-β-sulfonate (−218 mV), 1,1′-dimethyl-4,4′-dipyridylium dichloride (benzyl viologen, −360 mV), 1,1′-dimethyl-4,4′-dipyridylium dichloride (methyl viologen or Paraquat, −440 mV), 1,1′-trimethylene-2,2′-dipyridylium dibromide (Triquat, −540 mV), and 1,1′-trimethylene-5,5′-dimethyl-2,2′-dipyridylium dibromide (−670 mV). An anaerobic sodium dithionite (Fluka) solution in 1 mM NaOH was the titrant. All concentrations given refer to the final concentrations resulting in the samples after mixing. A combination Pt/Ag-AgCl electrode (Ingold) with a 7/15 inner ground joint† was used to measure the redox potential. The electrode was calibrated with quinhydrone at two pH values.

The redox titration was done in an anaerobic vessel fitted through an adaptor with the combination electrode, with an outlet for degassing and flushing, and an injection port with an air lock for additions, all located at the top of the vessel, and a two-way stopcock at the bottom.† The subchloroplast suspension in the vessel was thoroughly degassed before titration by five cycles of 1-min evacuation and reflushing with deoxygenated nitrogen. The titrant was delivered from a Hamilton 500-μl threaded plunger syringe (model 87001) through a 9-inch 26-gauge needle. The syringe was driven electrically in conjunction with a potentiostat which allows an automatic control of the titration process.†

Subchloroplast suspensions poised at desired potentials were transferred into quartz EPR tubes through additional three-way and two-way stopcocks connected to the two-way stopcock at the bottom of the vessel. The EPR tubes had an inner diameter of 4 or 3 mm and at the top a 7/15 standard taper joint (outer) for connecting the tube to the reaction vessel through the stopcock. To avoid contamination between transfers, the inner part of the stopcock was thoroughly cleaned, and the solution trapped in the channel of the stopcock plug was pushed back into the vessel by a slight positive pressure from the purified nitrogen before each new titration and transfer. The anaerobic conditions were considered satisfactory when this operation caused no change in the redox potential of the suspension. Before each transfer, the desired potential of the suspension was held for at least 5 min, although redox equilibrium between the electroactive species and the mediators appeared to be established very rapidly. The solution was constantly stirred with a glass-coated magnet bar, activated by a stirrer mounted on the side of the titration vessel.

All operations were done in dim room light. After transfer to the tube, the sample was stored in total darkness for 3 min before it was frozen in liquid nitrogen. Thereafter, the sample was always kept in total darkness, including manipulations during EPR measurements.

Reduction by dithionite alone or in the presence of methyl viologen was done with the aid of a sidearm apparatus described earlier (11). Where sample illumination was called for, a cylindrical flash illuminator† was used, which furnished uniform illumination around the EPR tube at a white light intensity of about 10^6 ergs/cm^2 per flash.

EPR spectroscopy was performed in a modified Varian spectrometer. Unless specifically mentioned, the following conditions were used: microwave frequency, 9.18 GHz; power, 3 mW; modulation frequency, 100 kHz; amplitude, 10 G; scanning rate, 200 G/min; time constant, 0.5 sec; and temperature, 13.30 ± 0.15°K at the sample position. The signal heights were evaluated as follows: the g = 2.05, 1.89, and 1.86 peaks were measured from the far left or right baselines, respectively. In addition, corrections were applied for overlap of the last two peaks and when required for background absorption, as seen in the untreated samples. This correction mainly applies to the g = 1.86 peak.

RESULTS

Redox Titration at pH 10 of Bound Iron–Sulfur Proteins of Photosystem I. A redox titration extending to a sufficiently low potential was performed at pH 10. Independent tests showed that neither the chemical nor the photochemical behavior of photosystem-I subchloroplast particles was affected by the high pH. Fig. 1 shows EPR spectra at four stages of the titration course. At the initial stage at −502 mV, an EPR spectrum (Fig. 1*A*) characteristic of a plant-type ferredoxin began to emerge. The spectrum had major lines centered at g of 2.05, 1.94, and 1.86. These values are only to indicate field positions, without the implication that they are true g factors. A large free-radical signal appeared in g = 2 region, which was presumably mostly due to the

† Ke, B.: Reports on the electrode adaptor, the syringe drive with potentiostatic control, and the flash illuminator are in preparation.

dipyridyl radicals formed from the redox mediators. At −532 mV, all major lines seen above increased further in magnitude. At −562 mV, in addition to a further increase of the same major lines, two new lines at g = 1.92 and 1.89 began to appear. Below −562 mV, the lines at g = 2.05, 1.92, and 1.89 continued to increase, the line at g = 1.94 changed only very little, and the peak at g = 1.86 began to decrease. As shown by spectrum D in Fig. 1, taken at −612 mV, the most negative potential obtainable at pH 10, the lines at g = 2.05, 1.94, 1.92, and 1.89 reached the maximum and that at g = 1.86 the minimum level.

The changes in the EPR spectra during the titration can be more clearly seen from plots of the heights of the various peaks against the redox potential (Fig. 2). The appearance of the g = 1.94 signal followed a two-electron titration course, with a midpoint potential of −533 mV, and became practically constant near −560 mV. The g = 2.05 signal increased during the entire titration up to −610 mV, with an apparent halt at about −550 mV. The first segment had a midpoint potential of −532 mV, and represented a two-electron change. This is practically identical with that derived from the behavior of the g = 1.94 signal. The upper segment had apparently not yet reached a plateau; the midpoint potential can only be estimated to be ≦−580 mV. The g = 1.86 signal reached the maximum level in the middle of the titration, and then decreased. The phases of increase and decrease are nearly symmetrical and each can be ascribed to a two-electron change, with midpoint potentials at −528 and −584 mV, respectively. The g = 1.92 and 1.89 signals did not begin to increase until at about −540 to −550 mV, but continued to increase up to −610 mV. These two peaks appear to change in unison. Only the signal at g = 1.89 was therefore plotted in Fig. 2. The slope of the change of the g = 1.89 signal again was consistent with a two-electron change, and the midpoint potential can only be estimated to be near −580 mV.

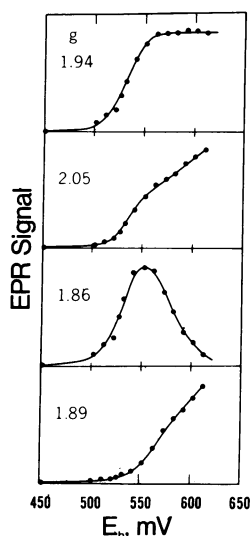

FIG. 2. Plots of EPR-signal heights of four prominent peaks for a redox titration at pH 10. Each set of points was derived from a separate spectrum of the kind shown in Fig. 1. The curves presented are the result of a least-square fit of segments of each curve similar to the procedure proposed by Morrey (16). A third-order polynomial was chosen as a model. Each segment consisted of a set of five consecutive points. For construction of the curves shown, a set of data obtained in a single extended titration was used. Data obtained in separate, less complete, but otherwise analogous experiments confirmed these results.

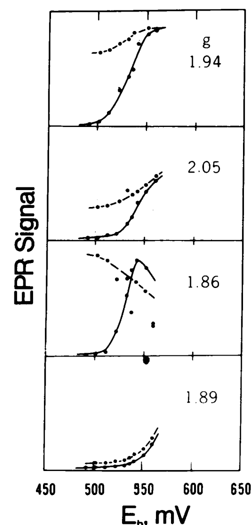

FIG. 3. Plots of EPR-signal heights as in Fig. 2 for a redox titration at pH 9 (*lower solid curves*). The other conditions were as for Figs. 1 and 2, except that the inner diameter of the tubes was 3 mm. The upper curves (*dashed*) represent the total signal heights shown by each sample after subsequent flash illumination at 77°K.

The titration behavior of the system appears to be reversible, as, whenever this was done, the same EPR spectra were obtained by titration of oxidized samples with dithionite or of reduced samples with ferricyanide.

Redox Titration at pH 9 of Bound Iron–Sulfur Proteins of Photosystem I. When samples were titrated at pH 9, the lowest redox potential that could be achieved was −550 mV, as dictated by the electrochemical relationship between potential and pH. If the redox reactions were independent of pH, then it would be expected that only the first half of the titration behavior as found at pH 10 would be seen at pH 9. This was indeed the case, as shown by the lower (*solid*) curves in Fig. 3. The titration course of the g = 1.94 signal was almost identical with that measured at pH 10: the profile represented a two-electron change, had a midpoint potential of −532 mV, and reached a constant level between −550 and −560 mV. The g = 2.05 signal developed to slightly beyond 50% of the maximum level; the estimated midpoint potential was near −530 mV and the slope was also consistent with a two-electron change.

Up to −550 mV, the g = 1.86 signal rose as it did at pH 10, with a midpoint potential estimated to be −530 mV and a slope consistent with a two-electron change. The signals at g = 1.89 and 1.92 rose at a lower potential than the other signals, with no significant development of a titration profile yet at −540 mV.

Illumination of Incompletely Titrated Subchloroplast Particles. Below the maximum negative potential, only a fraction of the iron–sulfur proteins was electrochemically reduced. Additional illumination is expected to cause photochemical charge separation and to bring the total signal to the maximum level. This was occasionally checked for samples titrated at pH 10 and more systematically checked for pH 9. The

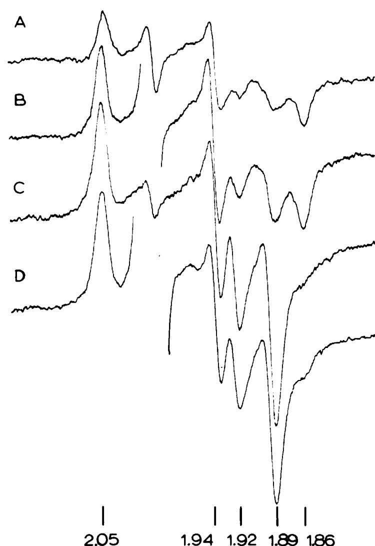

FIG. 4. EPR spectra of photosystem-I subchloroplasts (chlorophyll concentration, 1 mg/ml). (*A*) With 10 mM dithionite (final concentration) added to the sample kept under anaerobic conditions in the dark and frozen also in the dark; (*B*) the same sample used in *A* subsequently illuminated at 77°K; (*C*) the anaerobic subchloroplasts with 10 mM dithionite (final concentration) added, then illuminated for 1 min at room temperature, and then frozen in liquid nitrogen while being illuminated; and (*D*) the anaerobic subchloroplasts with 10 mM dithionite and 0.5 mM methyl viologen (both final concentrations); the sample was always kept completely in the dark.

latter results are shown by the upper (*dashed*) curves in Fig. 3, representing the total signal levels after the EPR tubes were flash-illuminated at 77°K.

For the $g = 1.94$ signal, 80–100% of the total magnitude was brought out by flash illumination. Incomplete photochemical reaction is possible here because of insufficient light penetration, since the optical density of the suspension from the wall to the center of the tube (tube diameter 3 mm) was nearly 10. For the $g = 2.05$ signal, the total height was about 50% of the maximum possible. For the $g = 1.86$ signal, flash illumination yielded a signal at the peak level of the bell-shaped curve (see Fig. 2). For the $g = 1.89$ signal, the curve was only slightly shifted upward from the electrochemically produced signal height. From these results it is reasonable to conclude that illumination at 77°K did not bring about photoreduction of the bound iron–sulfur proteins beyond what was accomplished electrochemically at a potential of about −550 to −560 mV.

Photo- and Chemical Reduction of the Bound Iron–Sulfur Proteins of Chloroplasts. Although the EPR signals attributed to bound ferredoxin were initially observed with chloroplasts illuminated at 77°K (1), 25°K (2), or at room temperature (5), but subjected to EPR spectroscopy near liquid-helium temperature, it was not clear whether the extent of the development of the various peaks depended on the temperature at which the material was illuminated. Furthermore, reports concerning the effect of dithionite on the appearance of the ferredoxin-type signal, either in the light (1) or in the dark (5), were also at variance. We, therefore, investigated these effects.

Presumably because of insufficient light penetration (see above) at the chlorophyll concentrations used here, the magnitude of the light-induced EPR signal was always smaller than the electrochemically produced maximum signal level. We also found that the amount of signal development was temperature dependent. Whereas samples preilluminated at room temperature and then frozen while still being illuminated developed a signal similar to that produced by electrochemical reduction at −600 mV, illumination of samples at 77°K only developed a signal with features seen after the first stage of electrochemical reduction at about −550 mV.

Contrary to a recent report (5) that dithionite alone produced no EPR signal of reduced ferredoxin when the sample was kept in the dark, we found that 10 mM dithionite under strictly anaerobic condition and in the dark brought about a partial reduction to the extent of the first-stage electrochemical reduction (spectrum *A* in Fig. 4). Subsequent flash illumination of the frozen sample containing dithionite increased the signals further with even some appearance of the peaks at $g = 1.92$ and 1.89 but no sign of diminution of the 1.86 signal (spectrum *B*, Fig. 4). On the contrary, if the anaerobic sample was reduced by dithionite and illuminated before and during freezing, the maximal signal developed (spectrum *C*, Fig. 4). In this case, the signals were larger than when dithionite was absent. Another interesting point in this spectrum is the almost complete absence of a free-radical signal due to $P700^+$. Presumably P700 was reduced by dithionite, as it is observed when $P430^-$ accumulates (12). On the other hand, if 0.5 mM methyl viologen was present in the anaerobic sample containing 10 mM dithionite, the maximal signal developed in *total* darkness (spectrum *D*, Fig. 4).

Contrary to an earlier report that the EPR signal due to the light-reduced iron–sulfur protein decays with a halftime of about 8 hr (1), we found that the EPR spectra of either light or chemically reduced iron–sulfur protein showed no change in 24 or 72 hr when stored at 77°K, and in some chemically reduced samples, we found no detectable change even after the samples were stored in the dark at 77°K for 10 days.

DISCUSSION

Previous studies (1–5), as well as the present one, have amply demonstrated that the iron–sulfur proteins in chloroplasts and subchloroplasts are firmly bound to the lamellae. The detergent-fractionated subchloroplasts used in the present work were apparently free of any detectable soluble ferredoxin, as they had no NADP-reduction activity without added soluble ferredoxin. The present work has also confirmed that the bound iron–sulfur proteins are associated with photosystem I (2). The fact that an identical EPR spectrum can be produced simply by illumination of the chloroplasts or subchloroplasts is consistent with the suggestion that these strongly reducing iron–sulfur proteins function as primary electron acceptor or reaction partners for the primary donor, P700, in photosystem I. We have further found that a photosystem-II reaction-center particle isolated from spinach (13) does not show ferredoxin-like EPR signals under comparable reducing conditions (unpublished experiments).

It is apparent from the EPR spectra obtained during the reported titrations of the photosystem-I subchloroplasts that there are several paramagnetic species involved. The conclusion is inescapable that the strong line at $g = 1.94$ (peaks at $g = 1.944$ and 1.933) is not due to the same struc-

ture as the line at g = 1.86, as has been previously inferred (1, 3, 5), unless the component giving rise to it is altered as reduction is carried beyond a certain point. There is no evidence incompatible with the assumption that the peaks at g = 1.89 and 1.92 belong to the same structure, and it appears that this structure as well as that represented in the line at g = 1.94 make a contribution at g = 2.05, as this latter line increases further on titration without concomitant changes at g = 1.94. Thus the minimum number of species represented is 3, namely, one with lines at g = 2.05 and 1.94, one with lines at g = 2.05, 1.92, and 1.89, and one for which we have only been able to resolve a line at g = 1.86. In spectra in which all lines are fully developed, we have been unsuccessful in distinguishing the different species by variation of microwave power or temperature. According to their properties and behavior, namely, appearance on reduction, field position, relatively efficient spin relaxation, and temperature sensitivity, the lines at g = 2.05, 1.94, 1.92, 1.89, and 1.86 may originate from iron-sulfur centers. Since the line at g = 1.86 appears and then disappears on progressive reduction, either it is due to an intermediate oxidation state or the structure giving rise to it undergoes a change as the potential is lowered. Heterogeneity of iron-sulfur proteins in a complex oxidation-reduction system appears now the rule rather than the exception (14, 15).

The appearance of multiple iron-sulfur species with highly negative potentials suggests that they are energetically capable of serving as primary electron acceptor or acceptors, and that one possible physiological role is to provide for a given donor more than one electron-transport pathway on the reducing side of photosystem I, e.g., the current possibility of noncyclic and cyclic pathways. The finding based on the slope of the titration curves, namely, that two-electron changes are involved in the reduction of the individual iron-sulfur species, raises additional problems of interpretation.

The midpoint potentials of the iron-sulfur species fall in two distinctly separate regions, one at -530 ± 5 mV, and the other at $\leqq -580$ mV. The redox potential of the iron-sulfur proteins reduced during the first stage, and presumably also the redox potential of those reduced during the second stage, are independent of pH. It is clear that either of these potentials would place these iron-sulfur proteins at the strongest reducing level known for a biological electron carrier. The chemical potential generated in photosystem I by the absorbed photon energy, as defined by the difference between the potential of photooxidized $P700^+$ and the highly negative potential found here for the reduced iron-sulfur proteins, would amount to 0.96–1.01 eV, respectively.

We have also demonstrated an apparent temperature dependence for the photoreduction of the iron-sulfur proteins, i.e., maximum reduction can be brought about at room temperature, whereas at 77°K only a photoreduction corresponding to the first-stage electrochemical reduction can take place. These phenomena naturally pose the question whether the iron-sulfur species reduced electrochemically at potentials beyond -560 mV but not photoreduced at 77°K can be involved in the primary photochemical event.

Although one of the original aims of the present work was to investigate the relationship of the bound iron-sulfur protein to the spectral species P430 by correlating their electrochemical behavior, this cannot yet be accomplished because of the unexpectedly low redox potentials found for the bound iron-sulfur proteins in the work reported here. With respect to the earlier spectrophotometric studies (9), it is worthwhile noting that while EPR spectroscopy is able to *directly* measure the amount of reduced iron-sulfur proteins, the flash-kinetic-spectroscopic method only *indirectly* measures the portion of the primary electron acceptor that is not yet electrochemically reduced and thus participates in the light-induced charge-separation reaction. As pointed out (9), under the conditions of these oxidation-reduction experiments, where a multitude of redox mediators is present in the reaction system the reduced forms of these mediators under the required anaerobic conditions could possibly serve as electron donors to the photooxidized P700, and consequently cause a premature attenuation of the amplitude of the absorption changes, leading to an underestimated redox-potential value.

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