EPR Characterization of the CP47-D1-D2-Cytochrome *b*-559 Complex of Photosystem II†

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ABSTRACT: A photosystem II complex consisting of a 47-kDa chlorophyll-binding protein (CP47), the reaction center proteins D1 and D2, and cytochrome *b*-559 was characterized. Trace amounts of plastoquinone were found, indicating that the primary acceptor quinone Q_A has been extracted during purification. However, in the presence of ferricyanide, an EPR signal with the characteristic line shape and *g* value of the tyrosine radicals associated with photosystem II could be photoaccumulated in the majority of the reaction centers; in the absence of ferricyanide, or under low-temperature illumination conditions, a 9.5-11-G wide signal with a Gaussian line shape was observed at $g = 2.003$. Neither signal is observed in D1-D2-*b*-559 complexes, indicating that retention of CP47 produces a more native, but quinone-depleted photosystem II reaction center. The tyrosine radical photogenerated at room temperature can be trapped at cryogenic temperatures; results are presented showing that this radical can arise from tyrosine Y_Z, from tyrosine Y_D, or from both species. Low-temperature EPR spectroscopy also revealed a pronounced split signal with contributions at $g = 6.05$ and $g = 5.75$, which is attributed to high-spin, non-heme Fe^{3+} with axial ligation symmetry which is probably the non-heme iron associated with the acceptor side of photosystem II.

Information on electron transport in the reaction center of photosystem II (PS II)[1] has increased significantly, owing to the development of methods for preparation of highly active PS II membrane fragments (Berthold et al., 1981) and subsequent research on structural and functional aspects of the photosystem (Andreasson & Vanngard, 1988; Brudvig et al., 1989; Babcock et al., 1989). It is now presumed that the photochemical reaction center of PS II is comprised of two polypeptides denoted D1 and D2. Nanba and Satoh (1987) first isolated these proteins in a complex which also binds cytochrome *b*-559. In this preparation, low-temperature illumination generates a characteristic spin-polarized triplet signal (Okamura et al., 1987) which is proposed to be a result of charge recombination of the primary radical pair, consisting of an oxidized Chl, P_{680}^+, and a reduced pheophytin *a* molecule, denoted I^-. In the presence of dithionite, it is also possible to photoreduce pheophytin *a* in the reaction center (Nanba & Satoh, 1987). These results implicate the D1-D2-*b*-559 complex as the binding site for the primary reactants P_{680} and I.

Further research on the D1-D2-*b*-559 complex has been hampered by its instability, which may be due to Triton X-100, the detergent used for purification. An alternate method combines the use of the milder detergent dodecyl maltoside and the chaotropic agent $LiClO_4$ to produce a complex characterized by improved room temperature stability (Ghanotakis et al., 1989), which binds about 10-12 Chl *a*, 2-3 Pheo *a*, and 2 Cyt *b*-559 molecules (Dekker et al., 1989). An intermediate derived from this procedure consists of a CP47-D1-D2-*b*-559 complex (Ghanotakis et al., 1989; Dekker et al., 1989). This material can be isolated with high purity, yield, and concentration, and is of interest in view of the isolation of two-dimensional crystals from the preparation (Dekker et al., 1990). As we report here, the CP47-D1-D2-*b*-559 complex is depleted of plastoquinone so that the reduced pheophytin acceptor cannot be reoxidized by the primary acceptor quinone Q_A, similar to the situation observed in preparations of D1-D2-*b*-559 which either lack plastoquinone (Nanba & Satoh, 1987; Ghanotakis et al., 1989) or contain only small amounts of nonfunctional quinone (Akabori et al., 1988). We also present a characterization of the CP47-D1-D2-*b*-559 complex by EPR spectroscopy and report features that differ substantially from those observed for D1-D2-*b*-559 complexes. Two of these differences are striking: first, we were unable to photoaccumulate the spin-polarized triplet in the CP47-D1-D2-*b*-559 preparation, and second, during illumination in the presence of the electron acceptor ferricyanide, we observed a tyrosine radical with the characteristic line shape and *g* value of the Y_Z^+ and Y_D^+ species.

Materials and Methods

Tris-treated reaction center complexes were prepared (Ghanotakis et al., 1987), washed with buffer A (20 mM Bis-Tris, 1.5% taurine, 20 mM NaCl, and 10 mM $MgCl_2$, pH 6.5), and stored in buffer A with 400 mM sucrose at −60 °C. Complexes containing CP47-D1-D2-*b*-559 or D1-D2-*b*-559 were prepared as described by Dekker et al. (1989) and stored in buffer A with 100 mM $MgSO_4$, 0.03% dodecyl maltoside,

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[1] Abbreviations: Chl, chlorophyll; Cyt, cytochrome; PS, photosystem; Y_Z, Y_D, redox-active tyrosine residues; RC, reaction center.

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Table I: Quantitation of Plastoquinone (PQ-9) Molecules in Various PS II Preparations[a]

Tris-washed RC complex	CP47–D1–D2–*b*-559	D1–D2–*b*-559
1.8–2.1	0.15–0.20	<0.01

[a] Values are expressed as PQ-9/RC, based on two cytochromes *b*-559 per RC and a differential extinction coefficient of 17 500 M^{-1} cm^{-1} for the reduced-minus-oxidized forms of cytochrome *b*-559 at 559 nm.

and 400 mM sucrose at −60 °C. Plastoquinone quantitation was performed by reversed-phase HPLC (Waters μBondpack, 0.3 × 30 cm) with a Shimadzu LC-6A system. Detection was at 254 nm; calibration of the peak area corresponding to plastoquinone 9 was achieved with Chromatopac CR4-A software and an HPLC-purified PQ-9 standard isolated from spinach. Samples (100 μg of Chl) were extracted with 0.5 mL of methanol, pelleted to remove insoluble material, and reextracted twice with 0.5 mL of methanol by resuspension in an ultrasonic water bath. Pooled extracts were dried under a stream of N_2 gas and dissolved in 1.0 mL of methanol; 20 μL was applied directly to the column. Pigments were separated by isocratic elution with 100% methanol at 1.5 mL/min to resolve PQ-9; control experiments showed that recovery of plastoquinone under these conditions was greater than 95%.

For EPR experiments, a Bruker ER200D spectrometer, equipped with a Bruker ERO35M Gaussmeter and Hewlett Packard 5245L electronic frequency counter (for the *g* value estimation) and an Oxford ESR liquid helium cryostat, was employed. Illumination was provided by a 800-W projector lamp.

Results

Plastoquinone Quantitation. The CP47–D1–D2–*b*-559 complex was isolated by solubilizing Tris-treated reaction center preparations at room temperature with dodecyl maltoside, followed by ion-exchange chromatography to remove pigment-free 22- and 10-kDa polypeptides and Chl-binding 28- and 43-kDa proteins (Dekker et al., 1989). The resulting material contains about 30 Chl and 2 cytochrome *b*-559 molecules per reaction center (Dekker et al., 1990) but almost no plastoquinone (Table I). This suggests that the primary quinone acceptor (Q_A) was released from the complex coincident with the removal of the Chl-binding 43-kDa protein (CP43), a hypothesis that is reinforced by the observation that highly resolved PS II preparations which retain CP43 in the presence of dodecyl maltoside also retain Q_A (Bowlby et al., 1988). Since no destabilizing agents, such as Triton X-100, are used for the purification of the complex, and a chaotropic agent is not required, the CP47–D1–D2–*b*-559 material should represent a relatively native reaction center lacking Q_A.

Room Temperature EPR Spectroscopy. For the initial photochemical characterization of the isolated CP47–D1–D2–*b*-559 complex, EPR experiments were performed at room temperature and compared to results with Tris-washed reaction center preparations and isolated D1–D2–*b*-559 complexes. Figures 1–3 show room temperature EPR signals in the free radical region; Tris-washed reaction center complexes (Figure 1) show signals similar to those observed in Tris-washed PS II membranes (Berthold et al., 1981). The dark stable radical arises from Y_D^+, the tyrosine-160 radical of the D2 polypeptide (Barry & Babcock, 1987; Debus et al., 1988a; Vermaas et al., 1988); continuous illumination generates the state Q_A^-–Y_Z^+–(Y_D^+), but Q_A^- is not seen at room temperature due to its magnetic interaction with the acceptor side non-heme iron. The 2-fold increase in signal intensity arises from formation of Y_Z^+, the oxidized tyrosine-161 of the D1 polypeptide

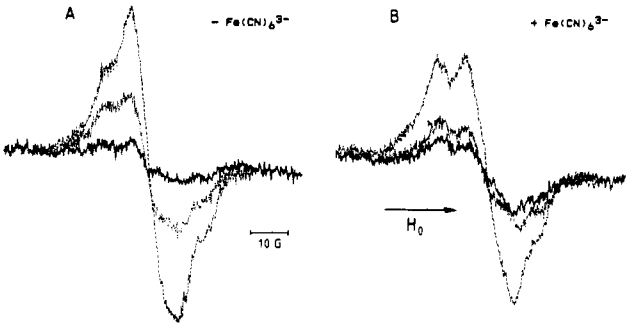

FIGURE 1: Room temperature EPR spectra in the $g = 2$ region of Tris-washed reaction center complexes in the absence (A) or presence (B) of 1 mM ferricyanide. Dashed lines, spectra recorded during continuous illumination; dotted lines, spectra recorded 2 min after terminating illumination; solid lines, spectra recorded in the dark before illumination. Chl concentration, 1.08 mM (0.017 mM PS II). Spectrometer conditions: (A) microwave power, 20 mW (10 dB); modulation, 4 G_{pp}; instrument gain, 1.25×10^6; (B) microwave power, 6.5 mW (15 dB); modulation, 4 G_{pp}; instrument gain, 2×10^6.

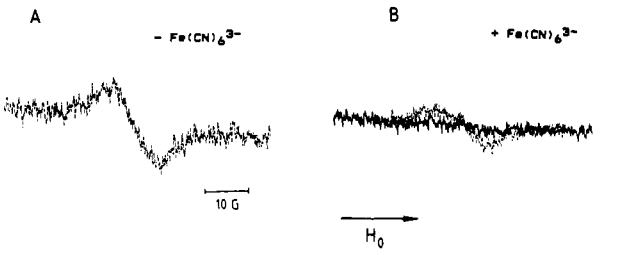

FIGURE 2: Room temperature EPR spectra of D1–D2–*b*-559 complexes in the absence (A) and presence (B) of 1 mM ferricyanide. Dashed lines, spectra recorded during illumination; solid line, spectrum recorded in the dark before illumination. Chl concentration, 0.35 mM (0.027 mM PS II). Spectrometer conditions are given in Figure 1B.

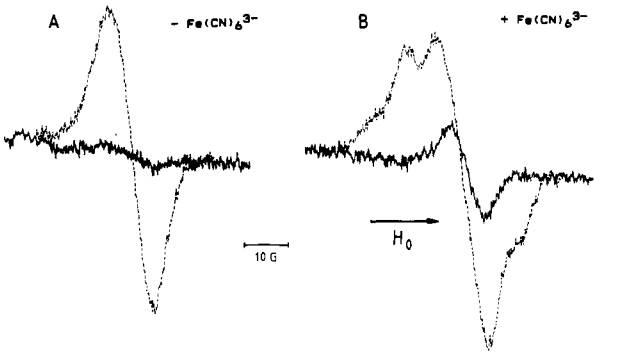

FIGURE 3: Room temperature EPR spectra of CP47–D1–D2–*b*-559 complexes in the absence (A) and presence (B) of 1 mM ferricyanide. Dashed/solid lines and spectrometer conditions are given in Figure 2. Chl concentration, 1.44 mM (0.045 mM PS II).

(Debus et al., 1988b; Metz et al., 1989). In the absence of ferricyanide, a small distortion is observed in the spectrum produced by illumination (Figure 1A, dashed line).

In quinone-depleted D1–D2–*b*-559 complexes, no significant EPR signals were observed (Figure 2). Thus, Y_D is reduced in the dark, and neither Y_D nor Y_Z can be photooxidized. Quite different EPR signals are observed for the CP47–D1–D2–*b*-559 complexes (Figure 3), which are also largely depleted of plastoquinone. The signal due to the dark-stable radical Y_D^+ is absent, but an unstructured, 9.5-G-wide signal is induced at $g = 2.003$ by light (Figure 3A, dashed line). The signal did not saturate up to microwave powers of 65 mW, formed within 300 ms (the instrument response time under these particular conditions), and showed a decay half-time of about 30–60 s.

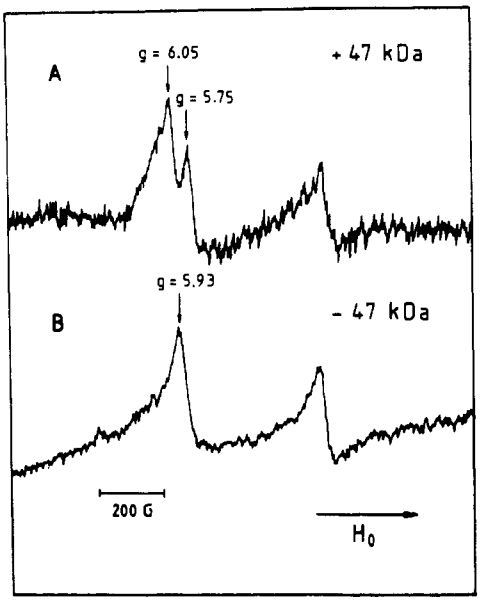

FIGURE 4: Low-temperature (5 K) EPR spectra of CP47-D1-D2-*b*-559 preparations (A) and of D1-D2-*b*-559 preparations (B), recorded in the dark. Spectrometer conditions: instrument gain, 6.3 × 10^5; microwave power, 5 dB; modulation amplitude, 20 G_{pp}.

The EPR characteristics of the CP47-D1-D2-*b*-559 complex differ even more from those of the D1-D2-*b*-559 complex if the electron acceptor ferricyanide is present. In the dark, a weak, unstructured $g = 2$ signal with a relatively narrow line width (about 7 G) was consistently observed (Figure 3B, solid line). This signal could be due to oxidized Chl in a small fraction of the centers. Continuous illumination did not result in the accumulation of the Gaussian signal at $g = 2.003$ seen in Figure 3A, but instead produced a signal with the characteristic hyperfine structure and g value of a tyrosine radical (Figure 3B). The rise time was again instrument-limited and showed a biphasic decay with half-times of about 0.5 and 5–10 s.

The spectra in Figure 3B were recorded with the same spectrometer settings as those used for Tris-washed reaction center complexes (Figure 1B); therefore, the latter material was used to obtain a preliminary spin count of the photoinduced tyrosine radical in CP47-D1-D2-*b*-559 complexes. The spin count was based on estimates of 63 Chl/reaction center for the Tris-washed material and 32 Chl/reaction center for the CP47-D1-D2-*b*-559 complex (Dekker et al., 1989). Assuming that continuous illumination can produce both Y_Z^+ and Y_D^+ in the reaction center complexes but only one of these species in CP47-D1-D2-*b*-559, we calculate that 65–70% of the latter centers contain an oxidized tyrosine. This number is significantly higher than the residual concentration of plastoquinone (Table I), and we therefore conclude that a tyrosine radical has been accumulated in a substantial amount of centers devoid of Q_A.

Low-Temperature EPR Spectroscopy. The spectroscopic properties of the PS II preparations were also analyzed at cryogenic temperatures in the low magnetic field region (which detects high-spin Fe^{3+}) and in the free radical region. Figure 4 presents low-field spectra of the CP47-D1-D2-*b*-559 and D1-D2-*b*-559 complexes. A signal at $g = 4.3$ due to rhombic high-spin Fe^{3+} is detected in both preparations; the amplitude of this signal, however, is smaller than in less purified preparations (Petersen et al., unpublished results), suggesting that it is due to nonspecifically bound, adventitious Fe^{3+}. In addition to the $g = 4.3$ component, a pronounced split signal at $g = 6$ appears in the CP47-D1-D2-*b*-559 complex with

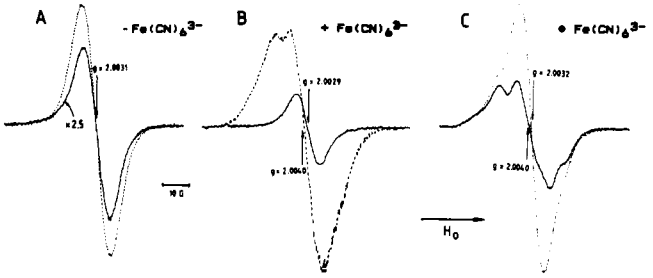

FIGURE 5: Low-temperature (7.3 K) EPR spectra of CP47-D1-D2-*b*-559 preparations in the absence (A) and presence (B, C) of 1 mM ferricyanide. Solid lines, spectra recorded in the dark before illumination (A, B) or in the dark of a sample that had been preilluminated at room temperature and rapidly frozen to 77 K (C). Dashed lines, spectra recorded in the dark (A, B) or in the light (C) of samples that had been preilluminated at 200 K (A, B) or at room temperature (C) and rapidly frozen to 77 K. Spectrometer conditions; (A) instrument gain, 6.3 × 10^5; microwave power, 15 dB; modulation amplidude, 2 G_{pp}; (B) instrument gain, 3.2 × 10^5; microwave power, 15 dB; modulation amplitude, 2 G_{pp}, $\delta = 9.46663$; (C) same as (B) but with a microwave power of 35 dB.

components at $g = 6.05$ and $g = 5.75$ (Figure 4A). An identical signal is observed in O_2-evolving particles retaining the intrinsic 43-kDa and extrinsic 33-kDa proteins (Bowlby, 1989).

The splitting of the $g = 6$ signal is not observed in the D1-D2-*b*-559 complex (Figure 4B), where a single component at $g = 5.93$ appears. The most likely explanation for the disappearance of the splitting is a collapse of strong anisotropy, resulting in a shift of the two components into one another. The $g = 6$ signals are detectable only at temperatures below about 8 K and with high microwave power (about 60 mW), suggesting the presence of an efficient relaxation channel. Therefore, the $g = 6$ signals are probably due to a high-spin, non-heme Fe^{3+} ion, decoupled from quinone(s) and ligated with axial rather than rhombic symmetry. Dark incubation with ferricyanide did not affect the $g = 6$ signal in either preparation (data not shown), and we believe that it originates from the Fe^{3+} associated with the reducing side of PS II; if the $g = 6$ signal were due to another species and the non-heme iron were reduced, ferricyanide incubation should oxidize the non-heme iron and change the signals in the $g = 5$–8 region (Diner & Petrouleas, 1987).

The $g = 2$ free radical region at low temperature is shown in Figure 5. The dark-adapted CP47-D1-D2-*b*-559 complex exhibits a weak, 13-G-wide signal at $g = 2.0031$ without resolved hyperfine structure; a tyrosine radical is not observed. Upon illumination at temperatures between 5 and 200 K (Figure 5A), signals with Gaussian line shapes are again produced. Very similar signals are also produced in the presence of ferricyanide, in contrast to the behavior at room temperature, where a tyrosine radical is accumulated. This behavior is consistent with the observation that low-temperature illumination of Tris-washed PS II membranes also fails to accumulate a tyrosine radical; instead, a 9-G-wide signal at $g = 2.0026$ is produced, which has been attributed to oxidized, monomeric Chl *a* (De Paula et al., 1985). The signal in the CP47-D1-D2-*b*-559 complex is characterized by a g value of 2.0032 (2) and a line width of 11.6 G. Figure 5B shows that the tyrosine radical produced at room temperature in the CP47-D1-D2-*b*-559 complex in the presence of ferricyanide can be trapped at low temperature. Subsequent low-temperature (5–200 K) illumination again produces a Gaussian signal at $g = 2.0032$ which is superimposed on a tyrosine radical signal (Figure 5C), showing that photooxidation of the tyrosine observed at room temperature does

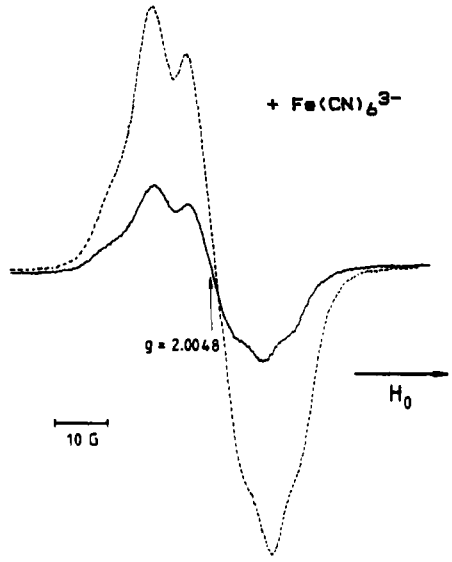

FIGURE 6: Low-temperature (9.2 K) EPR spectra of Tris-treated reaction center complexes in the presence of 1 mM ferricyanide. Solid line, Y_D^+ spectrum, generated by room temperature illumination, 2-min dark incubation, and rapid freezing to 77 K; dashed line, Y_Z^+ and Y_D^+ spectra, generated by room temperature illumination and rapid freezing during illumination to 77 K. Spectrometer conditions: instrument gain, 2.5×10^5; microwave power, 15 dB; modulation amplitude, 2 G_{pp}.

not prevent subsequent photoaccumulation of the species that gives rise to the Gaussian signal.

In principle, the tyrosine radical observed at low temperature can be due to either Y_D^+ or Y_Z^+. The latter component, however, has as yet not been observed at cryogenic temperatures because low-temperature illumination results in the oxidation of manganese, Chl, or cytochrome *b*-559, which prevents accumulation of the oxidized tyrosine. Therefore, in order to determine if Y_Z^+ can be accumulated at low temperature, experiments were performed with Tris-treated reaction center complexes isolated according to Ghanotakis et al. (1987). These preparations were illuminated at room temperature in the presence of ferricyanide, held for 2 min in the dark, and frozen and analyzed at 9.2 K (Figure 6, solid line). Under such conditions, Y_Z^+ should not accumulate because the decay half-times are usually much faster than 2 min; any signal observed should therefore arise from Y_D^+, which would not decay rapidly in the 2-min dark adaptation period. The signal observed at room temperature is in fact stable, showing a 20% decay after 5 min in the dark (data not shown). The dashed line in Figure 6 was recorded for a sample that has been illuminated for 2 min at room temperature and then frozen with continuing illumination; the amplitude at 9.2 K increases by more than 2-fold compared to the dark-adapted signal (solid line), indicating that under these conditions both Y_D^+ and Y_Z^+ are observed. No additional signals were detected in the free radical region. We conclude that Y_Z^+ formed at room temperature in the CP47–D1–D2–*b*-559 complex remains stable upon freezing; the tyrosine radical observed at low temperature in the CP47–D1–D2–*b*-559 complex must therefore be due either to Y_Z^+, to Y_D^+, or to both species.

We also examined the CP47–D1–D2–*b*-559 preparation for the presence of the photoinduced high-field signal of the quinone–iron complex; as predicted from the plastoquinone quantitation, no $g = 1.84$ signal could be accumulated. In addition, the $g = 2.0049$ signal, which should dominate the spectrum at low microwave powers in preparations where Q_A is decoupled from the non-heme iron (Petersen et al., unpublished results), was not observed, suggesting that the small nonstoichiometric amounts of plastoquinone still present in the preparations (Table I) do not function as electron acceptors. Finally, we could not accumulate the spin-polarized triplet (data not shown), in contrast to the situation in D1–D2–*b*-559 complexes (Okamura et al., 1987; Ghanotakis et al., 1989), a result which suggests that in the CP47–D1–D2–*b*-559 complex the radical pair does not form or decays too rapidly for the spin dephasing process to take place.

Discussion

Extraction of the 43-kDa Chl-binding protein denoted CP43 from the intrinsic protein core of PS II, to produce the CP47–D1–D2–*b*-559 complex, is accompanied by the loss of the primary acceptor quinone Q_A. Diner et al. (1988) have reported that elevated temperatures are required for the extraction and reconstitution of Q_A in detergent-solubilized CP43–CP47–D1–D2–*b*-559 preparations from the green alga *Chlamydomonas reinhardii*. Under our conditions for purification of CP47–D1–D2–*b*-559, the Chl-binding protein CP43 and plastoquinone were only released under room temperature conditions. It is therefore likely that detergent-induced extraction of Q_A is due to an effect of temperature on the association between CP43 and other PS II reaction center proteins, an observation which indicates that CP43 is an essential structural element of the PS II domain that binds Q_A.

Although both reaction center complexes are plastoquinone-depleted, the results presented in this paper reveal interesting differences in EPR characteristics of CP47–D1–D2–*b*-559 and D1–D2–*b*-559 preparations. In the presence of ferricyanide, a tyrosine radical is produced in the majority of the reaction centers of CP47–D1–D2–*b*-559 complexes at room temperature (Figure 3B), but not in D1–D2–*b*-559 complexes (Figure 2B). It is unclear whether this tyrosine radical is due to Y_D^+, Y_Z^+, or both species, since they produce identical EPR signals with very similar microwave power saturation characteristics in the absence of manganese (Warden et al., 1976; Yocum & Babcock, 1981; Babcock et al., 1989).

A tyrosine radical is the dominant photoaccumulated species in the $g = 2$ region of CP47–D1–D2–*b*-559 complexes (Figure 3B) when ferricyanide is present, a finding which excludes Pheo *a*, Chl *a*, or the non-heme iron of the acceptor side as terminal acceptors, even though the latter species is oxidized in the dark as evidenced by low-temperature EPR signals at $g = 6.05$ and $g = 5.75$ (Figure 4A). These EPR signals are not changed by illumination at room temperature (data not shown). Therefore, ferricyanide functions as the terminal electron acceptor in the CP47–D1–D2 complex, a conclusion supported by the experiments of Figure 3A. In the absence of ferricyanide, a 9.5-G-wide signal at about $g = 2.003$ dominates the spectrum, augmented by a weak signal in the low-field region which is due to a small contribution from a tyrosine radical. We attribute the $g = 2.0032$ signal to the reduced primary electron acceptor I^-, in agreement with other findings (Klimov et al., 1980); therefore the signal shown in Figure 3A is a composite of signals from I^- and a tyrosine radical. In experiments with ferricyanide, on the other hand, I^- is not detected and a tyrosine radical is the only species accumulated (Figure 3B).

The number of spins contributing to the $g = 2.0032$ signal is smaller than the number of spins generated by the tyrosine radical formed with ferricyanide (Figure 3A). This is not unexpected if the tyrosine radical arises from Y_Z^+ and photoaccumulation of the state $I^-Y_Z^+$ competes with the recombination process. If, in fact, the state $I^-Y_Z^+$ accumulates at room temperature, cryogenic illumintion should result in the

accumulation of the state I^-Chl^+ since in the frozen state a monomeric Chl is the preferred donor (De Paula et al., 1985); the signal shown in Figure 5A is compatible with this assignment. Preferential accumulation of I^- in the absence of ferricyanide also explains our failure to observe the spin-polarized triplet in CP47–D1–D2–*b*-559 complexes; with Pheo *a* and P_{680} both reduced, recombination is blocked, and triplet signals cannot form.

The formation of photoaccumulated EPR signals in CP47–D1–D2–*b*-559 complexes can be explained by kinetic models (Van Gorkom, 1985; Schatz et al., 1988) in which the radical pair $P_{680}^+I^-$ forms with low yield in centers containing Q_A^- and decays with a half-time of about 10 ns (Schlodder & Brettel, 1988). In the absence of Q_A^-, however, the radical pair might form with higher yield and decay more slowly, with a half-time (50 ns) similar to that observed in D1–D2–*b*-559 preparations (Danielius et al., 1987; Takahashi et al., 1987). In Tris-inhibited PS II membranes, reduction of P_{680}^+ by Y_Z occurs in about 5 μs at pH 6.5 (Boska et al., 1983), suggesting a yield of only 1% for the state $I^-Y_Z^+$. This state would accumulte only if its formation is faster than its decay; although the kinetics of such a decay have not been reported, a reasonable estimate can be made. The recombination of $Q_A^-Y_Z^+$ is about 500 times slower than that of $Q_A^-P_{680}^+$ (Babcock et al., 1989); if this recombination process is also 500 times slower than $I^-P_{680}^+$ in CP47–D1–D2, then decay kinetics of about 25 μs would be expected, which are sufficiently slow to permit an accumulation of the state $I^-Y_Z^+$. The decay rates we observe for both species, however, are much slower than 25 μs, suggesting that Y_Z^+ may also oxidize Y_D or another tyrosine.

Other plastoquinone-depleted CP47–D1–D2–*b*-559 preparations have been analyzed by EPR spectroscopy (Takahashi & Katoh, 1986; Boska et al., 1986; Yamaguchi, et al., 1988); the signals reported in such preparations were attributed to centers that retained Q_A. Takahashi and Katoh (1986) and Yamaguchi et al. (1988) reported the photoaccumulation of Gaussian EPR signals attributed to P_{680}^+ or to a tyrosine radical with altered line shape; ferricyanide was not employed as an electron acceptor in these studies. Boska et al. (1986) were able to photoaccumulate Y_Z^+ with the characteristic hyperfine-split line shape, but did not report results in the absence of ferricyanide. Our data provide an explanation for these inconsistencies and demonstrate that the EPR signals can, in fact, originate from centers lacking Q_A.

Unstructured 10-G-wide signals at $g = 2.003$ (Figures 3A and 5A) have been reported by Nugent et al. (1982), who observed such a signal coincident with the disappearance of a dark-stable tyrosine radical in preparations that had been aged, freeze-thawed, or treated with detergent, and ascribed the signal to an electron donor. Our results, on the other hand, suggest that the $g = 2.0032$ signal arises from photoaccumulation of I^- in the absence of Q_A, provided the donor side of the photoreaction is still functional. Recently, Frank et al. (1989) reported the photoaccumulation of I^- in dithionite-treated D1–D2–*b*-559 complexes; in this case, however, reduction of P_{680} by dithionite could prevent the recombination reaction.

It is not clear why tyrosine radicals cannot be detected in D1–D2–*b*-559 complexes. One explanation is that preparation of these complexes induces changes (caused by Triton X-100 or $LiClO_4$) which inhibit electron transfer from Y_Z to P_{680}^+. This would prevent accumulation of Y_Z^+, and therefore of I^-, so that charge recombination and formation of the spin-polarized triplet would take place. Alternatively, it is possible that the presence of the Chl-binding CP47 protein is required for formation of the tyrosine radical species. The EPR line shape of these radicals is sensitive to the unpaired spin density distribution and the orientation of the phenol ring with respect to the protein environment (Barry & Babcock, 1988; Babcock et al., 1989). The environment of the tyrosine radical observed in the CP47–D1–D2–*b*-559 preparation is virtually identical with that observed in less highly purified PS II preparations, suggesting that CP47 may indeed regulate the structure, as well as the function, of the oxidizing side of PS II.

Registry No. Tyrosine, 60-18-4.

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Photoinduced Electron Transfer within Complexes between Plastocyanin and Ruthenium Bisbipyridine Dicarboxybipyridine Cytochrome *c* Derivatives†

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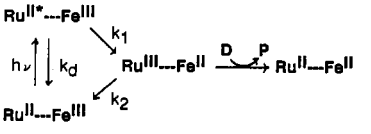
ABSTRACT: A new technique has been developed to measure intracomplex electron transfer between cytochrome *c* and its redox partners. Cytochrome *c* derivatives labeled at single lysine amino groups with ruthenium bisbipyridine dicarboxybipyridine were prepared as previously described [Pan, L. P., Durham, B., Wolinska, J., & Millett, F. (1988) *Biochemistry 27*, 7180-7184]. Excitation of Ru^{II} with a short light pulse resulted in the formation of the excited-state Ru^{II*}, which rapidly transferred an electron to the ferric heme group to form Fe^{II} and Ru^{III}. Aniline was included in the buffer to reduce Ru^{III} to Ru^{II}, leaving the heme group in the ferrous state. This process was complete within the lifetime of the light pulse. When plastocyanin was present in the solution, electron transfer from the ferrous heme of cytochrome *c* to Cu^{II} in plastocyanin was observed. All of the ruthenium cytochrome *c* derivatives formed electrostatic complexes with plastocyanin at low ionic strength, allowing intracomplex electron-transfer rate constants to be measured. The rate constants for derivatives modified at the indicated lysines were as follows: Lys 13, 1920 s^{-1}; Lys 8, 1480 s^{-1}; Lys 7, 1340 s^{-1}; Lys 86, 1020 s^{-1}; Lys 25, 820 s^{-1}; Lys 72, 800 s^{-1}; Lys 27, 530 s^{-1}. It is interesting that the derivative modified at lysine 13 at the top of the heme crevice had the largest rate constant, while lysine 27 at the right side of the heme crevice had the smallest. One possible explanation is that the bulky ruthenium complex at lysine 13 caused plastocyanin to bind toward the bottom of the heme crevice in a more favorable orientation for rapid electron transfer. The intracomplex rate constants for the derivatives modified at lysines 13 and 27 were actually larger at an ionic strength of 30 mM than at 5 mM, even though the binding constants were much smaller. High ionic strength may allow a greater range of binding orientations to occur, some of which are more favorable for rapid electron transfer.

Despite the importance of biological electron-transfer reactions, relatively few techniques are available to measure the rate of the actual electron-transfer step within a complex between two proteins. This is because the reactions are usually too rapid for stopped-flow techniques, and protein binding and dissociation steps are frequently rate-limiting. Several approaches have been developed to address this problem. Simondsen et al. (1982) developed the use of flavins to photochemically reduce the redox centers and initiate intracomplex electron transfer. This technique has been applied to a number of systems, including the complex between cytochrome *c* and cytochrome *c* peroxidase (Hazzard et al., 1987a,b, 1988a,b). Pulse radiolysis has been used to initiate electron transfer within the cytochrome *c*–cytochrome b_5 complex and the cytochrome *c*–plastocyanin complex (McLendon & Miller, 1985; Peerey & Kostič, 1989). In another approach, replacement of the native heme group with a zinc porphyrin allows electron transfer to be initiated photochemically. This technique was used to study electron transfer between cytochrome *c* and

Scheme I



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