An Electron Spin-Polarized Signal of the $P800^+A_1(Q)^-$ State in the Homodimeric Reaction Center Core Complex of *Heliobacterium modesticaldum*†

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Received August 10, 2007; Revised Manuscript Received February 9, 2008

ABSTRACT: The function of menaquinone as electron acceptor A_1 was identified by EPR in the purified type 1 homodimeric reaction center core complex (RC_{core}) of an anoxygenic photosynthetic bacterium, *Heliobacterium modesticaldum*. After illumination of the RC_{core} at 210 K in the absence and presence of dithionite, we detected the radical of a special pair of bacteriochlorophyll *g* molecules ($P800^+$) at $g = 2.0033$ and a quinone-type radical at $g = 2.0062$, respectively, at 14 K. Flash excitation of the dark-frozen RC_{core} at 14 K induced two types of transient EPR signals, i.e., the $P800^+$ radical that decayed with a time constant of 3.7 ms and a much faster decay component that showed the electron spin polarization (ESP) pattern of E/A (E, emission; A, absorption). The latter one was assigned to the $P800^+F_X^-$ radical pair state. A new ESP signal that had an apparent A/E/A/E pattern extended to the lower-magnetic-field side was transiently induced by the flash excitation in the RC_{core} that was preilluminated at 210 K in the presence of ascorbate and subsequently cooled to 14 K in the light. The 210 K preillumination of the RC_{core} in the presence of dithionite led to accumulation of the dark stable semiquinone-type signal at $g = 2.0062$ and increased the intensity of the light-induced P800 triplet signal. Flash excitation at 14 K induced the smaller A/E/A/E-type signal that had the greater contribution of the lower-magnetic-field envelope. This ESP signal could thus be ascribed to the $P800^+A_1^-$ radical pair. The kinetics and spectral shape of this ESP signal suggest that menaquinone serves as secondary electron acceptor A_1 with the molecular orientation of its ring being somewhat different from that of phylloquinone in photosystem I.

Heliobacteria belong to a family of strict anaerobic photosynthetic bacteria (*1, 2*) that have only a type 1 reaction center (RC), as do green sulfur bacteria (*3*). The structure and function of these RCs are supposed to resemble those of the photosystem I (PS I)[1] RC in plants and cyanobacteria (*4, 5*). However, the RCs of heliobacteria and green sulfur bacteria are homodimers made of two identical core polypeptides (*6, 7*), in contrast to all other RCs, namely, the PS I and PS II RCs of oxygenic organisms and the type 2 RCs of purple bacteria (*8–11*). In homodimeric RCs, the electron transfer reaction is assumed to proceed equally along two symmetrical pathways, although it has not yet been observed (*12, 13*).

The heliobacterial RC contains 35–40 bacteriochlorophyll (BChl) *g* molecules that exhibit the Q_y absorption peaks at about 800 nm (*14*). The light energy absorbed by the BChl *g* molecules is transferred to a special pair of BChl *g* molecules named P800 and produces the singlet excited state (P800*). P800* then gives an electron to the primary electron acceptor A_0, which is 8^1-hydroxychlorophyll *a* (8^1-OH Chl a_{670}) (*14, 15*), and, finally, to iron−sulfur centers F_A and F_B (*4, 16, 17*). Although it is well-known in PS I that an electron on A_0 (Chl *a* monomer) is transferred to phylloquinone (A_1) and then to F_X, F_A, and F_B (*18*), evidence regarding the functions of F_X and quinone in the heliobacterial RC has been scarce (*13*) and is still under debate (*19–21*).

The reoxidation of A_0^- after the photoexcitation of P800 was assumed to proceed with apparent time constants of 500–600 ps in heliobacterial (*22, 23*) and green sulfur bacterial RCs (*24*). The rates are significantly slower than the time constant of 20–50 ps reported for the reoxidation of A_0^- by phylloquinone in PS I (*25, 26*). Lin et al. (*23*) suggested that only the $P800^+F_X^-$ state is formed from the $P800^+A_0^-$ state in *Heliobacillus mobilis* by monitoring the 400–470 nm picosecond absorption spectroscopy. Transient spectroscopic analysis in the UV and blue regions by Brettel et al. (*27*) also showed no kinetics responsible for the quinone reaction from 2 ns to 4 s after laser flash excitation. Photovoltaic measurements indicated that A_0^- was reoxidized in a single phase with a time constant of 700 ps, suggesting the direct electron transfer from A_0 to F_X (*27*). Although the fast charge recombination kinetics (2–4 ms component) of $P800^+$ in a heliobacterial RC preparation at low temperatures

† Supported by Grants-in-Aid for Scientific Research for Young Scientists (B) (17740222) (to H.M.) and Scientific Research (B) (19370064) (to S.I.) and (C) (17570131) (to H.O.) and by the 21st Century Center of Excellence program for the Origin of the Universe and Matter (to T.K., H.M., and S.I.) from the Japanese Ministry of Education, Science, Sports, and Culture.

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[1] Abbreviations: Chl, chlorophyll; BChl, bacteriochlorophyll; EPR, electron paramagnetic resonance; ESP, electron spin polarization; PS I, photosystem I; P700, primary electron donor made of a special pair of chlorophylls *a* and *a*′ in photosystem I; P800, primary electron donor made of a special pair of bacteriochlorophyll *g* species in the heliobacterial reaction center; RC_{core}, reaction center core complex.

10.1021/bi701612v CCC: $40.75
Published on Web 03/19/2008

had once been ascribed to the re-reduction of $P800^+$ by A_1^- in analogy to that observed in PS I (*28*), this kinetic phase was attributed to the re-reduction by F_X^- (*13, 19, 31*). The direct measurement of the reaction kinetics of quinone as the electron acceptor A_1 has thus not been conducted in the heliobacterial RC.

The electron spin-polarization (ESP) dynamics gives the feature of the primary photochemical reactions showing the nonthermal distribution of electronic spin states, which are uniquely formed by the series of fast electron transfer reactions in the RC (*29*). The strong ESP signal with the emission−absorption−emission (E/A/E) pattern of the $P700^+A_1^-$ radical pair state in PS I has been explained to reflect the very fast electron transfer from A_0 to A_1 (*29*). An ESP signal with an E/A polarization pattern with net absorption was detected in green sulfur bacteria or heliobacteria (*30–34*). Heathcote et al. (*30*) first demonstrated this type of ESP signal in isolated membranes from *Chlorobium limicola*. van der Est and his colleagues (*31–34*) indicated that these ESP signals could be assigned to the contribution from the P^+ to the $P^+F_X^-$ state based on the recombination kinetics, the frequency dependence of the spin polarization, and the absence of an out-of-echo signal in both *Chlorobium tepidum* and *Hba. mobilis*. No transient ESP signal involving quinone has been identified yet.

The spatial arrangements of cofactors on the heliobacterial RC are considered to resemble those in PS I on the basis of the homology of amino acid sequences between the PshA subunit of the former RC and the PsaA and -B subunits of the PS I RC. The sequence of PshA has the more hydrophilic residues in the putative quinone binding sites compared to those in the PsaA and -B subunits (*19, 21*), as a result of which one can assume that menaquinone molecules might bind at the locations somewhat displaced from the binding sites of the phylloquinone molecules in PS I or that menaquinone is totally absent in the heliobacterial RC. However, if the electron transfer proceeds directly from A_0 to F_X in the heliobacterial RC over a distance approximately the same as that between A_0 and F_X in the PS I RC (about 2.0 nm), the distance seems to be too long to enable the fast electron transfer rate of 500–700 ps between them. According to the empirical law of Moser and Dutton (*35*), the electron transfer rate over 2.0 nm can be estimated to be slower than milliseconds. The time constant of 500–700 ps observed for the reoxidation kinetics of A_0^- thus requires the presence of any cofactor which plays an essential role in this reaction, which has been discussed many times (*13, 19, 21, 31–34*). The photoaccumulation of the semiquinone-type signal (*36, 37*) may suggest the involvement of the quinone molecule as the main or side path of the electron transfer reaction in the heliobacterial RC.

In this work, we studied the electron transfer reactions, especially focusing on the function of quinone as A_1, in the purified RC core complex (RC_{core}) of *Heliobacterium modesticaldum*, which lacks the PshB subunit (F_A/F_B protein), with EPR spectroscopy at cryogenic temperatures under different redox conditions. We succeeded in detecting a new type of ESP signal in the RC_{core}, which could be ascribed to the $P800^+A_1^-$ state. We will discuss the function of the quinone molecule as A_1 in the electron transfer pathway in the heliobacterial RC.

MATERIALS AND METHODS

Preparation of the RC Core Complex from Hbt. modesticaldum. Hbt. modesticaldum was grown anaerobically in a PYE medium under illumination with tungsten lamps at 47 °C for 18–20 h from a 1% inocular. The cells were harvested by centrifugation at 12000*g* for 10 min before cell lysis began, as mentioned previously (*19*). The RC_{core} was isolated basically according to the method previously described (*19, 38*) as follows. After cells had been disrupted with a French press, the cell debris was removed by centrifugation, and the supernatant was then subjected to ultracentrifugation to collect the membranes. The RC_{core} was solubilized with sucrose monolaurate and purified by stepwise sucrose density gradient centrifugation and subsequent hydrophobic chromatography. The purified RC_{core} contained a single PshA but no PshB subunit, as revealed by SDS−PAGE analysis (*19*). The RC_{core} was suspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM DTT, and 2 mM sucrose monolaurate.

For the EPR measurements, the RC_{core} was concentrated using a Minicon-B15 apparatus (Amicon) to give an absorbance of 350–500 at 788 nm. All the media were fully degassed and flushed with N_2 gas three times and kept in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) overnight before being used. The spinach PS I RC complex was prepared as reported previously (*25*). Appropriate amounts of solid sodium ascorbate and/or sodium dithionite were added to reduce cofactors in the RCs.

Measurements of ESP−EPR Signals. The EPR spectra were measured with a Bruker ESP-300 X-band spectrometer (Bruker Biospin) equipped with a liquid helium flow-type cryostat and a temperature control system (CF935, Oxford Instruments, Oxford, U.K.). A field modulation frequency of 100 kHz was used for the transient and CW EPR measurements. Continuous white light illumination at 35 mW/cm^2 on the sample surface was provided by a 500 W tungsten projector lamp through heat-cut glass filters and glass fiber for the preillumination and CW illumination studies. Repetitive xenon flash light with a 30 μs duration of nearly saturating intensity was given through a 1 m glass fiber light guide at 0.2–2 Hz for the measurement of the transient EPR signals. Signals were averaged to increase the signal-to-noise ratio as required.

RESULTS

EPR Spectra of Primary Electron Donor $P800^+$ and Acceptor A_1^- in the Isolated RC_{core} of Hbt. modesticaldum. The RC_{core} was isolated from cells of *Hbt. modesticaldum* as described previously (*19*). The RC_{core} contained only a PshA subunit when investigated by SDS−PAGE analysis and exhibited a charge-separated state of $P800^+F_X^-$, as revealed by EPR spectroscopy (*19*).

Illumination at 14 K of the RC_{core}, which was frozen in the dark in the presence of dithionite, induced an irreversible EPR spectrum at $g = 2.0033$ with a line width of 11.5 G (Figure 1, trace a). The spectrum was ascribed to photooxidized primary electron donor $P800^+$ (*39*). In the next experiment, the RC_{core} was preilluminated for 20 min at 210 K in the presence of a weak reductant ascorbate and then cooled to 14 K under illumination. In this case, we observed

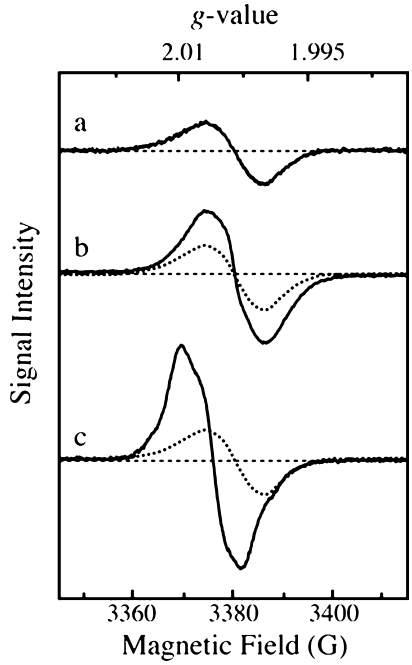

FIGURE 1: EPR spectra measured in the *Hbt. modesticaldum* RC_{core} at 14 K in the $g = 2.0$ region. (a) The sample was cooled to 14 K in the dark in the presence of dithionite. After illumination for 1 min at 14 K, measurement was carried out in the dark at the same temperature. (b and c) The samples were preilluminated for 20 min at 210 K and cooled to 14 K under illumination in the presence of sodium ascorbate (b) or sodium dithionite (c). Measurements were carried out in the dark at 14 K. Spectrum a is also represented by dotted lines for the comparison of the spectral shapes in b and c. The amplitudes of the spectra in parts a and b cannot be compared to that in part c directly due to the different saturation properties of $P800^+$ and A_1^-. Experimental conditions: temperature, 14 K; microwave power, 1 mW; microwave frequency, 9.479 GHz; modulation frequency, 100 kHz; modulation amplitude, 4 G.

the larger amplitude of $P800^+$ at 14 K in the dark (Figure 1, trace b). The result indicated that the 14 K illumination of the dark-adapted RC_{core} was insufficient to fully accumulate $P800^+$. This was consistent with the observation of the fast decay (2–3 ms) of $P800^+$ at cryogenic temperatures after flash excitation, presumably due to the charge recombination reaction between $P800^+$ and F_X^-, as estimated previously (*19*). The illumination at 14 K did not induce any other EPR spectra over the wider magnetic-field region, indicating the complete absence of F_A and F_B (not shown). This result was in agreement with the lack of a small PshB subunit in the RC_{core} preparation.

When the RC_{core} was illuminated for 20 min at 210 K and subsequently cooled to 14 K under illumination in the presence of a stronger reductant, dithionite, a different radical spectrum with a higher *g* value of 2.0062 and a 12 G line width was detected at 14 K in the dark (Figure 1, trace c). The spectrum exhibited a sharp outline with a *g* value clearly different from that of $P800^+$ and was similar to the semiquinone type signal that was photoaccumulated in the presence of dithionite in the heliobacterial membranes but exhibited a slightly different shape and smaller *g* value of 2.0038–2.0046 (*36, 37*). We further studied the effect of various preillumination conditions by recording the flash-induced EPR spectra.

Measurements of the Spectrum of the Spin-Polarized Radical Pair Signal. Figure 2 shows the transient decay kinetics of the flash-induced EPR signal observed in the

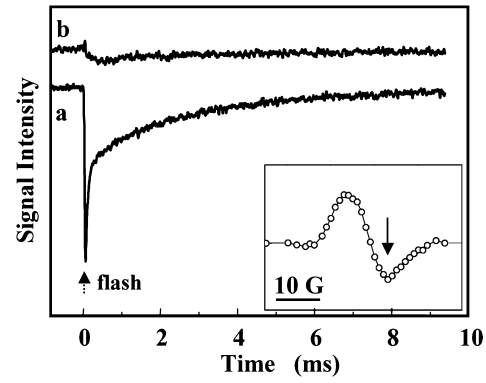

FIGURE 2: Flash-induced changes of the EPR signal measured at the magnetic field of the negative peak of $P800^+$ in the *Hbt. modesticaldum* RC_{core}. (a) The RC_{core} in the presence of dithionite was cooled in the dark and excited with the flash at 14 K. (b) The RC_{core} in the presence of ascorbate was preilluminated for 20 min at 210 K and cooled to 14 K under illumination. After the samples had been kept in the dark briefly, the flash-induced signal changes were measured at 14 K. The inset shows the EPR spectrum of $P800^+$ measured 300 μs after flash excitation under the same conditions used for spectrum a. Experimental conditions: temperature, 14 K; microwave power, 1 mW; microwave frequency, 9.500 GHz; modulation amplitude, 4 G; time constant, 10 μs; magnetic field, 3384.1 G (at the position shown by an arrow in the inset).

magnetic field at the negative peak of $P800^+$, as indicated by an arrow in the inset. The RC_{core} frozen in the dark in the presence of dithionite exhibited biphasic decay phases with time constants of 120 μs and 3.7 ms, respectively (Figure 2, trace a). The time constant of the slower decay phase agreed well with that of the charge recombination reaction between $P800^+$ and F_X^- (*19, 31*). The fast decay phase, on the other hand, was attributed to the spin relaxation time of the ESP signal derived from the $P800^+F_X^-$ state (*31–34*). The intensity at 30 μs of this phase gave a complex spectral feature, which was significantly different from that of $P800^+$ (see Figure 3, trace a in panel I).

When the RC_{core} in the presence of ascorbate was preilluminated at 210 K and subsequently cooled to 14 K under illumination, the flash excitation induced a different flash-induced kinetics at the same magnetic field (Figure 2, trace b). The kinetics showed a 250 μs rising phase and a 6.0 ms decaying phase. The amplitude of the 250 μs component varied markedly depending on the magnetic field, as depicted in trace b of Figure 3 in panel I that plotted the extent 30 μs after the flash excitation. This spectrum was completely different from that of the 120 μs component shown in trace a. The 250 μs component, thus, seems to contain the decay phase of the ESP signal that is different from the $P800^+F_X^-$ state, namely of the $P800^+A_1^-$ state, as discussed below. The time constant (6.0 ms) of the slower decaying phase (decay of $P800^+$) was longer than that of $P800^+$ (3 ms) detected in the charge recombination reaction between F_X^- and $P800^+$ (trace a) observed in the RC_{core} without preillumination.

In membranes from *Hbt. modesticaldum*, the EPR spectrum of F_X^- was shown to give a transient broad signal with an $S = {}^1/_2$ ground spin state. It was reported to be unable to be detected in the isolated RC_{core} (*19*). Another group recently demonstrated a photoaccumulated broad EPR spectrum of F_X^- with an $S = {}^3/_2$ ground state in both membranes and a similar RC_{core} preparation from the same species of heliobacteria (*20*). We, therefore, measured the light-induced difference in the EPR spectrum at 14 K in the RC_{core} in this

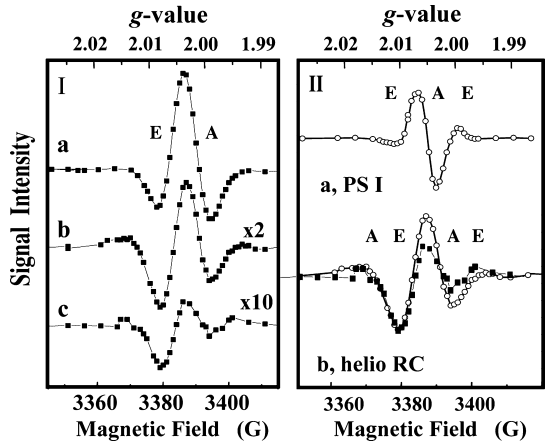

FIGURE 3: Flash-induced transient EPR spectra at 14 K in the *Hbt. modesticaldum* RC_{core} (I) and their comparison with the ESP pattern of PS I (II). (I) The signal intensity 30 μs after the flash excitation was plotted against a magnetic field. Before measurements were taken, each sample was cooled to 14 K in the dark in the presence of dithionite (trace a) or preilluminated for 20 min at 210 K and subsequently cooled to 14 K under illumination in the presence of ascorbate (trace b) or in the presence of dithionite (trace c). After the samples had been kept in the dark briefly, the flash-induced signal changes were measured. (II) Trace a was obtained by plotting the flash-induced transient EPR signals in the dark-frozen PS I particles measured under similar experimental conditions. (b) Traces b (○) and c (■) in panel I were normalized at the lower-magnetic-field peaks. Experimental conditions: temperature, 14 K; microwave power, 1 mW; microwave frequency, 9.500 GHz; modulation amplitude, 4 G; time constant, 10 μs. E and A in the figure indicate the emission- and absorption-type spectral features, respectively.

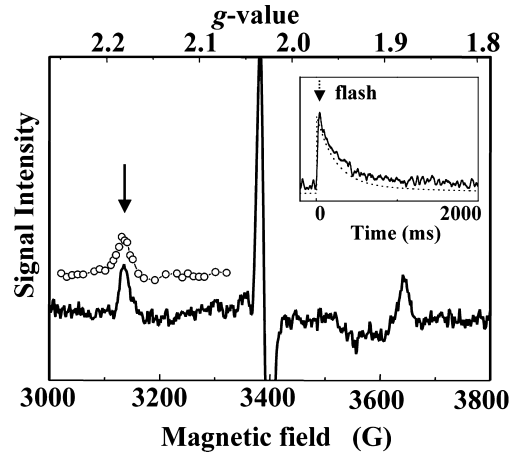

FIGURE 4: Light-induced transient EPR spectra at 4 K in the RC_{core} of *Hbt. modesticaldum* measured in the presence of dithionite. The solid line is the light-minus-dark difference EPR spectrum at 4 K of the RC_{core}. The RC_{core} was preilluminated for 20 min at 210 K and cooled to 4 K under continuous illumination, briefly kept in the dark, and then illuminated by continuous light or excited by the flash so the difference spectrum or kinetics could be measured. Circles show the transient EPR spectra 50 μs after laser flash excitation. The inset shows the kinetic trace of the $P800^T$ state detected at the *z* peak (3110 G) in a low magnetic field and the simulated curve (dotted line). Experimental conditions are the same as those described in the legend of Figure 1 except for the modulation amplitude of 4 G.

study, too. However, we detected no signal that could be ascribed to F_X^- even when samples were thoroughly scanned from a low- to a high-magnetic-field side.

Panel I of Figure 3 shows the flash-induced transient EPR spectra. The intensities of the EPR signal measured 30 μs after laser flash excitation in the RC_{core} under different redox conditions were measured and plotted against magnetic field at 14 K. Trace a represents the transient spectrum for the RC_{core} that was cooled in the dark in the presence of dithionite. Figure 3 also shows the flash-induced spectra obtained for the RC_{core} that was preilluminated for 20 min at 210 K and cooled to 14 K under illumination in the presence of ascorbate (trace b) and dithionite (trace c) before the flash measurements were taken at 14 K.

Trace a in panel I exhibited a distinct spectral pattern of E/A (E, emission; A, absorption). This type of spectrum, which indicated its origin was the radical pair-type ESP, was also detected in the membranes of *Hba. mobilis* and assigned to the $P800^+F_X^-$ state, as described previously (*31–34*). Contrary to this, traces b and c in panel I showed spectral changes extending to the lower-magnetic-field side, suggesting the relative increase in the intensity of a new signal with the ESP pattern of A/E/A/E, which was obviously different from the E/A pattern of trace a. Although the amplitude of the trace c spectrum was approximately one-fifth of that of the trace b spectrum and the spectral shape of trace c was slightly distorted due to the low signal-to-noise ratio, it is clear that trace c contains a new spectral component that is different from the one in trace a. It seems that trace b is a mixture of the two signals with spectral features that are contained in traces a and c. The A/E/A/E-type ESP pattern contained in traces b and c has never been reported before in RCs of heliobacteria, green sulfur bacteria, or PS I (*30–34*). The spectrum was very different from the ESP pattern of $P700^+A_1^-$ in PS I measured at 14 K that is shown in panel II of Figure 3.

The E/A ESP pattern in trace a and the A/E/A/E pattern in traces b and c seemed to remain the feature of the precursor $P^+A_0^-$ state with the net polarization developed during the lifetime of $P^+A_0^-$ (*31–34*). The broader A/E/A/E-type ESP pattern that extended to the lower-magnetic-field side, on the other hand, suggested the involvement of a component with a *g* value higher than that of $P800^+$, such as semiquinone$^-$, which was expected to contribute to the signal intensities around the $g = 2.0062$ region.

EPR Signal of P800^T. Figure 4 shows the light-minus-dark difference EPR spectrum measured by illumination at 4 K in the RC_{core} that was preilluminated for 20 min at 210 K and then cooled to 4 K under continuous illumination in the presence of dithionite. The reversible spectral change (solid line) exhibited a feature of the triplet-state EPR spectrum with the polarization of the spin sate, suggesting its origin from the charge recombination reaction in the RC (*29, 40*). The zero-field splitting parameter gave a $|D|$ value of 236×10^{-4} cm^{-1}. This value was smaller than the reported $|D|$ values for the triplet states of bacteriochlorophyll monomers (290×10^{-4} cm^{-1}) in *Chl. tepidum* or of carotenoids (290×10^{-4} cm^{-1}) in *Hba. mobilis* and almost agreed with the $|D|$ value of 208×10^{-4} cm^{-1} reported for $P840^T$ of *Chl. tepidum* (*31*) as well as with those for $P800^T$ (*4, 37*). The decay profile of the EPR signal after flash excitation, which was monitored at the *z* peak in the low-field side using the same RC_{core} sample, exhibited apparent time constants of 229 μs (80%) and 1.5 ms (20%) (Figure 4, inset). The former time constant was almost comparable to (a little longer than) the reported decay time of the P800 triplet state (*4, 37*). The spectrum obtained by plotting the

signal intensities 50 μs after flash excitation (circles) almost agreed with the spectrum measured under continuous illumination (solid lines), although the peaks other than *z* peaks are not strong in this spectrum. The $P800^T$ state would thus be formed by the charge recombination reaction between $P800^+$ and A_0^- (*4, 41*) because the signal intensity became higher after the photoaccumulation of the $g = 2.0062$ signal, although further analysis seems to be required for the more precise assignment. The preillumination also significantly increased the intensity of the flash-induced $P800^T$ signal detected optically (not shown).

DISCUSSION

Photoaccumulation of the $g = 2.0062$ A_1^- EPR Signal in the RC_{core} of Hbt. modesticaldum. Miyamoto et al. (*19*) recently identified the fast turnover of F_X in the membranes of *Hbt. modesticaldum*. The signal gave an EPR spectrum around $g = 2$, suggesting the $S = {}^1/_2$ ground spin state as in the case of PS I, and was detected only transiently in the range of several milliseconds even at 4 K. Therefore, the 3 ms decay time of $P800^+$ detected in the membranes below 150 K could be attributed to the electron transfer reaction from F_X^- (*19*). The EPR spectrum of F_X^-, however, could not be detected in the purified RC_{core}, although the RC_{core} exhibited a similar 3 ms decay kinetics of $P800^+$ below 150 K. It was, therefore, assumed that the spin relaxation time of F_X^- became too fast to be detected after the sulubilization of the RC_{core} with sucrose monolaurate (*19*). On the other hand, Heinnickel et al. (*20*) photoaccumulated an EPR signal around $g = 5$ in both the membranes and RC preparation, which was solubilized with dodecyl maltoside, of *Hbt. modesticaldum* and assigned it to F_X^- (*20*). This signal was reported to be rather stable and to remain even in the dark after illumination at a cryogenic temperature. We could see neither the $S = {}^3/_2$ nor the $S = {}^1/_2$ ground state of F_X^- in our present RC_{core} (data not shown), in agreement with the previous report (*19*). We nevertheless detected the large flash-induced increase in $P800^+$ that decays in the charge recombination reaction with F_X^- even at 4 K, and the ESP pattern of $P800^+F_X^-$ as well (see Figures 2 and 3). The discrepancy between these two assignments of the F_X^- signal still remains to be resolved.

Another uncertainty in the heliobacterial RC has been the role of quinone as primary acceptor A_1 (*13, 42*). The phylloquinone in the PS I RC is well-known to be tightly bound in the hydrophobic pocket through the hydrogen bond between the carbonyl oxygen of quinone and the NH group of a leucine residue, and the $\pi-\pi$ interaction between the aromatic rings of quinone and a tryptophan residue. The putative binding site of quinone in the heliobacterial PshA subunit also has a conserved leucine residue but lacks the counterpart for the tryptophan residue (*19, 21*). However, we photoaccumulated the quinone-type radical at $g = 2.0062$ (Figure 1) in the purified RC_{core}, which resembled those so far reported in membranes (*36, 37*). We also detected a transient ESP signal that seemed to be able to be attributed to the $P800^+A_1^-$ radical pair state (Figure 3, trace c in panel I). The binding site and environment around the menaquinone (A_1) cannot be envisaged at present in heliobacteria, although its function has long been expected to be analogous to that of phylloquinone A_1 in PS I (*13, 21*).

In the heterodimeric PS I RC, the anion radicals at $g = 2.0033$ and 2.0051 accumulated under different conditions of illumination and reduction, and they have been assigned to Chl a^- (A_0^-) and phyllosemiquinone (A_1^-), respectively, together with the signal of $P700^+$ at $g = 2.003$ (*43, 44*). The condition of photoaccumulation of the A_1^--like radical at $g = 2.0062$ in the heliobacterial RC_{core} resembled that in PS I.

E/A- and A/E/A/E-Type Spin-Polarized Signals in the Heliobacterial RC. We detected two types of transient signals with the ESP patterns of E/A and A/E/A/E in the RC_{core} of *Hbt. modesticaldum* (see Figure 3). The E/A pattern (trace a in panel I) was assigned to arise from the $P800^+F_X^-$ radical pair (*31–34*). The newly found ESP signal with an A/E/A/E pattern (in traces b and c in panel I) was clearly different from the ESP signal of the $P800^+F_X^-$ state. The former spectrum extended toward a higher-*g* value side compared to the latter one. The A/E/A/E signal, therefore, seems to be able to be ascribed to the signal from the $P800^+A_1^-$ transient radical pair. This signal was detected upon flash excitation of the RC_{core} at 14 K only after the preillumination at 210 K in the presence of dithionite. This suggests that the preillumination resulted in the accumulation of F_X^-, although the EPR spectrum of F_X^- itself was invisible under these experimental conditions. The apparent A/E/A/E-type signal in trace c still contains a small contribution of the E/A-type ESP signal from $P800^+F_X^-$ at the higher magnetic field because the signal intensity at the higher magnetic field decreased more in trace c than in trace b, as shown in panel II. Further studies are now in progress to characterize the nature of the present A/E/A/E pattern.

Simulation Based on the ESP Pattern. The ESP signal that was tentatively assigned to the $P800^+A_1^-$ state was somewhat different from that of $P700^+A_1^-$, as compared in panel II of Figure 3. The $P700^+A_1^-$ state and the $P865^+Q_A^-$ state in the Fe^{2+}-depleted/replaced purple bacterial RC are known to exhibit E/A/E pattern signals. These RCs have essentially similar molecular orientations of the acceptor quinones with respect to the primary donor (P) as predicted by the analyses of the ESP signals with the CCRP model (*45*).

The EPR spectra of the $P800^+$ and photoaccumulated A_1^- radicals in the heliobacterial RC (Figure 1) resembled well those of the $P700^+$ and phylloquinone A_1^- radicals, respectively, in PS I. We can assume the spatial locations of P800 and A_1 in the *Hbt. modesticaldum* RC to be similar to those in PS I judging from the similarities of the amino acid sequences of the putative quinone binding sites between PshA of *Hbt. modesticaldum* and PsaA/B of PS I as well as of their probable folding motifs (*13, 19, 21*). However, the A/E/A/E pattern in the $P800^+A_1^-$ ESP signal detected in traces b and c in panel I of Figure 3 in the heliobacterial RC was different from the E/A/E pattern of $P700^+A_1^-$ in PS I as compared in panel II of Figure 3. The A/E/A/E pattern of a newly identified ESP signal, thus, would indicate the different orientation of the quinone A_1 ring in the quinone binding sites in the *Hbt. modesticaldum* RC. The distinctly different patterns may reflect their different dipolar principal axes and/or different distances between two radicals (*45*). With the dipolar principal axis fixed in the RC, the **g** tensor principal axes can be related to the molecular axes, as shown in the bottom panel of Figure 5 (*46*). We assumed the **g** tensor of A_1 in *Hbt. modesticaldum* to have an isotropic *g*

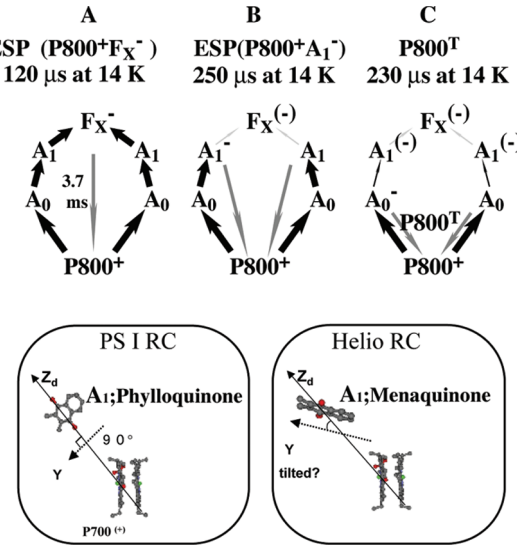

FIGURE 5: Schemes for the electron transfer paths in the RC_{core} of *Hbt. modesticaldum* at 14 K. Scheme A represents the sample frozen in the dark. Schemes B and C represent the cases after preillumination for 20 min at 210 K and subsequent cooling to 14 K under illumination in the presence of ascorbate and dithionite, respectively. Flash excitation is assumed to induce mainly the $P800^+F_X^-$ and $P800^+A_1^-$ states in schemes A and B, respectively, and the $P800^+A_0^-$ state that forms $P800^T$ in scheme C. In the bottom panel is shown the proposed geometry of menaquinone in the heliobacterial RC (right) compared to that of phylloquinone in PS I (left). The latter was drawn according to the molecular geometry of phylloquinone in the PS I RC (*47*).

factor ($g_{iso} = 2.0062$), as seen in trace c in Figure 1, and anisotropic g factors, as reported for A_1 in PS I (*46*). A preliminary simulation that assumed the quinone ring plane to be tilted compared to that of the phylloquinone A_1 ring in PS I, as represented schematically in the bottom panel of Figure 5, gave a better interpretation of the ESP pattern in traces b and c in panel I of Figure 3 which will be demonstrated elsewhere in detail. The ESP pattern, however, is also known to be affected by the electron transfer rates (*45, 46*). Though we can assume the reduction rates of menaquinone or F_X are sufficiently fast to produce the ESP signals, we have no concrete evidence of their reaction times in the heliobacterial RC at cryogenic temperatures at present. Studies on the high-frequency EPR spectroscopy and/or the out-of-phase spin–echo measurement will provide more precise information concerning the orientation of A_1 within the heliobacterial RC.

Electron Transfer Reactions in the Hbt. modesticaldum RC. The spatial arrangements of cofactors in the heliobacterial RC are assumed to be essentially similar to those of their counterparts in the PS I RC, judging from their homologous core subunits, the PshA of the heliobacterial RC, and PsaA and PsaB of PS I (*19, 21, 47*). In addition, the cofactors on the two electron transfer pathways are supposed to be located almost symmetrically along the C2 axis in the homodimeric heliobacterial RC, although this has not yet been directly shown. The quinone content has recently been estimated to be one to two menaquinone molecules per P800 in the present RC preparation by HPLC analysis (M. Kobayashi et al., manuscript in preparation).

Figure 5 shows a scheme of the electron transfer reactions in the *Hbt. modesticaldum* RC based on the results of the study presented here. The scheme is essentially similar to that in PS I. The flash excitation of the dark-frozen RC induced a transient E/A-type signal of $P800^+F_X^-$, which decayed with a time constant of 120 μs. The $P800^+$ was then rereduced with a time constant of 3.7 ms due to the charge recombination reaction with F_X^-. It is not clear, however, whether this reaction process occurs directly or via A_1. After preillumination at 210 K in the presence of ascorbate, $P800^+$ accumulated to some extent as seen in Figure 1. The subsequent flash excitation at 14 K induced the smaller E/A pattern signal and induced a new A/E/A/E-type signal that could be attributed to the $P800^+A_1^-$ state. This seems to be due to the partial preaccumulation of F_X^-. The 210 K preillumination in the presence of dithionite led to accumulation of the semiquinone-type A_1^- radical (see Figure 1, trace c). This sample showed the smaller extents of the E/A pattern signal and the E/A/E/A pattern signal induced by the flash excitation at 14 K (Figure 3, trace c in panel I). This condition, on the other hand, enhanced the $P800^T$ signal as well (Figure 4), suggesting the enhanced charge recombination between $P800^+$ and A_0^-. The electron transfer rates from the $P800^+A_0^-$ state to the $P800^+F_X^-$ and $P800^+A_1^-$ states, thus, can be assumed to be fast enough to give these ESP signals. The oxidation rate of A_0^- was estimated to be 500–700 ps at room temperature (*23, 27*). The rate, therefore, seems to be fast also at cryogenic temperatures.

Function of Menaquinone in the Homodimeric RC. In PS I, A_1 is known to be reduced by A_0^- with a time constant of 30–50 ps and reoxidized by F_X with a time constant of 25–200 ns at room temperature (*18, 25, 48*). The latter rate decreases when the sample is cooled and becomes nearly negligible below 150 K (*18*). Thus, the ESP signal of the $P700^+A_1^-$ radical pair dominates in PS I at low temperatures. However, in the heliobacterial RC, we detected only the signal of $P800^+F_X^-$ even at 14 K in the dark-frozen sample. Only after preillumination in the presence of ascorbate or dithionite could we succeed in the detection of the signal of $P800^+A_1^-$ at 14 K. The rate of electron transfer from A_0 to A_1 as well as that from A_1 to F_X in the heliobacterial RC was thus supposed to be rather fast even at cryogenic temperatures.

The A_1^- radical has never been detected as the transient state in the heliobacterial or green sulfur bacterial RCs. However, the detection of the transient EPR signal of $P800^+A_1^-$, as demonstrated here, suggested the rate of electron transfer from A_0 to A_1 to be sufficiently fast to give the spin polarization. The time constant of A_0^- reoxidation has been estimated to be around 500–700 ps at room temperature (*23, 27*). According to the empirical law concerning the distance−rate relationship, which was proposed by Moser and Dutton on the basis of Marcus' electron transfer theory (*35*), the maximum rate of the electron transfer reaction is expected to be faster with a shorter edge-to-edge distance between the reactant molecules. However, the rate is not fully predictable even at a fixed distance because it also depends on the free energy gap of the relevant reaction, as demonstrated in the reconstituted systems using various quinone molecules in PS I (*49*) and the purple bacteria RC (*35*). If the quinone ring is tilted or displaced, as proposed in Figure 5, the geometry may give longer and shorter edge-to-edge A_0-A_1 and A_1-F_X distances, respectively, compared to those in PS I. If the rate of electron transfer from A_1 to F_X is faster than that from A_0 to A_1 because of the short

distance, then we would observe a very small transient accumulation of A_1^- even when A_1 mediates the electron transfer between A_0^- and F_X. The latter rate may limit the overall reoxidation rate of A_0^- as well as the apparent rate of reduction of F_X. This type of mechanism might interpret the time constant of 500–700 ps for the reoxidation of A_0^- (*22, 23, 27*). With this assumption, we expect the accumulation of A_1^- to occur only after the accumulation of F_X^-, as detected in this study. The fast rate of electron transfer to F_X even at 14 K seems to indicate the reaction from A_0 to A_1 and to F_X to occur with almost no activation energy and suggests it is energetically downhill. This situation is somewhat different from that in PS I, in which the reaction from A_1 to F_X stops at around 100 K (*18, 48*).

In conclusion, we detected a new ESP signal with an A/E/A/E pattern that could be attributed to the $P800^+A_1^-$ radical pair in the heliobacterial RC. The precise spectral shape as well as its production mechanism still remains to be studied. This finding strongly suggests the function of menaquinone as A_1 in the heliobacterial RC and that its reaction mechanism is somewhat different from that of phylloquinone, which functions as A_1 in PS I, in terms of the molecular orientation and/or distance. The rates of electron transfer from A_0 to A_1 and to F_X (presumably via A_1) in the heliobacterial RC are still fast at 14 K and are expected to be different from those of the corresponding steps in PS I because of the different orientations and/or redox properties of A_1 (*19*). It is demonstrated that all type 1 and type 2 RCs of prokaryotes and eukaryotes ubiquitously use quinone molecules as secondary electron acceptors.

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BI701612V