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Spectroscopic characterization of a semi-stable, charge-separated state in Cu^{2+}-substituted reaction centers from *Rhodobacter sphaeroides*

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Abstract

In reaction centers from *Rhodobacter sphaeroides* exposed to continuous illumination in the presence of an inhibitor of the Q_A^- to Q_B electron transfer, a semi-stable, charge-separated state was formed with halftimes of formation and decay of several minutes. When the non-heme iron was replaced by Cu^{2+}, the decay of the semi-stable, charge-separated state became much slower than in centers with bound Fe^{2+} with about the same rate constant for formation. In Cu^{2+}-substituted reaction centers, the semi-stable state was associated with an EPR signal, significantly different from that observed after chemical reduction of the acceptor-side quinone or after illumination at low temperature, but similar to that of an isolated Cu^{2+} in the absence of magnetic interaction. The EPR results, obtained with Cu^{2+}-substituted reaction centers, suggest that the slow kinetics of formation and decay of the charge-separated, semi-stable state is associated with a structural rearrangement of the acceptor side and the immediate environment of the metal-binding site.

Keywords: Bacterial photosynthesis; Conformational change; Reaction center; Stabilized charge-separated state; Copper substitution

1. Introduction

The reaction center (RC) from the bacterium *Rhodobacter sphaeroides* is a membrane protein complex that consists of three subunits and nine co-factors. Most of these co-factors are involved in the electron transfer that converts light into chemical energy [1]. Although the electron transport has been studied for many years, several aspects of the process are still poorly understood. One such detail concerns the role of the protein in the regulation of electron transfer rates.

Slow, light-induced reactions in RCs, not clearly related to the normal, very fast electron transfers, were reported several years ago [2,3]. An accumulation of a stabilized, charge-separated state, not further characterized, was noted when RCs were illuminated while being cooled to cryogenic temperatures [4]. The slow reactions and the stabilization were interpreted as resulting from a conformational change of the RC protein, although no further evidence was presented. Kálmán and Maróti [5] reported a slow light-induced proton release in RCs at acidic pH, which they interpreted as a response to a switch in conformation. van Mourik et al. [6] found that RCs, illuminated with continuous light or several single-turnover flashes, accumulated long-lived, charge-separated states, which they attributed to a change in conformation. Electron transfer between Q_A and Q_B at cryogenic temperatures was observed in RCs frozen under illumination but not in RCs frozen in the dark [7]. This was interpreted as a change in structure as a response to charge separation. RCs frozen during illumination have been suggested to relax from an active to an inactive conformation at temperatures above 120 K [8]. Limited trypsination of RCs resulted in different peptide fragments from the acceptor side of the H and M subunits [9,10], depending on whether the proteolysis was performed in the dark or under illumination [9], suggesting the occurrence of light-induced conformational changes. The reduction of Q_A was observed to lead to rapid conformational relaxation of the protein structure, with time constants ranging from 1 ps to 1 ms at 300 K, in a process that was suggested to involve several conformational sub-states [11].

We have earlier shown that the slow accumulation of a long-lived, charge-separated state was associated with changes in the appearance of the EPR signal of the

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Fe^{2+}-semiquinone pair, which may be interpreted as an effect of a structural change on the acceptor side [12]. EPR analysis of the acceptor side of native RCs after charge separation requires temperatures near the boiling point of liquid helium due to the rapid relaxation of the EPR signal from the interacting Fe^{2+}-semiquinone radical pair. Furthermore, an EPR signal is generated only after single-electron reduction of an acceptor-side quinone while no signal is associated with the resting state defined as the relaxed, dark-adapted state. However, by replacing the non-heme Fe^{2+} by Cu^{2+}, the environment of the metal site in the resting state [13] and interactions with the Q_A^- radical, generated by chemical reduction [14] or charge-separation [10,15] can be studied by EPR at considerably higher temperatures. In the present study, we have used EPR with Cu^{2+} as a spectroscopic probe, replacing the non-heme Fe^{2+} in *R. sphaeroides* reaction centers, to further investigate the slow electron transfer reactions, induced by charge separation, and their possible relation to changes in the conformation of the RC.

2. Materials and methods

2.1. Materials

RCs from *R. sphaeroides* R26 were prepared as described in Ref. [16], except that the final dialysis was made against a buffer containing 40 mM Tris–HCl; pH 8.0; 0.05% *n*-dodecyl-β-D-maltoside. The concentration of RCs was determined optically using the molecular absorption coefficient $\varepsilon_{802} = 288\ mM^{-1}\ cm^{-1}$ [17].

Cu^{2+} substitution was carried out according to Utschig et al. [15]. Quantification of the amount of incorporated Cu^{2+}, by double integration of the EPR spectrum of dark-adapted RC and comparison with a known Cu^{2+} standard, showed a replacement of about 80%. The EPR spectrum of the Cu^{2+}-substituted RCs was essentially identical to earlier published spectra [14,15].

2.2. EPR measurements

Liquid nitrogen (77 K) X-band EPR was performed using an ESP 300 E (Bruker) with an X-band ESP 380–1010 (Bruker) bridge and a Bruker 4102 ST/9311 cavity. Spectra were recorded with a modulation amplitude of 2 mT at different microwave powers. EPR spectra at 10 K were recorded using the same instrument, equipped with a helium cryostat (Oxford Instruments). Actinic light was provided by filtering light from a 250 W halogen lamp through 10 cm of water placed in front of an optical fiber connected to the cavity. The maximum light intensity at the sample, as measured with a YSI-Kettering Model 65A Radiometer, was 0.3 W/cm^2. The light was further filtered with a 590 or 780 nm high-pass filter to prevent photodamage of samples exposed to prolonged illumination while still allowing a high light intensity and a efficient conversion to the semi-stable state. Simulations of EPR spectra were made with the program packet WIN-EPR (Bruker).

2.3. Optical measurements

Light-induced absorbance changes were measured at a sampling frequency of 1 Hz using a UV-2501 PC spectrophotometer (Shimadzu) with a temperature controller. The actinic light was applied perpendicularly to the measuring beam utilizing the same source as in the EPR-measurements with an 860 nm interference filter in front of the fiber.

3. Results

The kinetics of formation of the oxidized special pair, $P870^+$, in dark-adapted RCs with Cu^{2+} replacing Fe^{2+} to serve as an EPR spectroscopic probe, was followed spectrophotometrically at 870 nm (Fig. 1). The RCs were treated with 100 μM terbutryn, which inhibits Q_A^- to Q_B electron transfer by binding to the Q_B site. When the actinic light from a continuous source was turned on, an unresolved kinetic phase of rapid charge separation was observed, which was followed by a much slower phase of oxidation of P870 with a halftime of several minutes. Both the relative and absolute amplitudes of the two phases were dependent on the light intensities [12]. Also, when the light was turned off, the kinetics had a biphasic appearance with an unresolved fast phase of charge recombination followed by a slow re-reduction of $P870^+$. With continuous illumination, the kinetic behavior of Cu^{2+}-substituted RCs was similar to that of native centers (Fig. 1, dotted line) [12], but the observed halftimes for formation and disappearance of the light-induced, semi-stable state were significantly longer (Fig. 1, solid line). On prolonged illumination, an equilibrium situation is eventually attained. However, the position of the equilibrium is shifted far towards the stabilized state in comparison with Fe-containing RCs, as evident from the smaller amplitude of the fast recovery of absorbance when the light is turned off after 10 min of illumination. Slow accumulation and disappearance of a long-lived, charge-separated state, following continuous illumination of native reaction centers were observed earlier by Kálmán and Maróti [5], who could detect the biphasic kinetics only at lower pH values than that used here, and by van Mourik et al. [6]. These workers proposed that the electron transfer was limited by slow conformational changes in the RC proteins.

The kinetics is consistent with a mechanism involving a rapid, reversible step of charge separation and recombination followed by reversible, slow formation and decay of a semi-stable, charge-separated state, $[P^+Q_A^-]^*$ [12]:

$$[PQ_A] \underset{k_{AP}}{\overset{I_{h\nu}}{\rightleftharpoons}} [P^+Q_A^-] \underset{k^*_{-A}}{\overset{k^*_A}{\rightleftharpoons}} [P^+Q_A^-]^* \quad (1)$$

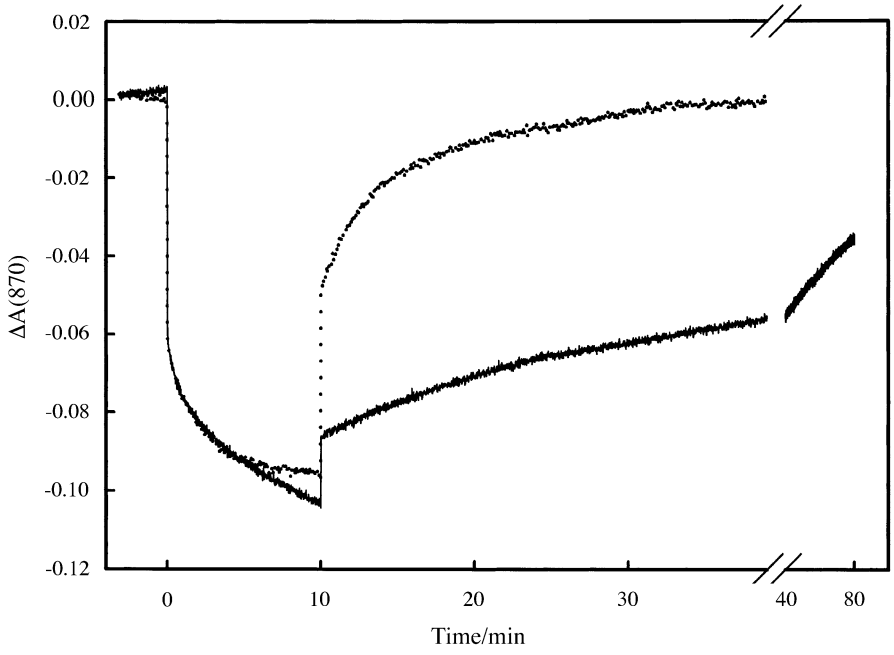

Fig. 1. Light-induced absorbance changes at 870 nm in native (dotted line) and copper-substituted (solid line) RCs. The actinic light was on for a period of 10 min with start at $t=0$. Conditions: 1.7 μM RC in 40 mM Tris–HCl; pH 8.0 and 0.05% *n*-dodecyl-β-D-maltoside in the presence of 100 μM terbutryn; temperature, 20 °C. Illumination and spectrophotometric detection as described in Materials and methods with a 590 nm high-pass filter in the light path.

In the above equation, the light intensity, I_{hv}, determines the rate of the primary charge-separation reaction. Compared to the corresponding phase in native reaction centers, the rapid charge recombination in the Cu^{2+}-substituted RCs was significantly smaller while the amplitude of the slow recombination phase was correspondingly larger. A closer analysis of the kinetics show that the Cu^{2+}-substituted RCs are divided in a major (about 80%) and a minor (20%) fraction, as was earlier shown to be the case with native RCs [12], with k_A^* and k^*_{-A} equal to $(310\ s)^{-1}$ and $(7600\ s)^{-1}$, respectively, for the major fraction and with the corresponding values $(14\ s)^{-1}$ and $(1800\ s)^{-1}$ for the minor fraction. Whereas the rate constant k_A^* in Cu^{2+}-substituted RCs is similar to that of Fe^{2+}-containing RCs [12], k^*_{-A} is smaller by more than a factor of 25. The decrease in k^*_{-A} relative to k_A^* shifts the equilibrium of the slow reaction in Eq. (1) to the right, which explains the large size of the slow charge recombination phase.

For further characterization of the semi-stable state, low-temperature EPR spectra were taken during various stages of the reaction. The correct incorporation of Cu^{2+} was confirmed by the EPR spectrum of the dark-adapted, resting state (Fig. 2a), which was essentially identical to spectra published earlier, with *g* values and hyperfine splitting constants (Table 1) similar to those reported by Calvo et al. [14] and Utschig et al. [15]. Furthermore, illumination of the Cu^{2+}-substituted reaction centers in the EPR cavity at 77 K produced a strong EPR signal at $g=2$ from $P870^+$ and a new signal characteristic of magnetic interaction between Cu^{2+} and the Q_A^- radical as has been observed after

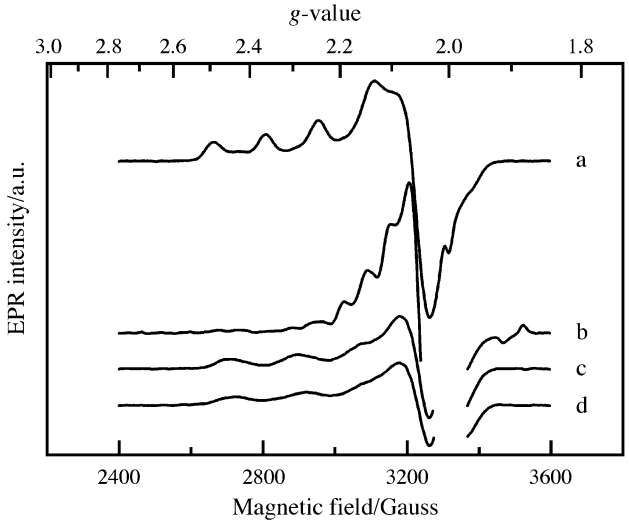

Fig. 2. EPR spectra at 77 K of copper-substituted RCs subjected to different illumination histories. The spectra represent RCs, 203 μM, treated with 1.5 mM terbutryn in the same buffer as in Fig. 1. Terbutryn was added from a stock solution of 400 mM of the inhibitor in acetone. (a) Dark adapted, (b) frozen in the dark and recorded while illuminated in the cavity with no filter in the light path, (c) frozen at 77 K under illumination and recorded in the dark, (d) frozen in the dark after 1 h illumination at 20 °C and recorded in the dark. In c and d, the intensity of the light was reduced with a 780 nm high-pass filter in the light path, see Materials and methods. Spectra b, c and d were obtained by subtraction of a fraction of spectrum a from the raw data until the characteristic hyperfine lines of spectrum a disappeared. Conditions for EPR: microwave frequency, 9.29 GHz; microwave power, 2.1 mW; modulation amplitude, 2 mT; modulation frequency, 100 kHz. All spectra were recorded with the same gain.

Table 1
EPR parameters for the Cu^{2+} signal in Cu^{2+}-substituted RCs subjected to different illumination histories

Acceptor side	$g_\perp$	$g_\parallel$	$A_\parallel$/gauss	10 K		77 K	
				$P_{1/2}$/mW	b	$P_{1/2}$/mW	b
Oxidized[a]	2.053	2.301	147	0.19	1.5	65	1.0
Reduced[b]	–	–	–	1.58	1.9	300	2.0
Reduced[c]	2.065	2.220	185	0.06	1.0	18	1.3
Reduced[d]	2.065	2.210	185	nd	nd	nd	nd

[a] Frozen in the dark.
[b] Frozen in the dark and recorded during illumination in the cavity at low temperature.
[c] Frozen under illumination.
[d] Frozen in the dark after 1 h illumination.

chemical reduction of ubiquinone [14] or after low-temperature illumination [15]. As with the corresponding state in native reaction centers, the signal disappeared rapidly by charge recombination when the illumination ceased.

Interestingly, several of the main lines in EPR spectrum of the charge-separated state were better resolved here than in the spectrum from RCs with isotopically pure copper, where Q_A had been chemically reduced [14]. The better resolution is at least partially accounted for by the significantly larger separation between the main lines in our case. As the reduction of the primary quinone was accomplished by different methods, chemical reduction by Calvo et al. [14] vs. light-induced charge separation in our case, this may indicate that the structure around the Cu^{2+} site is slightly affected by the total charge configuration in the RCs. A wider separation between the main lines in the EPR spectrum of $Cu^{2+}Q_A^-$ can also be discerned in a spectrum published by Utschig et al. [15], where illumination was used to reduce Q_A. These authors performed the illumination in the absence of terbutryn, whereas the inhibitor was present in our experiment. Therefore, the similarity between our results and those of Utschig et al. [15] suggests that the presence of terbutryn does not contribute significantly to the difference between the shape of the signal observed by Calvo et al. [14] and that seen here. This is in contrast with results from native RCs obtained with *o*-phenantroline, another inhibitor of the electron transfer between Q_A and Q_B, which affects the structure on the acceptor side, as indicated by a slight narrowing of the $Fe^{2+}Q_A^-$ signal in native RCs [18]. The weak double-line structure, centered around 2700 G, consistently appeared in spectra from different preparations and most likely belong to a paramagnetic impurity.

In RCs, cooled to 77 K during illumination, the original EPR signal from isolated Cu^{2+} was partially replaced by a new Cu^{2+} signal, stable even at 77 K and characterized by different EPR parameters and broad hyperfine lines. Direct quantification of this signal is made difficult because of interference from the strong P^+ radical. Instead, the double integration was performed on a simulated spectrum, fitted to the experimental one, after subtraction of the remaining resting state spectrum. The amount formed by the new, light-induced state corresponded well to the decrease in the original, dark-stable signal. The conversion efficiency, which was between 50% and 70%, depended on factors such as light intensity, the duration of the illumination and the freezing procedure. In RCs frozen in darkness immediately after illumination for 1 h, a similar signal with broad hyperfine lines was observed (Fig. 2d). A small difference in the EPR parameters of this signal (Fig. 2d) and that in Fig. 2c (Table 1) was discernible as a small but reproducible shift in the relative position of the hyperfine lines of the two spectra. The two states, represented by the EPR spectra c and d in Fig. 2, could also be trapped by freezing at 200 K under illumination or by illumination for 1 h prior to freezing in the dark at 200 K. The EPR parameters for the different signals are given in Table 1. The semi-stable states decayed back to the resting state after thawing and relaxation in the dark for several hours at room temperature, as confirmed by the disappearance of the signal at $g=2$ from $P870^+$ and full return of the normal Cu^{2+} EPR signal (identical to that in

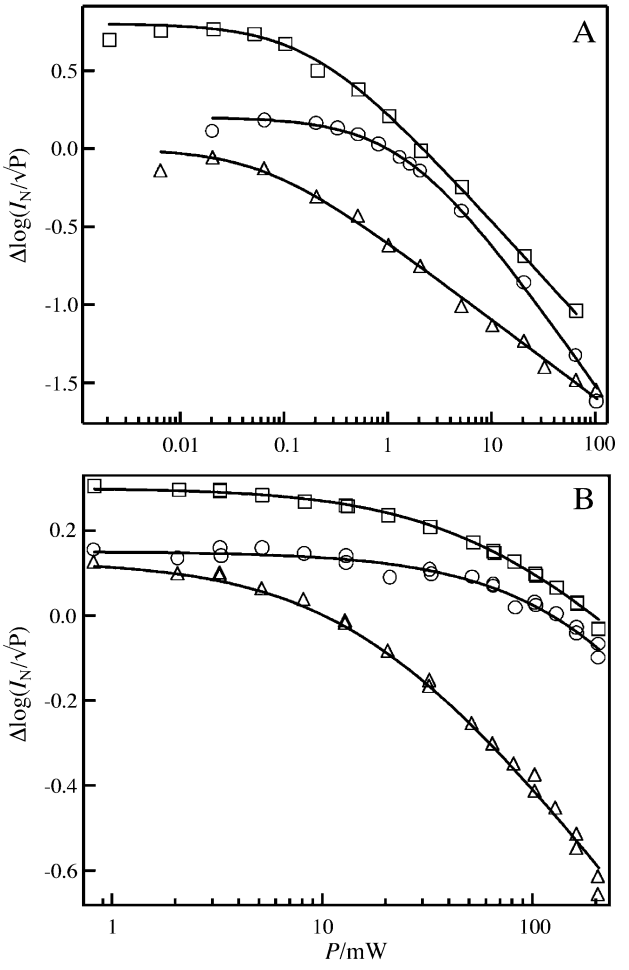

Fig. 3. Power saturation study at 10 K (A) and 77 K (B) of EPR signal from copper-substituted RCs subjected to different illumination histories. The squares, circles and triangles represent the spectra (a), (b) and (c) in Fig. 2, respectively. The solid lines are the best fit to Eq. (2) with the parameters given in Table 1. For reasons of clarity, the normalized data for each sample, $I_N = I/I_0$, are arbitrarily offset.

Fig. 2a). The normal charge-separated state could then be generated by low-temperature illumination, illustrating the semi-stability of the new charge-separated states and the complete reversibility of the reaction. Spectra recorded at 10 K were indistinguishable from those at 77 K.

The power dependence of EPR signals may give valuable information about the environment of the paramagnetic center. The amplitudes of the EPR spectra of Cu^{2+}-substituted RCs exposed to different illumination conditions, as those in Fig. 2, were measured at different microwave powers and the results are presented in Fig. 3 and Table 1.

The power-saturation properties of the signals were obtained using a fitting procedure based on the formula [19]:

$$I = \sum_i I_{0_i} \sqrt{\frac{P}{\left(1 + \frac{P}{P_{1/2_i}}\right)^{b_i}}} \qquad (2)$$

Here I is obtained as the difference between the signal amplitudes, at two different magnetic fields between $g = 2.1$ and 2.3 in the first-derivative EPR spectrum, P the microwave power in mW and $P_{1/2}$ the half-saturation parameter. I_0 is proportional to the concentration of unpaired spin of each component in the sample, while b varies with the ratio between inhomogeneous and homogeneous line broadening [20]. The summation runs over the number of components in the spectrum, one when only the dark-stable state is present and two, representing the light-induced signal and remaining dark signal, otherwise. A global fit using spectra from different illumination conditions was performed at each temperature (10 and 77 K). The power saturation behavior of the Cu^{2+} site in the different states is shown in Fig. 3.

The half-saturation parameter, $P_{1/2}$, at 10 K for the charge-separated $Cu^{2+}Q_A^-$ state, obtained by illumination at low temperature (Fig. 2b), was 1.58 mW compared to 0.19 mW for the relaxed, dark-stable state (Fig. 2a), consistent with relaxation enhancement due to strong magnetic interaction between the Cu^{2+} ion and the ubiquinone free radical in the former [14]. For the semi-stable, charge-

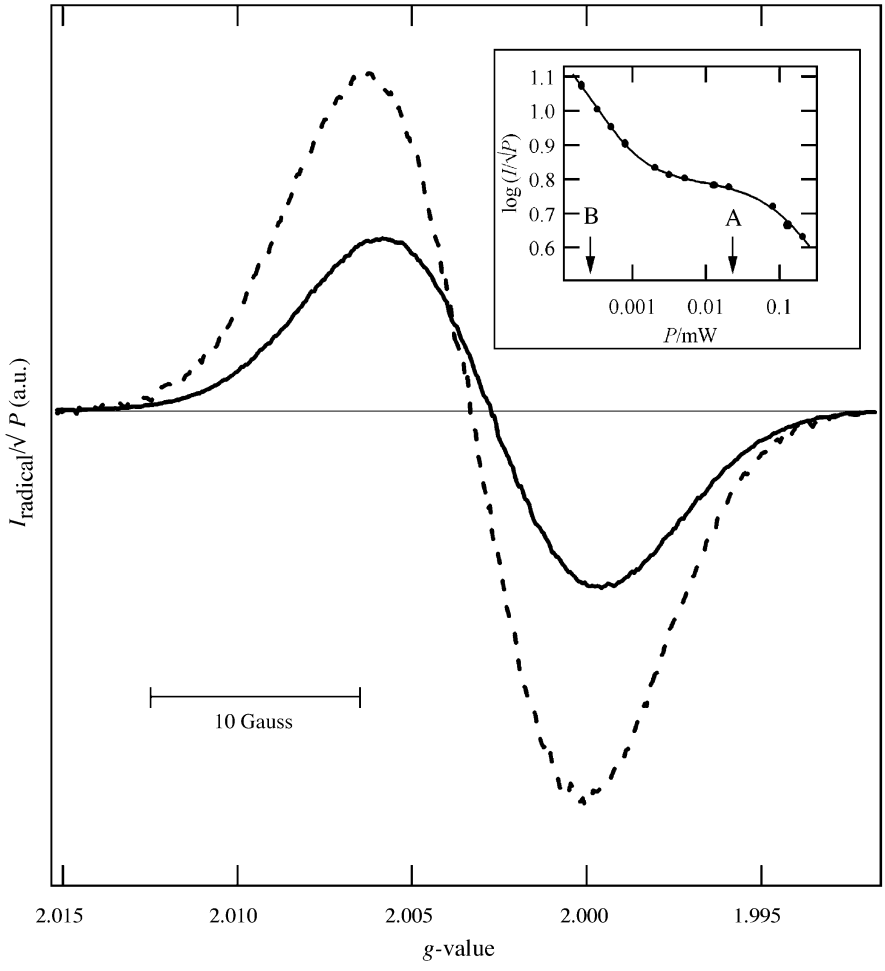

Fig. 4. EPR spectra at 77 K of the semi-stable radicals in copper-substituted RCs at different microwave powers. The same sample as in Fig. 2 was frozen under illumination as in Fig. 2c. Solid line, microwave power, 20.6 μW (arrow A in insert); dashed line, microwave power, 206 nW (arrow B in insert). The insert shows the power saturation behavior where the intensities, $I_{radical}$, were obtained from double integration of the signal. Conditions for EPR: microwave frequency, 9.2786 GHz; modulation amplitude, 0.5 mT; modulation frequency, 100 kHz.

separated state, represented by the spectrum in Fig. 2c, the corresponding value was 0.06 mW. Similar relations between $P_{1/2}$ for the various signals were found at 77 K although the signals were more difficult to saturate (Table 1) because of the enhanced spin–lattice relaxation at higher temperatures [21]. Light used to illuminate frozen samples in the EPR cavity may cause a slight increase in temperature, which should affect $P_{1/2}$. However, no increase in the temperature could be observed when measured by a thermocouple inserted in an illuminated RC sample at 77 K. Although different methods were used to cool the samples at 77 and 10 K (nitrogen dewar and helium gas flow, respectively) the temperature effect at 10 K is expected to be negligible. This is also suggested by the common relations between the $P_{1/2}$ of the different signals at 10 and 77 K. The power saturation properties at 10 and 77 K are summarized in Table 1.

If magnetic coupling between the Cu^{2+} ion and the semiquinone acceptor is weak or absent, the observed radical at $g=2$ should contain contributions from $P870^{+}$ and Q_A^{-}. Since the g values and power saturation properties of the two radicals are different [22], measurements at a series of microwave powers were carried out to de-convolute the radical spectrum. The measurements showed that the radical signal was clearly heterogeneous and partially saturated even at the lowest attainable microwave power (206 nW). As the power was raised, the g value of the signal shifted from 2.0036 at 206 nW to 2.0026 at 20.6 μW at the same time as the signal intensity leveled out at a lower amplitude (Fig. 4). As the microwave power was increased further, the remaining signal started to decrease without further shift in the g value (Fig. 4, inset). Clearly, the radical signal at low powers is composed of two components, one with a g value of 2.0026, which saturates at higher power and a second component, which, at very low power levels, contributes with a similar amount to the total signal intensity. Subtraction of the $g=2.0026$ component from the total spectrum gave a g value of 2.0046 for the second radical species. A comparison with published values identifies the $g=2.0026$ form as $P870^{+}$ while the properties of the more easily saturated $g=2.0046$ species agree well with those for Q_A^{-} [22].

4. Discussion

In bacterial RCs the semi-stable, charge-separated state, generated by extended continuous illumination, differs in several respects from the normal charge-separated state, i.e., that resulting from low-temperature illumination of dark-adapted RCs. In native RCs there appear to be two different, consecutively formed semi-stable states, which differ with respect to the spectroscopic properties of the reduced acceptor side from the normal charge-separated state [12]. The semi-stable nature of these states is underlined by their unusually high stability at cryogenic temperatures. A detailed description of the cause of these changes in properties cannot be provided with the information presently available but it appears likely that structural changes in the RC proteins are involved, such as have been suggested by the different results of partial tryptic digestion of dark-adapted and illuminated RCs [9,10]. The existence of an EPR signal very different from that of a free radical in the stabilized states [12] suggests that the unpaired electron on the semiquinone and the non-heme iron are close together.

Also in Cu^{2+}-substituted RCs a conversion to semi-stability occurs as a result of charge separation, with the sequential formation of two states with slightly different EPR spectra (Table 1). Normally, reduction of the Q_A ubiquinone, either by illumination of frozen RCs (Fig. 2b) [15] or by chemical reduction [14], leads to the formation of a completely different magnetic state, characterized by exchange and dipole couplings between the singly reduced ubiquinone and the Cu^{2+} ion [14] and by the drastically changed appearance of the Cu^{2+} EPR signal (Fig. 2b). In similarity with the situation in Fe^{2+}-containing, native centers, the charge-separated state, formed by low-temperature illumination of Cu^{2+}-substituted RCs (Fig. 2b), decayed rapidly by charge recombination with return of the resting state Cu^{2+} EPR signal (Fig. 2a) when the illumination was discontinued. In contrast, the charge-separated state, obtained when Cu^{2+}-substituted RCs were frozen during or after exposure strong light, did not show evidence of decay after several days at 77 K. In this state, the Cu^{2+} ion is located in a new environment as indicated by the change in the appearance of the Cu^{2+} EPR signal and its relaxation properties. The stabilization is also reflected in the very slow decay of the charge-separated state at room temperature (Fig. 1).

The Cu^{2+} EPR spectrum of the semi-stable state (Fig. 2c) does not show the expected complex structure, characteristic of magnetic interaction with the reduced quinone in RCs exposed to low-temperature illumination (Fig. 2b) or chemical reduction [14]. Instead, the clearly resolved copper hyperfine lines and their positions and separation are typical for magnetically isolated Cu^{2+} often found in proteins, as, for example, in the resting state of the RCs (Fig. 2a), and similar to Type 2 Cu^{2+} in blue oxidases [23]. The value of $P_{1/2}$ for the EPR signal from the stabilized state was even lower than the corresponding value for Cu^{2+} in dark-adapted, resting RCs and significantly lower than that of the charge-separated state after illumination at 10 or 77 K (Table 1). The high value in the latter is expected because of the relaxation enhancement due to exchange and dipolar couplings present between the quinone radical and Cu^{2+} ion [14].

The paramagnetic species with an EPR signal at $g=2.0046$ (Fig. 4), virtually identical to that from semiquinone in RCs with the non-heme iron removed [22], is easily saturated which leads to the conclusion that the semiquinone does not interact strongly with the Cu^{2+} in the semi-stable state, in agreement with the results obtained from the Cu^{2+} studies.

One interpretation of the EPR data is that the slow kinetics of accumulation of the charge-separated state (Eq. (1)) results from a slow change in the structure of the RCs, which increases the distance between the Cu^{2+} ion and the Q_A^- semiquinone radical and possibly also the relative orientation of their magnetic symmetry axes. Separation of the pair should lead to a magnetic decoupling and make the Cu^{2+} ion in all important respects appear as an isolated ion. Another explanation for the new EPR signal, although in our view less likely, is that the electron transferred to the acceptor side no longer resides on Q_A but on an unidentified secondary acceptor, either located at a more distant position on the reaction center or at an external redox component. A comparison with the corresponding situation in Fe^{2+}-containing RCs suggests that the magnetic interaction between the metal ion and the reduced acceptor in the altered charge-separated states also changes in that case. This was indicated by the change of the typical Fe-quinone radical signal in a sample trapped by freezing during illumination to the totally altered EPR spectrum of RCs shortly after long illumination [12].

The appearance of the EPR spectrum of the Cu^{2+} ion in the stabilized states (Fig. 2c and d) strongly indicates that not only is the magnetic coupling with the radical different from that after normal charge separation but also that the immediate environment of the Cu^{2+} ion has changed. This is evident from a comparison with the spectrum of the resting, dark-stable state (Fig. 2a). A likely explanation for the difference is that the geometric arrangement, possibly also the number of the protein ligands is different compared to the dark-adapted state. As noted above, the EPR spectrum is similar to that of Type 2 Cu^{2+} in blue oxidases. In these, the Cu^{2+} ion has a free position in the planar structure for the binding of external ligands such as water or inhibitors [24]. From the nitrogen hyperfine structure in reaction centers with isotopically pure Cu^{2+}, Calvo et al. [14] proposed that in substituted RCs the Cu^{2+} ion is surrounded by three imidazoles and one oxygen ligand in a planar coordination structure with a fourth, weakly interacting imidazole nitrogen in an apical position. The EPR spectrum of the stabilized state observed here may indicate that one of the planar ligands have been lost, resulting in a ligand environment mimicking that of Type 2 Cu^{2+}. It is not likely that the Cu^{2+} ion has been lost from its normal binding site to an external surface-exposed site, since the original dark-stable signal is completely restored after relaxation of the protein in darkness at room temperature and it appears unlikely that a removed Cu^{2+} ion would re-enter the site without considerable losses. The rearrangement of the Cu^{2+} site in the charge-stabilized state is consistent with the occurrence of a conformational change in the reaction center proteins on the acceptor side as was suggested by the EPR spectra from the Fe-containing RCs. Also, in both cases there seems to be a difference in the EPR signals from the state obtained by freezing under illumination and that trapped by freezing in darkness after an extended illumination period. This implies that the stabilized states may occur in at least two versions with different structures, although the EPR results suggest that this difference is smaller in the Cu^{2+}-substituted RCs than in native centers [12]. Since the sequential formation and decay of two different stabilized states is not obvious from the kinetics of the slow reaction, the transition between them must be rate-limiting for the whole reaction. The state, trapped when freezing during illumination, may then equilibrate relatively rapidly with the charge-relaxed ground state.

In summary, using RCs with Cu^{2+} replacing the non-heme iron as a spectroscopic probe we can show that charge separation at room temperature leads to the slow formation of a semi-stable state, with significant alterations in the environment of the metal site. The spectroscopic properties of the copper in the stabilized state suggest that a change in the coordination of the copper occurs as a result of its formation, together with an increase in the distance to the unpaired electron. This is in contrast to the case when Q_A is reduced chemically or by charge separation at cryogenic temperatures where there is a strong interaction between the unpaired spins on Q_A^- and Cu^{2+} and where the distance between the members of the pair is suggested to decrease rather than increase as a result of the reduction [14].

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