# The Bound Electron Acceptors in Green Sulfur Bacteria: Resolution of the *g*-Tensor for the $F_X$ Iron-Sulfur Cluster in *Chlorobium tepidum*

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ABSTRACT The photosynthetic reaction center (RC) of green sulfur bacteria contains two [4Fe-4S] clusters named $F_A$ and $F_B$, by analogy with photosystem I (PS I). PS I also contains an interpolypeptide [4Fe-4S] cluster named $F_X$; however, spectroscopic evidence for an analogous iron-sulfur cluster in green sulfur bacteria remains equivocal. To minimize oxidative damage to the iron-sulfur clusters, we studied the sensitivity of $F_A$ and $F_B$ to molecular oxygen in whole cells of *Chlorobium vibrioforme* and *Chlorobium tepidum* and obtained highly photoactive membranes and RCs from *Cb. tepidum* by adjusting isolation conditions to maximize the amplitude of the $F_A^-/F_B^-$ electron paramagnetic resonance signal at $g = 1.89$ (measured at 126 mW of microwave power and 14 K) relative to the $P840^+$ signal at $g = 2.0028$ (measured at 800 $\mu$W of microwave power and 14 K). In these optimized preparations we were able to differentiate $F_X^-$ from $F_A^-/F_B^-$ by their different relaxation properties. At temperatures between 4 and 9 K, isolated membranes and RCs of *Cb. tepidum* show a broad peak at $g = 2.12$ and a prominent high-field trough at $g = 1.76$ (measured at 126 mW of microwave power). The complete *g*-tensor of $F_X^-$, extracted by numerical simulation, yields principal values of 2.17, 1.92, and 1.77 and is similar to $F_X$ in PS I. An important difference from PS I is that because the bound cytochrome is available as a fast electron donor in *Chlorobium*, it is not necessary to prereduce $F_A$ and $F_B$ to photoaccumulate $F_X^-$.

## INTRODUCTION

The photosynthetic reaction center (RC) of green sulfur bacteria is widely considered to be an analog of photosystem I (PS I) of green plants and cyanobacteria (for a review see Feiler and Hauska, 1996). The RC of PS I is a heterodimer of the PsaA and PsaB polypeptides, whereas the analogous RC of green sulfur bacteria is composed of a homodimer of the PscA polypeptide. Green sulfur bacteria (*Chlorobiaceae*) contain a so-called Type I or iron-sulfur type RC. Type I RCs contain two [4Fe-4S] clusters, termed $F_A$ and $F_B$, which serve as the terminal electron acceptors. In PS I, $F_A$ and $F_B$ are bound to PsaC, whereas in *Chlorobium* (*Cb.*), they are bound to a similar protein named PscB. The RC of PS I contains the rare instance of an interpolypeptide [4Fe-4S] cluster termed $F_X$. Unlike the nonheme iron coordinated to the heterodimer that comprises the Type II RC (or "quinone type" RC; represented by RC of purple bacteria, RC of green filamentous bacteria, and photosystem II of green plants and cyanobacteria), $F_X$ serves as indispensable component of the electron transfer chain of PS I (Moënne-Loccoz et al., 1994; Van der Est et al., 1994; Vassiliev et al., 1995). PscA in *Chlorobium limicola* f.sp. thiosulfatophilum contains a [4Fe-4S]-cluster binding motif highly analogous to the $F_X$ binding site found in PsaA and PsaB of PS I (Büttner et al., 1992). This suggests that an analogous $F_X$ cluster should exist in green sulfur bacteria. However, electron paramagnetic resonance (EPR) spectroscopic evidence for the presence of an $F_X$-like iron-sulfur cluster in the RC of green sulfur bacteria remains equivocal. The evidence relies solely on the existence of a weak high-field trough around $g = 1.76$, which has been assigned to $F_X$ by analogy with PS I (Nitschke et al., 1990; Oh-oka et al., 1993; Kusumoto et al., 1994). Studies of the sensitivity of the iron-sulfur clusters to oxygen, EPR detection of the low-field and midfield resonances of $F_X$, and the determination of the *g*-tensor of $F_X$ constitute the major goals of this work.

Most of the information on electron transfer to $F_A$ and $F_B$ in *Chlorobium* is based on EPR measurements of isolated membranes and RCs. A low-potential iron-sulfur cluster with a midpoint potential of −550 mV was first identified in chromatophores from *Chlorobium limicola* (Knaff and Malkin, 1976; Jennings and Evans, 1977). Later, a set of resonances with a trough at about $g = 1.85$ typical of iron-sulfur clusters was uncovered by illuminating *Cb. limicola* membranes (Nitschke et al., 1990), *Cb. limicola* RCs (Feiler et al., 1992; Oh-oka et al., 1993), *Cb. vibrioforme* membranes and RCs (Kjær et al., 1994), and *Cb. tepidum* RCs (Kusumoto et al., 1994). A spectrum with mid- and high-field *g*-values of 1.91 and 1.89 was found by a photoaccumulation protocol that promotes more than one electron in *Cb. limicola* RCs (Feiler et al., 1992; Oh-oka et al., 1993), *Cb. tepidum* RCs (Kusumoto et al., 1994) in *Cb. limicola* membranes (Knaff and Malkin, 1976), and *Cb. vibrioforme* membranes (Kjær et al., 1994; Miller et al.,

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1992). A single iron-sulfur cluster was shown to be irreversibly photoreduced by a photoaccumulation protocol that promotes one electron in RCs from *Cb. vibrioforme*, and a second iron-sulfur cluster was uncovered by a photoaccumulation protocol that promotes more than one electron (Scott et al., 1997). The two clusters had midpoint potentials of −450 mV and −502 mV and were found at equal spin concentrations. It was proposed that the complex photoaccumulated and chemically reduced spectra arise from magnetic coupling between the two paramagnetic iron-sulfur clusters. Thus the two terminal iron-sulfur clusters in *Chlorobium* are considered to be highly similar to $F_A$ and $F_B$ in PS I.

Except for small, systematic differences in the *g*-tensor between cyanobacteria and higher plants (Mehari et al., 1991), the EPR spectra of $F_A$ and $F_B$ in PS I of a given kingdom are relatively invariant between thylakoids and PS I complexes isolated with either Triton X-100 or *n*-dodecyl-β-D-maltoside. In green sulfur bacteria, however, there is considerable variability in the spectrum of the terminal iron-sulfur clusters (Nitschke et al., 1987, 1990; Feiler et al., 1992; Oh-oka et al., 1993; Miller et al., 1992; Hager-Braun et al., 1997). One difference between PS I and the *Chlorobium* RC is that the linewidths of the individual *g*-components are considerably broader in the latter, although this assessment has since been questioned and attributed to detergent-induced alterations (Hager-Braun et al., 1997). Although a high-field feature around $g = 1.79$ that was attributed to an $F_X$ analog was reported in the spectra measured under strongly reducing conditions (Hager-Braun et al., 1997), the complete *g*-tensor of this acceptor and its electron transfer properties have not been reported. In addition, our knowledge of the photochemical properties, RC stability, and spectral properties of the iron-sulfur clusters in the green sulfur bacterial RC is incomplete, and detailed resolution of the properties of the electron acceptor system is necessary for a full understanding of its structure and function.

We decided to approach the issue of cluster intactness in a systematic manner. We first determined the EPR properties of $F_A$ and $F_B$ in freshly frozen whole cells of *Chlorobium vibrioforme* and *Chlorobium tepidum,* the two strains in which we have previously studied the EPR spectra of bound [4Fe-4S] clusters (Hager-Braun et al., 1997; Scott et al., 1997). Because genome sequencing of *Cb. tepidum* is nearly completed, this species is fast becoming the organism of choice for contemporary biochemical studies of green sulfur bacteria, and the present work is focused on studies of bound [4Fe-4S] clusters in this strain. We used the spectra of the whole cells as a benchmark to optimize isolation conditions for intact iron-sulfur clusters in isolated membranes and RCs of *Cb. tepidum* and then analyzed the properties of the EPR spectra in isolated membranes at different temperatures. By comparing the spectra of the RCs isolated from different *Chlorobium* strains (Scott et al., 1997; Hager-Braun et al., 1997) and the spectra of *Cb. vibrioforme* cells (this work), we found that the individual *g*-components of the interaction spectrum of $F_A^-/F_B^-$ in green sulfur bacteria have linewidths that are naturally broader than the interaction spectrum of $F_A^-/F_B^-$ in PS I. In *Chlorobium tepidum*, differences in the relaxation properties allowed us to make a distinction between the $F_A^-/F_B^-$ interaction spectrum and $F_X^-$ and made it possible to determine its complete *g*-tensor.

## MATERIALS AND METHODS

Cell cultures of *Chlorobium vibrioforme* (strain NCIB 8327) were grown in 2-L screw-cap bottles in modified Pfennig medium (Wahlund et al., 1991) at room temperature in an anaerobic chamber (Coy Products, Grass Lake, MI) filled with 95% nitrogen/5% hydrogen. The cells were grown under fluorescent light (10 W/m$^2$) until the cell density reached $16 \times 10^6$ cells/L. *Chlorobium tepidum* cells were grown as described previously (Hager-Braun et al., 1995).

The cells were harvested inside the anaerobic chamber in a benchtop centrifuge at 8000 × *g* for 30 min, washed with 25 mM Tris-HCl (pH 8.3), and centrifuged again at 8000 × *g* for 20 min. Approximately 5.5 ml of packed cell pellet was obtained from 1 L of culture. The pellet was resuspended at a ~1:3 (v/v) ratio in a medium containing 25 mM Tris-HCl (pH 8.3) and 70% glycerol and placed in airtight cryogenic vials, anaerobically flash-frozen in liquid nitrogen, and stored at −80°C. The concentration of Bchl *a* in the samples determined from acetone/methanol extracts was 5–8 μg/ml. For EPR measurements the samples were thawed inside the anaerobic chamber, and 120 μl of the cell paste was loaded into a quartz EPR tube (2-mm inner diameter), which was then closed with an air-tight stopper.

Chlorosome-containing membranes were isolated from *Chlorobium tepidum* by differential centrifugation of the homogenate obtained by passage through a French pressure cell, and the reaction centers were solubilized from these membranes, using Triton X-100, according to the method of Hager-Braun et al. (1995). The isolation procedure was adapted to meet strictly anaerobic conditions at all stages: all manipulations with the samples were performed inside the anaerobic chamber, the centrifugation was carried out in airtight polycarbonate tubes that were loaded inside the anaerobic chamber, the French press cell was loaded inside the anaerobic chamber, and the homogenate was collected from the French press into a nitrogen-filled closed volume reservoir with a pressure relief outlet. All of the buffers used during the isolation or for preparing solutions were first degassed using a vacuum pump and then purged initially with nitrogen, followed by argon gas (for 40–60 min at each step). All plastic and glassware were equilibrated with nitrogen inside the anaerobic chamber for at least 1 day before use. Isolated membranes and RCs were resuspended in 25 mM Tris-HCl buffer (pH 8.3) in the presence of 20% glycerol and stored in airtight glass vials at −80°C. The concentration of Bchl *a* was 38 μg/ml in the membranes and 14 μg/ml in the RCs, respectively.

EPR measurements were made with a Bruker ECS-106 X-band spectrometer equipped with either an ER462 ST cavity (resonant frequency, 9.48 MHz) or an ER 4116 DM cavity (resonant frequency, 9.62 MHz). Cryogenic temperatures were maintained with an ESR900A liquid helium cryostat and an ITC4 temperature controller (Oxford Instruments, Oxford, UK). The microwave frequency was measured with a Hewlett-Packard 5340A frequency counter that was interfaced to the spectrometer with an IEEE-488 bus. The common spectrometer settings were as follows: center field, 3480 G; sweep width, 1200 G; receiver gain, $6.3 \times 10^4$; conversion time, 4.096 ms; time constant, 2.048 ms; number of averages, three to five. Other spectrometer conditions are specified in the figure legends. To scan the microwave power dependency of the spectra, the spectrometer was programmed to change the microwave power in 5-dB decrements upon

completion of each scan. The data files were transferred from the spectrometer to a Power Macintosh 8500/250 computer and processed using a program written in Igor Pro, version 3.14 (Wavemetrics). The spectra were plotted against a *g*-value axis by converting the data points' magnetic field reference values, using the formula $g = 714.484 * f/H$, where $f$ is the microwave frequency in MHz, and $H$ is the magnetic field in Gauss. Implementation of this formula in a computer program allowed direct visual comparison of the *g* values of EPR spectra measured in different experiments, using the different resonant frequencies of the two cavities. Computer simulations of EPR spectra were performed using WINEPR SimFonia, version 1.25 (Bruker Analytische Messtechnik GmbH). The results of the simulation were ported to Igor Pro and converted into spectra that were plotted against *g*-values instead of magnetic field. Vertical offsets are applied in the figures, where necessary, to separate multiple spectra. The half-saturation values of microwave power ($P_{1/2}$) were determined in Igor Pro by a nonlinear regression fit of the EPR derivative signal amplitude ($S$) as a function of the microwave power to the equation (Rupp et al., 1978) $S = A\sqrt{P}/(1 + P/P_{1/2})^{b/2}$, where $P$ is the microwave power, $A$ is the proportionality constant, and $b$ is a constant that varies from 1 for inhomogeneously broadened lines to 4 for homogeneously broadened lines.

To illuminate the samples inside the cavity at a desired temperature, light from a 300-W halogen lamp was passed through a 2-cm water filter and focused to cover the entire aperture of the resonator; the incident light intensity was 1100 W/m$^2$. For illumination outside the cavity, the sample tube was placed in a 100-ml quartz dewar in direct contact with the dewar inner wall and illuminated with an identical light source for 5 min. The dewar was quickly filled with liquid nitrogen, and the sample was kept inside the dewar for another 2 min and then quickly transferred into the spectrometer cavity, which was illuminated as described above. The spectra designated as "photoinduced" were obtained as the difference between spectra measured in either of the conditions described above and spectra of the same samples that were dark-adapted for 5 min, frozen in the dark, and measured in the dark.

## RESULTS

Our main objective was to measure the photoinduced EPR spectra of the native iron-sulfur clusters $F_X$, $F_B$, and $F_A$ in the photosynthetic reaction center of *Chlorobium*. To distinguish between the iron-sulfur clusters, we measured the photoinduced spectra of *Cb. vibrioforme* cells and *Cb. tepidum* cells and isolated membranes and RCs at microwave power levels of 0.4, 1.26, 4, 12.6, 40, and 126 mW and at temperatures from 5 to 30 K. This approach provides a more comprehensive view of the EPR spectral signatures than the application of a fixed temperature and microwave power (see Introduction) because it can differentiate redox components based on their spin relaxation properties.

### Photoinduced EPR spectra of *Chlorobium* cells

Whole-cell samples of *Cb. tepidum* were illuminated for 5 min at room temperature with subsequent freezing to 5–30 K inside the EPR cavity, and spectra were measured at microwave power levels from 400 $\mu$W to 126 mW. Resonances with apparent *g*-values of 2.07, 1.94, and 1.89 were obtained at microwave powers greater than 10 mW (Fig. 1 *A*). These resonances are typical of [4Fe-4S] clusters and are most prominent in the temperature range from 11 K to 23 K. At temperatures higher than 20 K the amplitudes of the resonances decrease because of linewidth broadening, and the spectra become relatively simple in lineshape. Because of the similarity to the spectrum of isolated RCs of both *Cb.*

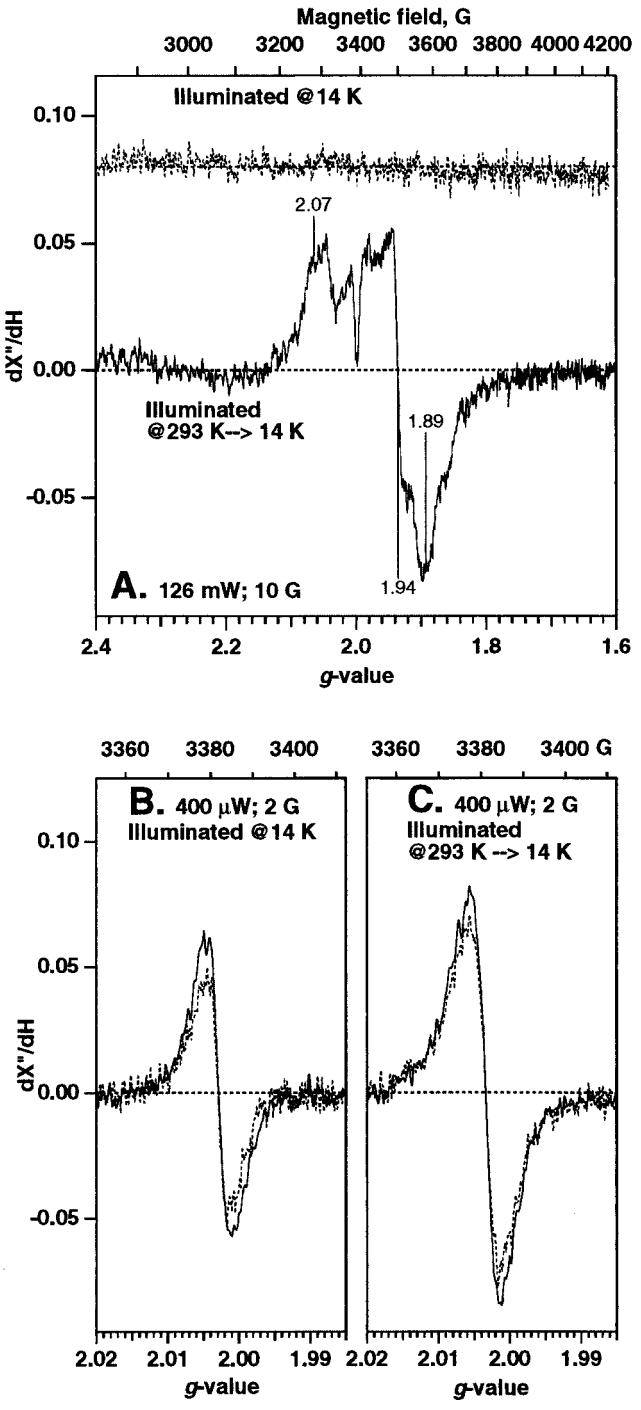



FIGURE 1 Photoinduced EPR spectra of *Cb. tepidum* cells measured at 14 K at 126-mW microwave power and 10-G modulation amplitude (*A*) and 400-$\mu$W microwave power and 2-G modulation amplitude (*B* and *C*). The spectra were photoaccumulated either by in-cavity illumination at 14 K (*B*) or by illumination at room temperature with subsequent freezing to 14 K (*C*). The spectra measured 10 min after termination of the in-cavity illumination are depicted by dashed lines (*B* and *C*).

*tepidum* (Hager-Braun et al., 1997) and *Cb. vibrioforme* (Scott et al., 1997), the resonances shown in Fig. 1 *A* can be assigned to the interaction spectrum of $F_A^-/F_B^-$. By fitting the dependency of the EPR spectrum amplitude at $g = 1.89$ as a function of microwave power to the power saturation equation (assuming $b = 1$; see Materials and Methods) the half-saturation parameter $P_{1/2} = 206$ mW at 14 K is obtained, a value that is consistent with the identification of the resonances as fast-relaxing iron-sulfur clusters.

Unlike our experience with isolated RCs (Hager-Braun et al., 1997), we were not able to detect any resonances in the $g = 1.9$ region by illumination of the whole cells at 14 K (Fig. 1 *A, top*). However, illumination at 14 K still results in a resonance at $g = 2.0028$ that can be detected at low microwave power. We studied two conditions: illumination at 14 K, where no more than one electron is promoted from P840, and illumination at room temperature followed by freezing in the light, where at least two electrons are promoted from the bound cytochrome and P840. In *Cb. tepidum*, the resonance at $g = 2.0028$ has a linewidth of 6.5 G when the sample is illuminated at 14 K, and 25% of the signal is lost in subsequent darkness 10 min after termination of in-cavity illumination (Fig. 1 *B*). Derivative-shaped photoinduced EPR signals have been measured in RCs from *Prostechochloris aestuarii* at 5 K and low microwave power levels with $g = 2.0025$ and a 9-G linewidth (Swarthoff et al., 1981) and in membranes from *Cb. limicola* at 35 K with $g = 2.0025$ and a 9.3-G linewidth (Wasielewski et al., 1982). These signals were attributed to $P840^+$. A narrower signal with a 7.7-G linewidth ascribed to $P840^+$ was measured at 77 K by Rigby et al. (1994) in membranes from *Cb. limicola*. By way of comparison, $P700^+$ in cyanobacterial PS I complexes has a *g*-value of 2.0028 and a linewidth of 7.8 G when measured by X-band EPR (Yang et al., 1998). The $g = 2.0028$ resonance is only slightly larger when the sample is photoaccumulated at room temperature and measured at 14 K, but the linewidth increases to 7.5 G (Fig. 1 *C*). The amplitude of the signal was decreased by 14% in subsequent darkness (Fig. 1 *C, dashed line*). Because the photoaccumulation protocol is expected to oxidize the entire population of $P840^+$, the implication is that the majority of reaction centers undergo irreversible charge separation when illuminated at 14 K. This irreversible behavior is similar to P700 in PS I, where irreversible charge separation occurs between P700 and the terminal iron-sulfur clusters, $F_A$ and $F_B$ (Jung et al., 1997). However, because the linewidth is ~1 G broader after photoaccumulation at 290 K than illumination at 14 K, this may indicate partial reduction of yet another species with a higher *g* value. Further study at higher microwave frequencies will be required to resolve the *g* anisotropy of any composite signals.

The photoinduced EPR spectra of *Cb. vibrioforme* cells detected at 14 K and high microwave power (Fig. 2 *A*) are similar to those of *Cb. tepidum*. In addition to performing measurements at 14 K and at 290 K, we have also measured

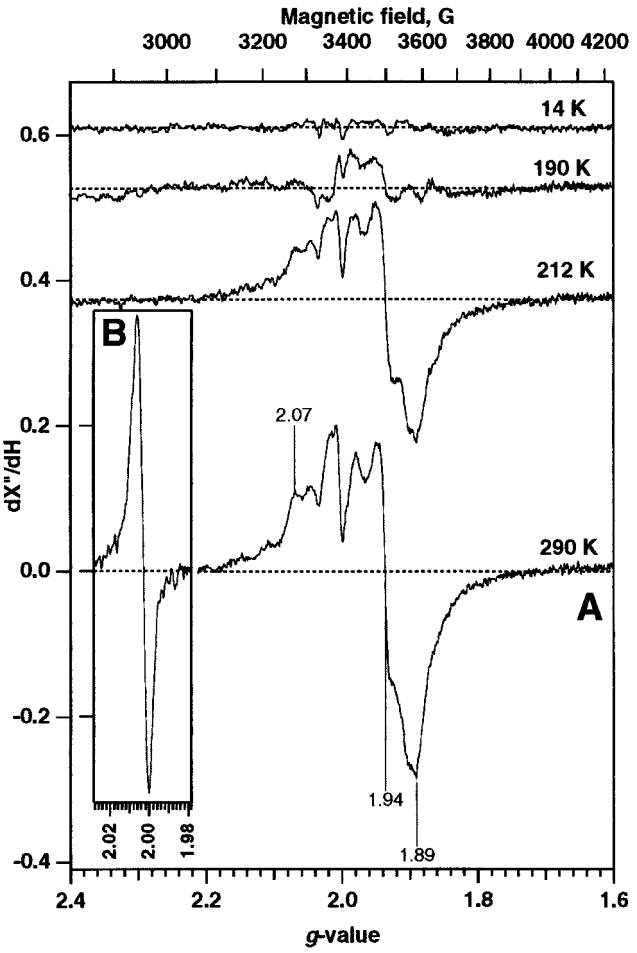



FIGURE 2 Photoinduced EPR spectra of *Cb. vibrioforme* cells measured at 14 K at 126-mW microwave power and 5-G modulation amplitude (*main panel, A*) and 800-$\mu$W microwave power and 2-G modulation amplitude (*inset, B*). The spectra were photoaccumulated by 10-min illumination at different temperatures (indicated near the spectra) with subsequent freezing to 14 K. The spectrum in the inset (*B*) was photoaccumulated at 290 K with subsequent freezing to 14 K.

the spectra of *Cb. vibrioforme* cells at intermediate temperatures. We found virtually no photoinduced signals in the $g = 1.8$–$1.9$ region after illumination at temperatures between 14 and 200 K. As in to *Cb. tepidum* cells, a narrow derivative-shaped photoinduced signal centered at $g = 2.0028$ was detected in the whole temperature range tested at microwave powers below 1 mW (Fig. 2 *B*), which was formed largely irreversibly in the light (not shown).

## Effect of air exposure on the iron-sulfur clusters in *Chlorobium* cells

To study the effect of oxygen on the $F_A/F_B$ iron-sulfur clusters a small volume of the sample was gently bubbled with air and then with nitrogen gas. As shown in Fig. 3, air exposure of whole *Cb. tepidum* cells led to the loss of the

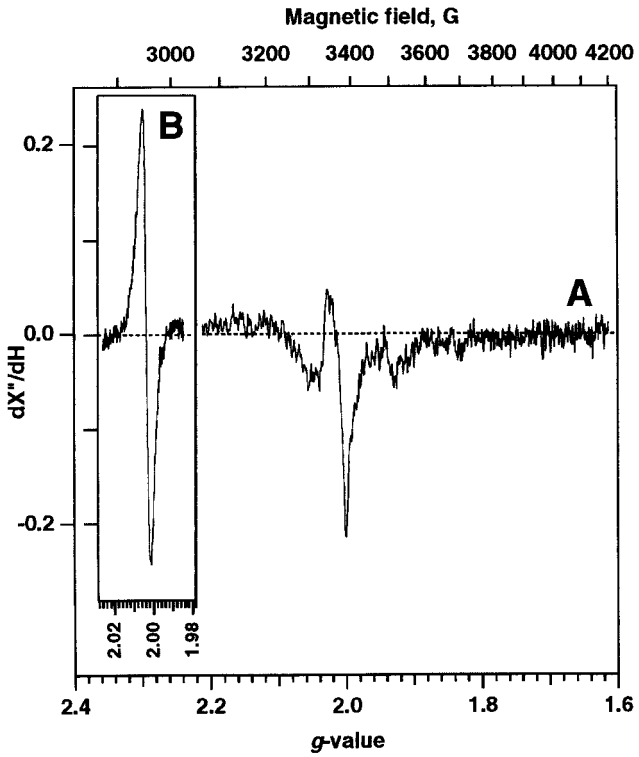



FIGURE 3 Photoinduced EPR spectra of aerobic *Cb. tepidum* cells measured at 14 K at 126-mW microwave power and 5-G modulation amplitude (*main panel, A*) and at 800-$\mu$W microwave power and 2-G modulation amplitude (*inset, B*).

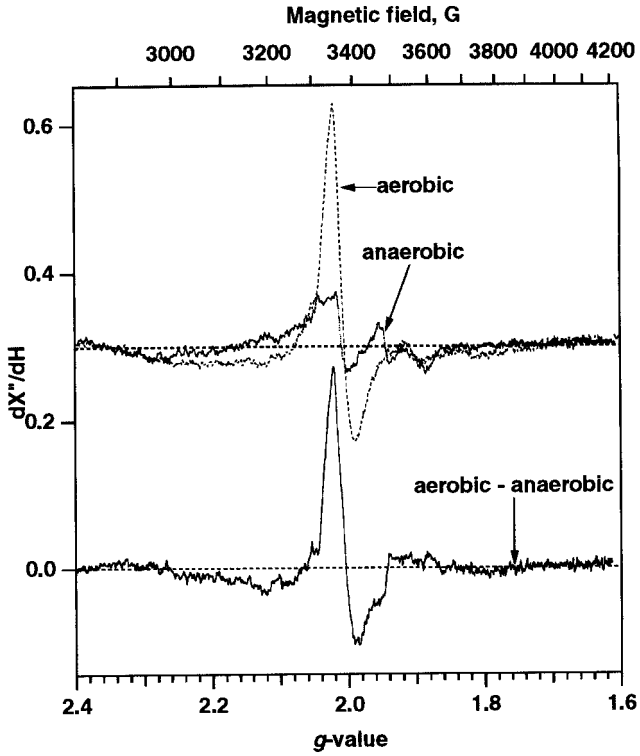



FIGURE 4 EPR spectra of dark-frozen anaerobic and aerobic *Cb. tepidum* cells (*top*) and the difference (*bottom*) between the two spectra. The spectra were measured in the dark at 14 K at 126-mW microwave power and 5-G modulation amplitude, and the background spectrum arising from the empty cavity was subtracted from the original spectra of the dark-frozen cells.

photoinduced signal in the $g = 1.85–1.9$ region (measured at a microwave power of 126 mW). A 20–30-min exposure completely eliminated this photoinduced signal in *Cb. tepidum*; however, a 5-min exposure was sufficient to completely eliminate the signals in *Cb. vibrioforme*. The addition of sodium dithionite (50 mM) did not restore the resonances in this sample (not shown). However, the amplitude of the photoinduced signal centered at $g = 2.0028$ (measured at a microwave power of 800 $\mu$W) decreased by only 20% relative to control on exposure to air (Fig. 3 *B* compared to Fig. 1 *C*). A similar result was obtained when a liquid culture of *Cb. vibrioforme* was bubbled with air for 10–15 min, and the cells were sedimented and resuspended to a concentration appropriate for EPR measurements (not shown). After exposure of the liquid culture to air, the cells did not grow after they had been washed with anaerobic buffer and resuspended in fresh anaerobic media. Hence, exposure of *Chlorobium* cells to oxygen does not affect P840 photooxidation, but rather leads to the irreversible loss of $F_A$ and $F_B$ and, consequently, to cell death.

To understand the mechanism of inactivation of the [4Fe-4S] clusters we have compared spectra of the dark-frozen cells before and after air exposure, measured in the darkness (Fig. 4). It is notable that in both *Chlorobium* strains the air exposure gives rise to a relatively broad dark signal in the $g = 2$ region that is distinguishable from photoinduced $P840^+$ by its *g*-value, linewidth, and relaxation properties (microwave power greater than 10 mW is necessary for its detection). The difference between the spectrum of the aerobic and anaerobic cells (Fig. 4) resembles the spectrum of an oxidized [3Fe-4S] cluster in the NarH iron-sulfur protein from *Escherichia coli* (Guigliarelli and Bertrand, 1999). The spin concentration of the putative [3Fe-4S] cluster based on double integration of the above-mentioned spectrum of *Cb. tepidum* cells is ~15% of the spin concentration of $F_A^-/F_B^-$ determined from the photoinduced EPR spectrum at the same measurement conditions (14 K, 126 mW microwave power) before air exposure. The same ratio of the spin concentration values of ~4% occurs in the case of *Cb. vibrioforme* cells. Hence we propose that a [3Fe-4S] cluster may act as an intermediate in the route of destruction of the [4Fe-4S] clusters.

Based on different air sensitivities of P840 and RC-bound [4Fe-4S] clusters, we defined an arbitrary quality metric as the amplitude of the $F_A^-/F_B^-$ signal at $g = 1.89$ (measured at a microwave power of 126 mW) relative to the $P840^+$ signal at $g = 2.0028$ (measured at a microwave power of 400–800 $\mu$W). This metric was found to be equal to 1.0–

1.2 in several batches of both *Cb. tepidum* and *Cb. vibrioforme* cells collected and measured under strictly anaerobic conditions. This ratio represents an arbitrary parameter and does not reflect the absolute stoichiometry of the components, but rather serves as a criterion of the intactness of the RC under the conditions specified here. We used this metric to optimize the conditions for the isolation of membranes and RCs from *Chlorobium tepidum* and to monitor the intactness of the preparations during each stage of the isolation procedure.

### EPR spectra of isolated membranes and reaction centers of *Cb. tepidum*

The original protocols of membrane and RC isolation (Hager-Braun et al., 1995) were modified to ensure strict anaerobic conditions, and EPR spectra were measured at all stages of the procedure from whole cells to the preparation of membranes and RCs. Throughout the isolation of membranes and RCs, we changed the conditions to maximize the above-defined quality metric to ensure the maximum amount of intact $F_A$ and $F_B$. To confirm the enrichment in Bchl *a* content in the isolated RCs, optical absorbance spectra were also measured at all stages of the isolation (not shown). An increase in the ratio of the Bchl *a* peak at 810 nm relative to that of Bchl *c* at 745 nm in the isolated RC indicates the removal of the chlorosomes and the enrichment of the sample in the photosynthetic RC. We found that when strict anaerobic conditions were maintained throughout the procedure, the ratio of the amplitudes of the derivative at $g = 2.0028$ and the trough around $g = 1.89$ was retained in the membrane (Fig. 5) and RC (Fig. 6) preparations. This indicates that the samples were largely intact and that the RC-bound [4Fe-4S] clusters are the sole components that contribute to the spectral features observed in the $g = 1.9$ region in whole cells and isolated membranes.

The resonances attributed to $F_A/F_B$ in isolated membranes show virtually the same relaxation properties as determined by a microwave power and temperature matrix as the resonances attributed to $F_A/F_B$ in whole cells (the whole data set is not shown; only the most representative spectra are depicted and discussed below), although some batches of isolated membranes and RCs show spectra with narrower linewidths of the individual *g* components than are found in the whole cells. This is reminiscent of our previous studies, in which resonances with narrower linewidths and more structural features were observed in *Cb. tepidum* RCs (Hager-Braun et al., 1997) compared to *Cb. vibrioforme* RCs (Scott et al., 1997).

Similar to the whole cells (Fig. 1, *B* and *C*), the amplitude of the derivative-shaped signal centered at $g = 2.0024$ (linewidth 7.6–7.8 G) measured at 400 $\mu$W in isolated membranes was larger when it was illuminated at room temperature than at 14 K (Fig. 5, *B* and *C*). The decrease in the amplitude after termination of in-cavity illumination was more pronounced in the isolated membranes than in the whole cells, i.e., a 52% decrease in the case of EPR spectrum photoaccumulated at 14 K (Fig. 5 *B*).

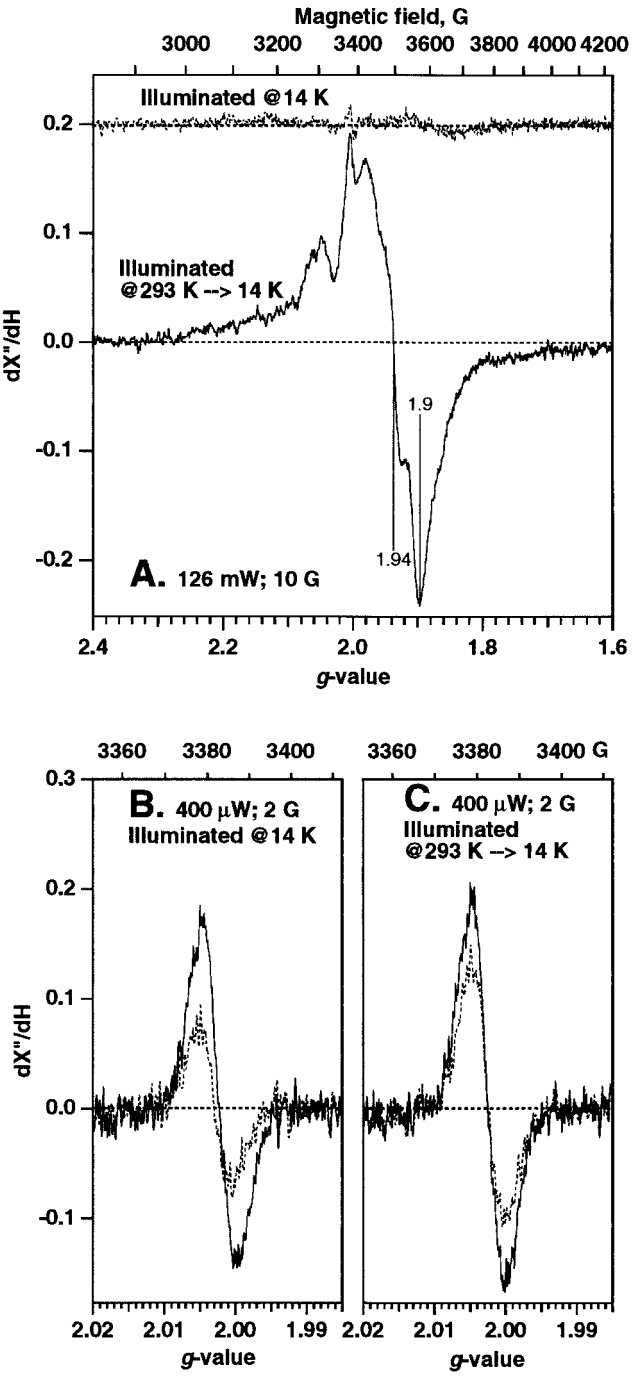



FIGURE 5 Photoinduced EPR spectra of isolated *Cb. tepidum* membranes measured at 14 K at 126-mW microwave power and 10-G modulation amplitude (*A*) and 400-$\mu$W microwave power and 2-G modulation amplitude (*B* and *C*). The spectra were photoaccumulated either by illumination at room temperature with subsequent freezing to 14 K (*B*) or by illumination at 14 K (*C*). The spectra measured 10 min after termination of the in-cavity illumination are depicted by dashed lines (*B* and *C*).

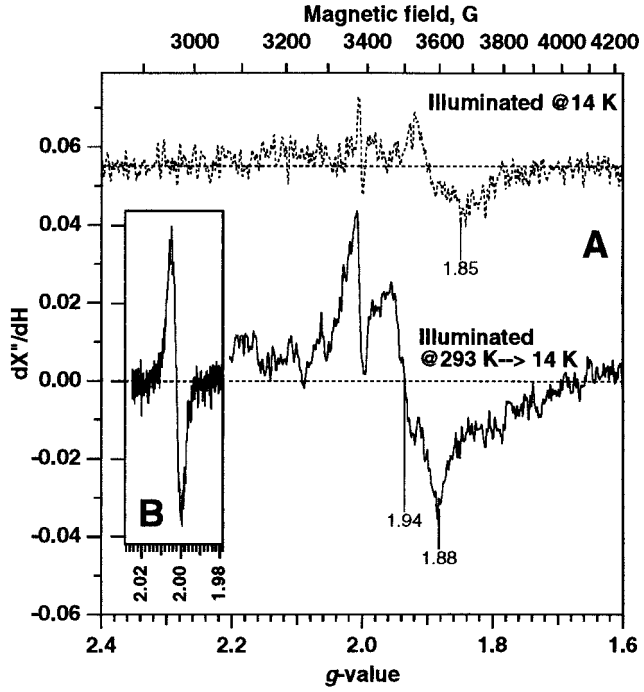



FIGURE 6 Photoinduced EPR spectra of isolated *Cb. tepidum* reaction centers measured at 14 K at 126-mW microwave power and 10-G modulation amplitude (*main panel, A*) and 400-$\mu$W microwave power and 2-G modulation amplitude (*inset, B*). The spectra were photoaccumulated either by illumination at room temperature with subsequent freezing to 14 K or by illumination at 14 K as denoted near the spectra. The spectrum in the inset (*B*) was photoaccumulated at 290 K with subsequent freezing to 14 K.

Although no photoinduced EPR resonances of iron-sulfur clusters were observed upon illumination of *Cb. tepidum* and *Cb. vibrioforme* at 14 K, a spectrum similar to that reported under low-temperature illumination of chlorosome-depleted membranes of *Cb. tepidum*, *Cb. vibrioforme,* and *Cb. limicola* (Hager-Braun et al., 1997; Scott et al., 1997) was observed in isolated chlorosome-containing membranes (Fig. 5) and RCs (Fig. 6). The amplitude of the $g = 1.86$ trough (upon illumination at 14 K) relative to the $g = 1.89–1.90$ trough (when photoaccumulated at room temperature with subsequent freezing) was almost an order of magnitude higher in the RCs than in the membranes. The latter trough, which represents the $F_A^-/F_B^-$ interaction spectrum, showed the greatest amplitude at 14 K (microwave power 126 mW). Addition of an exogenous electron donor (50 $\mu$M *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine in the presence of 10 mM sodium ascorbate) had no effect on the spectra of the membranes and RCs described above.

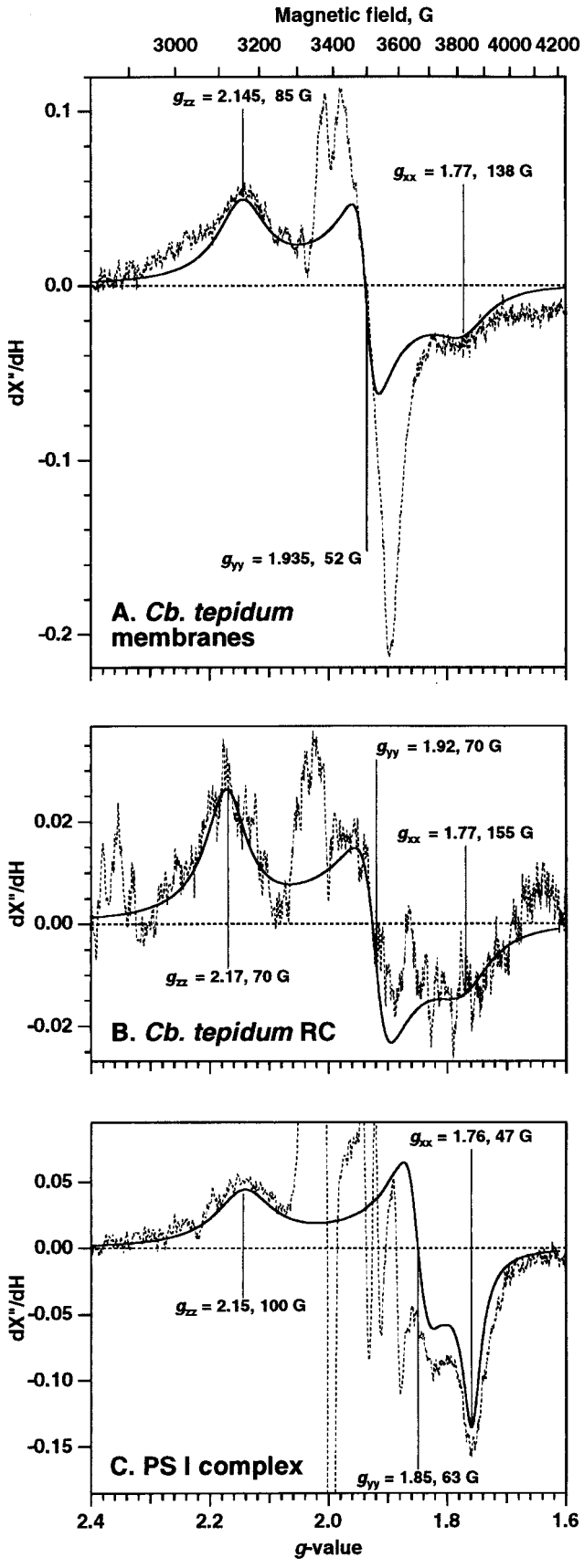
## EPR spectrum of $F_X$ in isolated membranes and reaction centers of *Cb. tepidum*

In addition to the interaction spectrum of $F_A^-/F_B^-$, a low-field peak and a high-field trough were present in the photoinduced EPR spectrum of membranes at temperatures below 9 K and were most pronounced at 4.5 K (Fig. 7 *A*). A simulated spectrum with *g* values of 1.77, 1.935, and 2.145 closely matches both the low-field peak and a high-field trough observed in the membranes at 4.5 K, although there is ambiguity in the position of the midfield resonance due to the contribution from the $F_A^-/F_B^-$ interaction spectrum in this region. In the isolated RCs, however, the amplitude of the $g = 1.89$ trough from $F_A^-/F_B^-$ was less prominent at 4.5 K, and the simulated spectrum with *g* values of 2.17, 1.92, and 1.77 was found to closely match the low-field peak, the midfield derivative, and the high-field trough (Fig. 7 *B*). The differences from the whole-cell spectrum can be accounted for by small changes in the linewidths of the individual *g*-components. To further address the analogy between the RCs of PS I and *Chlorobium* we have revisited the EPR spectral properties of the $F_X$ spectrum in PS I. A photoinduced spectrum of $F_X$ in isolated PS I complex of *Synechocystis* sp. PCC 6803 (with $F_A$ and $F_B$ prereduced with sodium dithionite) is shown for comparison in Fig. 7 *C*. This spectrum has a more distinct low-field peak than the spectra depicted and simulated in our previous study (Vassiliev et al., 1995) and therefore permits a precise numerical simulation of the *g*-tensor with principal values of 2.15, 1.85, and 1.76 (Fig. 7 *C*).

## DISCUSSION

In this work photoinduced EPR spectra of RC-bound iron-sulfur clusters were measured for the first time in whole cells of green sulfur bacteria. The amplitude, *g* value, and linewidth of these spectra represent a reliable benchmark by which the quality of isolated membranes and RCs could be judged. We found that exposure of *Chlorobium* cells to oxygen does not affect primary charge separation, as manifested by the signal at $g = 2.0028$ that arises because of the $P840^+$ cation. However, a short-term exposure (seconds) to air leads to irreversible damage to $F_A$ and $F_B$. We defined a "quality metric" to estimate the intactness of the RCs of green sulfur bacteria based on the ratio of the $g = 1.89$ resonance from the iron-sulfur clusters to the $g = 2.0028$ resonance from $P840^+$ measured at a microwave power appropriate for each signal.

The photoinduced EPR spectra measured in the whole cells at a microwave power of 126 mW are attributed entirely to the RC-bound [4Fe-4S] clusters. There are two important points to note. First, the spectrum of the iron-sulfur clusters photoinduced at 212 K is identical to the spectrum photoinduced at 290 K, and the amplitude of the spectrum photoinduced at 212 K is no less than 60% that of that photoinduced at 290 K (Fig. 2 *A*). Therefore, the major contributors to these spectra are the [4Fe-4S] clusters bound to the RC; electron transfer from the RCs to soluble ferredoxins (which contain the amino acid motif for [4Fe-4S] clusters in *Chlorobium*; Hase et al., 1978; Tanaka et al.,




1974, 1975) is unlikely at 212 K. Second, the ratio between the amplitude of the trough in $g = 1.9$ region (measured at 126 mW) to the amplitude of the derivative signal at $g = 2.0028$ (measured at 800 $\mu$W) remains constant in the course of the membrane and RC isolation procedure, which would remove all soluble proteins (Figs. 1, 5, and 6).

No photoinduced signals in the $g = 1.9$ region were found in the whole cells of either *Cb. vibrioforme* or *Cb. tepidum* upon illumination at 14 K (Figs. 1 and 2). This is an interesting phenomenon, and it is different from the EPR behavior of $F_A$ and $F_B$ in PS I. We infer either that in whole cells $F_A$ and $F_B$ are frozen in a spin state that does not allow the spectrum to be observed easily around the $g = 1.9$ region, or that $F_A$ and $F_B$ are preceded by an electron acceptor that can be reduced at 14 K without passing the electron forward to $F_A$ or $F_B$. Because no signals could be detected at 4.2 K around $g = 2.15$ or 1.76, this putative acceptor is probably not $F_X$ (assuming $S = 1/2$). Rather, it may be the quinone acceptor $A_1$ or an intermediate acceptor of a different chemical nature following $A_0$, which is photoaccumulated in its reduced form under these conditions. The major difference between whole cells and isolated membranes and RCs is that no photoreduction of the iron-sulfur clusters occurs at 14 K in the former, while photoreduction of one of the iron-sulfur clusters has been reported in the latter (Scott et al., 1997; Hager-Braun et al., 1997; and the present study). Because the amplitude of the $g = 2.0028$ signal photoinduced at 14 K comprises 60–70% of the signal photoinduced at room temperature, it is unlikely that the optical properties of whole cells would prevent penetration of the light at 14 K and the consequent photoreduction of the clusters. In support of this assertion, one should note also that the contribution of the spectrum attributed to

FIGURE 7 (*A*) Photoinduced EPR spectrum of *Cb. tepidum* membranes in 25 mM Tris-HCl buffer (pH 8.3) and 20% glycerol measured at 126-mW microwave power and 10-G modulation amplitude at 4.5 K (*dotted line*). Photoaccumulation of the spectrum was carried out by illumination during cooling of the samples from 293 K to 14 K. The simulated EPR spectrum with $g_{xx} = 1.77$ (138 G), $g_{yy} = 1.935$ (52 G), $g_{zz} = 2.145$ (85 G), is shown by a solid line. (*B*) Photoinduced EPR spectrum of *Cb. tepidum* reaction centers in 25 mM Tris-HCl buffer (pH 8.3) and 20% glycerol measured at 126-mW microwave power and 10-G modulation amplitude at 4.5 K (*dotted line*). Photoaccumulation of the spectrum was carried out by illumination during cooling of the samples from 293 K to 14 K. The simulated EPR spectrum with $g_{xx} = 1.77$ (138 G), $g_{yy} = 1.935$ (52 G), $g_{zz} = 2.145$ (85 G) is shown by a solid line. (*C*) Photoinduced EPR spectrum of PS I trimeric RC complex of *Synechocystis* sp. PCC 6803 was measured at 20-mW microwave power and 20-G modulation amplitude at 10 K (*dotted line*). The photoinduced spectrum was obtained by in-cavity illumination of the samples suspended to a chlorophyll *a* concentration of 800 $\mu$g/ml in 100 mM glycine buffer (pH 10.0), 100 mM sodium dithionite, and 20% glycerol; preilluminated for 5 min at room temperature; and subsequently frozen at 77 K. The spectrum of dark-frozen sample in the presence of sodium dithionite was used as a background for subtraction. The simulated EPR spectrum with $g_{xx} = 1.76$ (47 G), $g_{yy} = 1.85$ (63 G), $g_{zz} = 2.15$ (100 G) is shown by a solid line.

reduction of one of the terminal clusters ($g = 1.86$) relative to the interaction spectrum of $F_A^-/F_B^-$ ($g = 1.89–1.90$ trough) is considerably higher in the RC than in the membranes (Figs. 5 and 6). We propose that extraction of the RC from the membrane may lead to a change in the protein or a slight alteration in PscB position relative to the $(PscA)_2$ homodimer that may facilitate photoreduction of the cluster proximal to the heterodimer. This assumption, along with the fact that the transition to photoreduction takes place near the temperature of glass formation of glycerol (200 K; see Fig. 2), is in a reasonable agreement with models describing the temperature dependencies of electron transfer in PS I on the basis of conformational effects (Brettel, 1997). Alternatively, as in bacterial reaction centers (Stowell et al., 1997), freezing in the dark or in the light may result in different conformations of the binding sites for the iron-sulfur clusters, which may lead to different spin states. If the spin state of the "dark" conformation is $S > 1/2$, then these clusters would be difficult to detect by EPR (particularly if the spin state is $S = 3/2$; see Golbeck, 1999). If the spin state of $F_A^-$ and $F_B^-$ is $S = 1/2$, then we must conclude that photoreduction of both $F_A$ and $F_B$ is blocked below 200 K. Further study is needed to decide between these possibilities. The slower relaxation of the photoinduced signal around $g = 2.0028$ in the isolated membranes frozen under illumination compared to the sample illuminated at 14 K (Fig. 5) may be also related to the existence of these different conformational and/or spin states.

Although weak lateral bands were documented for the $F_A^-/F_B^-$ interaction spectrum of the PS I RC (Guigliarelli et al., 1993), the resonance at $g = 2.12$ in *Chlorobium* cells is unlikely to be of a similar origin because of its different relaxation behavior, as deduced by its appearance at temperatures different from those optimal for the interaction spectrum of $F_A^-/F_B^-$. It has been suggested that the high-field trough at $g = 1.79$ measured at 8 K in the membranes of *Cb. tepidum* in the presence of dithionite corresponds to a cluster analogous to $F_X$ of PS I (Nitschke et al., 1990; Oh-oka et al., 1993; Kusumoto et al., 1994). Although there is a close match to the high-field $g$-value of $F_X$ in PS I (Hager-Braun et al., 1997), the complete $g$-tensor of this putative electron acceptor was not previously resolved. We show in the present work that the EPR spectrum of $F_X^-$ in *Cb. tepidum* membranes or isolated RC can be distinguished from the $F_A^-/F_B^-$ interaction spectrum by their different relaxation properties. The slight differences between the spectra seen in the isolated membranes and in the RC can be rationalized by minor changes of the conformation of the $F_X$ binding niche upon extraction of the RC from the membrane. It is important to note that the midfield and high-field features of the $F_X$ spectrum were hardly distinguishable in the whole cells of the two strains, which implies that the properties of $F_X$ are affected by detergent treatment, making it possible to reveal the whole $g$-tensor in the isolated membranes and RCs with highly intact iron-sulfur clusters.

As seen from Fig. 7, the $g$-tensor of $F_X^-$ in the PS I RC and that in the *Cb. tepidum* RC are not identical. It should be noted that the $g$-tensor of $F_X^-$ in PS I is not invariant, and mutations in amino acids adjacent to the $F_X$ cysteine ligands affect both the linewidths and the principal $g$-values (Vassiliev et al., 1999). For instance, alterations of the aspartate amino acids adjacent to the $F_X$ cysteine ligands in PS I were shown to alter the high-field trough $g$-value from 1.744 in $D566K_{PsaB}$ to 1.794 in $D566A_{PsaB}$. Because the amino acids that surround $F_X$ in *Chlorobium* are not identical to those that surround $F_X$ in PS I, a difference in the $g$-tensor might be expected. Chemical reduction of $F_A$ and $F_B$ before illumination is necessary to observe photoaccumulated $F_X^-$ in PS I. In *Chlorobium*, however, we were able to photoaccumulate $F_X^-$ by freezing from room temperature to 4.5 K without prereducing $F_A$ and $F_B$. Most likely, this difference in the behavior of the $F_X^-$ spectrum is due to the presence of a tightly bound cytochrome $c_{551}$, which serves as an efficient electron donor to $P840^+$. We propose that this cytochrome donates electrons via P840 to $F_X$ at a faster rate than $F_X^-$ can be reoxidized by $P840^+$. In PS I the natural membrane-bound electron donor, cytochrome $c_6$, is lost during isolation, and a relatively inefficient artificial donor must be used to provide electrons to $P840^+$.

The issue of the oxygen sensitivity of $F_X$ in *Chlorobium* requires further exploration, which is best approached by time-resolved optical spectroscopy, along with low-temperature EPR spectroscopy. Thus far we have not observed an EPR spectrum resembling $F_X$ in air-inactivated *Chlorobium* cells or in isolated membranes at the microwave power and temperatures used in this study. This does not necessarily indicate that $F_X$ in *Chlorobium* is as sensitive to oxygen as $F_A$ and $F_B$; this result can be alternatively explained by an inability to photoaccumulate $F_X^-$ in the absence of $F_A$ and $F_B$.

The resolution of the EPR spectrum of $F_X$ in *Chlorobium*, along with the analogy of the [4Fe-4S]-cluster binding motif in PscB to the $F_X$ binding site in PsaA and PsaB of PS I (Büttner et al., 1992), provides evidence of yet another instance of an interpolypeptide [4Fe-4S] cluster serving as an electron carrier and further strengthens the evolutionary relationship between the reaction center of green sulfur bacteria and photosystem I.

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