Electronic and Vibrational Structure of the Radical Cation of P_{840} in the Putative Homodimeric Reaction Center from *Chlorobium tepidum* As Studied by FTIR Spectroscopy†

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ABSTRACT: Light-induced FTIR difference spectra of P_{840} upon its oxidation (P_{840}^+/P_{840}) have been measured with the reaction center complex from the green sulfur bacterium *Chlorobium tepidum*. A broad band centered near 2500 cm^{-1} was observed in P_{840}^+, which is comparable to the band near 2600 cm^{-1} previously observed in P_{870}^+ of purple bacteria and assigned to the electronic transition in the bacteriochlorophyll *a* (BChl*a*) dimer [Breton et al. (1992) *Biochemistry 31,* 7503−7510]. The intensity of this electronic band found in P_{840}^+ was about the same as that in P_{870}^+. The P_{840}^+ spectrum also showed several intensified vibrational modes, which are characteristic of the P_{870}^+ spectrum as well. These similar features of the electronic transition and the intensified lines indicate that P_{840}^+ is a BChl*a* dimer whose electronic structure is similar to P_{870}^+. Based on the previous theoretical works, the possibility that P_{840}^+ has an asymmetric structure as P_{870}^+ was suggested. Also, two strong positive bands at 1707 and 1694 cm^{-1} probably assigned to the keto C_9═O stretching modes of P_{840}^+ were observed in the P_{840}^+/P_{840} spectrum. Three different interpretations are possible for the presence of the two C_9═O bands: (i) P_{840}^+ is an asymmetric dimer cation. (ii) P_{840}^+ has a symmetric structure, and the time constant of positive charge exchange between the two BChl*a* molecules coincides with that of IR spectroscopy (10^{-13} s). (iii) The electric field produced by the positive charge on P_{840}^+ affects the C_9═O frequency of the neutral BChl*a* in P_{840}^+ itself (when the charge exchange time is slower than the time scale of 10^{-13} s) or of a BChl*a* in the close proximity of P_{840}^+. The negative bands at 1734 and 1684 cm^{-1} were assigned to the ester C_{10}═O and the keto C_9═O of neutral P_{840}, respectively, both of which are free from hydrogen bonding. These results and interpretations regarding the structural symmetry and the molecular interactions of P_{840} and P_{840}^+ are discussed in the framework of the "homodimeric" reaction center of green sulfur bacteria.

Reaction centers (RCs)[1] of photosynthetic systems can be classified into two types, i.e., "quinone type" (type II) RCs that possess a terminal electron acceptor of a mobile quinone molecule, and "iron−sulfur type" (type I) RCs that possess iron−sulfur clusters as terminal electron acceptors (Golbeck, 1993; Barber & Andersson, 1994). The former type includes RCs of purple bacteria, green filamentous bacteria, and photosystem (PS) II of cyanobacteria and plants, whereas the latter type includes RCs of green sulfur bacteria (Feiler & Hauska, 1995; Sakurai et al., 1996), heliobacteria (Amesz, 1995), and PS I.

The striking feature of these photosynthetic RCs is the dimeric structure of the core proteins. The structures of the RCs of purple bacteria have been clarified by X-ray crystallography revealing pseudo-C_2 symmetry, which consists of the L and M subunits (Deisenhofer et al., 1985; Allen et al., 1987; Chang et al., 1991). The arrangement of cofactors also holds the symmetry; the primary donor (P), a BChl dimer, is located on the C_2 axis between the two subunits, and two sets of monomer BChl, BPhe, and quinone are symmetrically arranged as well. This structural concept is considered to be conserved in PS II, which consists of D1 and D2 subunits instead of L and M (Barber & Andersson, 1994). The core of PS I is also a dimer of homologous subunits, PsaA and PsaB, and the X-ray crystallography at 4.5 Å resolution (Schubert et al., 1995) showed a roughly symmetrical arrangement of chlorophyll and vitamin K1 pigments (including A_0 and A_1) around the axis through a chlorophyll dimer (P700) and a FeS center (F_x).

In contrast to these RCs, only a single gene of RC core protein could be identified in green sulfur bacteria (*psc*A) (Büttner et al., 1992a,b) and in heliobacteria (*psh*A) (Liebl et al., 1993). Since the putative two cysteine ligands of F_x, which consists of four cysteines offered by two polypeptides, were conserved in these sequences, the finding of only a single gene lead to the idea that the RCs in these bacteria may form a "homodimer" consisting of the two identical polypeptides, in contrast to the other RCs forming a "heterodimer" (Büttener et al., 1992a,b; Liebl et al., 1993).

A puzzling matter in the primary photosynthetic process is that the electron flows only in one direction in spite of the presence of two branches, as has been best characterized in RCs of purple bacteria (Woodbury & Allen, 1995). This

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[1] Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; BV, benzyl viologen; DTT, dithiothreitol; FTIR, Fourier transform infrared; IR, infrared; P, primary electron donor; P_{840}, primary electron donor of *Chlorobium*; PS, photosystem; RC, reaction center.

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unidirectionality in heterodimeric RCs has been considered to arise from different protein environments around P and the electron acceptors between the two subunits. However, attempts to change the electron flow branch by manipulating polypeptide sequences by site-directed mutagenesis have not been successful so far (Woodbury & Allen, 1995). A clue to answer this question is to investigate homodimeric RCs of green sulfur bacteria or heliobacteria. It is expected that in a homodimeric RC P is exactly symmetric and environments around the cofactors are identical. If this is the case, the electron should flow in both branches with the same ratio. On the contrary, if this is not the case for some reason, the asymmetric nature and unidirectional electron flow may have some essential meaning in the photosynthetic processes.

The primary donor in RCs of green sulfur bacteria, so-called P_{840}, consists of two BChl*a* molecules like P_{870} in most purple bacteria. However, the nature of P_{840} differs from that of P_{870} in several ways; the midpoint potential is +240 mV (Fowler et al., 1971), which is much lower than that of P_{870} of about +500 mV. Also, the absorption maximum of the Q_y band is at 830−840 nm in P_{840} (Sybesma &Vredenberg, 1963; Fowler et al., 1971; Olson et al., 1973; Vasmel et al., 1984) in contrast to at about 870 nm in P_{870}. Thus, the structure and electronic coupling of P_{840} should be somewhat different from those of P_{870}.

Recently, various spectroscopies have been applied to study the structures of P_{840} in RCs of green sulfur bacteria. Rigby et al. (1994) showed by ENDOR and special TRIPLE that P_{840}^+ is a cation of BChl*a* dimer with a highly symmetrical distribution of electron spin density. Feiler et al. (1995) indicated by FT-Raman spectroscopy that the acetyl C_2═O and keto C_9═O groups of the two BChl*a* in P_{840} are free from a hydrogen bond and the positive charge is equally shared between the two BChl*a* molecules in P_{840}^+, and hence concluded that P_{840} has a symmetric structure. On the other hand, Olson et al. (1995) observed CD anisotropy in the 1160 nm band of P_{840}^+ and suggested that the configuration of P_{840} is significantly different from that of P of purple bacteria. However, the observed asymmetry of P_{840}^+ was attributed to the localization of the positive charge on one side of the two BChl*a*s on the time scale of 10^{-15} s.

FTIR spectroscopy has been extensively used to investigate the structures and molecular interactions of P in purple bacteria [reviewed by Mäntele (1995)], PS I (Nabedryk et al., 1990), and PS II (Noguchi et al., 1993). The light- or electrochemically-induced difference spectra upon formation of P^+ or ^3P have shown various vibrational modes that provide information on the interactions of C═O groups and the central Mg as well as protein structural changes. Moreover, Breton et al. (1992) found a broad absorption band near 2600 cm^{-1} in *Rhodobacter sphaeroides* and near 2750 cm^{-1} in *Rhodopseudomonas viridis* in the P^+ spectra. This band was not observed in monomeric BChla^+ in organic solution and also in the RCs of the mutants that have a BPhe in place of either P_L or P_M of the special pair BChls (Breton et al., 1992; Nabedryk et al., 1992; Davis et al., 1992), so that the band was assigned to the electronic transition characteristic of the BChl dimer (Breton et al., 1992). Their calculation (Breton et al., 1992; Parson et al., 1992) provided a substantial energy difference between the states in which a positive charge is localized on either P_L or P_M, and hence indicated the asymmetric structure of P^+. Recently, two other theoretical works on rather different bases have been reported (Reimers & Hush, 1995; Gasyna & Schatz, 1996) and argued about the electronic coupling, charge localization, and symmetry.

In this work, we applied light-induced FTIR difference spectroscopy to investigate the structure of P_{840} in the RC of a green sulfur bacterium. The used RC preparation from *Chlorobium tepidum* contains 50−70 BChl*a* molecules and consists of 5−6 polypeptides including cyt c_{551} (PscC), F_A/F_B (PscB), PscD, and the FMO protein in addition to the PscA core protein (Kusumoto et al., 1994). It has been shown that this RC preparation retains the photochemical activities to reduce the F_A/F_B centers and to oxidize cyt c_{551} (Kusumoto et al., 1994, 1995). From the obtained P_{840}^+/P_{840} spectra, we will discuss the structural symmetry, C═O interactions, and charge delocalization in P_{840} and its cation.

MATERIALS AND METHODS

The RC complex from *C. tepidum* was prepared as described previously (Kusumoto et al., 1994). Briefly, cells were disrupted using a cell disintegrator (BioNebulizer). Chromatophore membranes were obtained by centrifugation, and RC complexes were solubilized from the membranes with Triton X-100. The RC complexes were then purified by sucrose density gradient centrifugation, DEAE chromatography, and hydroxyapatite chromatography. Chlorosome-depleted membranes were prepared following the method reported previously (Fowler et al., 1971; Klughammer et al., 1995). All operations were conducted under anaerobic conditions.

Light-induced FTIR difference spectra were measured on a JEOL JIR 6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101) as described previously (Noguchi et al., 1994). The RC preparation (OD_{814} = 20; 25 mL), in a buffer containing 20 mM Tris-HCl (pH 7.8), 0.1 mM EDTA-2Na, 5 mM sodium ascorbate, 2 mM DTT, and 2.5 mM $MgCl_2$, or the membrane fragments (OD_{814} = 15; 50 mL), in a buffer containing 50 mM Tris-HCl (pH 7.8) and 5 mM sodium ascorbate, were lightly dried on a BaF_2 plate (13 mm ϕ) with N_2 gas flow, and then the wet film was covered with another BaF_2 plate. For the experiments in the presence of BV, the buffers containing 0.5 mM BV were used. The sample temperature was controlled in a cryostat (Oxford DN1704) with a controller (Oxford ITC-4). Difference spectra were obtained by subtraction between the two single-beam spectra (300 scans; 150 s accumulation for each) recorded before and after continuous-light illumination (5 s) by a halogen lamp equipped with heat-cut and red (>600 nm) filters (~30 mW/cm^2 at the sample). Spectral resolution was 4 cm^{-1}.

A light-induced visible and near-IR spectrum was measured on a Shimadzu UV-3100PC spectrophotometer. The sample preparation for the measurement, the cryostat system, and the illumination source were the same as those for the FTIR measurements. Two spectra before and after illumination (30 s) were measured, and the difference spectrum was calculated.

RESULTS

Figure 1 shows a light-induced FTIR difference spectrum (after minus before illumination) of the RC complex from *C. tepidum* in the 3200−1500 cm^{-1} region obtained at 220 K. The inset shows an absorption change in the visible and

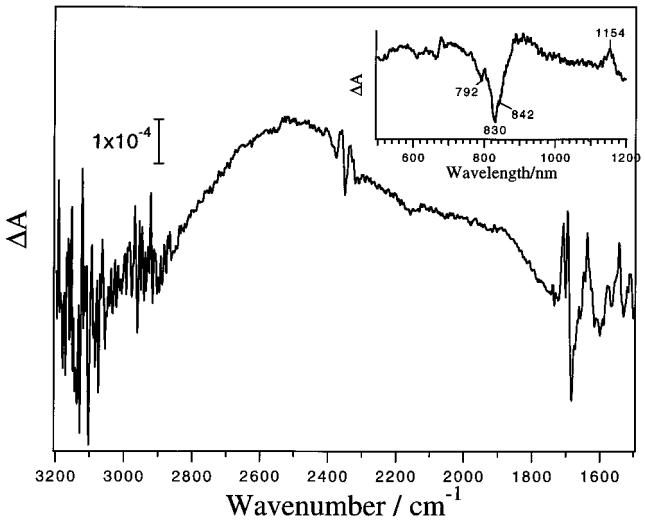

FIGURE 1: Light-induced FTIR difference spectra ($P_{840}^+FeS^-/P_{840}$FeS) of the RC complex from *C. tepidum* in the 3200−1500 cm^{-1} region. The temperature was 220 K. Small peaks at 2400−2300 cm^{-1} are due to the residual structure after subtraction of the CO_2 bands. Inset: Light-induced difference spectrum in the visible and near-IR region (500−1200 nm) measured essentially under identical conditions.

near-IR region (500−1200 nm) measured essentially under identical conditions. The band shape of the negative peak at 830 nm with minor peaks at 792 and 842 nm is very similar to the previously reported absorption changes upon P_{840}^+ formation at room temperature (Fowler et al., 1971; Olson et al., 1973; Swarthoff & Amesz, 1979; Okkels et al., 1992; Feiler et al., 1992; Kusumoto et al., 1994; Oh-oka et al., 1995a,b). Also, the positive band at 1154 nm directly indicates the formation of P_{840}^+ (Olson et al., 1976, 1995). Since no bleaching at 550−555 nm was observed in the light-induced spectrum in the visible region (Figure 1, inset), cyt c_{551} that is bound to the RC and donates an electron to P_{840} at room temperature (Okkels et al., 1992; Kusumoto et al., 1994, 1995; Oh-oka et al., 1995b) was not oxidized at this cryogenic temperature. [Refer to the chemically oxidized-minus-reduced difference spectrum of the same RC preparation (Figure 1c in Kusumoto et al., 1994) that showed a negative peak at 551 nm by cyt c_{551} oxidation.] This is in agreement with the previous observation by Swarthoff et al. (1981). Thus, P_{840} is the only species that is photooxidized in this sample. Since no exogenous electron acceptor was present in the sample, an electron was probably trapped on the terminal Fe-S acceptor, F_A/F_B. This idea was confirmed by FTIR measurement in the presence of an exogenous electron acceptor, BV (see below).

The FTIR difference spectrum ($P_{840}^+FeS^-/P_{840}$FeS) (Figure 1) showed a broad positive band centered near 2500 cm^{-1} with a shoulder at about 1900 cm^{-1}, which closely resembles that of P^+/P of purple bacteria (Breton et al., 1992). (It is noted that small peaks at 2400−2300 cm^{-1} are due to the residual structure after subtraction of the CO_2 bands, and the significant noise above 2800 cm^{-1} is due to saturated absorption of water.) The bandwidth (fwhm) was 740 ± 50 cm^{-1} when the shoulder was included, or 650 ± 30 cm^{-1} when it was neglected. The intensity of this broad mid-IR band (as a ΔA value) was smaller than the 1154 nm band in the near-IR region (inset) by a factor of about 3, although the precise quantification was difficult because of the different measurement systems.

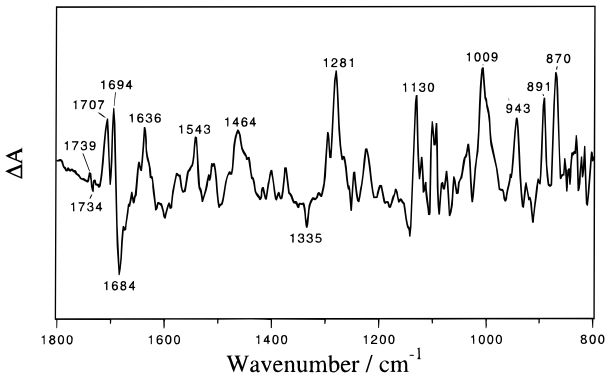

FIGURE 2: Light-induced FTIR difference spectrum ($P_{840}^+FeS^-/P_{840}$FeS) of the RC complex from *C. tepidum* in the 1800−800 cm^{-1} region. The temperature was 220 K.

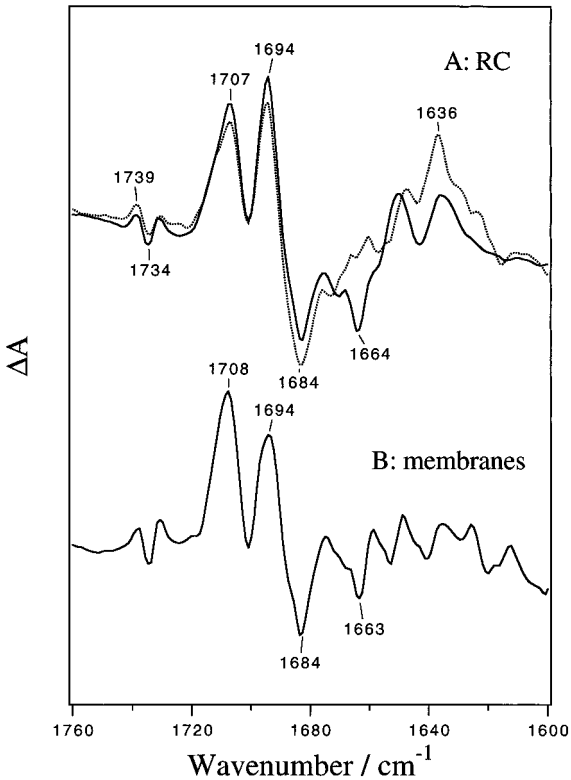

FIGURE 3: Light-induced FTIR difference spectra in the C═O stretching region of the RC complex from *C. tepidum* (A) in the presence of BV (P_{840}^+/P_{840}, solid line) compared with that in the absence of BV ($P_{840}^+FeS^-/P_{840}$FeS, dotted line), and of the membrane fragments (B) in the presence of BV (P_{840}^+/P_{840}). The temperature was 220 K.

Figure 2 shows the lower frequency region (1800−800 cm^{-1}) of the $P_{840}^+FeS^-/P_{840}$FeS spectrum. Again, the spectral features were comparable to those of the P^+/P spectra of purple bacteria (Mäntele et al., 1985, 1988; Hayashi et al., 1986; Leonhart & Mäntele, 1993; Nabedryk et al., 1993), i.e., differential signals near 1740 and 1700 cm^{-1} due to ester C_{10}═O and keto C_9═O bands of BChl*a*, respectively, and relatively strong positive signals near 1550, 1470, and 1280 cm^{-1}. The bands below 1200 cm^{-1}, e.g., the bands at 1130, 1009, 943, 891, and 870 cm^{-1}, seem more enhanced than those in the P^+/P spectra of purple bacteria (Mäntele et al., 1985).

Figure 3 shows the expanded view (1760−1600 cm^{-1}) of the C═O stretching region of the light-induced FTIR spectra. In order to investigate the contribution of the acceptor-side signal (Fe-S) to the spectrum, the difference spectrum of the

RC sample in the presence of an exogenous electron acceptor, BV, was measured (solid line) and compared with the spectrum without BV (dotted line). In this case, an electron will be abstracted out of the protein by BV, and the P_{840}^+/P_{840} spectrum free from Fe-S signals should be obtained. In fact, the spectrum substantially changed at 1670−1620 cm^{-1} by the presence of BV. The difference spectrum of BV in H_2O from its reduced form (chemically reduced by sodium dithionite) showed only a differential signal at 1628(+)/1639(−) cm^{-1} in the wavenumber region of Figure 3A (data not shown). Thus, the observed spectral changes at 1670−1620 cm^{-1} are probably attributed to the signals from the Fe-S center as well as those of BV. The reduction of the Fe-S center may cause the protein conformational changes around it, which result in changes in the amide I bands. It is noted that the spectrum in the other region of 3200−800 cm^{-1} was little changed by the BV presence (not shown), and hence the contribution of the Fe-S center in this region is much smaller compared with the P_{840}^+/P_{840} signals. This is consistent with the prediction that the main changes upon reduction of the Fe-S center will take place in the Fe-S (<450 cm^{-1}) and S-C (750−550 cm^{-1}) vibrations except for the conformational changes.

The assignments of the bands in P_{840}^+/P_{840} (Figure 3A solid line) can be achieved by analogy with the assignments of the previous P^+/P spectra of purple bacteria (Nabedryk et al., 1992, 1993) with the aid of IR and Raman data of BChl*a* and its cation *in vitro* [reviewed in Lutz and Mäntele (1991)]. The 1739/1734 cm^{-1} bands are assignable to the ester C_{10}═O, which shows a band at 1737 cm^{-1} in neutral BChl*a* in THF and slightly upshifts upon cation formation (Mäntele et al., 1988). The band position at 1734 cm^{-1} indicates that this ester C_{10}═O in P_{840} is basically free from a hydrogen bond.

The strong positive bands at 1707 and 1694 cm^{-1} are probably assigned to the keto C_9═O modes of P_{840}^+, which result from the upshift of this mode upon oxidation of BChl*a* (Mäntele et al., 1988). In fact, in various mutants of *Rb. sphaeroides*, in which hydrogen bonding of the keto C_9═O in P has been manipulated, the C_9═O frequencies of P^+ ranged from 1718 to 1678 cm^{-1} (Nabedryk et al., 1993). The implication of the appearance of two C_9═O bands in P_{840}^+ will be discussed later. The negative band at 1684 cm^{-1} is most probably the C_9═O mode of neutral P_{840}. This position is almost the same as that of C_9═O of BChl*a* in THF (1984 cm^{-1}) (Mäntele et al., 1988) and C_9═O of P_M in *Rb. sphaeroides* that is free from a hydrogen bond (1683 cm^{-1}) (Nabedryk et al., 1993). Thus, we may conclude that at least one C_9═O in P_{840} is free from a hydrogen bond.

For the negative band at 1664 cm^{-1}, there are at least three possible sources: (1) The hydrogen-bonding keto C_9═O of the other BChl*a* in P_{840}. BChl*a* in MeOH showed a C_9═O band at 1652 cm^{-1} (Mäntele et al., 1988), and the C_9═O of P in the mutants of *Rb. sphaeroides* to which hydrogen bonding was introduced showed a band at 1664−1657 cm^{-1} (Nabedryk et al., 1993). (2) The acetyl C_2═O of P_{840}. BChl*a* in THF showed this mode at 1659 cm^{-1} with an intensity a little weaker than the keto C_9═O (Mäntele et al., 1988). Upon oxidation, this band seems to upshift to near 1670 cm^{-1} [see Figure 1 in Mäntele et al. (1988)], but does not appear as an apparent positive band in the BChla^+/BChl*a* spectrum probably because it overlaps the strong negative band of the keto C_9═O (Mäntele et al., 1988). (3) The amide I band of the protein. The formation of P_{840}^+ may rearrange the structure of the surrounding protein, and this rearrangement will be reflected in the amide I changes. In fact, the negative bands near 1665 cm^{-1} in P^+/P spectra of purple bacteria have been interpreted as contributions of amide I changes (Nabedryk et al., 1992).

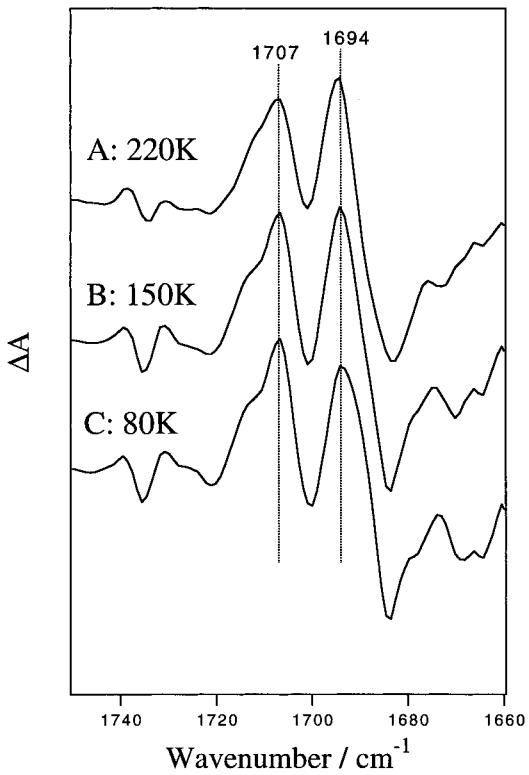

FIGURE 4: Temperature dependence of the C═O bands in the P_{840}^+-$FeS^-/P_{840}FeS$ FTIR spectrum of the RC complex from *C. tepidum*. (A) 220 K; (B) 150 K; (C) 80 K.

The P_{840}^+/P_{840} spectrum was also measured with the chlorosome-depleted membranes in the presence of BV (Figure 3B). The spectral features were basically identical between the RC and the membranes. Although the relative intensity of the two strong positive bands at 1708 and 1694 cm^{-1} differs a little from that in the RC sample, their band frequencies were essentially unchanged. This indicates that only a small difference is present in the structure of P_{840} and its protein environments between the RC and membrane preparations.

Figure 4 shows the temperature dependence of the C═O bands in the $P_{840}^+FeS^-/P_{840}FeS$ spectrum of the RC. As the temperature was lowered from 220 to 80 K, the intensity ratio of the 1707 cm^{-1} band to the 1694 cm^{-1} band increased without altering the band positions. Although some spectral changes were also observed at 1675−1660 cm^{-1}, it is likely that these are ascribed to changes of the amide I bands coupled with P_{840}^+/P_{840} and/or FeS^-/FeS. The temperature dependence of the whole region of the $P_{840}^+FeS^-/P_{840}FeS$ spectrum is shown in Figure 5. These spectra are normalized on the basis of the magnitude in the keto C_9═O region (the intensity difference between the negative 1684 cm^{-1} band and the positive band either at 1707 or at 1694 cm^{-1} with stronger intensity). The broad band centered at 2500 cm^{-1} at 220 K gradually downshifted to 2480 cm^{-1} at 150 K and to 2460 cm^{-1} at 80 K. At the same time, the bandwidth became narrower as the temperature was lowered; when we neglect the contribution of the shoulder near 1900 cm^{-1}, the

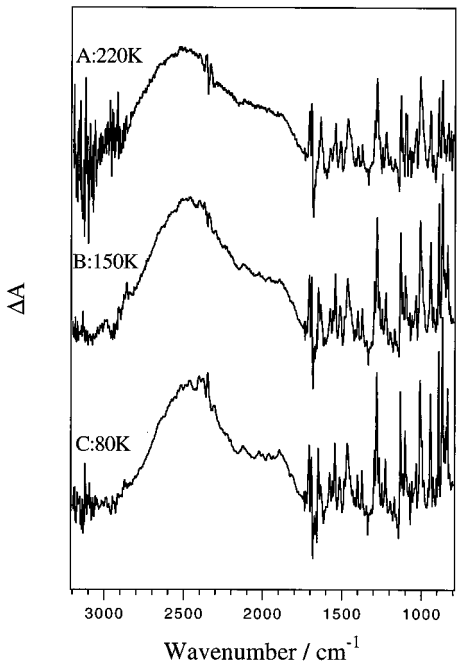

FIGURE 5: Temperature dependence of the $P_{840}^+FeS^-/P_{840}FeS$ FTIR spectrum of the RC complex from *C. tepidum* in the whole region (3200−800 cm^{-1}). (A) 220 K; (B) 150 K; (C) 80 K.

bandwidths are calculated to be 650 ± 30 cm^{-1} at 220 K, 570 ± 30 cm^{-1} at 150 K, and 500 ± 30 cm^{-1} at 80 K. By contrast, the intensity of this broad band does not seem to change by lowering the temperature. Another prominent feature is that the enhanced vibrational lines in P_{840}^+ (e.g., the bands at 835, 870, 891, 943, 1009, 1130, 1281, 1464, and 1543 cm^{-1}) increased in their intensities as the temperature was lowered. This temperature dependence seems not to be mode-specific, but rather depend on the band frequency. The intensities of the bands at 1600−1000 cm^{-1} uniformly increase by a factor of about 1.5 as the temperature is lowered from 220 to 80 K. However, the temperature effect is more pronounced for bands lower than 1000 cm^{-1}: the 943 cm^{-1} band is 2.1 times stronger, the 891 and 870 cm^{-1} bands 2.3 times stronger, and the 835 cm^{-1} band 3.1 times stronger at 80 K than at 220 K.

DISCUSSION

Broad Electronic Band Centered Near 2500 cm^{-1} and Intensified Bands in P_{840}^+. In the $P_{840}^+FeS^-/P_{840}FeS$ FTIR difference spectrum of the RC from *C. tepidum*, we observed a broad positive band centered near 2500 cm^{-1} with a shoulder at about 1900 cm^{-1} (Figure 1). This band is comparable to the band previously observed in P_{870}^+/P_{870} spectra of *Rb. sphaeroides* and *Rb. capsulatus*, which is centered at 2600 cm^{-1} with a shoulder near 2100 cm^{-1} (Breton et al., 1992). The band intensities were about the same between *C. tepidum* (Figure 1) and purple bacteria (Breton et al., 1992) when they are compared on the basis of the intensity in the BChl*a* C_9═O region. Temperature dependence was also similar; the band narrowed by about 150 cm^{-1} as the temperature was lowered from 220 to 80 K (140 K decrease) in *C. tepidum* (Figure 5), while it narrowed by about 170 cm^{-1} as the temperature was lowered from 260 to 100 K (160 K decrease) in *Rb. sphaeroides* (Breton et al., 1992). [Note that the bandwidths were estimated by omitting the contribution of the 1900 cm^{-1} shoulder in this work, whereas the shoulder was included in the estimation by Breton et al. (1992)].

Another similar feature of the P_{840}^+/P_{840} spectrum to the P_{870}^+/P_{870} spectrum is the several intensified vibrational modes of P_{840}^+. In addition to the relatively strong bands at 1543, 1464, and 1281 cm^{-1} (Figure 2), which probably correspond to the bands at 1551, 1479, and 1284−1295 cm^{-1} in *Rb. sphaeroides* (Nabedryk et al., 1993), we observed intensity enhancement in the lower-frequency bands at 1130, 1009, 943, 891, and 870 cm^{-1} as well (Figure 2). The intensification of these bands was more pronounced at lower temperatures (Figure 5).

These features of the broad band and the intensity enhancement are the characteristics of the radical cation of a BChl dimer. They were not seen in monomeric BChla^+ in organic solution and in the heterodimeric P^+ of the mutants, in which either P_L or P_M is replaced by BPhe (Breton et al., 1992; Nabedryk et al., 1992; Davis et al., 1992). Thus, the observation in this study provides strong evidence that P_{840} of green sulfur bacteria is in fact a BChl*a* dimer like P of purple bacteria.

Breton, Nabedryk, and Parson (1992) originally interpreted the broad band of P^+ as an electronic transition between supermolecular eigenstates obtained by mixing the basis states in which the positive charge of P^+ is localized on either P_L or P_M. They showed that the observed dipole strength of this band indicates a substantial difference in energy between the two basis states, $P_L^+P_M$ and $P_LP_M^+$ (the energy gap was estimated to be about 2000 cm^{-1}), implying the asymmetric structure of P^+ (Breton et al., 1992; Parson et al., 1992).

Recently, Reimers and Hush (1995) analyzed this transition of P^+ by employing the view of a weakly interacting dimer. They calculated the energy difference between $P_L^+P_M$ and $P_LP_M^+$ as 1990−2200 cm^{-1} assuming two different base lines in the spectra. They also discussed the intensified vibrational modes as the phase-phonon lines, which had been typically observed in the organic linear chain semiconductor TEA-$(TCNQ)_2$ (Rice et al., 1977), and calculation was done by taking these lines into consideration. They mentioned that whether or not the long tail beyond 3500 cm^{-1} exists is important to their calculation. It is noted that in our FTIR system, unfortunately, the base line beyond 3000 cm^{-1} is quite unstable and not reproducible, and hence we could not give information about this issue. On the other hand, Gasyna and Schatz (1996) more recently analyzed this electronic band of P^+ of purple bacteria on the basis of the unsymmetrical mixed-valence dimer. In their calculation, they were able to reproduce the band shape with a lower-frequency shoulder as well as the intensified lines below 1700 cm^{-1}. They concluded that in P^+ the positive charge is strongly delocalized.

According to the above theories, the nature of the broad band (i.e., the position, intensity, and width) and the intensified phase-phonon lines should be sensitive to the strength of electronic coupling and the asymmetric structure in P^+. Thus, the closely similar features between the P_{840}^+ and P_{870}^+ spectra indicate that they have similar electronic structures and coupling. It may be even possible that P_{840}^+ has a rather asymmetric nature like P_{870}^+, because the former two theories required a relatively large energy gap between $P_L^+P_M$ and $P_LP_M^+$ (∼2000 cm^{-1}) to fit the experimental data. In particular, according to Parson and co-workers (Parson et al., 1992; Breton et al., 1992), if the BChl dimer had a

symmetric structure (the energy gap = 0) keeping the same interaction energy, the intensity of the mid-IR electronic band would be more than 2 times larger. The fact that the peak positions of the broad band and its shoulder are lower by about 100 and 300 cm^{-1}, respectively, in P_{840}^+ than in P_{870}^+ of *Rb. sphaeroides* might reflect a slightly smaller energy gap in P_{840}^+, namely, the less asymmetric structure. Clearly, further theoretical work will be necessary to draw a more distinct conclusion about the symmetry of P_{840}^+. Calculation assuming a symmetric dimer to fit the data may be needed. On that occasion, the temperature-dependent behavior of the broad band and the intensified lines that were observed in this study will help the calculation.

Very recently, the independent study by Nabedryk et al. (1996), which reports a P_{840}^+/P_{840} FTIR spectrum of the membranes from *Chlorobium limicola* focusing on the mid-IR electronic band and intensified vibrational bands, has been published. The band features were basically identical to ours in the present study. They concluded that the structure of P_{840}^+ is close to that of purple bacterial P^+, being consistent with our conclusion.

Keto C_9=O Bands of P_{840}^+. There appeared two distinct positive bands at 1707 and 1694 cm^{-1} that are most probably assigned to the keto C_9=O of P_{840}^+ (Figure 3). Feiler et al. (1995) previously observed a keto C_9=O band at 1707 cm^{-1} in their FT-Raman spectrum of chemically oxidized P_{840} in the RC from *Chlorobium limicola*. Although this band has the same position as one of the bands observed in the present FTIR study, they did not report the other band at 1694 cm^{-1}. This discrepancy may be, of course, partly due to the difference in the bacterial species used in the two studies. However, it might be also possible that in their oxidized-minus-reduced difference Raman spectrum (Feiler et al., 1995) the other band is canceled by the large negative band around 1691 cm^{-1} that is due to the bleach of neutral P_{840}. In their original (before subtraction) Raman spectrum of P_{840}^+ [Figure 3, upper spectrum in Feiler et al. (1995)], a shoulder seems to exist at 1690−1700 cm^{-1}.

As for the presence of the two bands, the following possibilities will be considered:

(1) Heterogeneity exists in the RC preparation to provide two different states in P_{840}. This possibility is unlikely because the membrane preparation also showed similar two bands at almost the same positions (Figure 3B). Further, another RC preparation from *C. tepidum* obtained by different procedures using sucrose monolaurate (Oh-oka et al., 1995b) showed an identical light-induced FTIR spectrum (Noguchi and Oh-oka, unpublished data).

(2) Another BChl*a* molecule is photooxidized. Although it has been reported that in some RC preparations contaminating or unspecific antenna BChl*a* can be chemically oxidized (Kusumoto et al., 1992; Feiler et al., 1995), there has been no report that such a species is oxidized by light illumination. The light-induced near-IR spectrum of the present RC sample (Figure 1, inset) was basically identical to the P_{840}^+-minus-P_{840} spectra at room temperature reported so far in various RC and membrane samples (Fowler et al., 1971; Olson et al., 1973; Swarthoff & Amesz, 1979; Okkels et al., 1992; Feiler et al., 1992; Kusumoto et al., 1994; Oh-oka et al., 1995a,b). Again, the observation of the similar FTIR difference spectrum in the membrane preparation (Figure 3B) gives evidence against such contamination of unspecific BChl*a* in the FTIR spectra of the RC preparation. Thus, the possibility of photooxidation of another BChl*a* can be exclude.

(3) P_{840} has an asymmetric structure. Two cases are assumed: the positive charge is moving back and forth between the two BChl*a*s either slower or faster than the time scale of 10^{-13} s (resolution time of IR spectroscopy). In other words, the positive charge is either localized or delocalized on this time scale. In the localized case, the frequencies of the two bands reveal the difference in molecular interaction of the individual C_9=O groups, and thus the 13 cm^{-1} difference (1707 vs 1694 cm^{-1}) means considerably different environments around the two C_9=O groups. The ratio of the band intensities directly indicates the probability of finding the charge on one side, which depends on the energy gap and the temperature (Morita et al., 1993). The observed temperature dependence of the intensity ratio without changing the positions (Figure 4) is consistent with this view. In the delocalized case, on the other hand, the band positions reveal the charge distribution in the BChl*a* dimer in addition to the difference in the molecular interactions of the C_9=O groups. The larger upshift of the C_9=O frequency (from the C_9=O of neutral P_{840}) is expected for the more charged BChl*a*. When the temperature change affects the charge distribution, this effect will result in the shifts of the band positions. However, the band positions were actually little affected by the temperature change from 220 to 80 K (Figure 4).

(4) P_{840} has a symmetric structure, and the charge moves between the two BChl*a* molecules on the time scale of 10^{-13} s. Under this time scale condition, the two C_9=O bands can appear separately in P_{840}^+ even if it is completely symmetric. It has been known that oxidation of monomeric BChl*a* in THF shows an upshift by 32 cm^{-1} from 1684 to 1716 cm^{-1} (Mäntele et al., 1988). If the charge exchange is much slower than this time scale, then P_{840}^+ will have the C_9=O bands at the positions of cationic and neutral BChl*a* monomer. Since the band of neutral BChl*a* should be canceled in the difference spectrum, a large upshift of only one C_9=O band by ∼30 cm^{-1} will be observed. On the contrary, if the charge exchange is much faster than this time scale, the positive charge is just equally shared between the two BChl*a*s and hence the C_9=O groups will show only one band at the center between the cationic and neutral BChl*a*. Now, when the charge is exchanging just on the 10^{-13} s time scale, an intermediate situation will be expected; i.e., two bands with relatively large and small upshifts appear in the range between the cationic and neutral BChl*a*. If we assume that the negative band at 1684 cm^{-1} (Figure 3) is due to both C_9=O groups of neutral P_{840}, the 1707 and 1694 cm^{-1} bands in P_{840}^+ imply 23 and 10 cm^{-1} upshifts, respectively. Interestingly, the sum of these values gives a 33 cm^{-1} upshift, which is almost the same as the upshift of monomeric BChl*a* in THF (32 cm^{-1}). Also, it has been shown that the positive charge in P_{840}^+ is delocalized on the EPR time scale (10^{-10} s) (Rigby et al., 1994) but localized on the CD time scale (10^{-15} s) (Olson et al., 1995), and hence it is reasonable that the charge exchange time may be on the IR time scale (10^{-13} s).

(5) The charge on P_{840}^+ perturbs the C_9=O frequency of a nearby neutral BChl*a*. The electric field produced by the positive charge on P_{840}^+ possibly affects the vibrational modes of a nearby molecule through the so-called vibratinal

Stark effect (Bishop, 1993). When the charge on P_{840}^+ is localized on one BChl*a* in the dimer (the charge exchange time is slower than the time scale of 10^{-13} s), the C_9═O of the other neutral BChl*a* in P_{840}^+ may be perturbed to show a frequency shift. The 1694/1684 cm^{-1} band may be interpreted as this shift. The possibility cannot be excluded that such a perturbed BChl molecule is the one different from P_{840}, if it is present in close proximity of P_{840}. In either case, P_{840}^+ should have a symmetric structure; otherwise, more than two positive bands would appear in the P_{840}^+/P_{840} spectrum.

From the above considerations, interpretation (3), (4), or (5) remains as a possible mechanism that explains the presence of the two C_9═O bands in P_{840}^+. The case that P_{840} is asymmetric and the charge is moving slower than the IR resolution time is most consistent with the observed temperature difference of relative intensities with no band shift. At the present stage, however, it is difficult to decide which one is the correct mechanism.

Structure and Interaction of P_{840} in the Homodimeric RC of C. tepidum. It has been predicted that the coupling structure of the BChl*a* dimer in P_{840} is rather different from that of P_{870} of purple bacteria, because various different characteristics have been observed between P_{840} and P_{870}, e.g., the midpoint potential (+240 mV vs. +500 mV), the Q_y absorption peak (840 nm vs 870 nm), the near-IR peak of the cation state (1160 nm vs 1250 nm), and its CD anisotropy [-27×10^{-4} vs $+11 \times 10^{-4}$ (Olson et al., 1995)]. However, the electronic structure of P_{840}^+ was close to P_{870}^+ with respect to the broad electronic band in the mid-IR region and intensified vibrational modes. This close similarity as well as the presence of the two C_9═O bands of P_{840}^+ suggested the possibility of the asymmetric structure of P_{840}^+ as the case of P_{870}^+. The two C_9═O bands could be explained also with a symmetric structure, if the time constant of exchanging a charge is just on the IR time scale (10^{-13} s) or the electronic field by the charge on P_{840}^+ affects the C_9═O band of the other neutral BChl*a* in the dimer or of a nearby BChl*a*. If P_{840} has an asymmetric structure in the homodimeric RC, it is possible that this asymmetry is introduced by binding of peripheral proteins such as cyt c_{551} or F_A/F_B. Although it has been claimed that two cyt c_{551} are attached to the homodimeric core and both can donate an electron to P_{840} (Oh-oka et al., 1995b), F_A/F_B is originally asymmetric, and its binding to the RC may cause some asymmetry in the RC structure. Alternatively, the two identical polypeptides might favor an asymmetric assembly by themselves to diminish the overall potential. At the present stage, however, FTIR spectroscopy could not draw a clear conclusion about the symmetric nature. Further works on both the theoretical and experimental sides will be necessary.

From the negative bands of the P_{840}^+/P_{840} spectrum, the molecular interactions of neutral P_{840} were detected. The ester C_{10}═O band at 1734 cm^{-1} and the keto C_9═O band at 1684 cm^{-1} indicate that these C═O groups in P_{840} are free from hydrogen bonding. Previous FT-Raman data (Feiler et al., 1995) showed that acetyl C_2═O groups of P_{840} are also not hydrogen-bonded. Since it has been known that hydrogen bonding to the conjugated C═O groups of P_{870} increases its midpoint potential (Woodbury & Allen, 1995), free C═O interactions in P_{840} are basically consistent with its low potential (+240 mV). However, they cannot be the direct reason for the lower potential of P_{840} compared with that of P_{870} (+500 mV) of purple bacteria (e.g., *Rb. sphaeroides*), in which most of the C═O groups are also free from hydrogen bonding (Lancaster et al., 1995).

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