Fourier Transform Infrared Study on the Primary Donor P798 of *Heliobacterium modesticaldum*: Cysteine S−H Coupled to P798 and Molecular Interactions of Carbonyl Groups†

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ABSTRACT: Light-induced Fourier transform infrared (FTIR) difference spectra of the primary donor P798 upon its cation formation ($P798^+$/P789) were measured using the membranes and purified RC complex of *Heliobacterium modesticaldum*. A differential signal at 2550/2560 cm^{-1} was observed in the difference spectra and assigned to the S−H stretching mode of cysteine by an isotopic shift to 1854/1861 cm^{-1} upon deuteration. The observed frequencies indicate that this S−H forms a strong hydrogen bond and that the bond is further strengthened upon $P798^+$ formation. Polarized FTIR difference spectra showed that this S−H group is oriented at $<40°$ with respect to the membrane normal. It was proposed that the cysteine S−H is coupled to P798 through a hydrogen-bond network or by direct hydrogen bonding to either a P798 carbonyl or a ligand histidine. In the carbonyl stretching region, differential signals were observed at 1741/1737, 1725/1718, 1702/1693, and 1687/1666 cm^{-1}. In a dry membrane film, the signal at 1687/1666 cm^{-1} was mostly lost and hence was assigned to the amide I bands arising from the protein conformational change, which was suppressed upon dehydration of the membranes. The 1702/1693 cm^{-1} signal was assigned to the 13^1-keto C═O of P798, which was free from hydrogen bonding and had a nearly parallel orientation to the membrane plane. The upshift by 9 cm^{-1} upon P798 oxidation, which is much smaller than upshifts of monomeric (bacterio)chlorophylls [(B)Chls] in organic solution, indicates that the positive charge on $P798^+$ is significantly delocalized in a BChl*g* dimer. The signals at 1741/1737 and 1725/1718 cm^{-1} were assigned to a free and a hydrogen-bonded ester C═O group, respectively. The dichroism measurement showed that the C═O of 1741/1737 cm^{-1} was oriented nearly parallel to the membrane plane while that of 1725/1718 cm^{-1} was considerably tilted by $<31°$ to the membrane normal. It was proposed that one of the two ester signals arose from the 13^2-carbomethoxy C═O of P798 while the other arose either from the 17^2-ester C═O of P798 or from an ester C═O of adjacent BChl*g* or 8^1-OH-Chl*a* that was electrostatically influenced by oxidation of P798.

Heliobacteria are unique photosynthetic bacteria characterized by the presence of bacteriochlorophyll (BChl)[1] *g* as a major pigment [reviewed by Madigan and Ormerod (*1*) and Amesz (*2*)]. Heliobacteria possess neither specialized light-harvesting proteins nor chlorosomes, and all antenna pigments are directly associated with the reaction center (RC) protein. In *Heliobacillus mobilis*, only a single gene coding for a 68-kDa RC polypeptide has been identified, thus suggesting that the RC of heliobacteria has homodimeric organization (*3*). Similar to PS I and the RC of green sulfur bacteria, the RC of heliobacteria is an iron−sulfur type RC, which contains an iron−sulfur center as the terminal electron acceptor and a Chl*a*-like primary electron acceptor. The chemical structure of the primary electron acceptor of heliobacteria has been identified as 8^1-OH-Chl*a* (*4*).

The primary donor of heliobacteria, P798, has been thought to be a dimer of BChl*g* or its 13^2-epimer, BChl*g*′ (*5*). The redox potential of P798 is +225 mV (*6*), which is comparable to that of P840 of green sulfur bacteria (+240 mV) (*7*) but much lower than that of P870 of purple bacteria (∼+500 mV) (*8*), P700 of PS I (∼+500 mV) (*9*), or P680 of PS II (∼+1100 mV) (*10*). These potentials not only determine the electron transfer rates but are also directly related to RC functions such as oxygen evolution and NAD(P)H production. The factors that determine the redox potential of a primary donor remain unclear. While hydrogen bonding of the conjugated carbonyl groups significantly affects the redox potential (*8*), hydrogen bonding alone cannot explain the very low potential of P840 (*11, 12*) and the very high potential of P680 (*13*). Hence, the potential must be elaborately controlled by the protein environment around the primary donor through hydrogen bond networks and electrostatic effects.

Light-induced FTIR difference spectroscopy is a powerful tool with which to investigate the structure and molecular

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[1] Abbreviations: BChl, bacteriochlorophyll; BV, benzyl viologen; Chl, chlorophyll; FTIR, Fourier transform infrared; *H., Heliobacterium*; *Hc., Heliobacillus*; IR, infrared; P, primary electron donor; P798, primary electron donor of heliobacteria; PS, photosystem; RC, reaction center; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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interactions of cofactors in photosynthetic RCs (for reviews, see refs 14 and 15). This kind of analysis provides information not only of the cofactor itself but also of surrounding protein moieties including side-chain interactions and subtle changes in secondary structures. Light-induced FTIR difference spectroscopy is also an appropriate monitor to detect the formation and breakage of hydrogen bonds and the changes in the electronic states of pigments upon site-directed mutation (*16*−*18*). Primary donors in purple bacteria (reviewed in refs 14 and 15), PS I (*19, 20*), PS II (*13*), and green sulfur bacteria (*12, 20*) have been studied by detecting the light-induced differences upon cation or triplet formation. Recently, Nabedryk et al. (*20*) reported a $P798^+$/P798 FTIR difference spectrum, obtained using the membranes from *Hc. mobilis*, that focused on the broad electronic band in the mid-IR region and the phase-phonon bands, which are characteristic of the dimeric nature of a primary donor. However, they did not discuss the molecular interactions of P798 and structural changes in the protein. On the other hand, Liebl et al. (*21*) obtained Soret-excitation resonance Raman and near-IR-excitation FT-Raman spectra of the membranes from *Hc. mobilis*. Although they found that keto carbonyl groups of bulk BChl*g* molecules were engaged in hydrogen bonding, the bands specific to P798 were not observed. Thus, molecular interactions of P798 and its protein environment have yet to be clarified.

In the present study, we obtained light-induced FTIR difference spectra of P798 upon oxidation from the membranes and purified RC complex of *Heliobacterium modesticaldum*. In the spectra, we found a cysteine S−H signal that was structurally coupled to P798 and changed its hydrogen bond strength upon $P798^+$ formation. Molecular interactions of the carbonyl groups of P798 were also investigated. Measurement of polarized FTIR difference spectra with an oriented membrane film aided the assignments of the C═O bands and provided information on the orientations of the S−H and C═O groups.

MATERIALS AND METHODS

H. modesticaldum was grown anaerobically for 40 h in PYE medium (*22*) in a 1-L bottle. Although the optimal temperature for the growth of *H. modesticaldum* is 50−52 °C, cultivation was performed at 46 °C in order to avoid accumulation of appreciable amounts of lysed cells late in the logarithmic growth phase. For cultivation of partially deuterated cells, *H. modesticaldum* was grown in medium containing 50% (v/v) D_2O. To achieve this, cells were cultivated first in medium with 15% D_2O, then 30% D_2O, and finally 50% D_2O.

The following procedure that describes the preparation of the membranes and RC complex was performed under anaerobic conditions as described previously (*23*). All buffers were fully degassed and flushed with N_2 gas before use.

Cells were harvested by centrifugation and washed once with buffer A [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mM ascorbate]. They were then suspended in 20 mL of buffer A and disrupted by sonication. After removing unbroken cells by centrifugation at 12000*g* for 10 min, the membranes were collected by ultracentrifugation at 200000*g* for 1 h and resuspended in buffer A. For preparation of the partially deuterated membranes, a 50% (v/v) D_2O buffer containing the same components as buffer A was used.

The RC complex was solubilized from the membranes by adding sucrose monolaurate (SM-1200, final concentration of 20 mM) to the membrane suspension (OD_{790} = 30). The solution was stirred for 1 h in the dark at room temperature, followed by ultracentrifugation at 250000*g* for 1 h. The green supernatant was applied to a stepwise sucrose gradient (30 and 35% (w/v)) in buffer A containing 2 mM SM-1200 and centrifuged at 50000*g* for 2 h. The dark green band that formed just below the interface between the 30 and 35% (w/v) sucrose solutions was collected and mixed gently with a saturated $(NH_4)_2SO_4$ solution (100%) to a final concentration of 40% saturated $(NH_4)_2SO_4$. The resultant solution was loaded onto a 1.5 × 4.0 cm Toyopearl HW-65F column equilibrated with buffer B [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM ascorbate, 2 mM SM-1200, and 40% saturated $(NH_4)_2SO_4$]. After the column was washed with one-column volume of buffer B, the green fraction was eluted by decreasing the saturated $(NH_4)_2SO_4$ concentration from 40 to 30%. The purified RC complex thus obtained consisted of a single core polypeptide as revealed by SDS−PAGE analysis. The RC sample was desalted and concentrated using Microcon-100 (Amicon).

For spectroscopic measurements, wet samples and oriented dry films were prepared. For a wet sample, the membrane suspension (OD_{790} = 180; 5 μL) in buffer A or the RC complex (OD_{790} = 390; 10 μL) in buffer A with 2 mM SM-1200 was mixed with 10 μL of 3 mM BV solution and lightly dried on a BaF_2 plate (13 mm Φ) under N_2 gas flow. The wet sample was then covered with another BaF_2 plate. For an H/D exchange experiment and measurement of the partially deuterated membranes, a D_2O buffer (pD 8.0) containing the same components as buffer A was used. An oriented dry membrane film was prepared by slowly drying the membranes (OD_{790} = 180; 10 μL) suspended in 5 mM Tris-HCl (pH 8.0), 2 mM ascorbate, and 1.5 mM BV on a CaF_2 plate under a N_2-gas atmosphere. During the drying procedure, the sample was kept at 0 °C on ice in the dark.

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 (*24*). 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). Spectra were measured at 220 K with a 4 cm^{-1} resolution except where otherwise noted.

Polarized FTIR difference spectra were measured with the oriented dry membrane films by tilting the sample at 45° with respect to the direction of the IR beam. IR light was polarized using a wire grid polarizer (ST-Japan STJ-1001) either perpendicular to the normal of the membrane plane or parallel to the incidence plane. The dichroic ratio, R, was defined as $\Delta A_{\parallel}/\Delta A_{\perp}$, in which $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are the absorbance changes in parallel and perpendicular polarization, respectively. Analysis of the polarized FTIR spectra was performed according to previously described methods used for purple membranes (*25, 26*) and photosynthetic membranes (*27*). A value of n = 1.5 was used for the refractive index of the dry membranes (*27*).

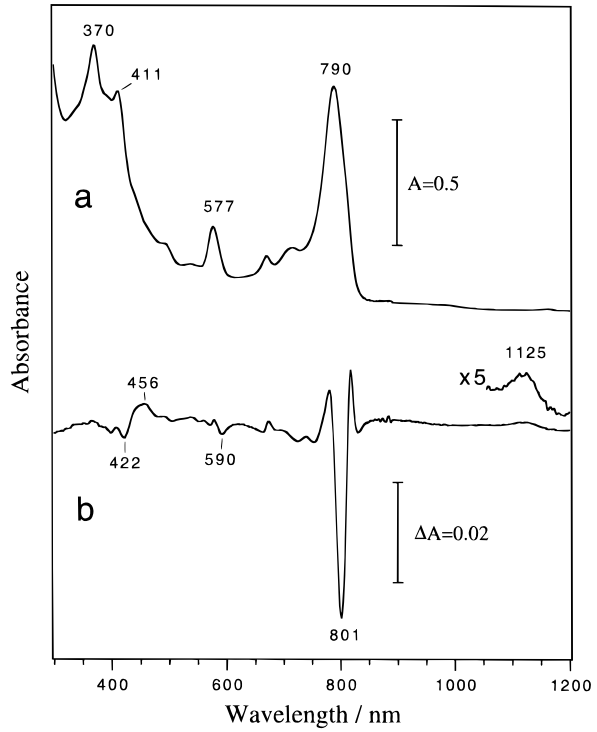

FIGURE 1: Electronic absorption spectrum in 300−1200 nm of the membranes of *H. modesticaldum* (a) and its light-induced (after-minus-before illumination) difference spectrum (b) measured 220 K. BV was added as an exogenous electron acceptor.

Electronic absorption spectra in the 300−1200 nm region were measured on a Shimadzu UV-3100PC spectrophotometer. Sample preparation for measurement, the cryostat system, and the illumination source were the same as those used for FTIR measurement. For light-induced difference spectra, spectra before and after illumination (30 s) were measured and the differences were calculated.

RESULTS

The electronic absorption spectrum between 300−1200 nm and its light-induced difference spectrum of the membranes of *H. modesticaldum* are shown in Figure 1. The sample and measuring conditions were the same as those used to obtain the FTIR difference spectrum shown in Figure 2a, that is, a wet membrane sample in the presence of BV as an exogenous electron acceptor and a temperature of 220 K. The difference spectrum (Figure 1b) is almost identical to the $P798^+$/P798 spectrum previously reported for the membranes and RC complexes from *H. chlorum* and *Hc. mobilis* (*28*). A large negative band at 801 nm indicates the photooxidation of P798. A small positive band was observed at 1125 nm, which is probably a near-IR absorption band of $P798^+$ comparable to the near-IR bands that have been seen in the cation states of other primary donors. Absence of a band at 553 nm indicates that cytochrome *c*-553 (*29*) did not donate an electron to P798 at this low temperature. Similar blockage of electron donation from cytochrome *c*-553 to $P798^+$ at cryogenic temperatures has been observed in *H. chlorum* (*6*). A difference spectrum obtained 10 min after illumination (data not shown) was almost the same as the spectrum obtained immediately after illumination (Figure 1b), indicating that $P798^+$ was stably stored at 220 K. When the sample was warmed to 278 K to relax the charged state and then cooled down to 220 K again, exactly the same light-induced difference spectrum was produced with the same intensity. This indicates that no irreversible change occurred in the membrane preparation upon illumination at 220 K.

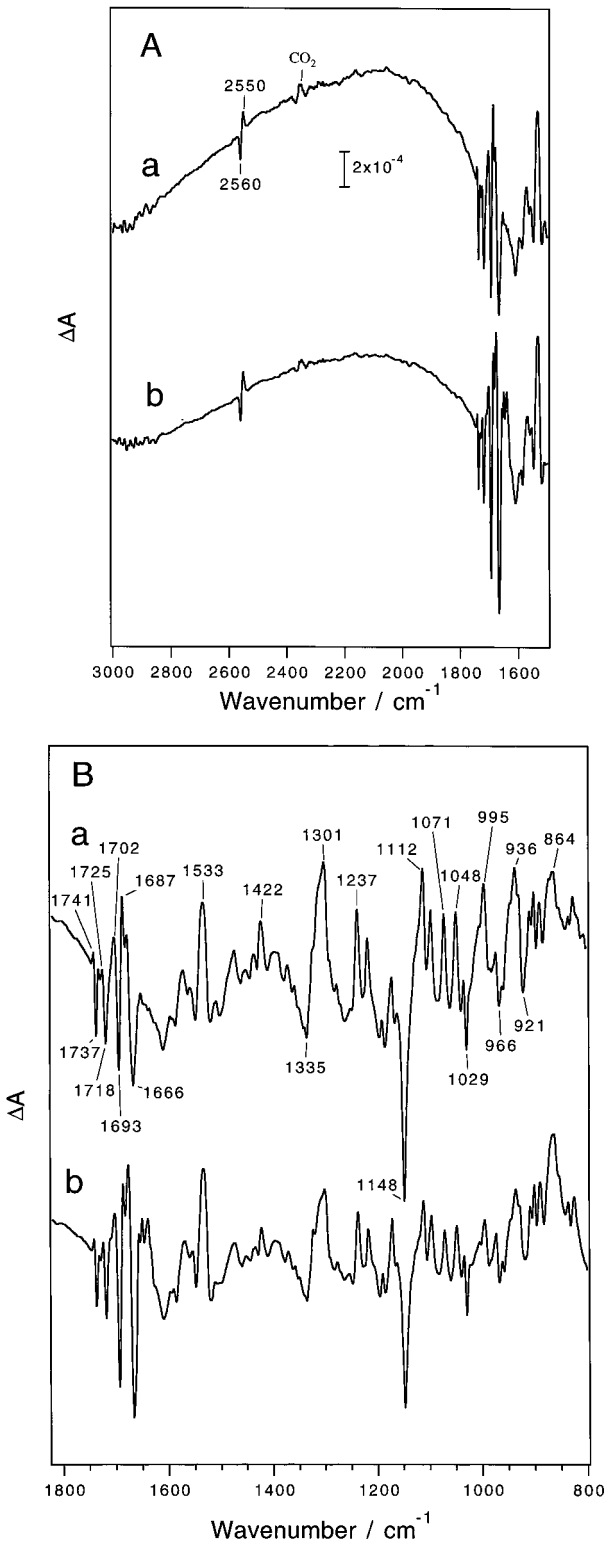

FIGURE 2: Light-induced FTIR difference spectra of P798 upon its cation formation ($P798^+$/P798) measured with the membranes (a) and the purified RC complex (b) of *H. modesticaldum.* (A) 3000−1500 cm^{-1}; (B) 1800−800 cm^{-1}. Temperature was 220 K. BV was added as an exogenous electron acceptor. Small bands at about 2300 cm^{-1} are artifacts by CO_2 absorption.

Figure 2 shows light-induced FTIR difference spectra of (a) the membranes and (b) the RC complex of *H. modesti-*

caldum upon $P798^+$ formation ($P798^+/P798$). All the bands are observed at identical positions between the membranes and the RC complex, although relative intensities are somewhat different. Because the RC complex comprises only a single polypeptide and does not contain cytochrome *c*-553 and F_A/F_B proteins, the identical band positions in the two spectra indicate that changes of neither cytochrome *c*-553 nor F_A/F_B are included in these difference spectra. Also, because the formed $P798^+$ was stably stored at 220 K, BV must have abstracted an electron out of the RC protein. A difference spectrum between neutral BV in solution and chemically-reduced (by dithionite) BV showed major bands at 1639 and 1450 cm^{-1} with negative intensities (neutral BV) and at 1628, 1591, and 1493 cm^{-1} with positive intensities (reduced BV) (data not shown). No appreciable bands can be observed at these positions in Figure 2B, indicating that the contribution of BV bands in the spectrum is small and does not interfere with the $P798^+/P798$ bands. The difference in the relative band intensities between the membranes and the RC complex might be related to the absence of peripheral proteins in the RC preparation. However, as will be shown later, the relative intensities of some bands are highly sensitive to the hydration status of the samples, and hence the real effect of peripheral proteins on the spectrum is unknown at present.

The most prominent feature of the $P798^+/P798$ spectrum is a broad positive band around 2100 cm^{-1} (Figure 2A). An almost identical band has been reported for the $P798^+$ spectrum of the membranes of *Hc. mobilis* by Nabedryk et al. (*20*) and was assigned to the electronic transition characteristic of the cationic state of dimeric BChls. Comparable mid-IR electronic bands have been observed in the cations of primary donors in purple bacteria (*16*) and green sulfur bacteria (*12, 20*).

Complex band features are evident in the 800−1500 cm^{-1} region (Figure 2B), which were most likely due to the various macrocycle ring modes of BChl*g*. One explanation for the predominance of positive bands over negative bands may be the intensity enhancement mechanism of phase phonon lines (30), which is characteristic of the cations of dimeric BChls (*12, 20*). The bands around 1533 cm^{-1} are ascribable to the amide II modes (NH bending modes of polypeptide backbones) as well as the macrocycle C═C modes of BChl*g*.

Several positive and negative bands are evident at 1600−1750 cm^{-1} (Figure 2B), which is the C═O stretching region of BChl*g* and protein backbones. The C═O modes of (B)Chls have been extensively investigated both *in vitro* and *in vivo* (reviewed in refs 31 and 32), and approximate assignments can be performed according to general criteria. Also, it has been reported that the C═O bands of conjugated carbonyl groups shift to higher frequencies upon cation formation (*19, 33*). The negative bands at 1666 and 1693 cm^{-1} and the positive bands at 1687 and 1702 cm^{-1} are candidates for the 13^1-keto C═O stretching modes (for the carbon numbering of BChl*g*, see ref 5) of P798 and its cation. The contribution of the amide I bands (C═O stretching bands of polypeptide backbones) is also expected in this region. On the other hand, the negative bands at 1718 and 1737 cm^{-1} and the positive bands at 1725 and 1741 cm^{-1} are candidates for the 13^2-carbomethoxy C═O modes of P798 and its cation. Assignment of the C═O bands is discussed later in more detail.

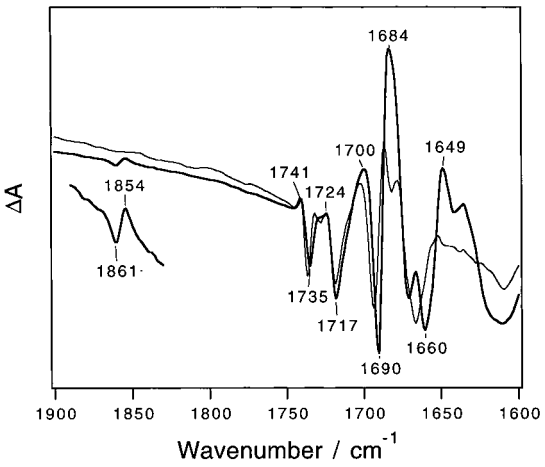

FIGURE 3: $P798^+/P798$ FTIR difference spectrum of the partially deuterated membranes of *H. modesticaldum* cultured in a 50% D_2O medium (thick line), compared with that of the normal membranes (thin line). Temperature was 220 K.

A unique feature in the $P798^+/P798$ spectra is the presence of a differential signal at 2550/2560 cm^{-1} (Figure 2A). These vibrational frequencies are in the region typical of the S−H stretching mode of a cysteine side chain, 2520−2600 cm^{-1} (*34*−*38*). Because no other compounds included in the protein complexes have fundamental frequencies in this region (*39*), this signal most probably represents the cysteine S−H mode.

In order to confirm this assignment and exclude the possibility of overtones and combinations of lower-frequency modes, an H/D exchange experiment was performed. A membrane sample suspended in D_2O buffer was incubated at 6 °C overnight or at room temperature for 3 h. However, in the $P798^+/P798$ spectra of these samples, no band was evident in the S−D stretching region of 1820−1900 cm^{-1} (*34, 35, 37, 38*) (data not shown). In the original FTIR spectra (before taking difference) of both the membrane samples, about half of the amide II band around 1550 cm^{-1} was converted to an amide II′ band around 1450 cm^{-1} due to the H/D exchange of backbone amides, indicating that deuteration of the hydrophilic region of the RC protein had satisfactorily occurred.

In order to deuterate the cysteine S−H in the hydrophobic region of the protein, *H. modesticaldum* was cultivated in a 50% D_2O medium. A $P798^+/P798$ spectrum obtained using the membranes prepared from these partially deuterated cells is shown in Figure 3 (thick line). A differential signal appeared at 1854/1861 cm^{-1} ascribable to the S−D stretching mode. The isotopic frequency shifts (ν_{SD}/ν_{SH}) were 0.7271 and 0.7270 for the 2550 and 2560 cm^{-1} bands, respectively, which are identical to the reported shift of L-cysteine in $H(D)_2O$, 0.7270 (*37*). This result confirms the assignment of the 2550/2560 cm^{-1} signal to the cysteine S−H. The intensity of the S−D signal was smaller than the S−H signal by a factor of 5 (with respect to the peak height), when the spectra were normalized at the 1735−1737 cm^{-1} band. Because 50%, at most, of S−H would have changed to S−D, for practical purposes, the S−D signal was smaller than the S−H signal by a factor of 2.5. This is in good agreement with a previous report that the S−H band in hemoglobin lost its intensity by a factor of about 3 upon deuteration (*35*). It should be noted that the S−H band in the partially deuterated spectrum could not be detected due to the overlap

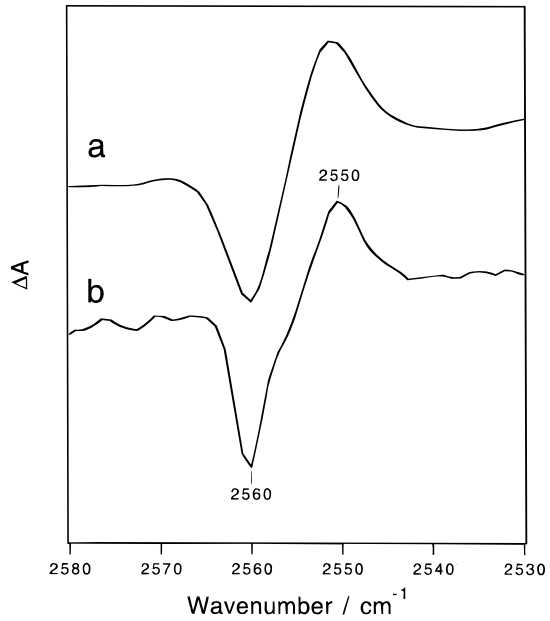

FIGURE 4: S−H stretching region in the $P798^+$/P798 FTIR spectra of the membranes measured at 220 K with a 4 cm^{-1} resolution (a) and at 80 K with a 2 cm^{-1} resolution (b).

of a saturating D_2O band in the same region. In the spectrum of partially dueterated $P798^+$/P798, most of the bands in the C═O stretching region showed slight downshifts, which may have been due to minor mixing of the C═O modes with hydrogen-involving modes of BChl*g* or the protein.

An expanded view of the S−H signal in the $P798^+$/P798 spectrum is shown in Figure 4. Measurement at the lower temperature of 80 K with a higher resolution of 2 cm^{-1} (Figure 4b) resulted in clearer resolution of band shape than that at 220 K with a 4 cm^{-1} resolution (Figure 4a). The positive band at 2550 cm^{-1} is a little broader than the negative band at 2560 cm^{-1}; the width (full width at half maximum) of the 2560 cm^{-1} band is 4.1 cm^{-1} while that of the 2550 cm^{-1} band is 5.7 cm^{-1} (Figure 4b). The spectral shape in Figure 4b also shows that the peak frequencies indeed indicate the respective band positions and are not apparent values resulting from a difference between only slightly separated bands.

Figure 5 shows a $P798^+$/P798 difference spectrum of a dry membrane film (thick line) compared with that of a wet sample (thin line: the same spectrum as Figure 2a). Because the light-induced difference spectrum of electronic absorption (300−1200 nm) of a dry film (not shown) was almost identical to that of a wet sample (Figure 1), no changes in the redox reactions in the protein are thought to be induced by the drying procedure. As evident in Figure 5A, the broad electronic band becomes much larger when the membrane sample was dried. Also, in the C═O stretching region, the bands at 1687/1666 cm^{-1} in the wet sample spectrum lost most of their intensity in the dry film spectrum (Figure 5B). Because the bands of the BChl*g* dimer should remain in the spectrum in any case, these diminished bands very likely represent the amide I bands of protein backbones. This description is supported by the observation that the positive band at 1533 cm^{-1}, which is most likely the amide II band, also lost its intensity (Figure 5A). Thus, the remaining negative band at 1693 cm^{-1} in the keto C═O region (1700−1650 cm^{-1}) can be automatically assigned to the keto C═O of the neutral P798. The bands at frequencies lower than

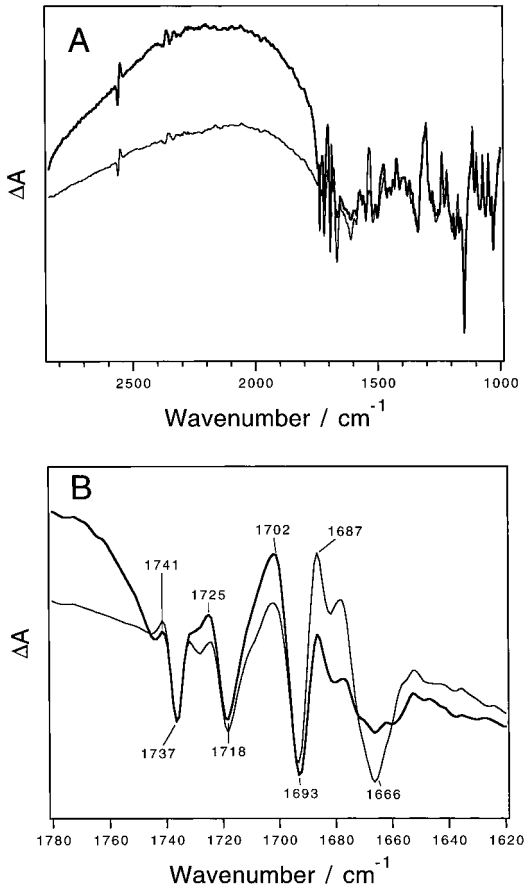

FIGURE 5: $P798^+$/P798 FTIR difference spectrum of a dry membrane film measured at 220 K (thick line), compared with that of a wet sample (thin line). (A) Whole region; (B) C═O stretching region. The two spectra were approximately normalized with the bands in the 1000−1400 cm^{-1} region.

1500 cm^{-1}, which may arise from the complex ring modes of BChl*g*, were not affected by the drying procedure.

The presence of the amide I bands in the $P798^+$/P798 difference spectrum of the wet membranes indicates the occurrence of protein conformational changes upon $P798^+$ formation. Although the reason why such conformational changes were suppressed in the dry membrane sample is unknown at present, a similar observation has been reported for the $P870^+$/P870 spectrum of the *Rhodobacter sphaeroides* RC, in which the amplitude of the 1665/1658 cm^{-1} bands, which were assigned to the amide I modes, was sensitive to the hydration state of the sample (*20*). The increased intensity of the broad electronic band may be due to a subtle change in the electronic coupling of the BChl*g* dimer caused by dehydration of the membranes.

Figure 6 shows polarized FTIR difference spectra of $P798^+$/P798 measured for a dry membrane film. The broad electronic band around 2100 cm^{-1} showed almost no dichroism (Figure 6A), indicating that this transition dipole was oriented nearly parallel to the membrane plane. This observation is in agreement with that of the similar electronic band of purple bacterial P^+, in which the dipole is oriented at an angle of greater than 60° with respect to the membrane normal (*16*). The S−H signal at 2550/2560 cm^{-1} exhibited relatively strong dichroism, and a dichroic ratio (*R*) of 1.4 was obtained for both the positive and negative peaks (Figure 6B). The extent of mosaic spread of the dry membranes is unknown, and thus only the maximum value of the dipole

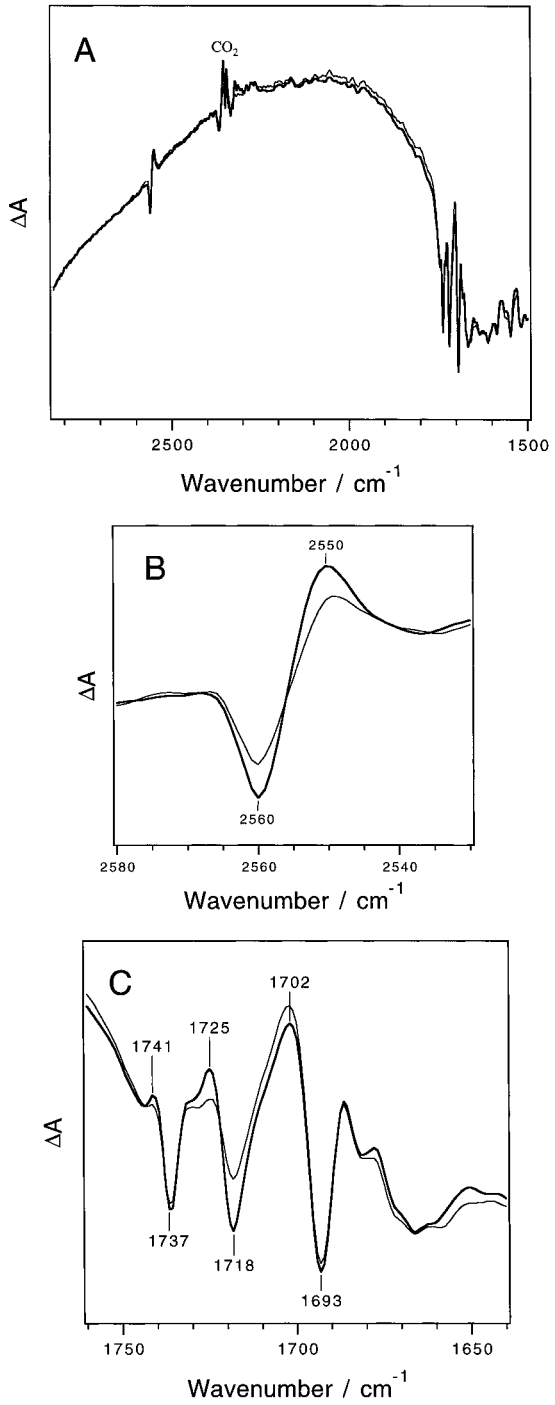

FIGURE 6: Polarized FTIR difference spectra of $P798^+$/P798 measured with a dry membrane film at 220 K. (A) Region of a broad electronic band; (B) S−H stretching region; (C) C═O stretching region. The oriented membrane film on a CaF_2 plate was tilted at 45° with respect to the direction of an IR beam. The spectra were measured with IR light polarized either perpendicular to the normal of the membrane plane (thin line) or parallel to the incidence plane (thick line). Bands at about 2300 cm^{-1} are artifacts by CO_2 absorption.

angle could be estimated. The angle of the S−H group was estimated to be less than 40° with respect to the membrane normal. In the C═O stretching region, a pair of positive and negative bands at 1725/1718 cm^{-1} showed strong dichroism, while pairs at 1702/1693 and 1741/1737 cm^{-1} did not. [Although the small positive band at 1741 cm^{-1} appears to show some dichroism, at 80 K this band appeared with a much higher intensity with almost no dichroism (data not shown).] Because the C═O orientation can be assumed not to change significantly upon radical formation, this result indicates that the positive and negative bands in each pair originate from the same C═O group. From the dichroic ratio of the 1725/1718 cm^{-1} signal, $R = 2.0$, this C═O group is estimated to be tilted by less than 31° to the membrane normal. In contrast, both the C═O groups that show signals at 1741/1737 and 1702/1693 cm^{-1} are oriented nearly parallel to the membrane plane.

DISCUSSION

Cysteine S−H Coupled to P798. In the $P798^+$/P798 difference spectrum, the S−H signal of cysteine was found at 2550/2560 cm^{-1} (Figure 4), and the S−H assignment was confirmed by its deuteration (Figure 3). Thomas and co-workers extensively studied the effects of hydrogen bonding and C−C−S−H geometry on the S−H frequency using model mercaptans and L-cysteine (*36*, *37*). When the S−H group is non-hydrogen bonded, its stretching band was found at 2585 ± 5 cm^{-1}, while the band was found at 2575−2580 cm^{-1} for a weakly hydrogen bonded S−H, at 2560−2575 cm^{-1} for a moderately hydrogen bonded S−H, and at 2525−2560 cm^{-1} for a strongly hydrogen bonded S−H (*36*). Also, rotamers with respect to the C−S torsion yielded S−H frequencies that were separated by <10 cm^{-1} (*36*, *37*). Thus, the S−H frequencies at 2550 and 2560 cm^{-1} found in the present study indicate that this S−H group is strongly hydrogen bonded both before and after $P798^+$ formation. The downshift of the S−H band by 10 cm^{-1} upon $P798^+$ formation indicates either that the hydrogen bonding was further strengthened or that the conformation of C−S torsion changed. The idea that this shift is simply caused by the electrostatic effect of $P798^+$ formation can be excluded by the relatively large frequency shift that accompanied the change in bandwidth (Figure 4). Because the dichroic ratios of the 2560 and 2550 cm^{-1} peaks were almost identical (Figure 6B), i.e., the orientation of the S−H (<40° to the membrane normal) did not change upon $P798^+$ formation, a change in the C−S conformation seems unlikely. In addition, Li and Thomas (*36*) suggested that the above two cases can be distinguished by the width of an S−H band, i.e., the broader the band, the stronger the hydrogen bond. In agreement with this, we observed a broader width for the 2550 cm^{-1} band than for the 2560 cm^{-1} band (Figure 4), supporting stronger hydrogen bonding of the 2550 cm^{-1} band. However, the possibility that the broad 2550 cm^{-1} band resulted from the overlap of the two S−H bands with slightly different frequencies cannot be excluded (see below).

Although protein conformational changes upon $P798^+$ formation were suppressed in dry membranes, the S−H signal was basically unaffected (Figure 5), thus indicating that the S−H movement was not a secondary effect of protein conformational changes. The cysteine S−H is most likely involved in a hydrogen bonding network connected to P798 through its C═O groups or a ligand histidine. The relatively large frequency shift of 10 cm^{-1} suggests that this S−H is located rather close to P798. An attractive idea is that this cysteine is directly hydrogen bonding to the ligand histidine or to the carbomethoxy C═O that shows a hydrogen bonding frequency of 1718 cm^{-1} (see below). The close structural relationship between the cysteine S−H and P798 in turn indicates that this cysteine may have some influence on the structure of P798 and its electron transfer function. Such a

coupling of cysteine to the primary donor has not been observed in other RCs and photosystems. Hence, this coupling might be related to the specific properties of P798, such as its extremely low redox potential (~+225 mV).

The S−H signal was also observed in the $P798^+$/P798 spectrum of the purified RC complex that lacks peripheral proteins (Figure 2A), and thus this cysteine residue is a component of the 68-kDa RC polypeptide. In addition, the deuteration experiments showed that this cysteine is present in the hydrophobic region of the protein. The amino acid sequence of the RC protein of heliobacteria has been reported only for *Hc. mobilis* (*3*). In the $P798^+$/P798 FTIR spectrum of *Hc. mobilis* recently measured by Nabedryk et al. (*20*), a differential signal at about 2550 cm^{-1} that is almost identical to the S−H signal in the present study was observed. Although they did not mention the presence of this signal, it most probably originated from the same cysteine residue as in *H. modesticaldum*. Hence, the cysteine residue coupled to P798 in *H. modesticaldum* should be conserved in *Hc. mobilis*. Eight cysteine residues are present in the RC protein of *Hc. mobilis*, among which Cys-471 at the border of helix IX, Cys-534 in helix X, and Cys-602 and Cys-605 in helix XI are found in the predictable hydrophobic region (*3*). Recent mutational studies in *Chlamydomonas* PS I proposed the conserved histidine residues in helix X as candidates for the ligands of P700 (*40*). Also, helices IX, X, and XI in PS I are thought to correspond to helices C, D, and E, respectively, in the RC of purple bacteria (*41*, *42*). X-ray crystallography has shown that helix D coordinates P and that helices C and E are proximate to P (*43*−*45*). The residue that is a candidate for a ligand of P798 in *Hc. mobilis* is His-538 in helix X, and all four cysteine residues mentioned above are located on the periplasmic side of helices IX, X, and XI. Therefore, all these cysteines are candidates for the cysteine coupled to P798. Mutational studies of the RC protein of heliobacteria are required to identify this cysteine residue as well as to clarify its roles in the structure and function of P798.

Because only a single gene for an RC polypeptide has been found in *Hc. mobilis*, the RC of heliobacteria is thought to be a homodimer comprising two identical polypeptides (*3*). The S−H signal observed in the present study most likely originates from the two quasi-equivalent cysteine residues in the homodimeric RC. Whether the homodimeric RC of heliobacteria as well as that of green sulfur bacteria do indeed have C_2 symmetry with equivalent electron flow reactions in the two pigment branches is an intriguing question when one considers the evolution of heterodimeric RCs and the essential importance of this evolution in photosynthesis. In the present study, however, no positive evidence of an environmental difference between the two cysteines such as splitting of the S−H signal was found. Although the broader S−H band at 2550 cm^{-1} (Figure 4) possibly resulted from the overlap of two S−H bands with slightly different frequencies caused by symmetry breakdown upon $P798^+$ formation, an environmental difference between the two S−H groups, if any, would not be large.

An S−H stretching band is not interfered with by other vibrational modes and thus can be a useful marker for monitoring the protein environment. This has been demonstrated in studies on various proteins such as hemoglobin (*34*, *35*, *46*) and rhodopsin (*38*, *47*). The S−H signal found in the present study will also be a useful monitor to detect the changes in the environment around P798 upon various site-directed mutations, which will help elucidate the structure of P798 and the factors that determine its redox potential.

Molecular Interactions of the C═O Groups of P798. The 13^1-keto C═O band of P798 was observed at 1693 cm^{-1} in its neutral state, indicating that this C═O is free from hydrogen bonding (*48*−*51*). As shown in purple bacteria, a hydrogen bond of the keto C═O of P increases its potential by 60−125 mV (*8*). Thus, the absence of hydrogen bonding in the keto C═O of P798 is consistent with its low redox potential of +225 mV (*6*). However, other factors must be involved in determining the potential, because P870 of wild-type *Rb. sphaeroides*, which has a potential of about +500 mV, also does not form hydrogen bonds in its keto C═O groups.

Upon oxidation of P798, the keto C═O frequency upshifted by 9 cm^{-1} to 1702 cm^{-1}. Although an FTIR spectrum of BChlg^+ in solution has not yet been reported, the tendency of spectral change upon oxidation may not differ greatly among (B)Chl species; in spite of the differences in macrocycle structures, BChl*a*, BChl*b*, and Chl*a* in THF solution show similar upshifts of the keto C═O bands upon their oxidation by 32, 19, and 26 cm^{-1}, respectively (*19*, *33*). The 9 cm^{-1} upshift observed in the P798 spectrum upon oxidation is much smaller than these values of monomeric (B)Chls. This suggests that the positive charge on $P798^+$ is significantly delocalized in a BChl*g* dimer.

Polarized FTIR measurement showed that the keto C═O of P798 is oriented nearly parallel to the membrane plane (Figure 6C). This is in agreement with the keto C═O orientation of P in purple bacteria clarified by X-ray crystallography (*43*, *52*). This agreement suggests that the orientation of the BChl*g* molecules in P798 is basically similar to that of the BChl*a* molecules in purple bacterial P.

There are two candidates for the 13^2-carbomethoxy C═O band of P798; the signals at 1741/1737 and 1725/1718 cm^{-1}. The former is free from hydrogen bonding while the latter is hydrogen bonded (*48*, *49*). Also, the dichroism measurement of these signals (Figure 6C) indicated that the former is oriented nearly parallel to the membrane plane, while the latter is tilted by $<31°$ to the membrane normal. This significant difference in the interactions and orientations suggests that these two signals do not correspond to each of the two carbomethoxy C═O groups in P798. At the very least, one of the two signals must arise from P798, because the carbomethoxy C═O is weakly conjugated to the macrocycle and an upshift in its frequency has been observed upon oxidation *in vitro* (*19*). The other signal may arise either from a 17^2-ester C═O of P798, which is usually insensitive to oxidation *in vitro* (*19*), or from an ester C═O of an adjacent pigment, BChl*g* or 8^1-OH-Chl*a*. In this case, the band appearance in the $P798^+$/P798 spectrum may have resulted from an electrostatic effect of cation formation. The half-deuterated $P798^+$/P798 spectrum (Figure 3) showed only slight downshifts in the 1737 and 1718 cm^{-1} bands (<2 cm^{-1}), which were probably due to minor mixing with hydrogen-containing macrocycle modes. Because a COOH band shows a downshift by ~10 cm^{-1} upon its deuteration (*53*, *54*), a large contribution of COOH modes of amino acid side chains to the above two signals is unlikely.

In the RC of purple bacteria, the carbomethoxy C═O groups of P have roughly parallel orientations with respect

to the membrane plane (*43*, *52*). If P798 has the same molecular arrangement, the 1741/1737 cm^{-1} signal is likely attributed to the carbomethoxy C═O of P798. However, P798 may consist of BChl*g*′, an epimer of BChl*g* with respect to the carbomethoxy group (*5*). In addition, the carbomethoxy C═O can change its orientation by rotation around the $C(13^2)$-$C(13^3)$ bond. Thus, an unambiguous assignment of the carbomethoxy C═O of P798 cannot be made at present.

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