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FTIR studies on crystals of photosynthetic reaction centers

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It is shown that high-quality light-induced FTIR-difference spectra can be obtained from reaction center crystals of *Rhodopsendomonas viridis*. Difference spectra between the ground state, PQ, and a light-activated state, P^+Q^-, have been recorded. The difference spectra are in good agreement with those reported previously for reaction centers reconstituted into lipid vesicles [(1985) FEBS Lett. 187, 227–232]. This good correspondence indicates that in both sample preparations the same intramolecular processes take place during this transition. In addition to measurements of absorbance changes in the visible spectral region, which indicate reactions of the chromophores and their microenvironments, those in the infrared spectral region also show that the protein side groups and backbone undergo the same light-induced changes in the crystals. It is observed that, besides the porphyrin ring system, the C_9 = O keto and ester groups of, most likely, the primary donor, undergo light-induced changes in charge distribution during oxidation of the primary donor. Large conformational changes of the protein backbone can be excluded for the observed transition.

Fourier transform infrared spectroscopy; Intramolecular process; Photosynthesis; Reaction center; Protein crystal

1. INTRODUCTION

Photosynthetic reaction centers catalyze light-induced electron transfer across photosynthetic membranes [1]. The reaction center from *Rhodopseudomonas viridis* has been successfully crystallized [2] and its structure determined to 3 Å resolution [3,4]. The primary electron donor (special pair, P) which absorbs at 960 nm consists of two closely associated bacteriochlorophyll *b* molecules (BC_{MP}, BC_{LP}). Two additional bacteriochlorophylls *b* and two bacteriopheophytins *b* are arranged symmetrically in the protein complex resulting in two branches of pigments which are more closely associated with either the L or M protein subunit. The electrons are conducted mainly through the L branch via bacteriopheophytin to a quinone (Q) [5].

From X-ray data the structure of the protein ground state has been very well characterized. In order to gain more insights into the intramolecular dynamics of the chromophore-protein complex, its function has been investigated using a non-invasive technique which displays reactions of single groups. Resonance Raman spectroscopy has provided information on the chromophores and their local environments [6]. However, this technique is restricted to vibrations of conjugated parts of the chromophores. In contrast, infrared spectroscopy is able to monitor all groups of the protein. In order to select the absorbance bands of the groups which show light-induced changes, difference spectra between different active states of the protein must be measured. To record absorbance changes of the order of 10^{-3} beyond the large background absorbance of the whole crystal, it is necessary to use Fourier transform infrared spectroscopy (FTIR). This approach has been successfully applied to rhodopsin [7,8] and bacteriorhodopsin using static [9–11] and time-resolved [12,13] FTIR techniques. The results of these experiments have provided insights into the light energy transduction mechanism of bacteriorhodopsin [9,14].

FTIR difference spectra of reaction center

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crystals from *Rps. viridis* were taken using an FTIR spectrophotometer equipped with an IR microscope. Crystals were grown directly on CaF_2 windows. Difference spectra in the visible and infrared spectral regions were taken between the ground state PQ and a charge-separated state corresponding to P^+Q^-, which is stabilized by steady-state illumination with actinic light >780 nm.

2. MATERIALS AND METHODS

Reaction centers from *Rps. viridis* were isolated and purified as described [2]. Fractions with an absorbance ratio (280 nm/830 nm) of 2.0–2.1 were pooled and crystallized as in [2] with the following alterations: triethylammonium phosphate was omitted and reaction center solutions containing 1.5 M ammonium sulfate were equilibrated with 1.9–2.1 M ammonium sulfate solutions by vapor diffusion.

Reaction centers were crystallized between CaF_2 windows separated by a 6.5 μm teflon spacer for infrared spectroscopy; the crystals typically had dimensions 1.0 mm × 0.5 mm × 6.5 μm. The crystals were analyzed for photochemical activity with a Zeiss UMSP 80 microspectrophotometer using actinic illumination of 900–1000 nm. Infrared spectra were taken on a Bruker IFS 88 instrument equipped with an infrared microscope with mercury cadmium telluride detector. The home-built sample holder for the two CaF_2 windows between which the crystals were grown was thermostabilized at 300 K. In order to monitor the baseline quality two different absorbance spectra (124 scans each) were recorded with a spectral resolution of 2 cm^{-1} in the dark and subtracted from each other. Only spectra showing high baseline quality were added. The same procedures were repeated during steady-state illumination. The difference was taken between absorbance spectra recorded in the dark and during steady-state illumination with actinic light >780 nm. In the difference spectrum shown (fig.3) five difference spectra taken from one crystal were added. Different crystals yielded the same difference spectra.

3. RESULTS

The optical absorbance spectrum of a typical *Rps. viridis* reaction center (thin) crystal is presented in fig.1 and the absorbance changes due to illumination of the same crystal with actinic light between 900 and 1000 nm are shown in fig.2. Both spectra agree well with published data for reaction center crystals [16,17].

The absorbance spectrum in the UV, visible and near-IR regions of reaction center crystals (fig.1) is characterized by a band at 960 nm arising from the bacteriochlorophyll *b* 'special pair' (not shown), and another 830 nm resulting from the Q_y transition of the two bacteriochlorophyll *b* monomers; these two molecules also contribute a Q_x absorbance at 610 nm and a Soret band at 375 nm. The two bacteriopheophytins *b* have Q_y transitions at 790 and 810 nm, as well as a Q_x transition at 530 nm. The two high- and two low-potential cytochromes absorb around 555 nm (α-band) and contribute a composite Soret band with a maximum at 415 nm.

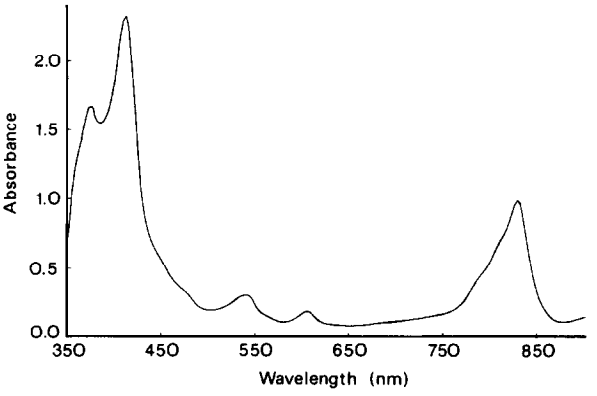

Fig.1. Optical absorbance spectrum of a single thin crystal of reaction centers from *Rps. viridis* taken on a microspectrophotometer.

During steady-state illumination the primary donor is oxidized (P^+), as indicated by complete bleaching of the 960 nm absorbance band (not shown) as well as bleaching of the Q_x absorbance at 610 nm, where a negative band is present in the difference spectrum (fig.2). The band pattern with negative bands at 412 and 385 nm and a positive band at 450 nm indicates that a P^+Q^- state is stabilized [15]. The Stark shift of the accessory monomers to higher energies appears to be responsible for the negative lobe at about 850 nm and the positive lobe at about 810 nm of the largest difference band [17].

In fig.3 the same difference spectrum in the infrared spectral region is shown. The negative bands correspond to the state PQ and the positive bands to P^+Q^-. The crystals contain only about 30% protein and about 70% water, detergent and crystallization buffer [2]. Therefore, large background absorbance of H_2O at 1640 cm^{-1} (OH bending vibration) and of ammonium sulfate and phosphate buffer between 1050 and 1150 cm^{-1} (PO_4^{3-}, SO_4^{2-}) increases noise in the regions at about 1640 and 1050–1150 cm^{-1}, compared to other parts of the difference spectrum. Excluding

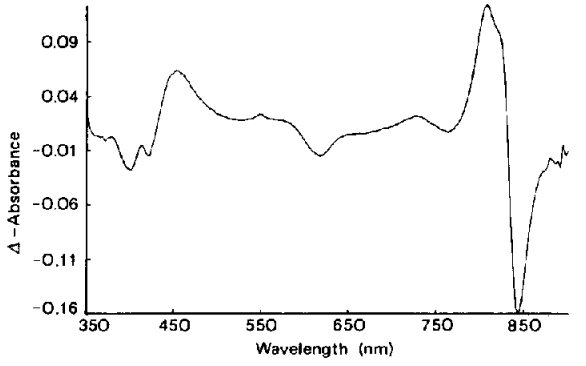

Fig.2. Difference spectrum of the same crystal as in fig.1 between PQ and P^+Q^-.

this frequency range, highly reproducible band patterns in the difference spectra could be measured.

The difference spectrum is dominated by positive bands of the P^+Q^- state. The greater polarity in the P^+Q^- state seems to lead to larger changes in dipole moment of the molecular groups involved, thereby increasing their infrared absorbance. The measured difference spectrum is in good agreement with that reported for reaction centers of *Rps. viridis* reconstituted into lipid vesicles taken under similar conditions [18]. The somewhat increased noise in the difference spectrum of the crystals compared to the spectra in [18] is due to the better spectral resolution used in the spectra of the crystals (2 vs 4 cm^{-1}). Not only the larger absorbance bands at 1754, 1744, 1715, 1548, 1477, 1305 and 1193 cm^{-1} but also the smaller ones

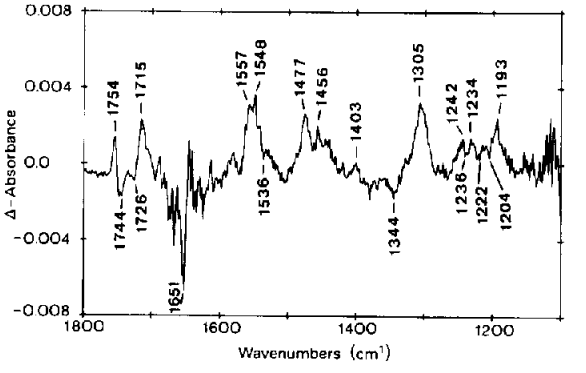

Fig.3. FTIR difference spectrum between PQ and P^+Q^- of a reaction center crystal from *Rps. viridis* at 2 cm^{-1} spectral resolution.

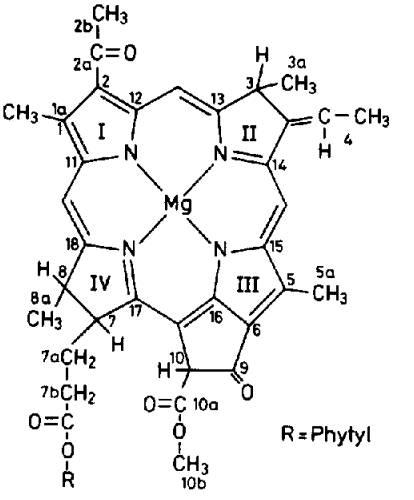

Fig.4. Bacteriochlorophyll *b*.

are in good agreement. The band pattern observed, e.g. between 1403 and 1193 cm^{-1}, shows surprisingly good agreement at 1403, 1344, 1305, 1242, 1234 and 1193 cm^{-1}.

4. DISCUSSION

In the following, the assignments of absorbance bands to molecular groups of the chromophore-protein complex will be discussed in comparison with the results obtained from resonance Raman experiments [6] and infrared spectra of model compounds [19,20]. Complete proof of the assignments requires isotopically labelled samples. In such cases, the absorbance bands of the labelled groups are shifted [14]. In this first approach such labelling has not yet been used.

In fig.3 a difference band with a negative peak at 1744 cm^{-1} and a positive peak at 1754 cm^{-1} is observed. Resonance Raman spectra of chlorophylls and reaction centers do not show such high-frequency bands [6]. Therefore, the band cannot belong to a group of the conjugated part of the chromophores (fig.4). Infrared spectra of bacteriochlorophyll recorded in *n*-butylcyclohexane show a band at 1739 cm^{-1} which was assigned to an ester carbonyl band [20]. Difference spectra between bchl *a* and bchl a^+ taken in an aprotic solvent also demonstrate a difference band shifting from 1737 to 1749 cm^{-1} which was assigned to an ester group [19]. Based on the agreement in band

positions, the difference band in fig.3 is assigned to an ester carbonyl absorbance band shifting from 1744 to 1754 cm^{-1} during oxidation of the primary donor (fig.4). The high wave number indicates a very hydrophobic environment for this ester group in the protein.

A spectroelectrochemical study [19] showed, during cation formation of bchl *a*, the appearance of a band at 1715 cm^{-1}, which was assigned to a $C_9 = O$ keto carbonyl band. In resonance Raman spectra of bchl *a*-containing reaction centers of *Rps. sphaeroides* an increase in the intensity of the $C_9 = O$ keto carbonyl band at 1709 cm^{-1} during cation formation is also observed [6]. Based on these results the positive band arising at 1715 cm^{-1} is assigned to a $C_9 = O$ keto carbonyl group (fig.4) in agreement with [18]. As in the case of the ester carbonyl vibration, this band is at unusually high frequency, indicating that the environment of this group is very hydrophobic. In general, the bands above 1700 cm^{-1} can also be caused by vibrations of protonated carboxylic acids of aspartic or glutamic acids [14]. However, the bands at 1754, 1744 and 1715 cm^{-1} are not shifted in D_2O [18], therefore excluding these possibilities. In the region below 1700 cm^{-1} assignment becomes more difficult because not only the chromophores (hydrogen-bonded C=O, C–C, C–N) [6], but also quinones (C=O) [21], protein side groups and the protein backbone [10,14] show absorbance bands in this region. In bacteriorhodopsin [7] and rhodopsin [9], the difference spectra are dominated by absorbance bands of the chromophore. The protein side groups show only minor absorbance bands. Therefore, the strongest bands most likely arise from chromophore vibrations. Furthermore, the greatest changes in dipole moment and therefore in infrared absorbance are expected for the primary donor: the observed ester and keto absorbance bands are thus most likely due to the primary donor.

Based on results obtained from resonance Raman experiments [6] and spectroelectrochemical studies of bchl *a* and bchl a^+ [20], the bands at 1557 and 1548 cm^{-1} could be caused by the C-C stretching vibrations of bacteriochlorophyll. Resonance Raman experiments with ^{15}N-labelled chlorophylls show only shifts of bands below 1350 cm^{-1} [6]. Therefore, the bands at 1305 and 1193 cm^{-1} are most likely due to C–N stretching vibrations. Carbonyl vibrations of the quinones are expected between 1600 and 1700 cm^{-1} [21]. At least part of the band at 1477/1456 cm^{-1} could be caused by vibrations of the quinone radical [22].

Based on these results, the following conclusions can be drawn:

(i) The excellent agreement between difference spectra for reaction centers of *Rps. viridis* reconstituted into lipid vesicles [18] and of crystals show that the same intramolecular processes take place in the chromophores, protein side groups and protein backbone for both sample preparations.

(ii) Since no large difference bands were observed at 1657 and 1545 cm^{-1} where the amide I and II bands of the protein backbone vibrations absorb, the possibility of large conformational changes in the protein during P^+Q^- formation can be excluded. The band at 1651 cm^{-1} could indicate only a small conformational change involving one or two groups of the protein backbone.

(iii) As discussed, the difference spectra appear most likely to be dominated by the P^+ absorbance bands. The protein side groups seem to show only minor absorbance bands.

(iv) The band pattern observed above 1700 cm^{-1} seems to be more indicative of radical formation and the concomitant change in charge distribution of the primary donor than of a change of the microenvironment of its ester and keto carbonyl groups. This conclusion is confirmed by spectroelectrochemical studies on cation formation of bchl *a*, which show similar patterns [19]. The observed bands of the keto and ester carbonyl vibrations are at unusually high frequencies, indicating a very hydrophobic environment. It is interesting to note that during cation formation large changes in charge distribution of the primary donor take place not only in the conjugated part of the chromophore but also at the $C_{10}=O$ or $C_7=O$ ester carbonyl groups (fig.4).

(v) Comparison with structural data [23] shows that the observed non-hydrogen-bonded $C_9=O$ keto carbonyl group can most likely be assigned to the Bchl *b* molecule of the primary donor belong-

ing more to the M subunit (BC_{MP}). The $C_9=O$ keto group of the L subunit bchl *b* molecule (BL_{LP}) is hydrogen-bonded and would be expected to absorb at lower frequencies.

REFERENCES

[1] Parson, W.W. (1982) Annu. Rev. Biophys. Bioeng. 11, 57–80.
[2] Michel, H. (1982) J. Mol. Biol. 158, 567–572.
[3] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, M. (1984) J. Mol. Biol. 180, 395.
[4] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, M. (1985) Nature 318, 618.
[5] Shopes, R.J. and Wraight, C.A. (1985) Biochim. Biophys. Acta 806, 348–356.
[6] Lutz, M. (1984) in: Advances in Infrared and Raman Spectroscopy (Clark, R.J.H. and Hester, R.E. eds) vol.11, pp.211–300, Wiley, Heyden.
[7] Siebert, F., Mäntele, W. and Gerwert, K. (1983) Eur. J. Biochem. 136, 119–127.
[8] Rothschild, K.J. and De Grip, W.J. (1986) Photochem. Photobiol. 13, 245–258.
[9] Gerwert, K. and Siebert, F. (1986) EMBO J. 5, 805.
[10] Roepe, P., Scherrer, P., Ahl, P.L., Das Gupta, S.K., Bogomolni, R.A., Herzfeld, J. and Rothschild, K.J. (1987) Biochemistry 26, 6708.
[11] Dollinger, G., Eisenstein, L., Lin, S.-L., Nakanishi, K. and Termini, J. (1986) Biochemistry 25, 6524–6533.
[12] Gerwert, K. and Hess, B. (1988) Microchim. Acta, in press.
[13] Braiman, M.S., Ahl, P. and Rothschild, K.J. (1987) Proc. Natl. Acad. Sci. USA 84, 5221–5225.
[14] Engelhard, M., Gerwert, K., Hess, B., Kreutz, W. and Siebert, F. (1985) Biochemistry 24, 100.
[15] Holten, D., Windsor, M.W., Parson, W.W. and Thornber, J.P. (1979) Biochim. Biophys. Acta 501, 112–126.
[16] Zinth, W., Kaiser, W. and Michel, H. (1983) Biochim. Biophys. Acta 723, 128–131.
[17] Knapp, E.W., Fischer, S.F., Zinth, W., Kaiser, W., Deisenhofer, J. and Michel, H. (1985) Proc. Natl. Acad. Sci. USA 82, 8463–8467.
[18] Mäntele, W., Nabedryk, E., Tavitian, B.A., Kreutz, W. and Breton, J. (1985) FEBS Lett. 187, 227–232.
[19] Mäntele, W., Wollenweber, A., Nabedryk, E., Breton, J., Rashwan, F., Heinze, J. and Kreutz, W. (1987) Progress in Photosynthesis Research (Biggens, D. ed.) vol.1, p.21, Nijhoff, Dordrecht.
[20] Ballschneider, K.H. and Katz, J.J. (1969) J. Am. Chem. Soc. 91, 2661–2677.
[21] Morton, R.A. (1965) Biochemistry of Quinones, Academic Press, New York.
[22] Tripathie, G.N.R. (1981) J. Chem. Phys. 74, 6044.
[23] Michel, H., Epp, O. and Deisenhofer, J. (1986) EMBO J. 5, 2445–2451.