Light-induced Fourier transform infrared (FTIR) spectroscopic investigations of the primary donor oxidation in bacterial photosynthesis

W. Mäntele+, E. Nabedryk, B.A. Tavitian, W. Kreutz+ and J. Breton

+*Institut für Biophysik und Strahlenbiologie der Universität Freiburg, Albertstrasse 23, D-7800 Freiburg, FRG* and *Service de Biophysique, Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette Cédex, France*

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Fourier transform infrared (FTIR) difference spectroscopy of the primary electron donor (P) photo-oxidation has been performed for reaction centers (RCs) and chromatophores of purple photosynthetic bacteria. In the 1800–650 cm^{-1} spectral region highly reproducible absorbance changes were obtained that can be related to specific changes of individual bond absorption. Several bands in the difference spectra are tentatively assigned to changes of intensity and position of the keto and ester C=O vibrations of the P bacteriochlorophylls, and a possible interpretation in terms of changes of their environment or type of bonding to the protein is given. Small difference bands in the amide I and II region allow only minor protein conformational changes.

Fourier transform infrared spectroscopy *Reaction center* *Bacterial photosynthesis* *Primary donor* *Chlorophyll*

1. INTRODUCTION

The primary processes of photosynthesis take place in membrane-bound chlorophyll-protein complexes, where a high degree of organization seems to be a prerequisite for the remarkable efficiency of energy migration, charge separation, and charge stabilization. The basic units called reaction centers (RCs) can still perform the last 2 processes. They have been isolated and are especially well-characterized in purple photosynthetic bacteria [1]. There is increasing evidence that the bacteriochlorophyll (Bchl) molecules in these complexes are kept in precise order by specific interactions with each other and with specific groups in the polypeptides [2,3].

For an understanding of the mechanisms of the primary steps on a molecular basis, it is necessary to gain information on the binding sites and on the nature of these interactions in vivo. At present, there are several approaches to these questions: X-ray analysis of the crystallized *Rhodopseudomonas viridis* RC [4] has shown the fixed arrangement of the Bchl molecules and will provide information on the side groups of the polypeptides that are possible partners for interaction. On the other hand, resonance Raman (rR) data have demonstrated the binding of some of the Bchls' carbonyl groups to their host site [3]. The contribution of the carbonyl groups to rR scattering, however, is strongly dependent on the degree of conjugation they share with the main π-electron system. For example, the ester carbonyl groups do not contribute at all to rR spectra. For the same reason, no information is obtained directly on either the binding sites or conformational changes of the proteins. This high selectivity thus somehow limits the amount of information that can be obtained by rR spectroscopy.

In contrast, infrared (IR) spectroscopy is non-selective and is thus able to monitor all bonds of

Abbreviations: I, intermediate acceptor; Q_A, Q_B, primary, secondary quinone

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the chlorophyll (Chl) molecules – conjugated or side groups – as well as all bonds of the polypeptides and lipids. Furthermore, the measuring beam, unlike in rR spectroscopy, has no possibility of causing photochemistry or altering the sample. IR spectroscopy in the field of photosynthesis has been used to study the organization of protein secondary structures in intact membranes and in isolated antennae and RC [5–7]. A large amount of transmembrane α-helices has been found in all these systems [7]. On the other hand, IR studies of Chls in vitro [8,9] have indicated specific aggregation and interaction among these molecules. The non-selectivity of IR spectroscopy, however, makes the in vivo investigation of single pigment molecules in a large Chl-protein complex a rather hopeless enterprise with conventional techniques.

The principle of IR difference spectroscopy is thus to obtain a certain selectivity by only monitoring small changes in absorption on a high, but constant background absorption caused by the bulk membrane. Fourier transform (FT) and time-resolved IR difference spectroscopy have been successfully applied to the investigation of the light-induced molecular processes in rhodopsin [10,11], bacteriorhodopsin [12–15] and photosynthetic membranes [16,17]. Here, static FTIR difference spectra of the primary electron donor (P) oxidation in bacterial RCs from *Rps. sphaeroides, Rps. capsulata* and *Rps. viridis* are presented.

2. EXPERIMENTAL

The principle of FTIR difference spectroscopy, using low temperature to stabilize a light-induced intermediate, has been described in [10–15]. In the present work, spectra were recorded in the presence and absence of continuous actinic light (715 nm $< \lambda <$ 1100 nm) on a Nicolet 60 SX FTIR spectrophotometer equipped with MCTA and MCTB type detectors. Further details will be given in a subsequent paper. Control spectra were taken on a Cary 14 (IR 2 mode) and a Cary 17 spectrophotometer.

RCs were reconstituted in lipid vesicles according to [6] and air-dried on IR windows. For the IR spectra, films were mounted in a closed hydration cell controlled thermostatically between room temperature and −60°C.

3. RESULTS AND DISCUSSION

The IR absorbance spectrum from *Rps. sphaeroides* RC presented in fig.1a is in close agreement with the *Rps. sphaeroides* RC spectrum discussed in [6]. In the inset, a control absorption spectrum is given in the dark and under continuous illumination, showing the well-known bleaching of

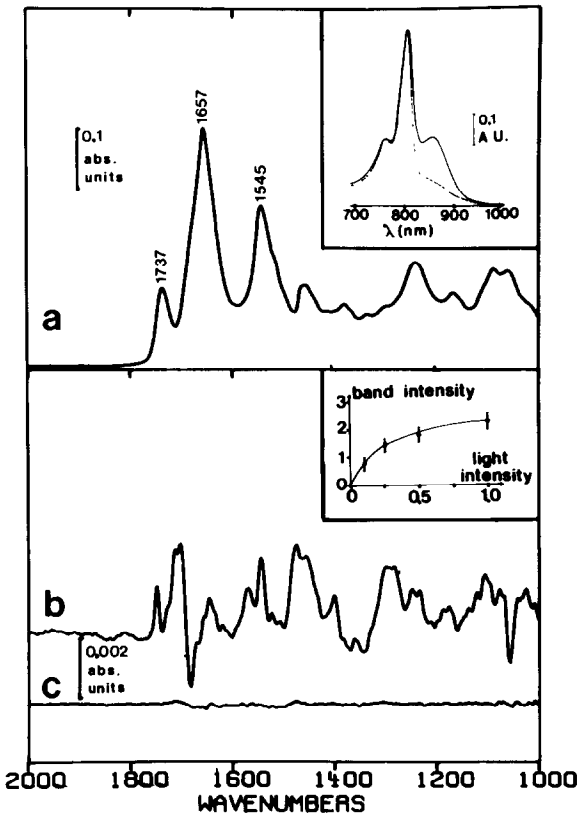

Fig.1. FTIR spectra of films of *Rps. sphaeroides* RC reconstituted in lipid vesicles and air-dried on CaF_2 discs. (a) Absorption spectrum. The inset shows the visible-near-IR absorption spectrum obtained with weak (——) and with strong (– – –) measuring light. (b) Light-induced difference spectrum obtained from a spectrum recorded with additional bleaching light and a spectrum without. Bands of the light spectrum appear positive. The inset shows the dependence of the highest frequency band (1750 cm^{-1}) upon actinic light intensity. (c) Difference spectrum obtained from spectra recorded before and after illumination. Measuring conditions: 64 interferometer scans were added; resolution: 4 cm^{-1}; temperature: −25°C. Note the expanded scale in (b) and (c) as indicated at the bar in absorbance units.

the primary donor band. Fig.1b shows the light-induced IR absorbance difference spectrum of the same sample. Note the ordinate expansion between fig.1a and b. The actual noise level is seen in the 2000–1800 cm^{-1} spectral region as well as in fig.1c, which was obtained from the 'dark' spectrum before and after illumination, showing complete back reaction. These difference spectra can be repeated many times within the noise level. Spectra from different samples, scaled in amplitude, were superimposable. The inset of fig.1b shows the dependence of a band at 1750 cm^{-1} upon actinic light intensity. The same behavior was found for all bands, indicating the accumulation of a single species. The light intensity used to record the photo-oxidized RC spectrum (fig.1a, inset) was higher than that used to achieve saturation in the IR experiments (fig.1b, inset). It is therefore reasonable to assume that the spectral changes shown in fig.1b represent molecular changes associated with the photo-oxidation of the primary donor. These P^+-minus-P spectra will further on be referred to as P^+ spectra, keeping in mind that the reduction of quinones may contribute to them. The spectrum in fig.1b does not show bands between 2000 and 1760 cm^{-1}. In the frequency region between 1760 and 1650 cm^{-1}, prominent bands are observed, presumably caused by carbonyl bonds. No strong contribution to the difference spectrum is observed in the region of the amide I and amide II bands of the protein (at 1657 and 1545 cm^{-1}, respectively), dominating in the absorbance spectrum (fig.1a).

Fig.2 shows the P^+ spectra obtained from RCs of *Rps. sphaeroides, Rps. capsulata, Rps. viridis* and from chromatophores of *Rps. viridis*. In the whole frequency region, a good agreement of the band features is observed for the RCs of *Rps. sphaeroides* (fig.2a) and of *Rps. capsulata* (fig.2b), both containing Bchl *a* and having a similar protein structure [18–20]. Throughout the 4 difference spectra, bands are observed at closely corresponding positions, but with different intensities. This is especially clear for the bands around 1550, 1475, 1400 and 1300 cm^{-1}, but also for a large number of small bands and shoulders. In the carbonyl frequency region, an isolated band is observed at 1750 cm^{-1} in both *Rps. sphaeroides* and *Rps. capsulata* P^+ spectra (fig.2a and b). This band appears shifted to 1755 cm^{-1} in the P^+ spectra of the Bchl *b* containing RC and chromatophores of *Rps. viridis* (fig.2c and d). Moreover, a differential band feature with a positive wing at 1702 cm^{-1} and a negative wing at 1682 cm^{-1} seems to be common in the spectra of *Rps. sphaeroides* and *Rps. capsulata* RCs, but is not found at comparable absorption strength in the *Rps. viridis* RC spectrum. At the lower end of the carbonyl frequency region, a positive band of medium intensity is observed both in fig.2a (at 1657 cm^{-1}) and in fig.2b (at 1660 cm^{-1}). In contrast, an extremely sharp dispersive band is found in the spectrum of *Rps. viridis* RC (fig.2c), its positive wing being at 1658 cm^{-1} and its negative wing at 1649 cm^{-1}. This band is located directly at the position of the strongly absorbing amide I band (fig.1a). In high-accuracy FTIR spec-

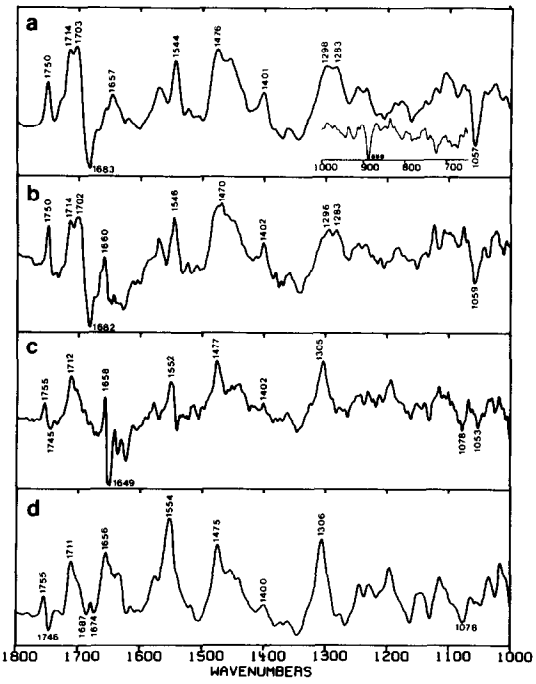

Fig.2. Light-induced FTIR difference spectra of reconstituted RC and chromatophore films recorded as described in fig.1. (a) *Rps. sphaeroides* RC. Inset shows extended wavenumber range. (b) *Rps. capsulata* RC. (c) *Rps. viridis* RC. (d) *Rps. viridis* chromatophores. Spectra (A) and (D) were recorded with an MCTA type detector. 64 interferometer scans were added. Spectra (B) and (C) were recorded with an MCTB type detector. 2048 interferometer scans had to be added for a signal-to-noise ratio comparable to that in spectra (A) and (D).

troscopy, one encounters the problem of phase errors resulting in inaccuracies of band positions (for a discussion of possible phase errors, see [13]). In the present case, slightly different band positions for the dark and 'light' spectra might result in baseline problems in the region of strong absorption bands. To check this, samples of different absorbance were investigated to control for possible artifacts. The dispersive band feature, however, was found throughout all P^+ spectra of *Rps. viridis* RC. It can be best explained by a sharp band which shifts its position upon RC photooxidation. If this dispersive band was to be interpreted in terms of conformational changes at all, it would only involve changes at the level of 1 or 2 peptide bonds as judged from the IR absorbance magnitude. The absence of strong positive or negative bands in the amide I or amide II region in all the spectra, however, makes the general idea of large conformational changes associated with P^+ formation rather unlikely.

It is reasonable to assume that, among the strong bands in the spectral region between 1640 and 1760 cm^{-1}, some are caused by carbonyl bonds of the Bchl molecules forming the primary donor. Their position and magnitude is determined by the nature of the group, i.e. ester-, keto-, acetyl-, for example, and the type of bonding to the protein [3]. A possible interpretation of the large differential feature in fig.2a and b could then be given in terms of a carbonyl vibration shifting from 1683 cm^{-1} (1682 cm^{-1}) in the P state to 1703 cm^{-1} (1702 cm^{-1}) in the P^+ state. According to model compound spectra [8] it may be attributed to the 9-keto C=O vibration on ring V of the Bchl molecule. The shift of this carbonyl absorption would then correspond to a change in its environment. It is worth noting, however, that changes in the environment of a carbonyl bond do not necessarily result in a differential feature. In the case of extensive hydrogen-bonding in the state P, but of a localized C=O bond in P^+, for example, only the appearance of a sharp positive band in the difference spectrum would be seen. This would be due to the disappearance of the hydrogen-bonded carbonyl band located at lower wavenumbers. This could be the case for the positive bands at 1750 cm^{-1} in the *Rps. sphaeroides* and *Rps. capsulata* P^+ spectra, where only a few corresponding negative bands are seen if any. In the *Rps. viridis* P^+ spectra (RC and chromatophores) however, a clear differential feature is present. A similar interpretation can be given for the positive bands at 1712 cm^{-1} in *Rps. viridis* RC and chromatophore P^+ spectra (fig.2c and d). The bands at 1750 cm^{-1} (fig.2a and b) and at 1755 cm^{-1} (fig.2c and d) might be interpreted as arising from the carbomethoxy or propionic ester C=O, which, for isolated Bchl *a* molecules, are found around 1740 cm^{-1} [8]. The difference in position between the P^+ spectra of RC containing either Bchl *a* or Bchl *b* as the primary donor constituents would then rather point to a slightly different type of bonding to the protein than to the difference between Bchl *a* and Bchl *b* itself, since coupling between substituents at ring II (where Bchl *a* and Bchl *b* differ) and molecular vibrations at ring IV or ring V can be expected to be rather weak.

One has to be careful, however, about the interpretation of the bands in the 1760–1640 cm^{-1} spectral region in terms of Chl carbonyl absorption only. Since IR difference spectroscopy, as applied here, is not selective to Chl molecular vibrations, carbonyl absorption changes from amino acid side chains, the peptide backbone (as discussed above) and also lipid esters have to be taken into account. Protonation of individual carboxyl groups in bacteriorhodopsin, for example, gives rise to IR difference bands [12] comparable in position and magnitude to the 1750 cm^{-1} (1755 cm^{-1}) band. Exchange of ^1H_2O against ^2H_2O performed through the vapor phase in the hydration cell for 24 h, however, did not change the 1750 cm^{-1} (1755 cm^{-1}) band position in the P^+ spectra (a downward shift of approx. 10 cm^{-1} in ^2H_2O would be expected for an accessible carboxyl group).

The P^+ spectra in the region below 1640 cm^{-1} seem very complicated. A positive band, located in the amide II band region around 1550 cm^{-1}, may correspond to the change observed in the amide I region discussed above. Further on, a broad positive band with a maximum at about 1475 cm^{-1} appears in a frequency range where mostly C-C vibrations from the Chl molecule are expected [3]. The negative band at 1057 cm^{-1} in fig.2a (at 1059 cm^{-1} in fig.2b) most probably arises from a C-N vibration, as preliminary spectra from *Rps. sphaeroides* RC labelled with ^{15}N show a splitting of this band. Using germanium instead of CaF_2 as

support for the films, spectra were extended to 650 cm^{-1} (fig.2a, inset). In the common spectral range they are in good agreement with those obtained from samples on CaF_2. A strong negative band at 888 cm^{-1} is observed in the P^+ spectra of both RC (fig.2a, inset) and chromatophores (not shown).

Possible contribution of the reduced quinone(s) to the difference spectra, especially in the carbonyl region, cannot be neglected, as the P^+ state is actually a $P^+Q_A^-(Q_B^-)$ state. A detailed investigation of the Q_A and Q_B contribution to the P^+ spectra, however, would require extraction and reconstitution with isotopically modified analogs and additional kinetic FTIR and time-resolved IR measurements of their back reactions.

4. CONCLUSIONS

The results presented above have demonstrated molecular changes associated with the formation of the photo-oxidized primary donor/reduced quinone acceptor couple in bacterial photosynthesis. The changes observed are of the order of 10^{-3} absorbance units but can be measured reproducibly at an accuracy which corresponds to the detection of single molecular bonds absorption per RC. At this level of sensitivity, large conformational changes of the protein would give rise to differential bands dominating in the spectra. From electron transfer kinetics, it has been recently proposed [21] that light-induced conformational changes occur upon photo-oxidation of the RC. Our results clearly demonstrate that large changes can be excluded, but, nevertheless, indicate that differential bands in the amide I and amide II spectral region can be attributed to minor specific changes in the conformation of the bacterial RC upon photo-oxidation. The carbonyl frequency region, which differs between the spectra of the Bchl *a*- and Bchl *b*-containing RC, presents a possible clue to the nature of bonding of the chlorophylls to their host site. From our data, we have to conclude that, for at least one Chl carbonyl group, the type of bonding or the environment is changed upon photo-oxidation.

It is clear that the analysis of the difference spectra is far from complete, and the comparison of difference spectra of isotopically labelled chromatophores and reaction centers will be necessary to obtain clear criteria for the assignment of bands. Selective isotopic labelling of the chromophores, as performed with bacteriorhodopsin [13], is not within reach. Preliminary isotope substitution ($^{14}N/^{15}N$) by growing bacteria on isotopically labelled substrates has shown that only a small number of bands is influenced or shifted, and $^{12}C/^{13}C$ substitution will probably be a more appropriate tool. Furthermore, it would be of interest to investigate IR spectra of the radical cation of isolated Bchl and to compare them to our P^+ spectra.

Apart from the accumulation of the P^+ state with additional bleaching light, the I^- state can be generated and stabilized in pre-reduced samples [22]. IR spectra of both states can be recorded with sufficient accuracy. Taken together, they can yield information that will help to understand the nature of the interactions that are experienced by the pigment molecules in bacterial photosynthesis, and their change upon charge separation.

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