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Time-resolved infrared studies of light-induced processes in plant thylakoids and bacterial chromatophore membranes. Evidence for the function of water molecules and the polypeptides in energy dissipation

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Spinach thylakoids and chromatophores from the photosynthetic bacterium *Rhodopseudomonas capsulata* were investigated by means of time-resolved infrared spectroscopy, using thin water-containing membrane films which fully maintained their photochemical activity. Upon flash excitation, reversible infrared absorbance changes were obtained and their difference spectra were recorded. In spinach thylakoids, these transient signals could be described by a sum of two exponential decay functions with half-times of about 2 and 30 ms, respectively. They were insensitive to the addition of benzyl viologen, ferricyanide or ferricyanide + DCMU. They are ascribed by their dependence on intensity and wavelength range of the actinic flash to processes in the antenna pigment-protein complexes. In chromatophores from photosynthetic bacteria, similar infrared signals in the millisecond time range were obtained. Their spectral distribution was investigated for three mutants of the photosynthetic bacterium and is different for membranes lacking carotenoids. Both signals, in thylakoids and chromatophores, reflect the proportion of absorbed flash energy which is neither channelled to the reaction center nor emitted as light, but is dissipated through radiationless decay. A common feature of the difference spectra from spinach thylakoids and bacterial chromatophores are bands identified by deuteration as being due to H_2O. Some bands are interpreted in terms of water going transiently from the hydrogen-bonded to the free state. Other bands are assigned to the polypeptides of the light-harvesting complexes, and thus indicate their participation in energy dissipation. Membranes from photosynthetic bacteria containing a photochemical reaction center show a distinct slow signal component decaying in about 1 s. It saturates at low flash intensity and is abolished upon chemical oxidation of the primary electron donor. Two bands in the difference spectrum of this component are tentatively assigned to the ester C = O and keto C = O vibrations of photooxidized bacteriochlorophylls in the reaction center. The data suggest that chromophoric and non-chromophoric infrared absorbance changes contribute to the difference spectra, and thus may represent a clue to the processes at the active sites of polypeptides in photosynthesis.

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Abbreviations: LHC_I and LHC_{II}, light-harvesting complex I or II; PS I and PS II, Photosystem I or II of higher plants; P-700, reaction center of Photosystem I; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; BChl, bacteriochlorophyll; S_0–S_3, singlet states.

Introduction

In photosynthetic membranes, light quanta are absorbed by the antenna pigment systems and channelled to the reaction centers, where light

energy is trapped and charge separation and stabilisation takes place. The combination of typically 300 antenna pigment molecules serving one reaction center allows the complex molecular machinery of photosynthesis to work efficiently at very low light intensities and requires fast energy dissipation at high light intensities, where excited Chl would be subjected to irreversible photooxidation in the presence of oxygen. A scheme of the pathways of collecting and dissipating energy transfer is given by Renger and Wolff [1].

Most evidence for energy collection and trapping mechanisms, as well as for charge separation in the reaction center and the dissipation of excess energy comes from flash spectroscopic techniques and from static difference spectroscopy [1–5]. These techniques cover the spectral range from near ultraviolet to near infrared, monitoring electronic levels and changes of these. We have extended the spectral range of the flash spectroscopic techniques into the mid-infrared region from about 3 μm wavelength (3300 cm^{-1}) to about 12 μm (850 cm^{-1}). Static infrared spectroscopy in this spectral region has already been used to study the organization of protein structures of thylakoid and chromatophore membranes [6–8], and of bacterial reaction centers reconstituted in lipid vesicles [9]. Apart from these investigations infrared studies on Chl and BChl model compounds were carried out [10,11].

In this paper we present an infrared spectroscopic study of light-induced reactions in spinach thylakoids and in chromatophores of photosynthetic bacteria. The preparation of samples, which, because of the low water content necessary, represents a crucial point in our experiments, is described in detail.

Several types of signals, i.e., time-resolved infrared absorbance changes, are described, and their transient difference spectra are discussed. The infrared absorbance changes are assigned to processes of energy dissipation and of photosynthetic electron flow based on their dependence on excitation intensity and excitation wavelength.

By comparison with published molecular spectra of the chromophores in situ [12–14] and of model compounds [10,11] a tentative assignment to chromophore and polypeptide groups is tried.

Materials and Methods

Spectroscopic techniques

The principles of time-resolved infrared spectroscopy and the apparatus developed by us is described in detail elsewhere [15–18]. Time-resolved infrared difference spectra were obtained point by point generating a kinetic signal every 5–10 cm^{-1} with a spectral resolution of about 5 cm^{-1}. Up to 100 signals were added to improve signal-to-noise ratio.

The sample was excited with light from two xenon flashlamps focused by elliptical mirrors onto the sample. Flash duration was between 30 μs and 100 μs, depending on the capacitors used for discharge. Filters were inserted to cut off the ultraviolet (below 320 nm) and the infrared (above 750 nm) part of the excitation light. Additional filters could be inserted to modify the spectrum of the exciting light. Excitation energy was varied between 1–100 J electrical energy varying the high voltage and the capacitor for the flashlamps.

For some experiments the sample was excited with a flash from a dye laser pumped by an excimer laser (Lambda Physik EMG 101), with a pulse duration of 10–20 ns and a pulse energy of about 100 mJ. Dyes were used to excite at 386 nm (BiBuQ), at 430 nm (Coumarin 120) or at 692 nm (Oxazin 170). Dyes were obtained from Lambda Physics, Göttingen. Signals were recorded in a Nicolet signal averager model 1070 with a time-resolution of up to 10 μs per channel, stored on a tape recorder and transferred to a computer. In almost all cases the decay signal time course could be described as a sum of two exponential decay functions. Amplitudes of the two components were either evaluated graphically or by using a non-linear least-square fit program on a computer.

To monitor photosynthetic electron flow in the infrared sample, a simple, home-built flash photolysis apparatus for the spectral region between 400 nm and 1000 nm was used. The oxidation of the primary donors of bacterial reaction centers was monitored at 864 nm. In all cases, suitable interference filters were used to reduce fluorescence. Spectra of thylakoid and chromatophore preparations and of infrared samples were run on a Cary 14 spectrophotometer.

Preparation of spinach thylakoids and bacterial chromatophores

Thylakoid membranes. Thylakoids were prepared from fresh spinach leaves essentially according to the method of Siggel et al. [19]. Spinach leaves were ground in a buffer containing ascorbate and filtered through a column of nylon sieves. Chloroplasts were washed twice and then broken osmotically in a hypotonic buffer. Centrifugation at low speed yielded thylakoids in the supernatant. These thylakoids were washed twice in the hypotonic buffer and resuspended in an isotonic buffer. Small aliquots with 5% dimethylsulfoxide were frozen in liquid nitrogen.

Chl concentration in the thylakoid preparation was determined according to the procedure of Arnon [20], and protein concentration according to the procedure of Lowry [21]. As a control of photosynthetic electron flow, oxygen production in continuous light was measured with a conventional Clark-type electrode.

Bacterial chromatophores. Chromatophores of three mutants of the BChl a containing photosynthetic bacterium *Rps. capsulata* were used.

Membranes from the carotenoid-free mutant A_1A^+ [22] and from the mutant Y5, which contains only LHC_{II}, but no reaction center [23,24], were kindly provided by Prof. Drews, Institut für Mikrobiologie, Freiburg. A culture of the mutant NK_3 was kindly provided by Norbert Kaufmann (NK) of the Institut für Mikrobiologie, Freiburg. Culture medium, growth procedure, and isolation of the membranes were the same as for the BChl *b* containing *Rps. viridis* and is essentially described elsewhere [24].

For a cross reference, the constituents of the different membranes can be summarized as follows:

		Reaction center	LHC_I	LHC_{II}	Carotenoids
Rps. capsulata	A_1A^+	×	×	/	/
	NK3	×	×	/	×
	Y5	/	/	×	×

All three different types of membranes were washed at least twice in 5 mmol/l NaCl (pH = 7), and resuspended in 1 mmol/l NaCl at a protein concentration of 5–10 mg/ml of suspension.

Infrared sample requirements and preparation

A severe problem of the infrared spectroscopy of biological molecules is the presence of water, generally necessary for the structure and function of these molecules. This holds particularly true for the complex photosynthetic membrane, and a compromise has to be found between the amount of water necessary for an undisturbed function and the amount of water that still allows infrared investigations even in the region of OH-absorption. This can be achieved by using thin humid films of photosynthetic membranes. For thylakoids and bacterial chromatophores, criteria for functional integrity are undisturbed light-induced electron-transfer reactions. It is shown in this study that in infrared samples prepared as thin humid membrane films, electron-transfer processes measured as oxidation and subsequent reduction of the primary electron donors, proceeded at the same extent and with reduction rates as in aqueous suspensions.

This thylakoid films were prepared by a centrifugation technique which had been used before for rod outer segments or purple membrane sheets [16]. A CaF_2 or BaF_2 infrared window was used as support for the film. It was sealed to a frame, thus forming a vessel with the window on the bottom which fitted into the centrifuge tubes of a large swing-out rotor. The vessel was filled with a suspension of thylakoids in the desired buffer, to which redox mediators or electron acceptors were added, and the membranes were spun down at moderate speed onto the infrared window. The supernatant was then soaked by use of a syringe and water on the film was carefully reduced with a stream of nitrogen.

Films of bacterial chromatophores were prepared by simply drying a drop of a concentrated suspension on a CaF_2 or BaF_2 window. Drying was performed in both cases only to a state in which the film stayed stable in a vertical position and could be transferred to the infrared spectrophotometer. The infrared window with the membrane film on it formed part of a cuvette built as a hydration chamber with a small reservoir on the bottom which could be filled with saturated salt solutions to provide constant relative humidity [25] in the gaseous phase above the salt solution. Further drying of the films to the desired humidity

was performed in situ in the infrared spectrophotometer by blowing a gentle stream of nitrogen through the hydration chamber and monitoring the OH-absorption band. Through a series of control experiments (see below) the degree of hydration was determined, at which electron transport reactions in the film sample were undistinguishable from those in suspensions. This was achieved with relative humidities of 90% to 100% in the hydration chamber, and the reservoir was filled with appropriate saturated salt solutions.

Hydrogen-deuterium exchange

Samples were washed twice in ^2H_2O-buffers and films were prepared as described above, with the exception that these films were kept in a ^2H_2O-atmosphere throughout the prepration. ^1H_2O-^2H_2O exchange was monitored in the infrared by measuring the intensity of the O-^{1}H and O-^{2}H stretching vibration and, in addition, by observing the shift of the amide-II band.

Control of photosynthetic electron flow in the infrared samples

The films prepared according to the centrifugation procedure were characterized by high homogeneity over the area covered by the infrared measuring beam. With the purification procedure for thylakoids and chromatophores described above about the same optical densities in the red absorption band of Chl (675 nm) and in the amide-I absorption band of protein (1655 cm^{-1}) were obtained. Control measurements in the visible part of the spectrum could therefore be performed on the same sample under the same conditions.

Electron transfer through PS I and PS II in thylakoids was controlled by monitoring the oxidation and subsequent reduction of P-700 at 706 nm. Without benzyl viologen as an electron acceptor for PS I, only a signal attributed to cyclic electron transfer around PS I is observed [26]. This signal is characterized by a two-exponential decay function and the fact that it cannot be blocked by DCMU.

Adding benzyl viologen as an electron acceptor resulted in a signal characterizing linear electron transport from water to benzyl viologen. The amount of benzyl viologen in the infrared sample was adjusted by adding benzyl viologen to the membrane suspension before centrifugation, and a concentration of 30 μmol/l was found to abolish cyclic electron transfer and yield maximum linear electron transfer. Starting from this situation, electron transfer was blocked by adding DCMU, or PS I was oxidized chemically by adding ferricyanide. In all cases the amplitude of P-700 oxidation was taken as a measure for inhibition.

Electron transfer in bacterial chromatophores was monitored as the oxidation and subsequent reduction of the primary donor at 860 nm. The time course of the reduction of the primary donor was found not to be influenced at relative humidities above 80% and became slowed down when the sample was further dehydrated. In addition, the absorption of the light-harvesting complex decreased irreversibly.

By adding small amounts of ferricyanide to the chromatophore suspension before drying onto the infrared window, the time course of the reduction was slowed down and films with chemically oxidized primary donor were obtained at high concentrations of ferricyanide. For films of chromatophores of *Rps. capsulata* mutant Y5 lacking the photochemical reaction center, the absorbance ratio A_{800}/A_{380} was compared to the one of suspensions and taken as a measure of functional integrity. For the infrared measurements, relative humidities between 90% and 100% were used in the hydration chamber.

Results and Discussion

Characterisation of infrared absorbance changes in thylakoids

Fig. 1 shows typical time-resolved infrared absorbance changes observed at 1460 cm^{-1} in spinach thylakoids at different hydration states. Upon flash excitation, a fast absorbance decrease is observed, which decays back to the original absorbance value in about 100 ms. In each case, 40 signals were added to improve the signal-to-noise ratio. The amplitude of a single signal in Fig. 1a–c corresponds to a relative intensity change at the detector of $\Delta I/I$ of about $2 \cdot 10^{-3}$ at a flash intensity high enough to saturate photochemistry.

At very high humidity of the sample as judged by the infrared spectrum, the signal decay (Fig. 1a) can be described by a single exponential function.

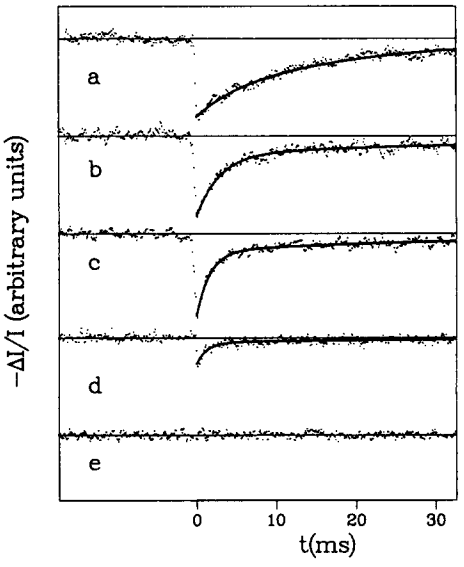

Fig. 1. Time-resolved infrared absorbance changes from a film of spinach thylakoid membranes measured at 1460 cm^{-1} at a resolution of 8 cm^{-1}. The sample was excited with light from a xenon flash with wavelengths from 350 to 800 nm. 40 signals were added to improve signal-to-noise ratio. Temperature 20°C. A negative signal corresponds to an absorbance decrease. The water content of the membrane film decreases from (a) to (e). The signal in (c) corresponds to a membrane film stored at about 90% relative humidity. With a dried sample, no signal is observed (e).

On going to lower humidity, a signal type seen in Fig. 1b, is observed. Now the time course can be described by a sum of two exponential decay functions, a fast one corresponding to a half-time of about 2 ms and a slow one with a half-time of about 30 ms. At still lower humidity corresponding to about 90% relative humidity, the slow component is reduced (Fig. 1c). Upon further drying, the fast component decreases too (Fig. 1d), and no signal at all is observed in a dry sample (Fig. 1e). Upon rehydration of the sample, only the fast component reappears. The disappearance of the slow component was also observed for films at high humidity stored for a longer time, i.e., for more than a few hours.

To measure the dependence of thylakoid electron-transfer processes on the humidity of the infrared sample, P-700 oxidation and reduction with benzyl viologen as an electron acceptor was followed at different hydration states. It was found that linear electron transfer was not influenced by relative humidities above 60% to 70% in the hydration chamber. Since the time-resolved infrared absorbance changes described below were already influenced at relative humidities below 80%, care was taken that a hydration at 90–100% relative humidity was maintained throughout all subsequent measurements.

To record the spectral dependence of the infrared signals, a hydration state at 95–98% relative humidity was chosen where both components of the signal could be observed. At this humidity, infrared kinetic measurements could be performed even in the region of OH-absorption around 1648 cm^{-1}. The difference spectrum of a sample con-

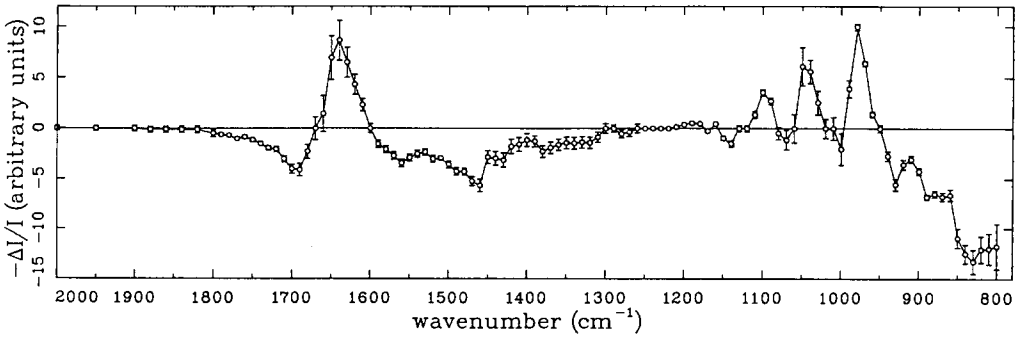

Fig. 2. Spectral dependence of the time resolved infrared signals from a thylakoid membrane film at 95–98% relative humidity. Linear electron transport through PS I and PS II was obtained using benzyl viologen as an electron acceptor. Benzyl viologen was added to the membrane suspension before centrifugation at a concentration of 30 μmol/l. Signals were recorded at intervals of 10 cm^{-1} at a resolution of 5–8 cm^{-1}. For each data point, between 20 and 50 signals were added to improve the signal-to-noise ratio. The total amplitude of the normalized signals is plotted. Measuring conditions are as in Fig. 1.

taining benzyl viologen as an electron acceptor for PS I is shown in Fig. 2. A positive band corresponds to an absorbance increase. The kinetic constants of the signals, i.e., the rates of the fast and the slow component and the ratio of their amplitude, were the same for all wavenumbers, so that only the maximum amplitude of the signal immediately after the flash is plotted in the difference spectrum. Within the limits of accuracy the time-resolved infrared difference spectra obtained from samples with the electron transfer modified or blocked by ferricyanide, DCMU or ferricyanide + DCMU (see Materials and Methods) were identical with the spectrum shown in Fig. 2. This immediately excludes a functional relation between the linear or cyclic electron-transport processes and the infrared absorbance changes observed.

In all time-resolved infrared absorbance changes shown above, the rise of the signal was limited in time to about 100 μs by the duration of the xenon flash. To obtain increased time resolution in the early phase of the signal, infrared absorbance changes were recorded with excitation by a laser flash of 15 ns duration. Excitation wavelenghts were chosen at 430 nm for the soret band and at 692 nm for the red absorption band of Chl. For both excitation wavelengths, measuring P-700 signal at 706 nm with neutral density filters in the exciting beam showed that saturating light intensity was obtained with the laser flash (see Fig. 3). When time-resolved infrared measurements were performed with excitation at 430 nm, the same signals were obtained as with xenon flashes. The rise of the signals could not be time-resolved and was faster than 10 μs, the resolution of the transient recorder.

No infrared signals were obtained, however, exciting the sample with a laser flash at 692 nm strong enough to saturate photochemistry. This gives rise to speculations on the pigments involved in infrared absorbance changes. To clarify this point, further experiments were performed in order to ascertain the dependence of the signal amplitude on the excitation wavelength.

Inserting long-wavelength-pass filters (Schott u. Gen.) with cut-off wavelengths spaced about 50 nm apart, the spectrum of the exciting xenon flash was successively narrowed down and an 'in-

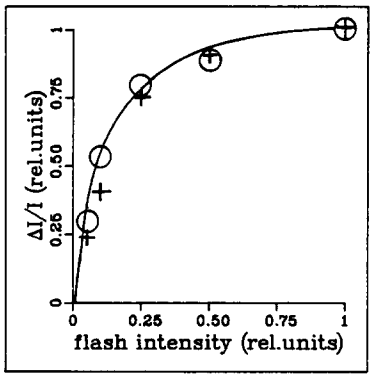

Fig. 3. P-700-absorbance changes of a thylakoid membrane film measured at 706 nm for different excitation with laser flashes. Excitation wavelength: ○, 430 nm; +, 692 nm.

tegrated action spectrum' was obtained [27]. The comparison with the 'differential action spectra' published in literature [28] showed that the action spectra for the infrared absorbance changes and for the photosynthetic activity were the same within the limits of accuracy, i.e., that both effects are related to light excitation by the same set of antenna pigments. It can thus be excluded that direct excitation of carotenoids is a source for the infrared signal observed, since excitation with $\lambda > 550$ nm, where carotenoids do not absorb, still causes a considerable infrared signal. In addition, the integrated action spectrum showed that, at excitation wavelengths $\lambda > 700$ nm, infrared signals can still be generated, provided that excitation intensity is much higher than the intensity needed to saturate photochemistry. These high excitation intensities could be obtained easily with the xenon flashes, but not with the laser flash. Infrared signals can thus be obtained under two conditions: (1) Excitation of the blue band of Chl with an intensity already below saturation. (2) Excitation of the red band of Chl with an intensity much higher than necessary for the saturation of photochemistry. These both conditions point directly to an excess energy dissipation mechanism being responsible for the infrared signals. The electronic ground state of Chl is a singlet state (S_0). (Here, the excited singlet states of Chl are named according to the nomenclature used by Shipman [29].) Absorption of red light leads to the first excited singlet state (S_1), while blue light gives rise to higher singlet states (S_n, $n > 2$). The contri-

tion of the S_2 state, which gives rise to an absorbance near 590 nm, can be neglected here because of its low oscillator strength. Fast radiationless processes quickly bring the molecules from these higher singlet states to S_1. The molecule gets rid of the difference in energy between the higher excited singlet states and the S_2 states by radiationless decay. When Chl is excited in its blue absorption band this dissipation occurs at any intensity of the exciting light, and would therefore give rise to infrared absorption changes even at intensities below saturation.

From the first excited singlet state (S_1) energy is transferred to the reaction centers and excess energy is dissipated via fluorescence or radiationless processes. This excess energy dissipation $S_1 \rightarrow S_0$ for high intensity excitation of the red band of Chl would also lead to infrared absorbance changes.

In both cases, the infrared signals do not show the primary dissipation processes, which occur much faster. It is more likely that it is the relaxation of the surrounding of the pigment molecules after energy transfer that gives rise to infrared absorbance changes.

For the correlation of excess energy dissipating mechanisms and infrared absorbance change discussed above, a different dependence of the infrared signal amplitude on excitation intensity should be obtained for the excitation in the blue or in the red band of the antenna pigments. This is illustrated schematically in Fig. 4. For the excitation in the soret band, infrared signals should be observed with their amplitude proportional to the intensity and a saturation intensity much higher than for photochemistry. For the excitation in the red band, no signal should be observed below or near saturation, and only at higher intensities should an infrared signal proportional to intensity be found. The hypothetical dependence of the infrared signals on red flash intensity drawn in Fig. 4 would correspond to the dependence of the carotenoid triplet signal amplitude on the actinic flash intensity measured by Renger and Wolff [1] in *Rps. sphaeroides*.

Two experimental limitations did not allow this hypothesis to be checked over the necessary range of excitation intensity. At low intensity of the exciting light, i.e., in the region of beginning excess of S_1 state population due to red excitation, infrared signals are beyond the limit of detection. On the other hand, the saturation of the infrared signal for red light or blue light excitation cannot be achieved with our excitation sources.

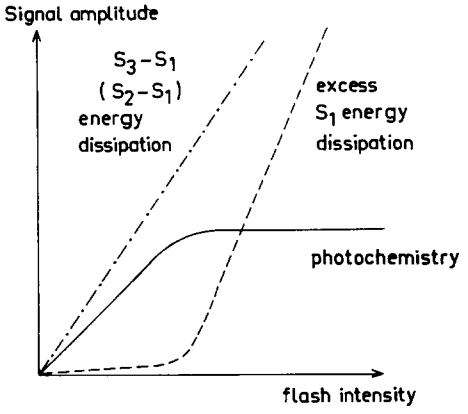

Fig. 4. Qualitative intensity dependence of signals indicating photosynthetic electron flow and hypothetical intensity dependence for excess energy dissipation mechanisms. The intensity dependence of photosynthetic electron flow is taken from Fig. 3. The states S_1, S_2 and S_3 denote excited states of the antenna Chl. The dashed line gives the hypothetical infrared signal amplitude for red light excitation, the dashed-dotted line for blue light excitation.

There remains the question about the molecular origin of the infrared absorbance changes. In the difference spectrum presented in Fig. 2, a strong positive band at 1640 cm^{-1} dominated in the band features observed. It can be assigned to the OH-bending vibration of water and its changes upon excess energy dissipation. This was confirmed upon the exchange of ^1H_2O by ^2H_2O. The difference spectrum of a sample in ^2H_2O together with one obtained from a sample in ^1H_2O is shown in Fig. 5. The spectral region was extended to 4000 cm^{-1} to cover the region of O^1H and O^2H stretching vibrations.

Deuteration changes tremendously the band structure in the difference spectra. In Fig. 5B the positive band at 1640 cm^{-1} disappears completely, and two bands at 1440 cm^{-1} and at 1200 cm^{-1} appear, at places where little or no absorbance change was observed with samples in ^1H_2O. In the high wavenumber region, a positive band at 3600 cm^{-1} and a negative band at 3300 cm^{-1} is observed, the largest part of which shifts upon deuteration to 2700 cm^{-1} and 2350 cm^{-1}.

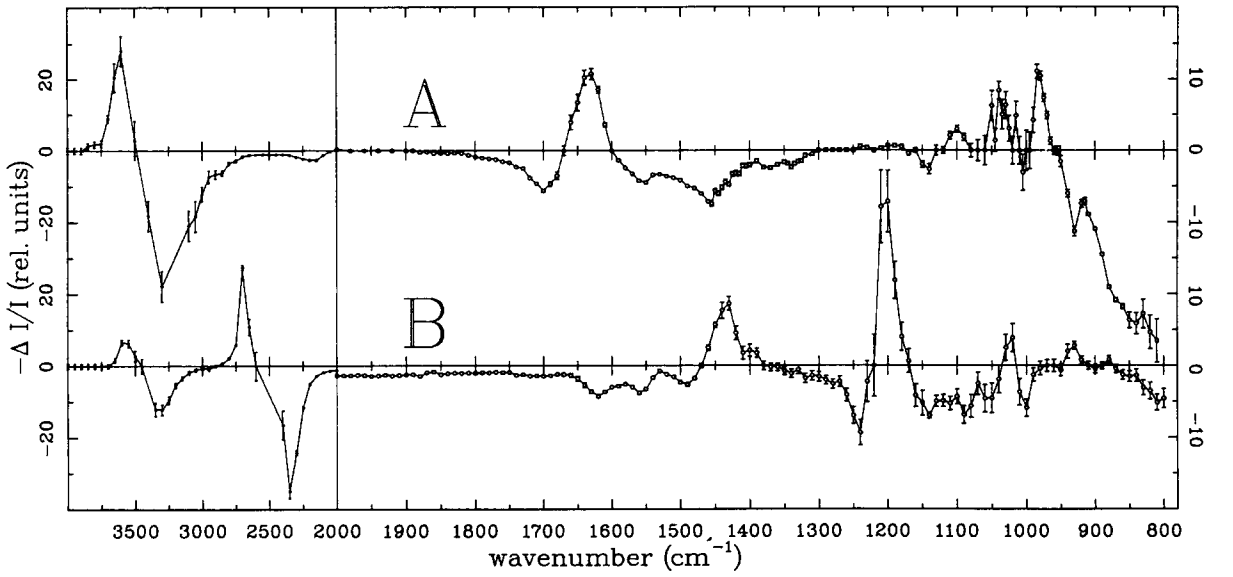

Fig. 5. Infrared difference spectrum of a thylakoid membrane film in ^1H_2O and in ^2H_2O. (A) Hydrated sample; (B) deuterated sample. The wavenumber region is extended to 4000 cm^{-1} to cover O^1H and O^2H stretching vibrations. Note changes in axis scaling at 2000 cm^{-1}.

According to band position and the shift observed upon deuteration, it seems reasonable to assign the positive band at 1640 cm^{-1} to the ^1H-O-^1H scissor vibration, the positive band at 1200 cm^{-1} to the ^2H-O-^2H scissor vibration, and the third band at 1440 cm^{-1} to the ^1H-O-^2H scissor vibration due to incomplete deuteration. The bands in the high frequency region can be assigned to the O^1H stretching vibration (3600 cm^{-1}/3150 cm^{-1}), and the O^2H stretching vibration (2700 cm^{-1}/2350 cm^{-1}). The residual bands at 3600 cm^{-1} and 3350 cm^{-1} in the spectrum of the sample in ^2H_2O are interpreted due to incomplete deuteration.

The bands attributed to O^1H and O^2H stretching vibrations show a clear differential feature, with a positive band at the high wavenumber side and a negative band at the low wavenumber side. For the bands attributed to the $^1HO^1H$, $^1HO^2H$ or $^2HO^2H$ scissor vibrations this differential feature is also present, although not very pronounced. Here we find the negative band at the high wavenumber side and the positive, much stronger band at the low wavenumber side. These differential band features can be seen in terms of the normal mode vibrations of water upon forming hydrogen bridges: if a water molecule is hydrogen-bridged to its neighborhood, the stretching vibration shifts to lower wavenumbers and the scissor vibration shifts to higher wavenumbers [30,31]. The differential band features can thus be understood as arising from water molecules that transiently go from a 'bound', i.e. hydrogen-bridged, state to a 'free', i.e. less hydrogen-bonded, state upon excess energy dissipation.

In all samples used to record difference spectra, sucrose was present for osmotic stabilisation. When sucrose was omitted, it was found that infrared signals could not be reproduced with the same accuracy in amplitude and in kinetics. A difference spectrum taken without sucrose [27] showed that the bands between 1250 cm^{-1} and 950 cm^{-1} had disappeared, whilst the other band features in the difference spectrum remained unchanged. The difference spectrin in Fig. 2 was compared to an infrared spectrum of sucrose published in Ref. 32. Strong bands in the sucrose spectrum appear at positions close to the bands at 1100 cm^{-1}, 1050 cm^{-1}, 980 cm^{-1} and 930 cm^{-1} in Fig. 2. Since sucrose molecules are known to replace water molecules at the protein-water interface, it seems likely that they participate in a similar way as the

water molecules in excess energy transfer. The water molecules giving rise to the infrared signal are therefore, possibly, located in the bulk water compartments.

Characterization of infrared absorbance changes in bacterial chromatophores

In spinach thylakoids, about 300 antenna chlorophyll molecules serve one reaction center [33]. This ratio is much smaller in *Rps. capsulata* chromatophores, where about 40–100 antenna bacteriochlorophyll molecules serve one reaction center [34]. This has important consequences for the infrared signals: if there was a contribution of reaction center photochemistry to infrared absorbance changes, then it was obviously too small to be detected in the infrared difference spectra of spinach thylakoids. Based on the same concentration of Chl, the contribution due to electron transfer processes should be at least 5-times larger in bacterial chromatophores. In addition, the background absorbance should be smaller due to the lower amount of polypeptides that are not functionally active in light-induced electron transfer.

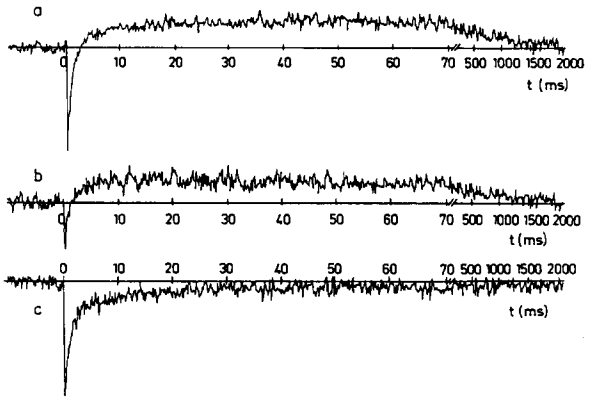

Fig. 6. Time resolved infrared absorbance change from bacterial chromatophore membrane films. (a) Chromatophores of *Rps. capsulata* mutant NK_3 containing the reaction center complex, LHC_I, and carotenoids. (b) Chromatophores of the *Rps. capsulata* mutant A_1A^+ containing the reaction center complex, LHC_I complex, but lacking carotenoids. (c) Chromatophores of the *Rps. capsulata* mutant Y5 containing LHC_{II} complex and carotenoids, but lacking a reaction center. Measuring wavenumber is 1460 cm^{-1} at a resolution of 6 cm^{-1}. Membranes were kept at 98% relative humidity at $T = 20°C$. Samples were excited with white light from a xenon flash ranging from 350–800 nm. 50 signals were added in each case to improve signal-to-noise ratio. Note the change in timebase at $t = 70$ ms.

Fig. 6a shows a time-resolved infrared absorbance change at 1460 cm^{-1} in chromatophores of *Rps. capsulata* NK_3 (reaction center, LHC_I, carotenoids, see Table I). Upon flash excitation, a fast absorbance decrease is observed, which decays with a half-time of about 1–2 ms to an absorbance value higher than before the flash. This positive component of the signal decays very slowly, within the time range of some hundred milliseconds. A similar signal is observed for chromatophores of the mutant A_1A^+ (reaction center and LHC_I but not carotenoids), Fig. 6b. Here the fast negative component seems smaller than that in the signal in Fig. 6a. An infrared signal obtained from chromatophores of the mutant Y5 (no reaction center, but LHC_{II} and carotenoids) is shown in Fig. 6c: only a negative component is seen, which can be separated in two components, one decaying with a half-time of 1–2 ms, the other with 10–20 ms. No positive long-persisting component is observed.

The spectral distribution of the signal components was recorded between 1750 cm^{-1} and 1300 cm^{-1}, with the exception of the region between 1600 cm^{-1} and 1680 cm^{-1}, which was obscured by the absorption of the amide-I band and the OH band of water. The slow and the fast component of the signals from membranes of the NK_3 and A_1A^+ mutants were separated, thus assuming two independently decaying exponential functions. For membranes of the mutant Y5, only the total amplitude is given, since the ratio of amplitudes of the two decay components was found to be the same over the whole wavelength range. The infrared difference spectra of the three different membranes are shown in Fig. 7a–7e.

The difference spectra of the fast relaxing component (Fig. 7a and c) varies markedly from one type of the membrane to the other and obviously depends on the composition of the membranes. The difference spectra of the slow components, however, are very similar for membranes of the mutants NK_3 and A_1A^+. The slow component is completely lacking for the Y5 mutant. The transmission changes corresponding to the slow components of the infrared signals are, in general, smaller than those of the fast component. Their spectral distribution shows a broad positive band around 1300 cm^{-1}, a small positive band around 1400

cm^{-1} and another positive band around 1480 cm^{-1}. The accuracy of the amplitude evaluation decreases in the spectral region between 1600 cm^{-1} and 1700 cm^{-1} due to the high amide-I and water absorption. This region is therefore omitted in the difference spectra. Above 1700 cm^{-1}, two further bands at 1710 cm^{-1} and at 1750 cm^{-1} appear.

The lack of the slow component for membranes of the Y5 mutant suggests the necessity of a reaction center as a prerequisite. A relation of the slow

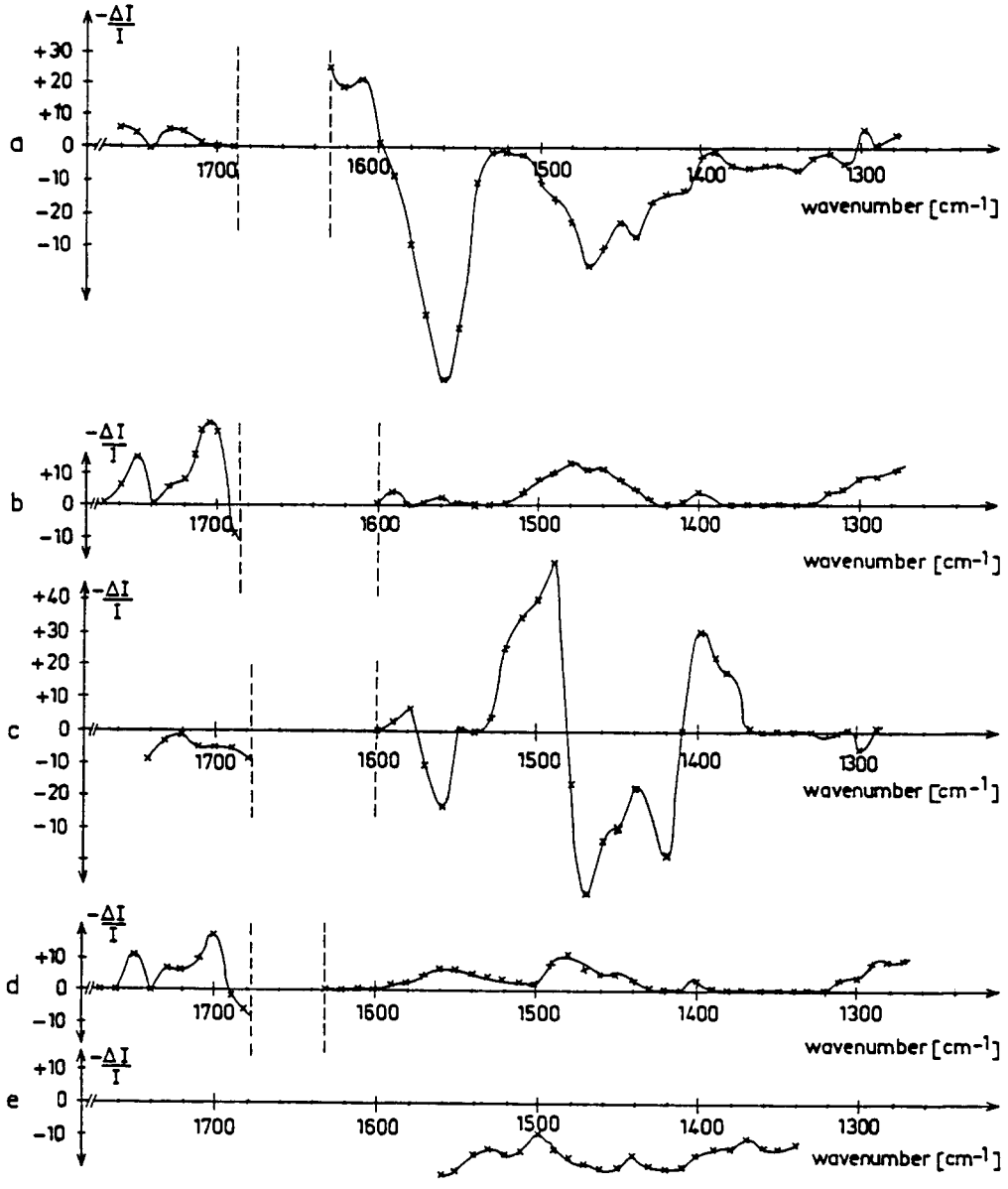

Fig. 7. Infrared difference spectra from films of chromatophore membranes of different *Rps. capsulata* mutants. The slow and fast signal components shown in Fig. 6 were separated. (a) Mutant NK_3 (reaction center, LHC_I, carotenoids), fast signal component; (b) mutant NK_3, slow signal component; (c) mutant A_1A^+ (reaction center, LHC_I), fast signal component; (d) mutant A_1A^+, slow signal component; (e) mutant Y5 (LHC_I, carotenoids), total signal amplitude. Signals were recorded at a resolution of 8 cm^{-1}. For each data point, 50 signals were added to improve the signal-to-noise ratio. The spectral region around 1650 cm^{-1} is obscured by O-H absorption and thus excluded in the difference spectra. Measuring conditions are as in Fig. 6.

decaying signal component and electron transfer reactions is also suggested by the fact that the kinetic constant of the slow signal component is very similar to the kinetic constant of the reduction of the primary electron donor observed at 860 nm or at 600 nm at the same samples.

A tentative assignment of the slow component to processes in the reaction center, based on the comparison of different mutants, was supported by recording time-resolved infrared difference spectra of isolated reaction centers incorporated into lipid vesicles following the procedure described by Nabedryk et al. [8]. These infrared difference spectra of isolated reaction centers (data not shown) showed only the slow component already seen in the difference spectra of NK_3 and A_1A^+ chromatophore membranes.

The fast infrared signal components present for membranes of all mutants show a decay time of 1–2 ms. It does not depend on the presence of a reaction centers but varies markedly according to the composition of the pigments and pigment-protein complexes in the membrane. We therefore assume that this component is caused by energy-dissipating processes in the antenna complexes, and thus corresponds to the infrared signals from plant thylakoids shown above.

Additional evidence for an assignment of the fast signal component to energy-dissipating processes and of the slow-signal component to reaction-center photochemistry comes from the different dependence of the signal components on flash intensity. In this experiment, time-resolved infrared absorbance changes were induced by intense xenon flashes of about 200 μs duration, where the time course of the discharge current was modified by a choke of appropriate inductivity to give a slow rise of the flash intensity. With this modification, the flash profile was symmetrical and maximum flash intensity was reached after about 100 μs. The time course of the flash intensity and of absorbance changes recorded at 1490 cm^{-1} and at 1560 cm^{-1} (bands of the fast component) as well as at 1700 cm^{-1} and at 1750 cm^{-1} (bands of the slow component) are shown in Fig. 8a–e. Whereas the rise of the signals at 1490 cm^{-1} and at 1560 cm^{-1} follows the flash profile and reaches its maximum value at about 100 μs, the signals at 1700 cm^{-1} and at 1750 cm^{-1} rise much faster, and thus indicate that they have already been generated by a small fraction of the flash intensity. The observation that the amplitude of the slow signal component has already reached

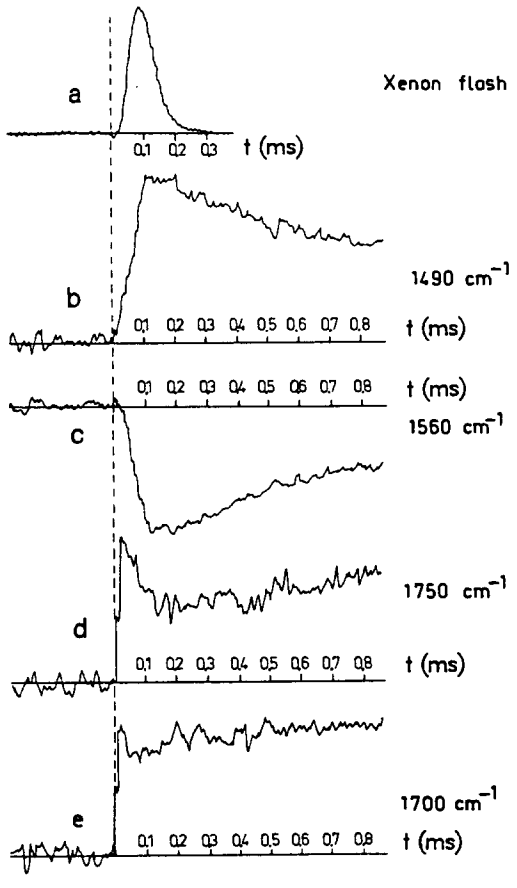

Fig. 8. Response of time-resolved infrared absorbance change from NK_3 chromatophores to an excitation flash profile. (a) Time course of the xenon flash intensity as measured with a photodiode detector at a time resolution of 1 μs. The rise of the flash intensity was slowed down by using a choke. Maximum intensity is reached about 100 μs after triggering of the flash. (b)–(e) Time course of infrared signals caused by the xenon flash with the profile shown in (a). The signals in (b) and in (c) were taken at 1490 cm^{-1} and at 1560 cm^{-1} at maxima of the bands in the difference spectrum of the fast component in Fig. 7c. The maximum amplitude of the signal is reached with the maximum intensity of the xenon flash. The signals in (d) and (e) were taken at 1750 and at 1700 cm^{-1} at maxima of the difference spectrum of the slow component in Fig. 7d. The maximum amplitude of the signals is reached 10–20 μs after triggering of the flash, indicating the saturation of these signals already at low intensity. Measuring conditions were as in Fig. 6.

saturation at moderate flash intensity and that the amplitude of the fast signal component increases with flash intensity even at high intensities strongly support the assignment of the slow components to photochemistry and the fast components to processes in the antenna complexes, possibly energy dissipation mechanisms. When all primary electron donors are photooxidized, an increase of the slow signal component cannot be expected. On the other hand, flash intensities high enough to excite most of the antenna molecules could not be reached in our experiments.

In addition to these assignments by the use of different mutants, by the comparison of the signal decay kinetics with the kinetics of the electron-transfer processes, and by the different saturation behaviour of the two components, further evidence for the origin of the slow components was obtained by investigating membranes with the reaction center oxidized with ferricyanide. Infrared absorbance changes from these membranes showed only the fast signal component. It was obviously not influenced by the chemical oxidation of the reaction center. The slow reaction component, however, was completely inhibited.

In order to establish a similar relation to excess energy dissipation for the fast signal observed in chromatophores as it was done for the corresponding signals in thylakoid membranes, the infrared signals were compared for different excitation wavelengths (data not shown). Since laser excitation at 750 nm was not possible at sufficiently high energy, xenon flash excitation was used with appropriate filters to cut off excitation light that would populate the states S_n, $n > 2$. The fast, negative component is reduced by a factor of at least 5 when the blue-green part of the excitation light is cut off. This experiment allows direct excitation of the carotenoids to be excluded as a reason of the fast signal component. Different signals for membranes with and without carotenoids, however, are observed (Fig. 7a and c).

In the difference spectra of thylakoids shown in Fig. 5, deuteration of the membrane induced drastic changes and was thus used to assign bands to vibrational changes of water molecules involved in the energy dissipation process. Deuteration also induces drastic changes in the difference spectra of bacterial chromatophores as seen in Fig. 9, mostly affecting the fast component of the signal. The effects of deuteration do not give any explanation for the positive bands at 1490 cm^{-1} and 1400 cm^{-1}, and the negative bands at 1470 cm^{-1} and at 1420 cm^{-1} in Fig. 9a in terms of vibrations of the hydroxyl groups of water. It is also not possible to assign these bands to molecular vibrations of Chl, that might change as a consequence of energy dissipation. The argument against an assignment to vibrations of Chl comes from the decay time of these signals: the infrared absorbance changes persist some milliseconds, orders of magnitude longer than the processes of energy conduction in light-harvesting complexes, and still much longer than the lifetime of triplet states.

There is a remarkable similarity between the ^2H_2O difference spectrum of bacterial chromatophores in Fig. 9b and the ^2H_2O difference spectrum of spinach thylakoids in Fig. 5b. The three negative bands at 1620 cm^{-1}, 1560 cm^{-1} and 1480 cm^{-1} are present in both spectra, as well as the positive band at about 1440 cm^{-1}. The latter was attributed to the ^1H-O-^2H scissor vibration in the spectra of spinach thylakoids. Another assignment for this band in the difference spectrum to the amide-II band (at 1450 cm^{-1} in ^2H_2O) can be excluded, since it would imply that in ^1H_2O a band of similar strength should be observed around 1450 cm^{-1}, the position of the amide-II band of a sample in ^1H_2O. The fact that there is no corresponding similarity between the ^1H_2O difference spectra of the bacterial chromatophore and spinach thylakoid spectra may be explained by the assumption that, in the bacterial chromatophore spectra, additional protein signal components are superimposed. Deuteration obviously shifts away these superimposed components.

The difference spectrum of the slow signal component (Fig. 9d) is not very much influenced by ^2H_2O. The bands between 1500 cm^{-1} and 1400 cm^{-1} seem to be stronger and broadened, and a negative band at 1620 cm^{-1} is observed, where, in the spectrum in H_2O, the absorption of the O-H scissor vibration obscured the spectra. The two bands at 1750 cm^{-1} and at 1700 cm^{-1} remain unchanged, as well as the band structure at about 1300 cm^{-1}. The two bands at 1750 cm^{-1} and at 1700 cm^{-1} can possibly be assigned to the ester C = O and keto C = O vibrations of BChl in reac-

tion centers, although they are located at a position somewhat higher (8–10 cm^{-1}) as found by Ballschmitter et al. [10] for bacteriochlorophylls in solution. Since these signals persist over the same time range as does the oxidized primary donor, it is reasonable to assume that these bands arise from carbonyl groups of the primary donor. The size of the absorbance change is rather high and no negative, disappearing band is observed close by. It may thus be concluded that the carbonyl groups absorb only very weakly in this region for the reduced primary donor. This is, possibly, a hint to hydrogen bonding between the carbonyl group and its counterpart, either in the poly-

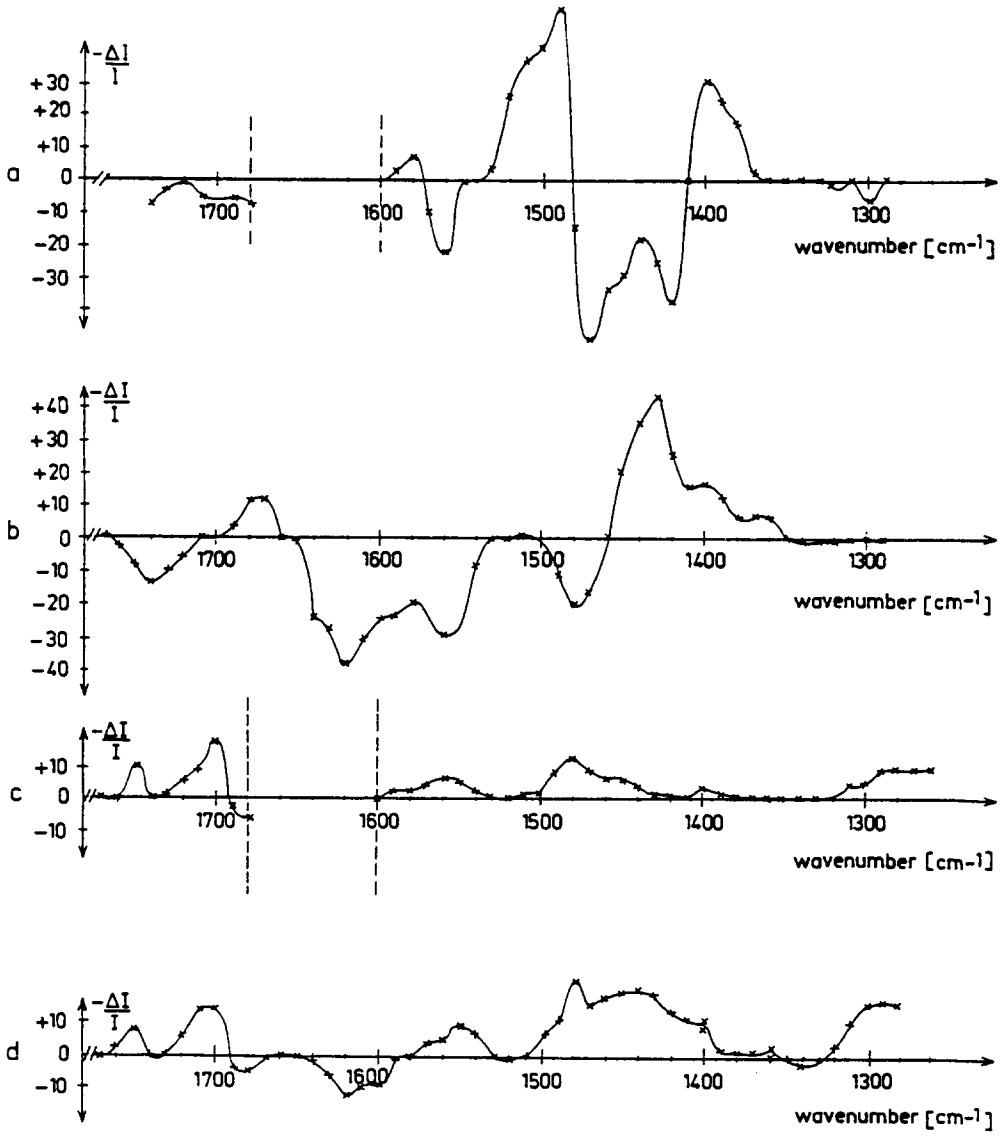

Fig. 9. Infrared difference spectra from films of bacterial chromatophore membranes from *Rps. capsulata* mutant A_1A^+ in ^1H_2O and in ^2H_2O. (a) fast signal component, ^1H_2O; (b) fast signal component, ^2H_2O; (c) slow signal component, ^1H_2O; (d) slow signal component, ^2H_2O. In (a) and (c), the spectral region around 1650 cm^{-1} is obscured by OH absorption, and is therefore omitted in the spectra. Measuring conditions were as in Fig. 7.

peptide or in a second BChl, which would give rise to a band position shifted to lower wavenumbers.

Concluding discussion

The results presented above have demonstrated that one type of transient infrared absorbance changes in the antenna pigments systems of plant thylakoids and bacterial chromatophores appears due to excess energy dissipation. A second type of transient infrared absorbance changes is functionally coupled to electron transfer processes in the bacterial reaction center. The relaxation of the infrared absorbance changes of the first type could be time-resolved and reflect processes in the millisecond time scale, strongly dependent on the water present in the membranes.

From flash photolysis investigations of thylakoids and chromatophores [1,4] transient optical absorbance changes not arising from photochemical electron transfer are known. These absorbance changes are, in general, interpreted as the formation of triplet states of carotenoids, and, in membranes lacking carotenoids, as triplet states of Chl. The decay times of the triplet states are in the order of microseconds for carotenoids and some tens of microseconds for Chl. Energy must be conducted from the chromophore molecules to their environment within that time, to prevent the chromophores from photooxidation and avoid destruction of the highly organized pigment structure. Although this primary energy dissipation occurs very rapidly, the environment of the chromophores relaxes much slower, in the millisecond time scale, as it is indicated by the infrared absorbance changes. Since we were not able to detect fast absorbance transients decaying in some tens of microseconds, we assume that triplet generation itself does not give rise to large changes of the molecular vibrations. For the infrared signals that we have attributed to processes in the antenna pigment system, the rise could not be time-resolved and thus must be faster than 10 μs. The transient infrared absorbance changes obtained from plant thylakoids indicate that these relaxation processes involve water molecules that transiently go from the hydrogen bridged to the free state. Additional transient absorbance changes, possibly arising from functional groups in the polypeptide are observed in chromatophores lacking carotenoids. In order to obtain fast and efficient energy dissipation in the light-harvesting pigment-protein complexes, a close protein-protein interaction and/or pigment-water interaction has to be claimed. In light-harvesting complexes of photosynthetic bacteria, this interaction is also claimed to account for the large red-shifts of more than 100 nm as compared to BChl in solution.

An approach to the molecular interpretation of the transient absorbance changes in chromatophores of photosynthetic bacteria, with or without carotenoids, presents serious difficulties. These transients presumably arise from functional groups in the polypeptide, and can therefore not easily be compared with model compounds. There is, however, some progress in crystallizing light-harvesting complexes [35], and it can be expected that soon more information will be available about the polypeptide structure around Chl. Up to now only the structure of a water soluble antenna complex from *Prosthecochloris aestuarii* is known in sufficient detail from X-ray diffraction data of Fenna, Matthews and co-workers [36,37]. Resonance Raman data obtained from this antenna pigment-protein complex by Lutz et al. [39] indicate that the influence of the polypeptide environment on the molecular structure of the chlorophylls is stronger than that of the aggregation state of the chlorophylls, and thus point out the importance of the binding site. One should be careful, however, in comparing this water-soluble complex too closely with the rather hydrophobic antenna complexes from purple photosynthetic bacteria and plant thylakoids.

The second type of infrared absorbance changes, which is functionally coupled to reaction-center electron-transfer processes, is most probably caused by molecular vibrations of the BChl molecules constituting the primary donor molecules and the groups in their polypeptide environment involved in bonding. The groups on the BChl molecules most likely involved in bonding are the keto and ester carbonyl groups. The latter do not participate in the conjugation of the π-electron system and are, therefore, not observed in resonance Raman spectra of reaction centers [14].

The band in the difference spectrum of the slow component at 1750 cm^{-1}, however, may well be

due to an ester carbonyl absorbance. A second band at about 1700 cm^{-1} correlates well with a band in the resonance Raman spectra obtained by Lutz [14] at 1708 cm^{-1}, assigned to a keto carbonyl group. Both bands are obviously weak in the reduced state and strong in the oxidized state of the reaction center.

It still remains unclear how these groups on the chlorophyll molecule are influenced by their environment, and how they can give such large changes upon photooxidation. With increasing knowledge on the molecular structure of the photosynthetic reaction center obtained by crystal analysis, it should be possible to identify the binding and interaction sites and learn more about the factors that influence the chlorophyll side groups. In further experiments, static Fourier transform infrared difference spectra of bacterial reaction centers have been obtained [39] that show a number of specific molecular changes upon photooxidation. It can be expected that they will contribute to the understanding of the interaction of the chlorophyll molecules with their environment.

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