Low-Temperature Femtosecond Spectroscopy of the Initial Step of Electron Transfer in Reaction Centers from Photosynthetic Purple Bacteria†

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ABSTRACT: The initial step of charge separation at 10 K has been monitored with 100-fs time resolution in reaction centers from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* as well as in reaction centers from the latter species in which one of the two monomeric bacteriochlorophyll (B) molecules has been removed by treatment with borohydride. Upon excitation at 870 nm, the absorbance changes measured at several wavelengths in the near-infrared absorption bands of the pigments, and notably at the absorption maximum of the B molecule(s), give no indication of a detectable concentration of B^-. Instead, the appearance of the cation radical of the dimeric primary electron donor (P) and of the bacteriopheophytin anion develops in concert with the decay of P*. An initial bleaching of the 850-nm band in reaction centers from *Rhodopseudomonas viridis* is consistent with an assignment of at least a large fraction of this band to the high-energy exciton component of P. Upon excitation of the B molecule(s) around 600 nm in the three types of reaction centers investigated, ultrafast energy transfer leads to the formation of P* in less than 100 fs. Under these conditions, a fast transient bleaching decaying with a 400-fs time constant is observed within the absorption band of B. This transient is also present upon preferential excitation of the bacteriopheophytins in the reaction center of *Rhodopseudomonas viridis*.

The very efficient transmembrane charge separation and stabilization processes which drive all the subsequent chemistry of photosynthesis occur in hydrophobic pigment–protein complexes named reaction centers. Following the isolation from photosynthetic purple bacteria of reaction centers in a pure and photochemically active form (Reed & Clayton, 1968), these complexes have been the object of many structural and mechanistic investigations using a variety of spectroscopic techniques (Parson, 1982; Breton & Vermeglio, 1982; Hoff, 1982; Kirmaier & Holten, 1987). The recent X-ray crystallographic determination of the structure of the reaction center from *Rhodopseudomonas* (*Rps.*)[1] *viridis* at 3-Å resolution (Deisenhofer et al., 1985; Michel et al., 1986) and similar studies on the reaction center of *Rhodobacter* (*Rb.*) *sphaeroides* (Chang et al., 1986; Allen et al., 1987) have shown the detailed organization of the prosthetic groups (4 bacteriochlorophylls, 2 bacteriopheophytins, 1 or 2 quinones, and 1 non-heme iron) anchored to a scaffold of 10 α-helices belonging to the 2 polypeptides L and M. These two subunits as well as the prosthetic groups are organized with approximate C-2 symmetry about an axis running from the iron atom to the center of the primary electron donor (P) which consists of a dimer of closely interacting bacteriochlorophylls. Two bacteriopheophytins (H_L and H_M) are located on either side of P, and the two remaining bacteriochlorophylls (B_L and B_M) are arranged approximately in between H_L or H_M and P. For reasons that are not yet elucidated, but which probably stem from a disymmetry in the proteic environment and in the bonding interactions of the chromophores with the protein, the electron transfer proceeds along the L "branch" of pigments, which extends toward Q_A (Michel-Beyerle et al., 1988).

The B_L molecule seems to form a bridge between P and H_L with center to center distances of ≈11 Å between B_L and P or H_L while the center to center distance between P and H_L is ≈17 Å. This is highly suggestive of an involvement of B_L in the electron transfer from P to H_L. Such a possible role of B_L has been the object of intensive experimental investigations. Using relatively long laser flashes (30 ps) with partial overlap of pump and probe pulses, Shuvalov et al. interpreted their data in terms of the creation of a state $P^+B_L^-$ prior to the formation of the better characterized state $P^+H_L^-$ (Shuvalov et al., 1978, 1986; Shuvalov & Klevanik, 1983; Shuvalov & Duysens, 1986). However, others using a similar experimental approach have found no convincing evidence for the state $P^+B_L^-$ (Borisov et al., 1983; Kirmaier et al., 1985). Femtosecond spectroscopy with excitation in the 600-nm region, where all four bacteriochlorophylls absorb, has variously led to interpretations either in favor of the existence of $P^+B_L^-$ (Zinth et al., 1985a; Chekalin et al., 1986, 1987), against this model (Woodbury et al., 1985; Zinth et al., 1986), or inconclusive regarding the presence of $P^+B_L^-$ (Holten et al., 1980). This ambiguity is due to the difficulty in discriminating between a transient bleaching of B due to the formation either of B*, the excited state of B, or of B_L^-. Such an ambiguity does not exist upon direct excitation of P in its lowest energy band with femtosecond pulses. Under these conditions, no transient bleaching of B_L could be resolved at room temperature, and it was concluded that there was no evidence for a state $P^+B_L^-$ lasting more than 100 fs [Martin et al., 1986;

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[1] Abbreviations: *Rps.*, *Rhodopseudomonas*; *Rb.*, *Rhodobacter*; P, primary electron donor; B, reaction center monomeric bacteriochlorophyll (B_L or B_M); H, reaction center bacteriopheophytin (H_L or H_M); Q_A, first quinone electron acceptor.

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Breton et al., 1986a,b,c; see also Wasielewski and Tiede (1986)].

Another difficulty in the interpretation of the fast absorbance changes associated with the initial electron transfer is due to the estimation of the relative contributions of the six pigments to each of the various bands in the absorption spectra of the reaction center. Although these spectra are usually described in terms of the main contribution from the P, B, or H pigments to each band, recent theoretical modeling of the spectra using the atomic coordinates of the chromophores obtained from X-ray spectroscopy (Deisenhofer et al., 1985) has led to the notion that the degree of excitonic coupling among all the pigments could be significantly larger than previously assumed (Parson et al., 1985; Knapp et al., 1985, 1986; Zinth et al., 1985b; Parson & Warshel, 1987; Lous & Hoff, 1987). In the near-infrared, the position and overlap of the absorption bands depend strongly on the chemical nature of the bacteriochlorin pigments, which is different in the reaction centers of *Rb. sphaeroides* and *Rps. viridis*, and also on the temperature so that cryogenic temperatures considerably improve the resolution of the spectra.

In the present report, we have investigated with 100-fs time resolution the spectral changes associated with the initial step of electron transfer in reaction centers from *Rps. viridis* and *Rb. sphaeroides* R-26. We have also studied reaction centers from the latter species after borohydride treatment, which is currently thought to remove the B_M molecule selectively (Maroti et al., 1985). We have worked at low temperature to improve the spectral resolution of the near-IR transitions of the chromophores. We have compared the results obtained by direct excitation of P with those obtained by exciting either the B or the H molecules.

Materials and Methods

Reaction centers from *Rb. sphaeroides* R-26 and *Rps. viridis*, prepared as previously described (Berger et al., 1984; Breton, 1985), were suspended in 0.1% sodium cholate and 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.8. The borohydride treatment of *Rb. sphaeroides* reaction centers was performed according to Maroti et al. (1985). In order to ensure oxidation of the high-potential cytochromes, 1 mM potassium ferricyanide was added to the *Rps. viridis* reaction centers. The reaction centers were diluted to a final concentration of 50–80 μM in 60% (v/v) glycerol. The samples, contained in 1-mm optical path-length cells, were cooled in the dark using a convection cryostat with a temperature-regulated (±0.5 °C) flow of helium gas.

Generation of 50-fs pulses and amplification of these pulses to power in the gigawatt regime have been described previously (Fork et al., 1983; Martin et al., 1983a,b, 1986; Petrich et al., 1987). Briefly, pulses of approximately 60-fs duration are obtained from an eight-mirror, colliding-pulse ring laser with four intracavity prisms to compensate for dispersion. These pulses are amplified to ≈1 mJ at 10 Hz by a four-stage dye amplifier. This beam is split in two parts and used to generate two stable continua. To obtain a tunable pump beam, we further exploit the technique of continuum amplification (Martin et al., 1983a,b, 1984, 1986) using rhodamine 6G to generate pump pulses at 600 or 585 nm, LDS 867 to generate pulses at 870 nm, and LDS 821 for excitation at 780 and 810 nm. Typical duration of the pump and probe pulses was 200 fs. Unless otherwise specified, 15–30% of the reaction centers were photooxidized on each pulse.

Data collection, analysis of the measured kinetics, and fitting of the curves were carried out essentially as described (Martin et al., 1983a,b, 1988).

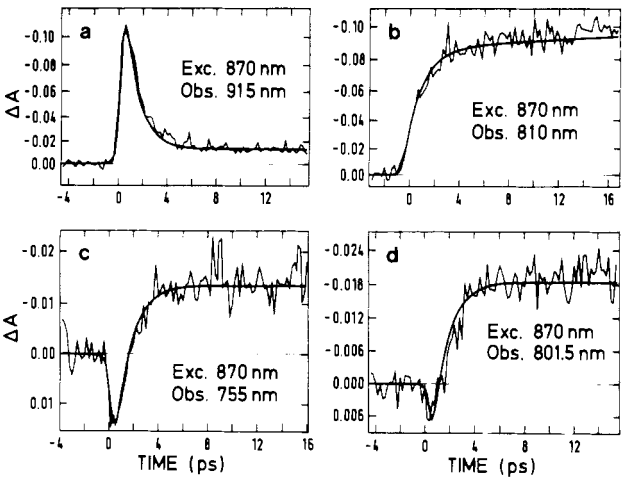

FIGURE 1: Kinetics of absorbance changes measured at 10 K for reaction centers from *Rb. sphaeroides* R-26 upon excitation at 870 nm. The smooth curve, which represents the best fit to the observed kinetics, includes the convolution of the pump and probe pulses (200 fs each). (a) Stimulated emission from P* relaxing with a time constant of 1.2 ± 0.1 ps. (b) Bleaching developing with a main 1.2-ps time constant and a small amount (10%) of a 100-ps kinetic component. (c and d) The initial absorbance increase, assigned to P* and decaying with 1.2-ps kinetics, is superimposed on a phase of bleaching developing with a 1.2-ps time constant.

Results

Excitation at 870 nm. Upon direct excitation of *Rb. sphaeroides* R-26 reaction centers in the long-wavelength transition of P at 870 nm, the decay of the stimulated emission from P* measured at 915 nm at 10 K (Figure 1a) proceeds with a 1.2-ps time constant (Fleming et al., 1988; Martin et al., 1988). The same time constant is observed for most of the bleaching at 810 nm (Figure 1b), assigned to the band shift to higher energies of the B molecules, and for the bleaching at 755 nm (Figure 1c), which corresponds to the reduction of H_L. This last bleaching is superimposed on an initial instantaneous (less than 100 fs) absorbance increase assigned to excited-state absorption from P*. Around 800 nm, near the isosbestic point for the band shift, a small initial absorbance increase is followed by a 1.2-ps phase of bleaching (Figure 1d). The amplitude of this last phase is significantly reduced compared to that observed at 810 nm. A component with a small amplitude (10%) and a lifetime of approximately 100 ps is consistently needed to obtain a satisfactory fit of the bleaching traces at 810 nm (Figure 1b). This component is ascribed to a change in the amplitude of the band shift occurring upon electron transfer from H_L to Q_A (Kirmaier & Holten, 1987).

Following the removal of B_M by borohydride treatment, the modified reaction center from *Rb. sphaeroides* R-26 still exhibits the same 1.2-ps time constant for electron transfer at 10 K as observed with the unmodified reaction centers. This can be seen in the decay of the stimulated emission at 915 nm (Figure 2a) as well as in the absorbance decrease and absorbance increase associated with the band shift of the B_L molecule monitored at 810 nm (Figure 2b) and 785 nm (Figure 2c), respectively. Upon detection close to the isosbestic point for the band shift around 800 nm, no evidence for a detectable bleaching of B_L can be found, and the kinetics (data not shown) are essentially identical with those observed for the unmodified reaction center (Figure 1d). Due to the limitation in the signal to noise ratio, it should be noticed that a satisfactory fit of the kinetic traces at 810 nm for both normal (Figure 1b) and modified (Figure 2b) reaction centers from *Rb. sphaeroides* could also be obtained by assuming up

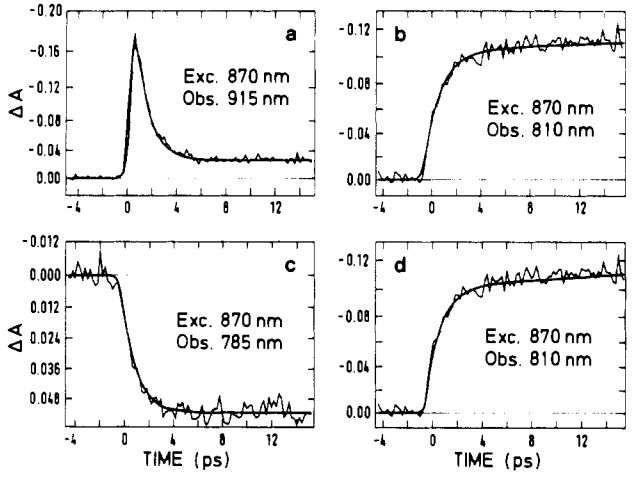

FIGURE 2: Same conditions as in Figure 1 but for borohydride-treated reaction centers from *Rb. sphaeroides* R-26. (a and b) Same fits as in the corresponding traces of Figure 1. (c) Induced absorption developing with a 1.2-ps time constant. (d) Same experimental curve as in (b), but the fit includes three components of bleaching developing with time constants of 0 ps (30% amplitude), 1.2 ps (60% amplitude), and 100 ps (10% amplitude).

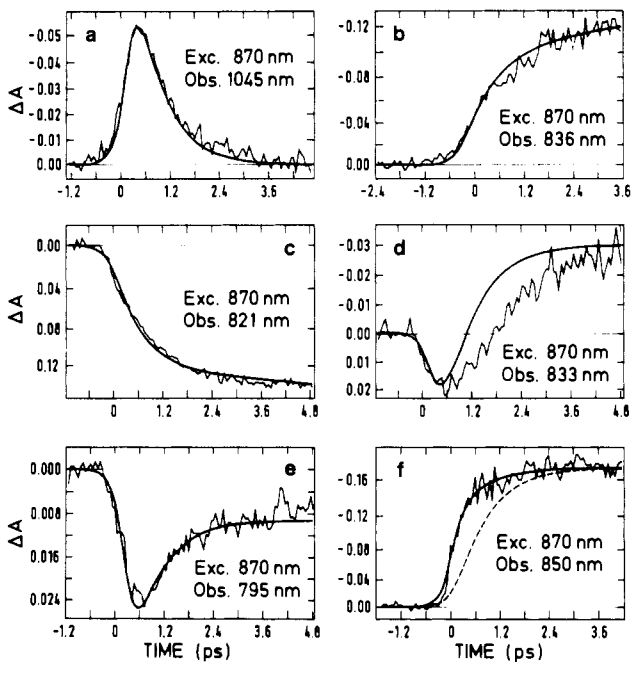

FIGURE 3: Same conditions as in Figure 1 but for reaction centers from *Rps. viridis*. (a) Stimulated emission from P* decaying with a 700 ± 100 fs time constant. (b) Apparent bleaching developing with time constants of 700 fs (80% amplitude) and 170 ps (20% amplitude). (c) Absorbance increase developing with time constants of 700 fs (85% amplitude) and 170 ps (15% amplitude). (d) Kinetics observed close to the absorption maximum (834 nm) of the reaction centers. The smooth curve shows a simulation assuming an initial absorbance increase decaying in 700 fs and a bleaching developing with the same time constant. (e) An initial absorbance increase assigned to P* and relaxing with a 700-fs time constant is superimposed on an absorbance increase developing with a 700-fs time constant. (f) The bleaching at 850 nm cannot be fitted with a single 700-fs time constant (---) but can be fitted when 70% of instantaneous bleaching is also included (—).

to about 30% of an instantaneous bleaching. This is demonstrated for the modified reaction centers by comparing the two fits of the bleaching at 810 nm shown in Figure 2b,d.

When reaction centers from *Rps. viridis* at 10 K are excited at 870 nm, the decay of the stimulated emission monitored at 1045 nm can be well fitted (Figure 3a) with a single exponential of 700-fs time constant (Fleming et al., 1988; Martin et al., 1988). Together with a minor component associated with the 170-ps kinetics of electron transfer from H_L to Q_A (Kirmaier & Holten, 1987), this same time constant is observed for the band shift of the 834-nm band, leading to an apparent bleaching on the long-wavelength side (Figure 3b) and an absorbance increase on the short-wavelength side (Figure 3c) of this band. At 833 nm, near the isosbestic point for the band shift, a rather complex kinetic pattern is observed (Figure 3d). As shown by the poor quality of the fit, the initial absorbance increase occurs too slowly to be ascribed solely to P*. We assign part of this absorbance change to the development of the band shift itself as observed from a wavelength located about 1 nm to the blue side of the maximum of the 834-nm band (Martin et al., 1988). Upon probing at 795 nm, a fast absorbance increase, assigned to P*, is followed by a 700-fs phase of recovery (Figure 3e). A bleaching is observed when monitoring in the 840–860-nm region. It develops with two kinetic phases: a fast phase that is not kinetically resolved and a 0.7-ps phase. The relative amplitude of the fast component increases from 840 to 860 nm. The trace monitored at 850 nm is shown in Figure 3f and can be well fitted with a 70% contribution of instantaneous bleaching (—). A 0.7-ps kinetic component alone (---) cannot fit the observed curve.

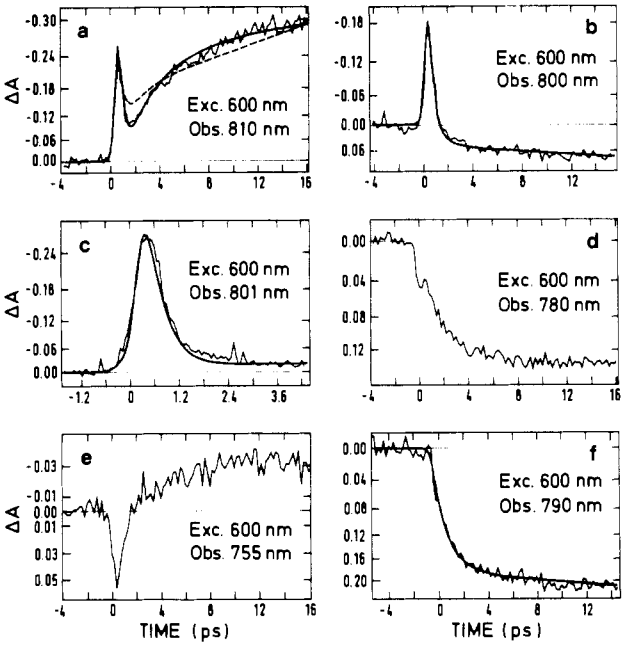

FIGURE 4: Kinetics of absorbance changes measured at 10 K for reaction centers from *Rb. sphaeroides* R-26 upon excitation at 600 nm. (a) The fits take into account the fast transient bleaching recovering in 400 fs and either the two components of bleaching used in Figure 1b (---) or a bleaching developing with a 1.2-ps time constant and an induced absorption rising in 400 fs and decaying with a 6-ps time constant (—). (b) The fit includes a fast transient bleaching relaxing with a 400-fs time constant and absorbance increases developing with time constants of 1.2 and 100 ps. (c) On a 6-ps full scale, the fast transient bleaching relaxes with a 400 ± 100 fs time constant. (d and e) These kinetics reveal a contribution of induced absorption from the 400-fs transient. (f) The fit describes absorbance increases developing with time constants of 1.2 ps (80% of the amplitude) and 100 ps (20% of the amplitude).

Excitation of the Monomeric Bacteriochlorophylls and of the Bacteriopheophytins. When reaction centers from *Rb. sphaeroides* R-26 are excited in the Q_X transition of the bacteriochlorophylls at 600 nm, the absorbance changes monitored at 10 K in the spectral range 820–800 nm show, in addition to the absorbance decrease or increase developing in 1.2 ps and described in the previous section, a fast transient

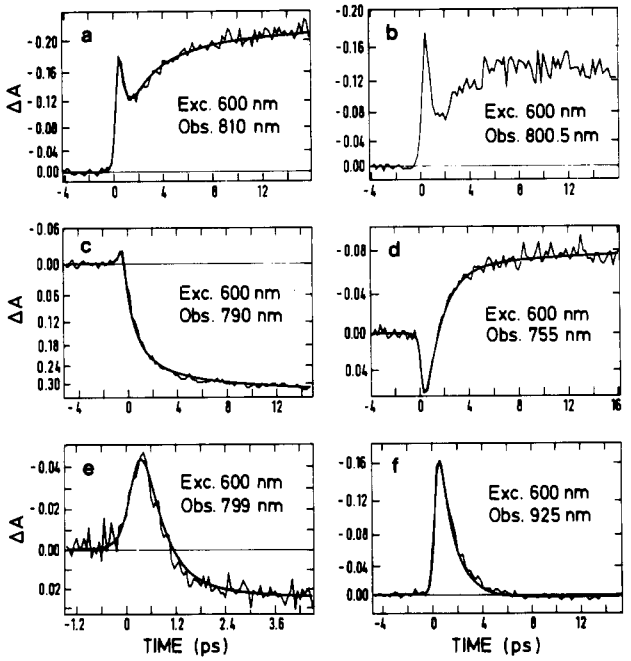

FIGURE 5: Same conditions as in Figure 4 but for borohydride-treated reaction centers from *Rb. sphaeroides* R-26. (a) The fit includes the same kinetic parameters as for Figure 4a (—) but with slightly different relative amplitudes. (b) Kinetics measured close to the maximum of the 800-nm band. (c) The fit includes the fast transient bleaching relaxing with 400-fs kinetics together with absorbance increases developing with the same kinetics as used in the fit of Figure 4f. (d) The fit includes only kinetic components of 1.2 and 100 ps, but equally good fits could be obtained with an additional phase of induced absorption relaxing with a 400-fs time constant. (e) For this trace, the pump energy was reduced by a factor of 4 compared to that used for the other kinetics. The fit includes the fast transient bleaching relaxing with 400-fs kinetics and an induced absorption developing with a 1.2-ps kinetics. (f) Decay of the stimulated emission from P* fitted with a 1.2-ps time constant.

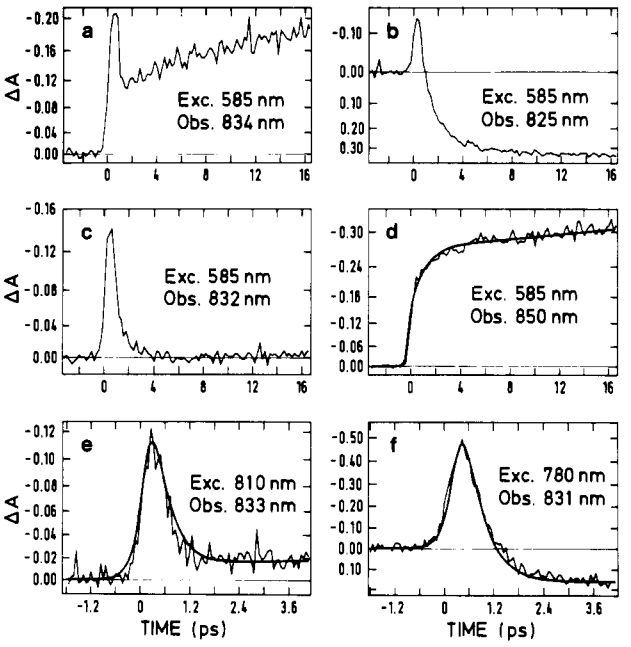

FIGURE 6: Kinetics of absorbance changes measured in reaction centers from *Rps. viridis*. (a–d) Temperature, 100 K; excitation, 585 nm. Observation of the 400-fs transient bleaching either isolated (c) or superimposed upon a bleaching (a) or an absorbance increase (b) assigned to the band shift of the monomeric bacteriochlorophyll(s). In (d), no fast transient bleaching is observed. The fit includes an instantaneous bleaching (amplitude 45%), a bleaching developing with a 1-ps time constant (amplitude 45%), and a bleaching developing with 170-ps kinetics (10% amplitude). (e and f) Temperature, 10 K. At these two excitation wavelengths, the fast transient bleaching relaxing with a 400-fs time constant is superimposed to an absorbance decrease (e) or increase (f) developing with a time constant of 700 fs.

bleaching (Figure 4a,b), the rise of which is not kinetically resolved. Close to the isosbestic point for the electron-transfer-induced band shift of the 800-nm band, the decay of this fast transient can be fitted with an exponential of 400-fs time constant (Figure 4c). At 780 nm (Figure 4d) and at 770 nm (data not shown), a fast transient absorbance increase is observed. Further to the blue, at 755 nm (Figure 4e), the fast initial transient absorbance increase which is detected probably contains contributions from both P* (see Figure 1c) and the fast transient spectral feature specifically detected upon 600-nm excitation. The isosbestic point for the transient absorbance changes relaxing in about 400 fs is located near 790 nm (Figure 4f) where the curve can be fitted with the main electron-transfer kinetics observed upon 870-nm excitation. However, at several other wavelengths in the 800-nm region, an additional kinetic component rising in 400 fs and decaying in 6 ps is required to fit the data taken under 600-nm excitation (Figure 4a).

The fast 400-fs transient feature is still present in the kinetic traces obtained for the modified reaction centers (Figure 5a–e). In Figure 5f, the rise and decay of the stimulated emission monitored at 925 nm are depicted together with a fit indicating that the 1.2-ps time constant for the decay of P* observed upon 870-nm excitation is unperturbed when the initial excitation is partially localized on the B_L molecule. An identical observation has also been made for the unmodified R-26 reaction centers (data not shown).

When reaction centers from *Rps. viridis* at 100 K are excited at 585 nm, a fast transient bleaching recovering in about 400 fs is observed around 810–840 nm. Several traces showing this transient are depicted in Figure 6a–c. Superimposed on this 400-fs transient, the band shift of the 834-nm band develops with a time constant of 1.0 ps, identical with that observed upon excitation at 870 nm at the same temperature (Fleming et al., 1988; Martin et al., 1988). On the other hand, no evidence of this fast transient bleaching can be found upon monitoring the bleaching at 850 nm (Figure 6d).

Upon excitation preferentially in the Q_Y absorption bands of the H_L or H_M molecules at 810 nm and at 780 nm, respectively, the fast transient bleaching decaying in 400 fs is clearly detected around the isosbestic point of the band shift (Figure 6e,f) for reaction centers from *Rps. viridis* at 10 K.

Discussion

In previous femtosecond spectroscopic studies of reaction centers from *Rb. sphaeroides* R-26 and *Rps. viridis* at room temperature (Martin et al., 1986; Breton et al., 1986a,b,c), we have determined that in both species electron transfer from P* to the state $P^+H_L^-$ proceeds with a 2.8-ps time constant. Upon direct excitation of P, we found no kinetic or spectral evidence for a transient bleaching of a B molecule in the time domain from 100 fs to 2.8 ps. Upon excitation in the Q_Y transitions of the B and H molecules, ultrafast energy transfer to P was observed, leading to the formation of P* in less than our time resolution of 100 fs. This state P* was characterized by its stimulated emission on the low-energy side of the long-wavelength transition of P and by a broad and featureless absorption spectrum extending over the whole spectral range investigated. However, upon selective excitation and observation in the near-IR absorption band of the B molecules, a fast transient bleaching relaxing with a 400-fs time constant was observed and tentatively ascribed to a small fraction

(<10%) of B molecules decaying more slowly than the main population. We also proposed that this fast transient bleaching was the cause of the previous discrepancies in the literature, being easily confused with a state such as $P^+B_L^-$. The observations on energy and electron transfer at low temperature reported in the present study confirm and extend these earlier conclusions.

400-fs Transient Bleaching of the B Molecules. When *Rb. sphaeroides* R-26 reaction centers are excited directly in P either at 870 nm or at 600 nm, where the Q_X transition both of the B molecules and of P absorb, the rise of P*, as seen either from the bleaching of P monitored at 890 nm or from the rise of the stimulated emission around 920 nm (Figures 1a and 5f and data not shown), occurs in less than 100 fs. This observation implies that (i) the relaxation from the S_2 state (Q_X) to the S_1 state (Q_Y) occurs in less than 100 fs and (ii) the energy transfer from B to P also occurs in less than 100 fs. The instantaneous (<100 fs) bleaching of P at 990 nm, which is observed upon excitation of reaction centers from *Rps. viridis* at 585 nm at 100 K, also demonstrates ultrafast energy transfer from both B_L and B_M to P. When exciting primarily H_M at 780 nm or H_L at 810 nm at 10 K, the fast (rise <100 fs) transient bleaching of B around 832 nm (Figure 6e,f) also demonstrates an ultrafast energy transfer from each of the H molecules to the B molecules. Furthermore, under these conditions of excitation, the bleaching of the 990-nm band occurs in <100 fs (data not shown). Thus, under several conditions in which the excitation energy has been deposited in various bands observable at 10 K and assignable at least partially to the B, H, and P chromophores, we have observed no evidence for an energy-transfer step lasting more than 100 fs between the pigments.

The fast transient bleaching that is observed upon excitation around 600 nm, where all the bacteriochlorophylls of the reaction center absorb, cannot be due simply to the decay by energy transfer of the excited state of the main population of B*. As just discussed, energy transfer generates P* in less than 100 fs. The transient bleaching, which decays in 400 fs at various temperatures (Breton et al., 1986a–c; Figures 4 and 6), has to be assigned to B_L and/or B_M. The observation that this fast transient is still present with the same kinetics in the modified reaction centers from *Rb. sphaeroides* R-26 (Figure 5) indicates that it is not due to B_M alone. At present, a quantitative comparison between the relative amplitudes of the fast transient bleaching and the 1.2-ps phase of bleaching at 810 nm for the normal and modified *Rb. sphaeroides* reaction centers does not appear justified because (i) the extinction coefficients of B_L and B_M relative to that of P at 600 nm are not known and (ii) the relative amplitudes of the two phases of bleaching are strongly dependent on the pump intensity, both at room temperature (Breton et al., 1986a) and at low temperature (this study; data not shown). It is thus not possible to decide whether the fast transient bleaching originates from B_L alone or from both B_L and B_M.

The observation that the fast transient bleaching is present with the same kinetics in all three types of reaction centers and at a variety of temperatures indicates that it is a genuine characteristic of these bacterial reaction centers and seems to exclude that it is due to a fraction of damaged reaction centers. Previous work at room temperature has shown that the transient bleaching is still present when the redox state of the reaction centers is altered (Breton et al., 1986a–c), thus lending support to the notion that this bleaching is not directly related to the electron-transfer process. The observation in the present study that the 400-fs relaxation kinetics are unchanged between room temperature and 10 K, while the electron-transfer kinetics are accelerated by factors of 2.3 (*Rb. sphaeroides*) to 4 (*Rps. viridis*), provides further support to this view.

It has been proposed (Breton et al., 1986a–c) that the fast transient bleaching could be due to a small fraction (about 10%) of the reaction centers in a different state (possibly conformational) in which B* decays in 400 fs. An alternative interpretation assumes that the 400-fs transient is due to the absorption of a second photon from the pump pulse by reaction centers which have already entered the state BP*. This scheme provides a rationale for the different intensity dependence observed at room temperature (Breton et al., 1986a), where the amplitude of the 400-fs transient is approximately linear with the pulse energy, while the signals assigned to the electron transfer appear to saturate at high intensity. It has been proposed recently (Scherer & Fischer, 1988) that the 400-fs transient bleaching in *Rps. viridis* reaction centers is due to the relaxation to the ground state of the charge transfer state $B_M^+H_M^-$. Our results on *Rb. sphaeroides* reaction centers, which show (i) no 400-fs bleaching of the H_M band at 755 nm (Figure 4e) and (ii) the presence of this fast transient bleaching after B_M has been removed (Figure 5a,b,e), do not support this proposal.

As previously suggested (Breton et al., 1986a), the fast transient bleaching of the B molecule, which is observed upon excitation of the B or H molecules but not upon direct excitation of P, could explain most of the previous observations suggesting B_L^- as a transient electron acceptor between P and H_L. As shown in Figures 4–6, this bleaching strongly perturbs the apparent kinetics in the region of the band shift of the B molecule(s). In addition to the 400-fs transient, a relaxation phase of ≈6 ps is required to fit the data (Figure 4a). As the amplitude of this component relative to that due to $P^+H_L^-$ seems to depend strongly on the pump intensity (data not shown), we have not attempted to characterize this transient further.

Initial Absorbance Changes Due to P at Low Temperature.* Upon excitation at 870 nm of *Rb. sphaeroides* R-26 reaction centers at 10 K, a fast (<100 fs) initial absorbance increase is clearly seen at 755 nm (Figure 1c). By analogy with observations at room temperature, this is assigned to P* (Woodbury et al., 1985; Martin et al., 1986). The following phase of recovery which occurs with a 1.2-ps time constant is attributed to the decay of P* together with the reduction of H_L, whose bleaching is partially compensated by the absorbance increase due to the blue shift of the absorption band of B around 800 nm. With *Rps. viridis* reaction centers excited under the same conditions, a similar fast transient absorbance increase followed by a relaxation phase of 700 fs is observed at 795 nm and can be interpreted similarly. A small contribution from an initial absorbance increase can also be detected at wavelengths close to the isosbestic point for the band shift of the B molecules with *Rb. sphaeroides* R-26 reaction centers either in the native state (Figure 1d) or after borohydride treatment (data not shown) and with *Rps. viridis* reaction centers (Figure 3d). The initial absorbance increase probably contains some contribution from P*. However, when the probe wavelength is slightly to the blue of a sharp band that undergoes a blue shift, an absorbance increase should appear as the band shift develops and the maximum of the band reaches the probe wavelength. For a pure band shift without change of amplitude of the band, this absorbance increase will be followed by an absorbance decrease as the band settles at its final position. Good fits of the traces at 833 nm (Figure 3d) and at 832 nm (data not shown) have been

obtained with the assumption of such a band shift together with some contribution from P* (Martin et al., 1988). As a general observation, the contribution of P* in the region of the band shift of B appears smaller at low temperature than in the corresponding measurements made at room temperature (Martin et al., 1986; Breton et al., 1986a). This is due at least in part to the shorter time constant of the charge separation at the lower temperature, which contributes to deplete the population of P* during the excitation pulse itself. Such an effect is likely to be responsible for the difference in the amplitudes of the initial absorbance increase at 755 nm measured at 295 K and at 80 K, as reported for reaction centers from *Rb. sphaeroides* (Woodbury et al., 1985).

In order to interpret the absorbance changes that accompany electron transfer in reaction centers, assignments of the various bands present in their absorption spectra are necessary. The availability of the molecular coordinates of the pigments in *Rps. viridis* reaction centers from the X-ray data (Deisenhofer et al., 1985) has led to theoretical attempts to model the absorption spectra (Knapp et al., 1985; Zinth et al., 1985b; Parson et al., 1985; Parson & Warshel, 1987; Lous & Hoff, 1987; Won & Freisner, 1988; Eccles et al., 1988; Pearlstein, 1988). While it is well agreed upon that the 990-nm transition in reaction centers from *Rps. viridis* at low temperature is due to the low-energy exciton transition of the dimer forming P, the location of the corresponding high-energy exciton component is more controversial. It has been proposed, on the basis of spectroscopy with polarized light, that the high-energy exciton component of P is responsible for the 850-nm band (Paillotin et al., 1979; Breton & Vermeglio, 1982; Vermeglio & Paillotin, 1982; Breton, 1985). However, the 850-nm band has also been attributed to one or more of the other bacteriochlorophylls (Shuvalov & Asadov, 1979; Shuvalov & Parson, 1981; Den Blanken et al., 1983; Zinth et al., 1985b; Knapp et al., 1985), and it has been proposed that the high-energy exciton band of P either is spectrally silent (Shuvalov & Asadov, 1979) or absorbs around 810–815 nm (Zinth et al., 1985b; Knapp et al., 1985). In recent theoretical analyses, the 850-nm band has been assigned to the high-energy exciton component of P, either almost pure (Won & Friesner, 1988; Eccles et al., 1988; Pearlstein, 1988) or mixed with some contribution (roughly 30–50%) from the accessory B_L molecule (Knapp et al., 1986; Parson & Warshel, 1987; Lous & Hoff, 1987). Thus, a pump wavelength at 870 nm excites mainly the primary donor both via the vibronic shoulder of the low-energy exciton component which is thought to be responsible for the broad pedestal in the 900-nm region (Breton, 1985; Knapp et al., 1986; Won & Friesner, 1987) and via the 850-nm component.

The initial absorbance changes that rise in less than 100 fs are expected to contain contributions both from P* and from the bleaching of P. The fast initial bleaching observed upon probing *Rps. viridis* reaction centers at 850 nm (Figures 3f and 6d) and which does not decay during at least the first 10 ps (Figure 6d) is thus consistent with the notion that at least a large fraction of the 850-nm band can be assigned to P. A fast transient bleaching of the B molecules recovering with a 400-fs time constant has been observed in *Rps. viridis* reaction centers upon excitation of the B molecules either around 830 nm at room temperature (Breton et al., 1986a) or at 585 nm at 100 K (Figure 6a–c) and upon excitation at 810 nm at 10 K (Figure 6e). This fast transient bleaching is not observed at 850 nm upon excitation at 870 nm (Figure 3f) or upon excitation at 585 nm (Figure 6d), thus lending further support to the view that the 850-nm band is due more to P than to a B molecule. A spectral component at 850 nm has been seen in the excitation spectrum of an accumulated photon echo effect probing the excited state of P in *Rps. viridis* reaction centers and has been taken to represent the high-energy exciton component of P (Meech et al., 1986). In the accumulated photon echo excitation spectrum, another band with an amplitude slightly smaller than that of the 850-nm band is seen around 830 nm. This second band has been assigned to an admixture of P character into the 834-nm band (Meech et al., 1986). Accordingly, a fast and long-lived bleaching should also be observed in this band upon formation of P*. The fact that no initial bleaching is observed at 833 nm (Figure 3d) is thus puzzling. However, recent calculations suggest that P contributes only very little to the 834-nm band (Parson & Warshel, 1987; Lous & Hoff, 1987) and it is possible that the accumulated photon echo excitation band observed around 830 nm originates from a species other than P. For an echo to be generated in these experiments, a grating must be built up in the ground state (Hesselink & Wiersma, 1981). It is essential that the optically excited transition should transfer some of its population directly (and not by energy transfer) into a long-lived bottleneck state. If the 834-nm band contains contributions from other species which are not part of P itself or do not transfer their energy to P but which are capable of directly populating some long-lived state, an echo could be generated. We tentatively suggest that the species responsible for the accumulated photon echo excitation band around 830 nm could be P^+ or ^3P, which both absorb strongly around 830 nm (Shuvalov & Parson, 1981; Den Blanken et al., 1983; Breton, 1985) and which could accumulate to some extent under the 80-MHz repetition rate of the photon echo experiment. Another candidate would be that fraction of the B molecules which, upon direct excitation, relaxes in 400 fs (Breton et al., 1986a) and could generate some long-lived state such as ^3B.

In the absorption spectrum of *Rb. sphaeroides* reaction centers at 10 K, a small shoulder around 810 nm, on the flank of the main 800-nm band, has been taken to represent the equivalent of the 850-nm shoulder in reaction centers from *Rps. viridis* (Breton & Vermeglio, 1982; Breton, 1985; Meech et al., 1986). The traces recorded at 810 nm (Figures 1b and 2d) are compatible with a contribution (of about 30%) of instantaneous bleaching. However, the larger overlap of the spectral components in reaction centers from *Rb. sphaeroides* compared to *Rps. viridis* (Breton, 1985, 1988) makes it more difficult to estimate the contribution of P in the main 800-nm band. In the case of *Rps. viridis* reaction centers at 10 K, it is possible to estimate the relative contributions of P (bleaching developing in less than 100 fs) and P^+ (bleaching developing with a 700-fs time constant) at various wavelengths within the 780–860-nm region. Such an analysis will be reported in a forthcoming publication.

Primary Electron-Transfer Step. Previous experiments on *Rb. sphaeroides* R-26 reaction centers at room temperature have shown that the quantum yield of charge separation, the transient bleaching of H_L, and the subsequent electron transfer to Q_A are essentially unaffected by the removal of the B_M molecule (Maroti et al., 1985). Our finding that the lifetime of P* and the formation of the radical pair $P^+H_L^-$ are also invariant following this chemical modification, both at room temperature (Breton et al., 1986b,c) and at 10 K (Figures 1 and 2), provides further evidence that B_M is not significantly involved in the primary electron-transfer process. In order to model the absorption properties of these reaction centers, Scherer and Fischer (1987) have proposed that upon boro-

hydride treatment P moves 2 Å toward the hole left by the removal of B_M. If the exponential decrease of the electron exchange coupling with the distance between donor and acceptor holds (Miller, 1987), a decrease of the rate of electron transfer by 1 order of magnitude would be expected when the distance between P and the acceptor increases by 2 Å. Thus, the proposal of Scherer and Fischer appears difficult to reconcile with our observations.

Upon excitation at 870 nm of the three types of reaction centers investigated in the present study, all the absorbance change kinetic traces can be well fitted with a fast component rising in less than 100 fs and a dominant slower component (1.2 ps in *Rb. sphaeroides*; 0.7 ps in *Rps. viridis*). In none of these traces, and notably those measured close to the isosbestic point in a region where the contribution of the absorption of B_L is large, can another kinetic phase which could reflect a sequential electron-transfer process be detected. The observation of fast transient bleaching upon excitation and detection in the B absorption band (Figures 4–6) clearly demonstrates that, when such a bleaching is present, we can indeed detect its presence, even at low temperature when the electron transfer is accelerated. These findings constitute compelling evidence against mechanisms in which B_L is a distinct, kinetically resolvable intermediary electron acceptor between P and H_L, such as those proposed in Shuvalov et al. (1978, 1986), Shuvalov and Klevanik (1983), Zinth et al. (1985a), and Shuvalov and Duysens (1986).

There are several possible mechanisms by which B_L^-, although present, would not be resolved kinetically, all of which require that the concentration of B_L^- either increases or decreases at the same rate as P* decays. Provided the concentration of B_L^- is large enough, such schemes should lead to a detectable bleaching of the band at B_L. One such possibility would be that some B_L^- (or B_L^+) is already present in the first 100 fs, for example, as a contribution to P* from a charge transfer state such as $P^+B_L^-H_L$ (or $PB_L^+H_L^-$). Assuming B_L contributes substantially to the 800-nm band of *Rb. sphaeroides* R-26 reaction centers and to the 834-nm band of *Rps. viridis*, an assumption which agrees with all the different models presented so far (Zinth et al., 1985b; Knapp et al., 1985; Parson et al., 1985; Breton, 1985; Parson & Warshel, 1987; Lous & Hoff, 1987; Won & Freisner, 1988; Eccles et al., 1988; Pearlstein, 1988), the absence of an initial bleaching at the isosbestic point for the band shift (Figures 1d and 3d) clearly demonstrates that the contribution of such putative charge transfer states to P* must be small.

Another mechanism, in which P* decays into a mixture of $P^+B_L^-H_L$ and $P^+B_LH_L^-$, has been proposed to rationalize femtosecond absorbance change measurements with 620-nm excitation of *Rb. sphaeroides* reaction centers at room temperature (Chekalin et al., 1986, 1987). This proposal is based upon the observation that the bleaching around 805 nm is larger than the absorbance increase at 785 nm, while a pure band shift should lead to positive and negative lobes of equal amplitude. Such a disymmetry in the amplitudes of the 805- and 785-nm components, which is also observed in other transient spectra taken a few picoseconds after the center of the flash with both native and modified reaction centers (Woodbury et al., 1985; Holten et al., 1987), is expected in the state $P^+H_L^-$ because the bleaching of H_L partially compensates the absorbance increase due to the band shift of the B molecules. This effect is enhanced upon modification of the reaction centers from *Rb. sphaeroides* R-26, as in this case only one of the two B molecules is left to undergo the shift. This effect is also more pronounced at room temperature than at low temperature due to the increased spectral overlap (Woodbury et al., 1985). Also, the contribution from the high-energy exciton component of P (Breton, 1985, 1988; Meech et al., 1986) leads to an additional bleaching around 810 nm both in the state P* and in the state P^+. Although the room temperature data of Chekalin et al. on the bleaching around 805 nm in modified reaction centers from *Rb. sphaeroides* could possibly indicate the buildup of some state $P^+B_L^-$, their data probably are affected by the fast transient bleaching of the B_L molecule resulting from their excitation at 620 nm, which could significantly perturb their initial kinetics. In contrast, our low-temperature data on reaction centers excited directly in P at 870 nm, which show an absorbance increase at 785 nm (Figure 2c) and an isosbestic point around 800 nm (Figure 1d), are clearly indicative of a shift rather than of a bleaching of the 800-nm band.

One should also consider the possibility that the apparent lack of involvement of B_L in the absorbance changes could be due to compensation effects whereby an instantaneous absorbance increase due to P* compensates bleachings due to both P^+ and B_L^-. In view of the rather small bandwidth (usually 10–15-nm full width at half-maximum) of the overlapping bands in the 750–860-nm spectral range at 10 K, such compensation effects are not consistent with the small magnitude of the initial absorbance increases attributable to P* as measured in the three types of reaction centers at a variety of wavelengths.

Thus, the absence of bleaching close to the isosbestic point of the band shift where B_L absorbs, together with the development with a main single time constant of all the absorbance changes in the near-infrared, provides compelling evidence against a kinetically or spectrally resolvable contribution of B_L^- to the electron-transfer process. There are several mechanisms by which B_L can be involved in this process without the buildup of a detectable concentration of B_L^-. For example, P* might decay with the characteristic kinetics into a very short-lived state such as $P^+B_L^-H_L$ (or $PB_L^+H_L^-$). Provided the second step, leading to the state $P^+B_LH_L^-$, is at least 50 times faster than the first one, no significant buildup in concentration of ionized B_L, and thus no bleaching of the absorption band of B_L, might be observed (Martin et al., 1988). The superexchange mechanism involving the quantum mechanical mixing of some $P^+B_L^-$ into P* has been compared to a two-step transfer, and a combined exciton–electron-transfer process has been discussed (Marcus, 1987, 1988; Bixon et al., 1987; Fischer & Scherer, 1987; Creighton et al., 1988; Warshel et al., 1988). The new spectroscopic data presented in this study for electron transfer at 10 K should enable a refinement of these schemes. In addition, although our data do not rule out a direct role of B_L in the primary electron-transfer step, it seems also important to assess other models in which the electron travels through preferential pathways in the matrix such as the residue tyrosine M208 in *Rps. viridis* reaction centers (M210 in *Rb. sphaeroides*) or the phytyl chain of the L-branch bacteriochlorophyll in the special pair, both of which are in van der Waals contact with H_L.

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Deactivation of CF_0–CF_1 ATP Synthase by Uncouplers†

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ABSTRACT: Energization of thylakoid membranes by illumination in the presence of dithiols induces a MgATPase activity which persists in the dark (Bakker-Grunwald & van-Dam, 1973). The relationship between the activated state of the ATP synthase (CF_0–CF_1) and $\Delta\tilde{\mu}_{H^+}$ has been investigated from effects of uncouplers on ATPase activity in reconstituted CF_0–CF_1 proteoliposomes. The results are inconsistent with the idea that persistence of the activated state requires a threshold $\Delta\tilde{\mu}_{H^+}$ for the following reasons: (1) Different uncouplers have different effects on steady-state ATPase activity: SF-6847 stimulates ATP hydrolysis at low concentration, in parallel with $\Delta\tilde{\mu}_{H^+}$ dissipation, but inhibits it at higher concentrations. Gramicidin inhibits ATP hydrolysis in parallel with $\Delta\tilde{\mu}_{H^+}$ dissipation. Nigericin just stimulates ATP hydrolysis. (2) Energization of proteoliposomes by an artificially induced $\Delta\tilde{\mu}_{H^+}$ activates ATP hydrolysis. Addition of SF-6847, gramicidin, or nigericin after energization further stimulates ATP hydrolysis. In contrast, the presence of these uncouplers during energization differentially inhibits ATP hydrolysis, and the inhibitions are not correlated with the effectiveness of the uncouplers in dissipating $\Delta\tilde{\mu}_{H^+}$. The results suggest that the inhibition results from deactivation, which is not purely energetic. (3) Deactivation of ATP hydrolysis by SF-6847 depends on the lipid composition. No deactivation is obtained without reconstitution with lipids, while glycolipids enhance the deactivation. Cholesterol inhibits H^+ uptake, but not the deactivation. (4) Low concentrations of ADP sensitize while phosphate stabilizes CF_0–CF_1 to deactivation by SF-6847, both with or without $\Delta\tilde{\mu}_{H^+}$ preactivation. The effect of ADP is not due to a direct inactivation, since by itself it does not inhibit but rather stimulates uncoupled ATP hydrolysis. It is suggested that deactivation of energized CF_0–CF_1 by certain uncouplers results from a specific interaction between the enzyme and the protonophores. This interaction is facilitated by ADP and by glycolipids and is inhibited by phosphate and during ATP hydrolysis. The results are consistent with the idea that activation involves occlusion of protons from the inner thylakoid space in hydrophobic sites of CF_0.

In energy-transducing membranes of mitochondria, chloroplasts, and bacteria, the transmembrane difference of electrochemical potential of protons ($\Delta\tilde{\mu}_{H^+}$)[1] provides the major driving force for ATP synthesis (Mitchell, 1977). The reaction is reversibly coupled to proton transport via a membrane-bound ATP synthase (F_0–F_1). In chloroplast ATP synthase (CF_0–CF_1), $\Delta\tilde{\mu}_{H^+}$ also activates the enzyme in addition to its role as a driving force for ATP synthesis (Carmeli & Liphshitz, 1972; Bakker-Grunwald & van Dam, 1974). This activation is manifested not only by the capacity of the enzyme to synthesize ATP in the light but also by its capacity to hydrolyze ATP in the subsequent dark period. The latter depends on the presence of either dithiol or trypsin during illumination (Lynn & Straub, 1969; McCarty & Racker, 1968; Mills & Mitchell, 1984; Bakker-Grunwald & van-Dam, 1973, 1974). It has been demonstrated that dithiols reduce two SH groups in the γ subunit of CF_1 which become exposed upon energization (Nalin & McCarty, 1984; Pick, 1983), while trypsin causes cleavage of the ϵ subunit of CF_1 which suppresses the activity and seems to be dislocated upon energization (Nelson et al., 1972; Richter et al., 1984; Finel et al., 1984). Marked changes in the binding and release of adenine nucleotides, particularly ADP, have suggested that high-affinity ADP binding sites in CF_1 may be involved in regulation of the activation of CF_0–CF_1 (Shulmann & Strotmann, 1981). It has also been demonstrated that the capacity to hydrolyze ATP induced upon energization persists long after complete decay of $\Delta\tilde{\mu}_{H^+}$, suggesting that maintenance of this capacity does not depend on persistence of $\Delta\tilde{\mu}_{H^+}$ (Bakker-Grunwald & van-Dam, 1974). The mechanism by which energization induces activation is not clear, but it has been suggested that it involves protonation of sites in the enzyme facing the inner thylakoid space (Schlodder et al., 1982; Mills & Mitchell, 1984). Support for this hypothesis comes from earlier studies by Jagendorff and collaborators (Ryrie & Jagendorff, 1971) that energization involves an irreversible exchange of hydrogens from water into CF_1 which persists even after isolation of the

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[1] Abbreviations: CF_0–CF_1, chloroplast ATP synthase; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical proton gradient; SF-6847, (3,4-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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