Trypsin Treatment of Reaction Centers from *Rhodobacter sphaeroides* in the Dark and under Illumination: Protein Structural Changes follow Charge Separation†

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ABSTRACT: Reaction centers from *Rhodobacter sphaeroides* R-26 were treated with trypsin in the dark and during illumination (in the charge-separated state). Trypsination resulted in a time-dependent modification of the reaction centers, reflected in changes in the charge recombination rate, in the inhibition of Q_A^- to Q_B electron transfer, and eventually to inhibition of charge separation. Comparisons of centers with ubiquinone or anthraquinone in the Q_A site, in which the charge recombination pathways are different, indicate that trypsination affects charges close to the Q_A^--binding site. Studies of light-induced voltage changes from moving charges in reaction centers incorporated in lipid layers on a Teflon film, a technique which allows the discrimination of effects on donor and acceptor sides, indicate that the acceptor side is preferentially degraded by trypsin in the dark. Tryptic digestion during illumination generally resulted in a marked strengthening and acceleration of the effects seen already during dark treatment, but new effects were also detected in gel electrophoretic peptide patterns, in optical spectra, and in the kinetic measurements. Optical kinetic measurements revealed that the donor side of the reaction centers became susceptible to modification by trypsin during illumination as seen in the value of the binding constant for soluble cytochrome c_2 which increased by a factor of 2, whereas it was much less affected after trypsination of reaction centers in the dark. The influence of illumination on the rate and mode by which trypsin acts on reaction centers indicates that changes in the protein conformation follow charge separation. The function of these light-induced structural changes may be to stabilize charge separation and facilitate forward electron transfer.

The photosynthetic reaction center from *Rhodobacter sphaeroides* is a membrane-bound protein complex which consists of three polypeptide subunits (H, L, and M) with molecular masses ranging from 28 to 35 kDa (Williams et al., 1986). The H subunit, which has a hydrophilic character and extends into the cytosol, is anchored in the membrane by one membrane-spanning α-helix. The L and M subunits are embedded in the membrane and bind the cofactors which are involved in the primary steps of the conversion of light energy into electrochemical energy. After absorption of light by a bacteriochlorophyll dimer (P),[1] an excited electron is transferred sequentially to a bacteriopheophytin (Φ_A), a primary ubiquinone (Q_A), and a secondary ubiquinone (Q_B). The oxidized donor, P^+, is re-reduced by a water-soluble cytochrome c_2 from the periplasmic side of the reaction center. Following a second excitation of P, Q_B becomes doubly reduced and picks up two protons from the cytoplasmic side of the reaction center. The dihydroquinone leaves the reaction center and is replaced by a quinone from a quinone pool in the membrane [for a review, see Feher et al. (1989) and Gunner (1991)].

At each electron-transfer step within the reaction centers, the electron-hole pair, separated by the protein−membrane dielectrics, is stabilized for progressively longer times. The forward transfer rates are at least 2 orders of magnitude faster than the recombination rates, making the charge separation a very efficient process: each absorbed photon produces one charge-separated state. The mechanism of this large quantum yield has been discussed previously [see Feher et al. (1989) and Moser et al. (1992)]. For example, in the initial charge separation from the excited dimer (P*) to Φ_A and from Φ_A^- to Q_A with time constants of 3 ps and 100 ps, respectively, the forward reactions have small driving forces and reorganization energies whereas the recombination reactions have relatively large driving forces. In terms of the Marcus theory for electron transfer [reviewed in Marcus and Sutin (1985)], this leads to back-reactions in the inverted region, which are slower than the forward reactions (Moser et al., 1992). The more long-lived charge-separated state $P^+Q_A^-$, on the other hand, may be stabilized by a structural change (Kleinfeld et al., 1984a; Brzezinski et al., 1992). Such changes have been noted in measurements of light-induced FTIR spectra of reaction centers (Nabedryk et al., 1990; Hienerwadel et al., 1992), in measurements of light-induced volume changes in reaction centers (Arata & Parson, 1981a; Malkin et al., 1994), in measurements of light-induced reaction center fluorescence (Arata & Parson, 1981b; Woodbury & Parson, 1984), and in measurements of time-resolved absorbance changes (Kirmaier et al., 1985; Sebban, 1988; Baciou et al., 1990; Gao

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[1] Abbreviations: P, bacteriochlorophyll dimer; Q_A and Q_B, primary and secondary ubiquinone acceptors, respectively; Φ_A bacteriopheophytin; UQ, ubiquinone; AQ, anthraquinone; k_B, Boltzmann's constant; LDAO, lauryldimethylamine *N*-oxide; Tris, tris(hydroxymethyl)aminomethane.

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& Wraight, 1990; Gao et al., 1991; Tiede & Hanson, 1992; Kálmán & Maróti, 1994). A particularly pronounced effect was observed by Kleinfeld et al. (1984a), who compared rates of light-induced electron-transfer reactions in reaction centers frozen in the dark and during illumination (in the charge-separated state). They found that the charge recombination kinetics $P^+Q_A^- \rightarrow PQ_A$ were slower in reaction centers frozen during illumination than in reaction centers frozen in the dark. In addition, the rate of $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transfer increased by several orders of magnitude when reaction centers were frozen during illumination. Recently, light-induced structural changes upon forming $P^+Q_A^-$ were resolved kinetically by measuring light-induced voltage changes (electrogenic events) associated with movement of charge in reaction centers incorporated in lipid layers (Brzezinski et al., 1992). The kinetics of these changes, as well as the pH and temperature dependencies, were the same as those of the electron transfer, $Q_A^-Q_B \rightarrow Q_AQ_B^-$, but the electrogenic events were observed also in Q_B-depleted reaction centers. These results indicate that light-induced structural changes may control the rate of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transfer. The involvement of a structural change associated with this electron transfer is also indicated by the relatively large enthalpy of activation (~0.6 eV) for this reaction (Mancino et al., 1984; Kleinfeld et al., 1984b). The same structural changes may also control the kinetics of charge recombination of $P^+Q_A^-$ [cf. Kleinfeld et al. (1984a)].

In this work, we have compared reaction centers treated with trypsin in the dark and under illumination. Trypsin cleaves proteins specifically at Arg and Lys residues. There are six such residues on the donor side, all located close to the reaction center surface. About 50 Lys or Arg residues are found on the acceptor side, 11 of these on the M subunit, 10 on the L subunit, and the remaining on the H subunit. Essentially no Arg or Lys residues are found in the membrane-spanning part of the reaction centers (Allen et al., 1987).

If the protein structure changes upon charge separation $PQ_A \rightarrow P^+Q_A^-$, the effect of trypsin may differ in the PQ_A and $P^+Q_A^-$ states. The trypsin-treated reaction centers were characterized using several different techniques such as SDS–gel electrophoresis, steady-state and time-resolved absorption spectroscopy, and time-resolved measurements of voltage changes in reaction centers incorporated in lipid layers.

As evidenced from all these measurements, the effect of trypsin was different in dark-treated and in light-treated reaction centers, indicating different structures under these conditions.

MATERIALS AND METHODS

Materials. Reaction centers from *Rhodobacter sphaeroides* R-26 were isolated in lauryl dimethylamine *N*-oxide (LDAO, Calbiochem) as described (Feher & Okamura, 1978). The concentration of reaction centers was determined from the 802 nm absorption using the absorption coefficient $\epsilon^{802} = 318\ mM^{-1}\ cm^{-1}$ (McPherson et al., 1993). The reaction centers contained 1.6 ubiquinones, as determined from the charge recombination kinetics [see, e.g., Kleinfeld et al. (1984b)]. Reaction centers containing ≥1.9 ubiquinones were prepared by addition of an excess of ubiquinone (ubiquinone-50, Sigma) at a molar ratio of 5:1 from a 1.7 mM solution in 1% dodecyl β-D-maltoside (Sigma). To prepare reaction centers with anthraquinone in the Q_A site, ubiquinone-depleted reaction centers were first prepared as described (Okamura et al., 1975). An excess of anthraquinone (Janssen Chimica), dissolved in ethanol at a concentration of 600 μM, was then added at a molar ratio of 5:1 to the reaction center solution.

Cytochrome c_2, isolated from *Rb. sphaeroides* as described by Bartsch (1978), was reduced to ~90% by hydrogen gas using platinum black (Aldrich) as a catalyst (Rosen & Pecht, 1976). Solutions of *o*-phenanthroline (Janssen Chimica) were prepared in ethanol prior to use.

Lipid vesicles containing reaction centers were prepared from a suspension of 10 mg/mL soybean lecithin (Sigma, type II), washed as described (Kagawa & Racker, 1971), in 10 mM Tris-HCl at pH 8.0, 10 mM KCl, supplemented with reaction centers to a final concentration of 1.5 μM. A volume of 1 mL of the reaction center–lipid suspension was sonicated (Bronson Sonicator, Model B-12) for ~5 min.

Trypsin Treatment. Bacterial reaction centers at 15 μM, in 40 mM Tris-HCl, pH 8.0, containing 0.05% dodecyl β-D-maltoside, in 3 mm inner diameter EPR tubes, immersed in a water bath thermostated at 20 ± 1 °C, were treated with 0.25 mg/mL trypsin in darkness or during illumination. After a predetermined time, the proteolysis was stopped by addition of 1 mg/mL soybean trypsin inhibitor. The illuminating light, 100 mW/cm², from a halogen lamp, was passed through a Baltzer B_1K_1 heat-reflecting filter and 10 cm of water.

SDS–Polyacrylamide Electrophoresis. After trypsin treatment, peptides from bacterial reaction centers were separated by electrophoresis on 4%/20% SDS–polyacrylamide gels according to the method of Laemmli (1970) and silver-stained as described (Goldman et al., 1980).

Optical Measurements. Absorption spectra were recorded on a Cary 4 spectrophotometer (Varian). Flash-induced absorbance changes were measured using a spectrophotometer of local design, modified from a setup described previously (Brzezinski & Malmström, 1987; Hallén & Brzezinski, 1994). The monitoring beam was provided by a 250 W halogen lamp, filtered through a heat filter (Oriel) or 3 cm of water. An interference filter (bandwidth 5 nm, Oriel) was placed before and a double monochromator (Oriel) after the sample. Changes in light intensity were measured using a photomultiplier tube (Hamamatsu R269 or R712) connected to a current-to-voltage converter (Hamamatsu C1053, bandwidth 3 MHz). The cuvette path length was 3.0 mm or 10.0 mm as indicated in the figure legends. Actinic light was provided by a Nd-YAG pulsed laser (Spectra Physics Model GCR 190) at 532 nm with a pulse width of approximately 10 ns and energy of approximately 100 mJ. All experiments were performed at 20 ± 1 °C.

For the tryptic treatment in light, the samples were illuminated in the optical cuvette with light from a fiber guide at right angles to the monochromatic measuring beam. At specified times, the illumination was switched off for kinetic measurements.

Electrical Measurements. The experimental setup is similar to a design used previously (Trissl, 1977; Feher & Okamura, 1984; Höök & Brzezinski, 1994) and has been described in detail (Brzezinski et al., 1994). Two compartments of a Teflon cell were separated by a 12 μm Teflon film (Saunders). The reaction center-containing vesicle solution (see above) was diluted 1:10 to a volume of 2.2

mL and supplemented with $CaCl_2$ to a final concentration of 10 mM. In the presence of Ca^{2+}, the vesicles break, and a monolayer is formed at the surface. The solution was added to one of the cell compartments to a level just below the Teflon film. After ~5 min the remaining volume was added slowly to allow the monolayer to attach to the Teflon film. Light-induced voltage changes were measured across Ag/AgCl electrodes immersed in the cell compartments, shielded from actinic light. Voltage changes were measured using an operational amplifier (OP128, Burr-Brown or LF356, National Semiconductors).

In the electrical measurements, the reaction centers were treated with trypsin, added to the solution facing the lipid monolayer. When treated in light, illumination was provided through the same port as was used for the excitation flashes.

Experimental Procedures. Voltages from both experimental setups were amplified using a preamplifier with a variable time constant (Stanford Research Systems, SR560) and recorded on a digital oscilloscope (Nicolet, Model 490). Rates were determined using a Nelder−Mead simplex algorithm in the Matlab software (Math Works).

RESULTS

Reaction centers were treated with trypsin in the dark or under illumination (in the charge-separated state). The native and trypsin-treated reaction centers were characterized using various techniques.

Absorption Spectra. Treatment of bacterial reaction centers with trypsin led to changes in the optical spectrum which differed in nature depending on the illumination conditions (Figure 1). Trypsination resulted in a time-dependent, gradual decrease in the main absorption bands around 365 and 802 nm, which was much more pronounced when the treatment was carried out during illumination compared to dark-treatment. Significant loss of absorption at wavelengths corresponding to the chlorophyll dimer of the primary donor at 865 nm and pheophytin at 760 nm was also observed. In addition to enhancing a general loss of absorption from the reaction center chromophores, proteolytic degradation during illumination induced optical changes with no correspondence in the dark-treated samples. In the light, trypsination caused a downshift in wavelength of the 865 nm band, which was evident already after 10 min and amounted to 10 nm after 30 min. In the range 400−500 nm, trypsination during illumination resulted in a gradual absorption increase. Essentially no changes in the absorption spectrum were recorded in reaction centers exposed to 15 min of illumination in the absence of trypsin (Figure 1B).

SDS−Polyacrylamide Electrophoresis. Figure 2 shows the effect of trypsin treatment on bacterial reaction centers. Trypsination during illumination did not affect the three subunits equally. After 10 min treatment in light, no traces remained of the H and M subunits at their original positions. Instead, new bands appeared at 27 kDa and at 18 kDa in addition to a weak band at 19 kDa and unresolved bands at lower molecular masses. After treatment for 30 min in light, also the L subunit was mostly degraded, and only the bands at 27 and 18 kDa were evident. The remaining band at the position of the L subunit (about 25 kDa) derives from trypsin while trypsin inhibitor is responsible for the diffuse band at 23 kDa.

When the trypsination was carried out in the dark, a similar pattern emerged although the degradation of the M subunit was less extensive. However, in addition, bands at 20 and 11 kDa were visible which were not detectable in the

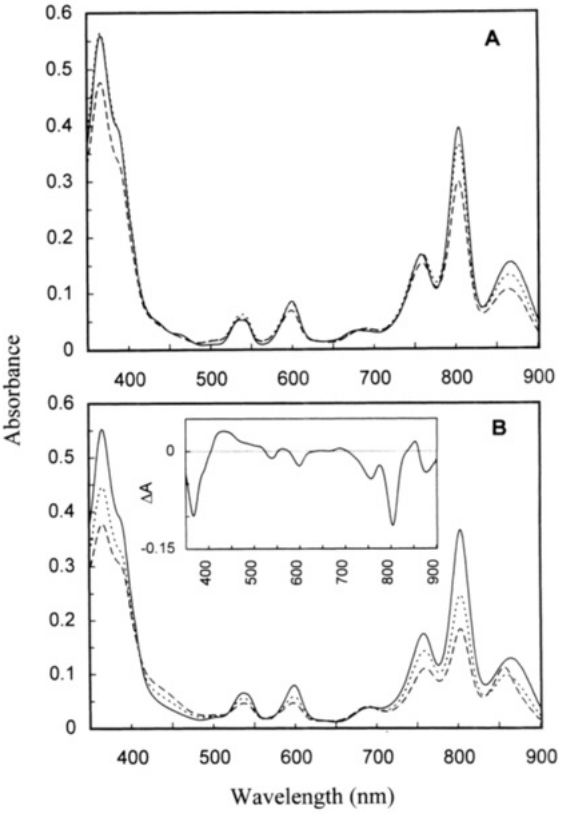

FIGURE 1: Effect of trypsin on the absorption spectrum of photosynthetic reaction centers from *Rb. sphaeroides*. Reaction centers, 13 μM, were treated with trypsin as described under Materials and Methods in the dark (A) or during illumination (B). After treatment, the samples were diluted 1:10 in 40 mM Tris-HCl, pH 8.0, 0.05% dodecyl β-D-maltoside. The cuvette path length was 1 cm. Spectra were recorded from native samples (—) and 10 min (•••) and 30 min (- - -) after addition of trypsin. The native sample in (B) was illuminated for 15 min. The inset in (B) shows an illuminated minus dark difference spectrum from samples trypsinated for 30 min.

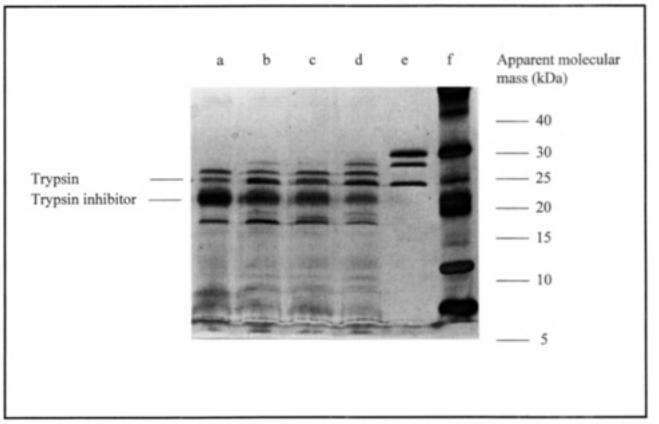

FIGURE 2: SDS−PAGE of reaction centers from *Rb. sphaeroides* after trypsin treatment in darkness and under illumination. Bacterial reaction centers were treated with trypsin as described under Materials and Methods. Lanes a−f represent(a and b) reaction centers treated with trypsin for 30 min in light and dark, respectively, (c and d) trypsin treated for 10 min in light and dark, respectively, (e) untreated reaction centers, and (f) molecular mass standards (ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; and aprotinin, 6.5 kDa). The positions of trypsin and trypsin inhibitor in lanes a−d are indicated.

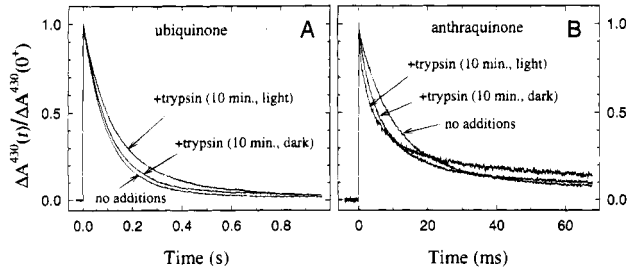

FIGURE 3: Flash-induced absorbance changes in native and trypsin-treated reaction centers (for 10 min) in the dark and under illumination with ubiquinone (A) and anthraquinone (B) in the Q_A site. The absorbance changes are normalized to the $PQ_A - P^+Q_A^-$ absorbance differences immediately after the flash (at $t = 0^+$). Conditions: 40 mM Tris-HCl, pH 8.0, 0.05% dodecyl β-D-maltoside, 15 μM reaction centers, 100 μM *o*-phenanthroline, 250 ng/mL trypsin, 20 ± 1 °C. The cuvette path length was 3.0 mm.

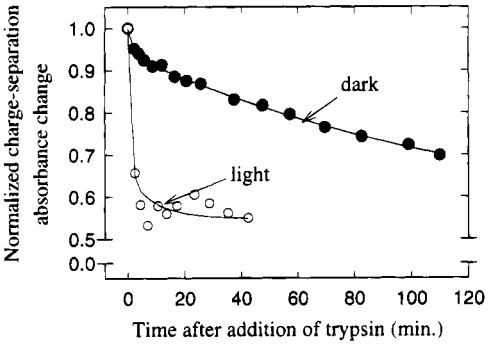

FIGURE 4: Charge separation absorbance change as a function of trypsin treatment time in the dark (●) and under illumination (○). The absorbance changes are normalized to the $PQ_A - P^+Q_A^-$ absorbance difference prior to trypsin treatment. Measuring conditions were the same as in Figure 3.

illuminated samples. Even after 30 min in the dark, the new bands were present although somewhat weaker while the band at the position of the L subunit appeared to be almost unaffected. Untreated reaction centers exposed to 15 min of light displayed an identical pattern as that of native reaction centers in the dark (Figure 2e).

Optical Kinetic Measurements. (A) Charge Separation and Recombination. The kinetics of primary electron transfer were studied optically after exposing the reaction centers to pulsed illumination. At 430 nm, the rapid increase in absorbance, due to charge separation, $PQ_A \rightarrow P^+Q_A^-$, was followed by a slower decrease associated with charge recombination (Figure 3). The amplitude of the charge separation absorbance change decreased with trypsin treatment time (Figure 4), showing that an increasing fraction of the reaction centers became photochemically inactive (i.e., the charge separation $PQ_A \rightarrow P^+Q_A^-$ did not take place). In the dark-treated reaction centers, the charge separation amplitude dropped to approximately 70% of the original value after approximately 100 min. In the light-treated reaction centers, the decrease in amplitude was much faster and reached approximately 55% of the original value after approximately 10 min.

The charge recombination kinetics were measured in reaction centers treated with trypsin in the dark or under illumination, respectively, with ubiquinone (Figure 3A) or anthraquinone (Figure 3B) in the Q_A site. Anthraquinone was used because it has a lower reduction potential than ubiquinone and, as a consequence, the charge recombination pathways are different (see Discussion). In reaction centers

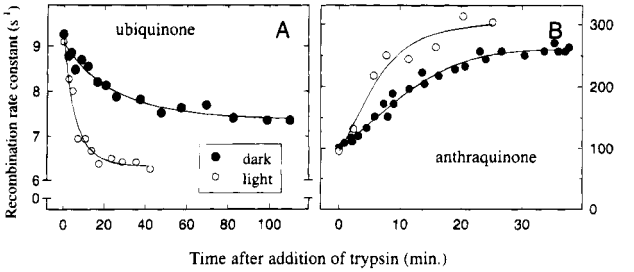

FIGURE 5: Charge recombination rate constants as a function of trypsin treatment time in reaction centers treated in the dark (●) and under illumination (○) with ubiquinone (A) and anthraquinone (B) in the Q_A site. Measuring conditions were the same as in Figure 3.

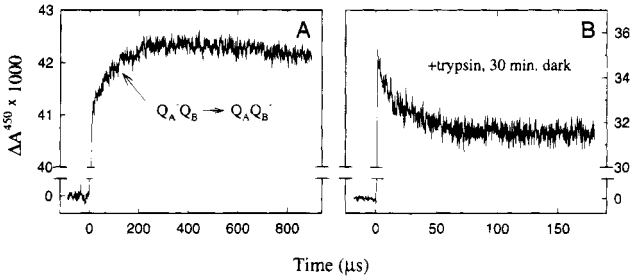

FIGURE 6: Flash-induced absorbance changes at 450 nm in reaction centers with 1.9 quinones. (A) Native reaction centers; (B) reaction centers treated with trypsin in the dark for 30 min. Note the different time scales. Conditions: 40 mM Tris-HCl, pH 8.0, 0.05% dodecyl β-D-maltoside, 2.5 μM reaction centers, 250 ng/mL trypsin (only in panel B), 20 ± 1 °C. The cuvette path length was 10.0 mm.

Table 1: Charge Recombination ($P^+Q_A^- \rightarrow PQ_A$) Rate Constants in Native and Dark/Light Trypsin-Treated Reaction Centers with Ubiquinone or Anthraquinone in the Q_A Site

	charge recombination rate constant (s^{-1})		
Q_A quinone	native	dark treated	light treated
ubiquinone	9.1 ± 0.2	7.4 ± 0.1	6.4 ± 0.2
anthraquinone	98 ± 2	260 ± 10	295 ± 15

with ubiquinone, the recombination rate constant decreased with treatment time (Figure 5A) while in reaction centers with anthraquinone this rate constant instead increased (Figure 5B). With both quinones, the changes in kinetics were larger in reaction centers treated during illumination than in the dark. The charge recombination rate constants, which reached essentially constant values after 30−60 min of trypsin treatment, are summarized in Table 1, together with those observed in native reaction centers.

A minor slower component in the recombination kinetics was also observed in trypsin-treated ubiquinone and anthraquinone reaction centers (Figure 3).

No significant changes in the recombination kinetics were observed when reaction centers were exposed to illumination for 15 min prior to the experiment.

(B) Electron Transfer $Q_A^-Q_B \rightarrow Q_AQ_B^-$. Figure 6A shows absorbance changes at 450 nm on a microsecond time scale. A rapid increase in absorbance, due to the charge separation $PQ_A \rightarrow P^+Q_A^-$, was followed by a further increase in absorbance with a time constant of ~200 μs, associated with electron transfer $Q_A^-Q_B \rightarrow Q_AQ_B^-$. Trypsin treatment led to a decrease in the initial fast absorption rise (see above) at the same time as the 200 μs absorbance change disappeared and was replaced by a fast decrease. After trypsination in the dark for 10 min, the time constant of this decrease was

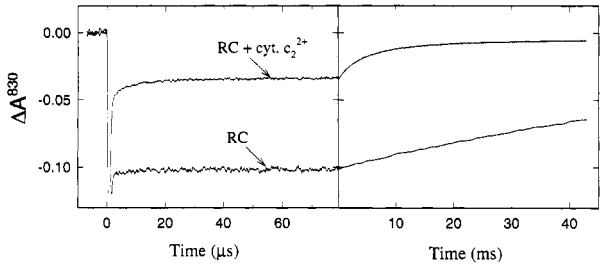

FIGURE 7: Flash-induced absorbance changes at 830 nm in native reaction centers without and with reduced cytochrome c_2. Note the different time scales before and after the solid vertical line. Conditions: 40 mM Tris-HCl, pH 8.0, 0.05% maltoside, 1.5 μM reaction centers, 1.5 μM cytochrome c_2, 20 ± 1 °C. The cuvette path length was 10.0 mm.

Table 2: Reaction of Cytochrome c_2 from *Rb. sphaeroides* with Trypsin-Treated Reaction Centers in Light/Dark for Different Times

sample	fraction rapid phases (%) (τ_1 < 10 μs)	fraction slow phase (%) (τ_3 ≅ 5 ms)
no treatment	70	30
10 min dark	70	30
10 min light	85	15
30 min dark	80	20
30 min light	100	0

~20 μs, and the amplitude was ~2% of the charge separation absorbance change. In light-treated reaction centers the decrease was biphasic with time constants of ~3 and ~20 μs at approximately equal amplitudes and a total amplitude of ~10% of the charge separation absorbance change. The relative amplitude of these rapid absorbance changes increased with treatment time. After 30 min in the dark, it was ~10% (Figure 6B), and in light, the total amplitude of the two kinetic phases was ~20%.

A similar absorbance change in the opposite direction to the charge separation change was also observed at 830 nm in trypsin-treated reaction centers (not shown). The amplitude after 10 min in the dark was ~8% of the charge separation absorbance change.

(C) Reaction with Cytochrome c_2. To study the effect of trypsin on the cytochrome c_2 binding site, the rate and extent of the reduction of P^+ by reduced cytochrome c_2 were measured in native and trypsin-treated reaction centers at 830 nm. In native reaction centers, a rapid decrease in absorbance, due to P^+ formation, was followed by a fast increase with time constants of <1 and ~10 μs, associated with reduction of P^+ by cytochrome c_2^{2+} in a fraction of reaction centers with bound cytochrome c_2 in an electrostatic complex [cf. Tiede et al. (1993)], followed by a slower increase in absorbance with a time constant of ~5 ms, associated with diffusion-controlled reduction of P^+ in a fraction of reaction centers without a bound cytochrome c_2 (Figure 7).

In trypsin-treated reaction centers, the relative amplitudes of the kinetic phases changed, but the time constants were essentially unaffected (Table 2). The observed increase in the relative amplitudes of the two fast components in the initial absorption rise is consistent with an increase in the binding constant of cytochrome c_2 during trypsin treatment. This effect was more pronounced when reaction centers were treated during illumination than in the dark.

Electrical Kinetic Measurements. Light-induced electrogenic events were measured in reaction centers incorporated

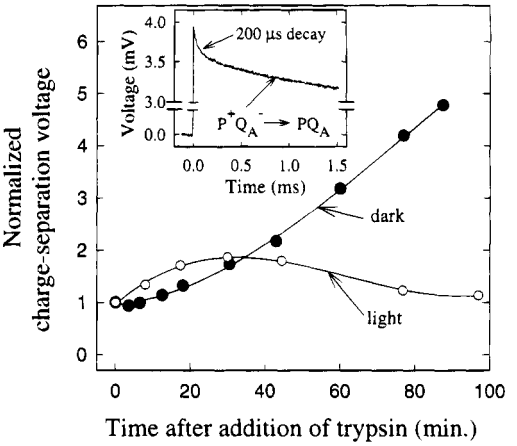

FIGURE 8: Initial charge separation voltage change (see inset) in reaction centers incorporated in a lipid layer adsorbed to a Teflon film as a function of trypsin treatment time in the dark (●) and under illumination (○). The trypsin concentration was 100 ng/mL. The voltage changes are normalized to the charge separation voltage in native reaction centers. The inset shows the time course of flash-induced voltage changes in native reaction centers. Conditions: 10 mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM $CaCl_2$, 0.5 μM reaction centers, 1 mg/mL lipids, 20 ± 1 °C.

in lipid layers adsorbed to a Teflon film. The reaction centers were oriented with either the donor side or the acceptor side toward the aqueous solution. The orientation was measured using a cytochrome *c*-based assay (Schönfeld et al., 1979) and was found to correspond to a distribution of 56/44% with the majority of the reaction centers with the donor side facing the aqueous solution.

The inset of Figure 8 shows the light-induced voltage changes in the reaction center−lipid layer. An increase in voltage, induced by the charge separation, $PQ_A \rightarrow P^+Q_A^-$, is followed by a decrease with a time constant of ~200 μs, previously ascribed to a structural change on the reaction center acceptor side (Brzezinski et al., 1992). The following slower voltage decrease, with a time constant of ~100 ms, is associated with charge recombination.

An observed voltage change is a sum of voltage changes with opposite signs originating from the two reaction center populations. Assuming that trypsin cannot penetrate the lipid layer, this allows separation of the effects of trypsin on the two reaction center populations; i.e., the effect on the donor and acceptor sides can be identified.

Light-induced voltage changes in reaction centers treated with trypsin in the dark and under illumination, respectively, were measured as follows: Two identical cells containing Teflon-adsorbed reaction center−lipid layers were prepared. Trypsin was added to the reaction center-containing compartment of each cell, and light-induced voltage changes were measured at different times after the addition. One of the cells was kept in the dark between the measurements whereas the other was illuminated through the same aperture as was used for the laser illumination. The CW-light intensity at the lipid layer was sufficient to keep on average half of the reaction center population in the charge-separated state during trypsin treatment.

Figure 8 shows the charge separation voltage as a function of digestion time in the dark and the illuminated samples, respectively. In the dark sample, the voltage increased with time. In the illuminated sample, the voltage increase was initially faster than in the dark, but the voltage reached a maximum value after about 30 min and then decreased.

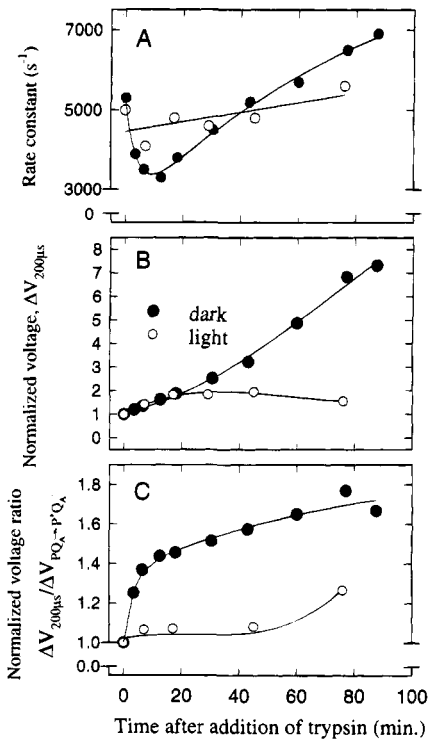

FIGURE 9: (A) Rate constant of the 200 μs voltage change as a function of trypsin treatment time. (B) Amplitude of the 200 μs voltage change as a function of trypsin treatment time, normalized to the amplitude before treatment. (C) Ratio of the 200 μs voltage change amplitude and of the charge separation voltage, normalized to this ratio before treatment.

Both the rate constant and the amplitude of the 200 μs voltage decay transient changed during trypsin treatment (Figure 9A,B). The change in rate was $\leq$30% in dark-treated reaction centers and $\leq$10% in light-treated reaction centers. The ratio of the decay voltage to the charge separation voltage increased with trypsin treatment time in both the dark-treated and the light-treated samples, but the increase was much less pronounced in the light-treated sample (Figure 9C). An increasing ratio is consistent with an increasing intrinsic amplitude of the 200 μs voltage change in the photochemically active reaction centers (see Discussion).

DISCUSSION

In reaction centers containing ubiquinone in the Q_A site, the recombination rate, measured at 430 nm, *decreased*, from ~9.1 s^{-1} in native reaction centers to ~7.4 and ~6.4 s^{-1} in trypsin-treated reaction centers in the dark and under illumination, respectively, whereas in anthraquinone reaction centers the recombination rate instead *increased* from ~98 s^{-1} in native reaction centers to ~260 and ~295 s^{-1} in trypsin-treated reaction centers in the dark and under illumination, respectively (Table 1).

Two important conclusions may be drawn from these observations. First, the influence of illumination on the trypsination effects indicates that light-induced changes in the protein structure are involved (see below). Second, the opposing effects of proteolysis on the recombination rates in ubiquinone- and anthraquinone-containing reaction centers suggest that trypsination changes the environment of the quinones on the acceptor side. The observed experimental behavior can be rationalized using an energy diagram such as that described in Gopher et al. (1985). Charge recombination of $P^+\Phi_AQ_A^-$ can proceed either directly from Q_A^- or indirectly through thermal population of the state $P^+\Phi_A^-Q_A$.

With the conditions used in these experiments, the recombination rate constant, k_{rec}, is (Gopher et al., 1985)

$$k_{rec} = k_{\Phi P}e^{-\Delta G^\circ/k_BT} + k_{AP} \quad (1a)$$

where $k_{\Phi P} = 8 \times 10^7$ s^{-1} and $k_{AP} = 9$ s^{-1} are the $P^+\Phi_A^-Q_A \rightarrow P\Phi_AQ_A$ and $P^+\Phi_AQ_A^- \rightarrow P\Phi_AQ_A$ recombination rate constants, respectively; ΔG° is the free energy difference between states $P^+\Phi_AQ_A^-$ and $P^+\Phi_A^-Q_A$.

With ubiquinone in the Q_A pocket, ΔG°_{UQ} is 500–600 meV (Arata & Parson, 1981b; Gunner et al., 1982a). Consequently, charge recombination occurs to >98% directly from Q_A^-, and eq 1a simplifies to

$$k_{rec} \cong k_{AP} \quad (1b)$$

With anthraquinone in the Q_A pocket, ΔG°_{AQ} is 340 meV (Gunner et al., 1982b; Gopher et al., 1985), and charge recombination from Q_A^- occurs instead to ~90% through thermal population of $P^+\Phi_A^-Q_A$. Equation 1a then simplifies to

$$k_{rec} \cong k_{\Phi P}e^{-\Delta G^\circ_{AQ}/k_BT} \quad (1c)$$

Consequently, in this case, the charge recombination kinetics are particularly sensitive to the free-energy difference between $P^+\Phi_A^-Q_A$ and $P^+\Phi_AQ_A^-$.

The observed increase in rate in trypsin-treated anthraquinone reaction centers is explained in terms of a raise of the energy level of $P^+\Phi_AQ_A^-$ relative to the level of $P^+\Phi_A^-Q_A$, for example, due to removal of positive charges from the vicinity of the Q_A site. The ratio of the recombination rates before and after trypsin treatment of anthraquinone-containing reaction centers is expressed in terms of a change in ΔG°_{AQ} of $\delta\Delta G^\circ_{AQ}$:

$$\frac{k_{rec}^{trypsin}}{k_{rec}} \cong e^{-\delta\Delta G^\circ_{AQ}/k_BT} \quad (2)$$

With recombination rates for the trypsin-treated reaction centers in dark and under illumination of ~260 and ~295 s^{-1}, respectively, the change in recombination rate from the original value of ~98 s^{-1} (Table 1) corresponds to $\delta\Delta G^\circ_{AQ}$ of 24 and 28 meV, respectively. A similar decrease in ΔG°_{AQ} was previously found in the pH dependence of the charge recombination rate in anthraquinone-containing reaction centers (Kleinfeld et al., 1985). The observed increase in rate with pH to a value of 230 s^{-1}, which corresponds to a $\delta\Delta G^\circ$ of 22 meV (Kleinfeld et al., 1985), close to those found in trypsin-treated reaction centers, was explained in terms of a change in the protonation of a site with a pK_a of 9.8 at a distance of >5 Å from the Q_A site. If this site carries a positive charge below pH 9.8 and is neutral above this pH, the enhancement in recombination rate in trypsin-treated reaction centers at pH 8.0 may be due to the loss of positive charge, resulting from, for example, removal of a lysine residue.

The relatively small decrease in the charge recombination rate in the trypsin-treated ubiquinone reaction centers can be explained, for example, in terms of a slight increase in distance between P and Q_A. The same should also apply to

the anthraquinone reaction centers, but this effect would be canceled out by the much larger increase in the recombination rate due to the changes in the energy levels of $P^+\Phi_A Q_A^-$ and $P^+\Phi_A^- Q_A$.

In trypsin-treated reaction centers, the 200 μs increase in absorbance following charge separation at 450 nm and attributed to electron transfer $Q_A^- Q_B \rightarrow Q_A Q_B^-$ was absent (Figure 6). This may result from a reduction of the electron-transfer rate by a factor of at least 100 (so that the rate is similar to the charge recombination rate) or from a total inhibition of the reaction. A considerable reduction of this electron-transfer rate was also found in reaction centers from which the H subunits were removed (Debus et al., 1985). The reduction of the electron-transfer rate in the trypsin-treated reaction centers may, therefore, be due to the rapid partial or total degradation of the H subunit in these reaction centers [cf. Bachmann et al. (1981)], which was observed after SDS−PAGE analysis (Figure 2). It is also possible that in trypsin-treated reaction centers the electron transfer is further impaired due to degradation of the Q_B binding site. A labilization of cofactor binding in trypsin-treated reaction centers is consistent with the kinetics at 450 nm where a decrease in absorbance followed the initial increase associated with charge separation (Figure 6B). The time constant of this decrease (~20 μs) was similar to that attributed to the decay of a triplet-state P in reaction centers with reduced Q_A, in quinone-free reaction centers (Parson et al., 1975; Chidsey et al., 1985), and in Fe^{2+}-depleted reaction centers (Kirmaier et al., 1984). One possibility is, therefore, that this absorbance decay was due to a fraction of reaction centers which had lost their quinones and/or the Fe^{2+} ion. Another possibility is that the decay was due to protein relaxation upon forming $P^+Q_A^-$ in reaction centers with $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron transfer blocked (Tiede & Hanson, 1992; Hienerwadel et al., 1992).

In reaction centers treated under illumination, the charge separation absorbance change decreased by about 45% during the first 10 min to reach an essentially constant value or a much slower decreasing value (Figure 4). This may be due to, for example, a lower binding constant for the Q_A quinone in the trypsin-treated reaction centers.

The slower component in the charge recombination kinetics in trypsin-treated reaction centers may, for example, be due to a small fraction of reaction centers with a different structure.

The experimental technique used in the electrical measurements offers a possibility to distinguish between effects of trypsin on the donor and acceptor sides. In these measurements, reaction centers are oriented with either the donor side (P-population) or the acceptor side (A-population) toward the aqueous solution. Since trypsin is not expected to penetrate the reaction center−lipid layer, we predict different degradation patterns for the two populations.

In the dark-treated reaction centers, the charge separation voltage increased with treatment time (Figure 8). In the light-treated reaction centers, the charge separation voltage first increased slightly and then decreased. Assuming that the number of reaction centers in the Teflon-bound reaction center−lipid layer did not change during treatment, the experimental behavior in the dark-treated reaction centers can be modeled in terms of a preferential inactivation of the A-population resulting in a larger apparent degree of orientation. Since initially the A-population is somewhat smaller than the P-population, the increasing imbalance results in an increase in the charge separation voltage. In the light-treated reaction centers, both populations are inactivated with similar rates by trypsin, resulting in only a small change in the charge separation voltage.

The voltage decay following charge separation (see inset of Figure 8) has the same time constant (~200 μs) as the $Q_A^- Q_B \rightarrow Q_A Q_B^-$ transfer (see Figure 6), but it is not associated with electron transfer because this reaction is parallel to the membrane surface and is not electrogenic [see, e.g., Feher and Okamura (1984)]. The 200 μs voltage decay has instead been attributed to light-induced structural changes on the acceptor side (Brzezinski et al., 1992). In the dark-treated reaction centers, the amplitude of the 200 μs voltage decay increased with treatment time (Figure 9B). The rate of change was larger than that of the charge separation voltage as evidenced from the increasing ratio with treatment time of these two voltage changes (Figure 9C). This shows that the amplitude of the 200 μs voltage transient increased in the photochemically active reaction centers with trypsin treatment time. A larger net amplitude is expected if the intrinsic amplitude increases in the P-population reaction centers or if it decreases in the A-population reaction centers. Since this voltage change has been suggested to originate from light-induced structural changes (i.e., movement of charges in the protein) on the acceptor side, it is more likely that the change in amplitude is due to a decrease in the amplitude in the A-population reaction centers with the acceptor side exposed to trypsin. A smaller amplitude is consistent with a smaller number of moving charges, which, in turn, is consistent with a partly degraded acceptor side in the photochemically active A-population reaction centers.

Assuming that the amplitude of the 200 μs decay transient decreases with treatment time only in the A-population reaction centers and that only the reaction centers in this population are inactivated by trypsin, the experimental behavior can be modeled. In the following, two time scales will be used: T is the time after addition of trypsin (in minutes), and t is the time scale in a flash-induced experiment (generally <1 s).

The orientation, Θ (=0.12, see Results), at time $T = 0$, i.e., before addition of trypsin, is defined by

$$\Theta = \frac{N_P(0) - N_A(0)}{N_P(0) + N_A(0)} \tag{3}$$

where $N_P(0)$ and $N_A(0)$ are the numbers of (photochemically active) reaction centers oriented with the donor side (P-population) and the acceptor side (A-population) toward the aqueous solution at $T = 0$.

The time course of the 200 μs voltage decay transient in the P-population reaction centers, at time T after the addition of trypsin, is modeled by

$$v_P(t,T) = v_0 \alpha_P(T) N_P(T) e^{-t/\tau_P(T)} \tag{4}$$

where v_0 is the charge separation voltage produced by each reaction center, $\alpha_P(T)$ is the fraction of v_0 that makes up for the 200 μs voltage decay, $N_P(T)$ is the number of photochemically active reaction centers in the P-population, and $\tau_P(T)$ is the decay time constant in the P-population reaction centers.

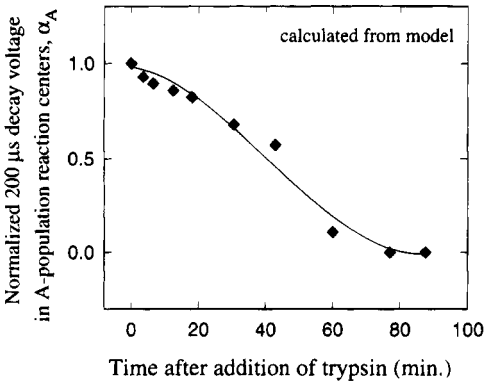

FIGURE 10: Amplitudes of the 200 μs voltage decay transient associated with a structural change on the acceptor side in the A-population reaction centers as a function of trypsin treatment time in the dark (♦), calculated using a model described in the text. The amplitude in native reaction centers is normalized to 1.

The net decay voltage is the difference between $v_P(t,T)$ and the corresponding voltage in the A-population reaction centers:

$$v_{net}(t,T) = v_P(t,T) - v_A(t,T) = v_0\alpha_P(T)N_P(T)e^{-t/\tau_P(T)} - v_0\alpha_A(T)N_A(T)e^{-t/\tau_A(T)} \quad (5)$$

The charge separation voltage, $v_{CS}(T)$, at time T after addition of trypsin is

$$v_{CS}(T) = v_0[N_P(T) - N_A(T)] \quad (6)$$

Equations 3 and 6 give

$$v_{CS}(0) = v_0N_P(0)\frac{2\Theta}{1+\Theta} \quad (7)$$

Assuming that only the A-population reaction centers are inactivated by trypsin treatment (see Results), i.e., $N_P(T) = N_P(0)$, that the intrinsic decay amplitude at $T = 0$ is the same in the A- and P-population reaction centers, i.e., $\alpha_P(0) = \alpha_A(0)$, and combining eqs 5–7:

$$v_{net}(t,T) = v_{CS}(0)\frac{1+\Theta}{2\Theta}\alpha_P(T)e^{-t/\tau_P(T)} - \left[v_{CS}(0)\frac{1+\Theta}{2\Theta} - v_{CS}(T)\right]\alpha_A(T)e^{-t/\tau_A(T)} \quad (8)$$

The measured voltage changes after different trypsin treatment times were fitted to eq 8. Adequate fits were obtained with $\tau_A(T) = \tau_P(T) = 210 \pm 50$ μs and with the intrinsic decay amplitude in the P-population reaction centers independent of trypsin-treatment time [$\alpha_P(T) = 0.09$] for $T \leq 60$ min. With this model, $\alpha_A(T)$, i.e., the intrinsic amplitude of the 200 μs decay in the A-population photochemically active reaction centers, decreased with trypsin-treatment time to reach a value of zero after about 1 h (Figure 10). For $60 < T < 85$, we had to use either a larger $\alpha_P(T)$ of 0.13 or a negative $\alpha_A(T)$, which is equivalent to a change in sign on the 200 μs decay. A zero amplitude can be found in A-population reaction centers in which the portion of the acceptor-side protein which is involved in the 200 μs decay is degraded so that the net charge of the protein region involved in the structural change is zero. Extensive degradation of this region is not unlikely considering the relatively large number of positively charged Lys/Arg residues in the corresponding part of the reaction center protein.

The observed changes in the absorption spectra suggest that illumination causes a further exposure of trypsination sites which were present already in the dark, but also that light induces additional structural changes which uncover sites not otherwise accessible to trypsin. A blue-shift of the dimer absorption peak has also been observed in trypsin-treated reaction centers from *Rps. viridis* (Moskalenko & Kuznetsova, 1993). A shift of the 865 nm peak in *Rb. sphaeroides* reaction centers may be due to changes in the dimer itself but may also result from loss of quinones (Debus et al., 1985).

The SDS–gel electrophoresis of trypsin-treated reaction centers also demonstrates that the proteolytic degradation occurs with rates which are dependent on the illumination conditions. The persistent differences in the observed polypeptide pattern between dark and illuminated samples also suggests that the degradation follows partially different pathways in the two cases.

Both the kinetic optical and electrical measurements support that at a given time after addition of trypsin there are cleavage sites that are accessible to trypsin during illumination which are not accessible in the dark. Since the rate of reduction of P^+ by cytochrome c_2^{2+} is not significantly affected by trypsin treatment, it is evident that in the *photochemically active* reaction centers there are only minor modifications on the donor side. This is consistent with the small number of Arg/Lys residues found on the donor side. The observed increase in binding constant after trypsin treatment, as evidenced by the increase in the rapid transient in P^+ reduction (Table 2), may result from a larger flexibility of the cytochrome c_2 binding surface, which allows formation of a tighter complex, or from elimination of positive charge on the reaction center, which increases the attractive forces between the cytochrome and its binding site.

The electrical measurements, which can discriminate between the effects on the donor and acceptor sides, indicate that in the dark trypsination of the donor side does not inactivate the reaction centers, whereas in the charge-separated state trypsin can inactivate reaction centers also from the donor side. The exposure of residues not accessible to trypsin in the dark may lead to distortion of the protein structure around the donor so that charge separation cannot take place.

Taken together, these results show that light-induced structural changes on both the donor and acceptor sides of the reaction centers follow charge separation $PQ_A \rightarrow P^+Q_A^-$. These changes may be important in stabilizing the charge-separated state and in facilitating electron transfer in the forward direction.

In addition, these results show that the reaction center integrity is not essential for light-induced charge separation [cf. Wiemken and Bachofen (1983) and Debus et al. (1985)]. However, even if the trypsin-removed parts of the protein are not directly involved in electron transfer, an intact reaction center is most likely necessary to achieve a high quantum yield.

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