LM Complex of Reaction Centers from *Rhodopseudomonas sphaeroides* R-26: Characterization and Reconstitution with the H Subunit†

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ABSTRACT: Reaction centers (RCs) from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* are composed of three subunits (designated L, M, and H) plus cofactors. An active and stable LM complex containing approximately one Fe per LM was obtained from these RCs by dissociating the H subunit with the chaotropic agent $LiClO_4$ in the presence of mild detergents such as sodium cholate. The LM was characterized and compared with native RCs in a variety of assays. RCs reconstituted from LM and H were indistinguishable from native RCs in all assays performed. Comparison of LM with native RCs showed that removal of H resulted in little or no change in (1) primary photochemical activity (i.e., bleaching of the absorption at 865 nm upon illumination), (2) Fe and quinone content, (3) narrow and broad EPR signals due to $(BChl)_2^+$ and the $Q_A^-Fe^{2+}$ complex, respectively, (4) kinetics of charge recombination between Q_A^- and $(BChl)_2^+$, and (5) kinetics of oxidation of ferrocytochrome *c* by $(BChl)_2^+$. These results show that the H subunit has little or no effect on the binding sites of Fe and cytochrome and that both primary and secondary quinone acceptors, Q_A and Q_B, bind to LM. However, electron transfer involving Q_A and Q_B was dramatically affected by the absence of the H subunit. Comparison of LM with native and reconstituted RCs showed that removal of H resulted in (1) reduction in the rate of electron transfer from Q_A^- to Q_B by a factor of 10^2–10^3, (2) reduction in the stability of the semiquinone anions Q_A^- and Q_B^- by a factor of 10^2–10^3, (3) elimination of the oscillations of the absorption of semiquinone in response to successive flashes of light, (4) reduction in the affinity of Q-10 for the Q_B site by a factor of $\sim$10, (5) reduction in sensitivity to inhibitors of electron transfer (e.g., *o*-phenanthroline and terbutryn) by a factor of $\sim 10^2$, and (6) reduction in stability to denaturation by detergents. These results suggest that the H subunit plays a major role in defining the environment of the Q_B site.

The primary events of photosynthesis, conversion of light into the energy of a charged donor–acceptor pair, take place in a membrane-bound complex of pigment and protein called the reaction center (RC).[1] RCs from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26 consist of three 30–35-kDa polypeptides present in a stoichiometry of 1:1:1 plus the following cofactors: four bacteriochlorophylls (BChl), two bacteriopheophytins (BPh), two ubiquinones (Q-10), and one atom of high-spin Fe^{2+}. A specialized dimer of bacteriochlorophyll, $(BChl)_2$ or D, serves as primary electron donor. Two ubiquinones, Q_A and Q_B, both magnetically coupled to the Fe^{2+}, serve as primary and secondary electron acceptors, respectively [for reviews, see Feher & Okamura (1978, 1984), Wraight (1982), Okamura et al. (1982a,b), Parson & Ke (1982), and Crofts & Wraight (1983)]. The RC binds Q_A much more tightly than Q_B. Between D and Q_A there are intermediate acceptors involving monomeric BChl and BPh [for reviews see Parson (1982) and Parson & Ke (1982)].

Neither the H subunit nor the Fe is required for primary photochemical activity (defined as reversible light-induced charge separation between D and Q_A): H can be dissociated from RCs to yield a photochemically active complex of LM (Okamura et al., 1974; Feher & Okamura, 1978), and Fe-depleted RCs have long been known to possess primary photochemical activity (Loach & Hall, 1972; Feher et al., 1972; Dutton et al., 1978; Blankenship & Parson, 1979). In addition, photochemically active LM can be prepared that contains <0.05 Fe/LM (Feher & Okamura, 1978; Okamura et al., 1980).

In this study we investigated the effects of the H subunit on the properties of RCs. This required the development of procedures to selectively dissociate the H subunit and subsequently to reassociate it. Existing methods for removing this subunit [e.g., treatment of RCs with $NaDodSO_4$ (Okamura et al., 1974; Agalidis & Reiss-Husson, 1983) or chaotropic agents (Okamura et al., 1974; Feher & Okamura, 1978) or treatment of chromatophores with $LiDodSO_4$ (Broglie et al., 1980) or with Triton X-100 and urea at alkaline pH (Hall et al., 1973; Loach, 1976)] yielded LM with low activity and/or low iron content or LM that lost activity rapidly with time. In the present study LM was isolated with the chaotropic agent $LiClO_4$ in the presence of low concentrations of the mild detergents sodium cholate or sodium deoxycholate. This preparation of LM retained full primary photochemical activity, was stable, and contained $\sim$1 Fe/LM. It was characterized, particularly with respect to its electron transfer properties and compared to native and reconstituted RCs. The development of a procedure to reconstitute RCs from LM and H was necessary to determine whether changes observed upon dissociation of the H subunit resulted from its absence or from

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[1] Abbreviations: RC(s), reaction center(s); Q-10, ubiquinone-50; 0Q, 1Q, and 2Q RC, RCs containing 0.12 ± 0.03, 0.95 ± 0.03, and 1.9 ± 0.1 Q-10 per RC, respectively; 0Q, 1Q, and 2Q LM, LM prepared from 0Q, 1Q, and 2Q RC, respectively; EPR, electron paramagnetic resonance; LDAO, lauryldimethylamine *N*-oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; cyt *c*, cytochrome *c*; ENDOR, electron nuclear double resonance.

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irreversible denaturation accompanying its removal.

Preliminary accounts of this work have been presented (Debus et al., 1981; Okamura et al., 1982a,b; Feher & Okamura, 1984). A companion study investigating the role of Fe^{2+} by reconstituting intact RCs from H and LM depleted of Fe will be reported separately.

Materials and Methods

Materials. Horse heart cyt *c* (type VI) and Q-10 were obtained from Sigma, $LiClO_4$ was from Alfa-Ventron, octyl β-D-glucopyranoside and CHAPS were from Calbiochem-Behring, and herbicides were from Chem Service, Inc. (West Chester, PA). LDAO was a gift from Onyx Chemical Co. (Jersey City, NJ). Cyt c_2 (*Rp. sphaeroides*) was isolated following the procedures of Bartsch (1978). ^{14}C-Labeled Q-10, with a specific activity of 0.1 μCi/μmol, was obtained as described by Okamura et al. (1975).

The concentration of a 3.3 M stock solution of $LiClO_4$ in H_2O was determined by atomic absorption spectroscopy (see Iron Analysis) using a 1000 ppm standard of lithium chloride (Alfa-Ventron).

Reduced cyt *c* or *cyt* c_2 was prepared by adding dithionite to a solution of cyt and chromatographing it on Sephadex G-25 (Pharmacia) or on a mixed-bed resin (Bio-Rad, RG 501-X8, 20–50 mesh) or by bubbling H_2 gas through a solution of cyt in the presence of platinum back (Aldrich). The reduced cyt (1 mM in 10 mM Tris-HCl, pH 8) was frozen in liquid nitrogen and stored at −70 °C. The concentrations were obtained with extinction coefficients of ϵ_{550}(reduced) = 27.6 and 30.8 mM^{-1} cm^{-1} for cyt *c* and cyt c_2, respectively (Margoliash & Frohwirt, 1959; Bartsch, 1978).

Q-10 was dissolved in detergent by the following procedure: Q-10 (in ethanol) was dried under N_2 gas onto the walls of a small vial. A solution of detergent was added and stirred for 1 h at 60 °C in darkness. Undissolved Q-10 was removed by filtration or centrifugation. The resulting solution (typically 1–2 mM Q-10 in 10% sodium deoxycholate) was frozen in liquid nitrogen and stored at −70 °C. For some experiments other detergents were substituted for sodium deoxycholate. The concentration of Q-10 was measured in ethanol from the absorption change at 275 nm following reduction with $NaBH_4$ [$\Delta\epsilon_{275}$ = 12.25 mM^{-1} cm^{-1} (Pumphrey & Redfearn, 1960)].

Reaction Centers. RCs were isolated following the procedures of Feher & Okamura (1978) as modified by Sutton et al. (1982). To minimize loss of Q_B, the concentration of LDAO was kept minimal (0.025–0.1%) following extraction of the RCs from the chromatophore membranes. Typical preparations contained 1.8–2.0 Q-10 per RC as assayed spectrophotometrically (Butler et al., 1980; Okamura et al., 1982a). The RCs (typically with A_{802}^{1cm} = 30 in 10 mM Tris-HCl, 0.025% LDAO, and 1 mM EDTA, pH 8) were frozen in liquid nitrogen and stored at −70 °C. Their concentration was determined with the extinction coefficient ϵ_{802} = 288 mM^{-1} cm^{-1} (Straley et al., 1973). RCs containing 0.12 ± 0.03 or 0.95 ± 0.03 Q-10 per RC (0Q RC and 1Q RC, respectively) were obtained by extracting Q-10 from RCs following the procedures of Okamura et al. (1975). RCs containing 2.0 ± 0.2 ^{14}C-labeled Q-10 per RC were prepared by adding excess ^{14}C-labeled Q-10 (dissolved in 1% LDAO) to 0Q RCs, dialyzing overnight at 4 °C, and separating RCs from unbound Q-10 by chromatography with DEAE-cellulose. The Q-10 content of these RCs was determined spectrophotometrically and by liquid scintillation counting.

LM Complex. The isolation of LM was based on a previously described method (Feher & Okamura, 1978). RCs were dialyzed for 1 or 2 days at 4 °C against 10 mM Tris-HCl, 0.1% sodium cholate, and 0.1 mM EDTA, pH 8, to remove LDAO. They were then incubated for 1 h at 25 °C (at A_{802}^{1cm} = 10) in 0.75 M $LiClO_4$, 50 mM $CaCl_2$, 10% (v/v) ethanol, 10 mM Tris-HCl, 0.03% sodium cholate, and 0.1 mM EDTA, pH 8. For some preparations sodium deoxycholate (0.2%) was added prior to incubation. Precipitated H subunits were removed by centrifugation (~5000*g*, 10 min). The resulting protein was dialyzed for 9–18 h at 4 °C against 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 8, frozen in liquid nitrogen, and stored at −70 °C. The concentration of LM was determined with the same extinction coefficient, ϵ_{802}, as for RCs.

H Subunit. The isolation of H subunits was based on a previously described method (Feher & Okamura, 1978). RCs were first dialyzed overnight at 4 °C against 50 mM Tris-HCl and 0.1% LDAO, pH 7.7. They were then layered (0.5 mL, A_{802}^{1cm} = 12) on top of 4.6 mL of 1.0 M $LiClO_4$, 50 mM Tris-HCl, and 0.1% LDAO, pH 7.7, and centrifuged for 20 h at 4 °C in a Spinco SW 65 Ti rotor at 60000 rpm (~250000*g*). H subunits were collected from the top portion of each tube and immediately frozen in liquid nitrogen for storage at −70 °C. The highest yields (~30% on a molar basis) were obtained at pH 7.7. Approximate concentrations were obtained with the extinction coefficient ϵ_{280} = 46 mM^{-1} cm^{-1} (Valkirs & Feher, 1982).

Reconstitution of RCs from H and LM. LM (A_{802}^{1cm} ~ 8.0) was mixed with H subunits (A_{280}^{1cm} ~ 0.3, still in $LiClO_4$ and LDAO) at an approximate molar ratio of 1.6 H/LM. The mixture was dialyzed against 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, for 1–2 days at 4 °C with several changes of buffer. For many experiments excess Q-10 was added (in 10% sodium deoxycholate) prior to dialysis. To maximize the Q_B activity of the reconstituted complex, it was necessary to minimize the time between mixing LM with H and initiating dialysis. In addition, it was necessary to prepare LM and H from RCs containing ~2 Q-10 per RC. Samples of LM and RC were dialyzed along with the reconstituting samples as controls. Samples were concentrated in Amicon ultrafiltration cells containing PM-30 membranes at 8 psi (N_2 gas).

Gel Electrophoresis in Agarose. Seakem ME agarose (FMC Marine Colloids) was dissolved in H_2O at 100 °C and cooled to 60 °C. Concentrated buffer (at 60 °C) was added to final concentrations of 1.5% agarose, 50 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate, and 0.1 mM EDTA, pH 8. (The presence of LDAO during electrophoresis was necessary to eliminate artifacts.) Gels were poured into 8 × 0.6 cm Pyrex tubes at 37 °C, capped with 0.1% LDAO (60 °C), and allowed to solidify at room temperature. The gels were secured in the tubes with dialysis membranes and preelectrophoresed at ~10 V/cm for 30 min at 4 °C. The buffer present in the electrode reservoirs was the same as in the gels. Samples (~10 μg in 50 μL of 10 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate, 0.1 mM EDTA, and 10% sucrose, pH 8), were loaded on top of the gels and electrophoresed at ~10 V/cm for 3.5 h at 4 °C. After electrophoresis the gels were scanned at 800 nm, fixed, stained with Coomassie Brilliant Blue R 250 (Inolex), and scanned at 560 nm as described previously (Okamura et al., 1974).

Iron Analysis. Fe contents were determined with an atomic absorption spectrophotometer (Varian, Techtron AA-5) in an acetylene/air flame. Standard solutions were prepared by diluting a 1000 ppm solution of $FeCl_3$ (Fisher Scientific) into 10 mM Tris-HCl, 0.025% sodium cholate, and 1 mM EDTA, pH 8.

Optical Measurements. Absorption spectra were recorded with a Cary 14-R or Cary 17-D spectrophotometer. Kinetic measurements were made with a modified single-beam spectrophotometer (McElroy et al., 1974; Kleinfeld et al., 1984b). Changes in optical transmission were recorded on a Nicolet 1090A digital oscilloscope. Data were converted to units of absorption with a Z-80A microprocessor-based computer system of local design. Exponential recovery rates and amplitudes were obtained after plotting the logarithm of the absorption changes vs. time. Cuvettes containing samples were held in a thermostated jacket. Measurements at cryogenic temperatures were performed as described previously (McElroy et al., 1974; Okamura et al., 1975).

Actinic flashes were produced with a pulsed dye laser (Phase-R DL-2100C) operated at λ 584 nm with a flash duration of ~0.4 μs and an energy of 0.2 J/pulse. For the experiments of Figure 6 the second flash was provided by a second pulsed dye laser (Phase-R DL-1000). Triggering and timing circuitry were of local design.

Continuous actinic illumination was provided by a Leitz-Prado 500 tungsten filament projector (500 W). The actinic illumination was filtered through H_2O (1-cm path length) and a Corning CS-2-64 color filter (λ > 660 nm passed). Light intensities were measured with a YSI Model 65 radiometer (Yellow Springs Instrument Co.).

Other Procedures. $NaDodSO_4$ gel electrophoresis in polyacrylamide was performed as described previously (Okamura et al., 1974), except that samples were prepared as described by Valkirs & Feher (1982). Rabbit antiserum to LM was obtained as described by Valkirs & Feher (1982). Anti-LM IgG was purified from antiserum by precipitation with ammonium sulfate (40% saturation) followed by chromatography with DEAE-cellulose (Levy & Sober, 1960). The IgG was stored at 4 °C (at A_{280}^{1cm} = 10) in 17.5 mM potassium phosphate, pH 6.3, in the presence of 0.04% sodium azide. Solutions of LM or RC containing ^{14}C-labeled Q-10 were precipitated by mixing with an equal volume of of antibody solution and incubating overnight at 4 °C. The precipitates were resuspended with 1% $NaDodSO_4$ and 10 mM Tris-HCl, pH 8, prior to liquid scintillation counting.

RESULTS

LM Complex

Purity and Yield. LM prepared by precipitating H with $LiClO_4$ in the presence of sodium cholate was >95% pure (it contained <0.05 H/LM) as determined by electrophoresis in polyacrylamide gels in the presence of $NaDodSO_4$. The same purity was obtained when sodium cholate was replaced by a number of other detergents: LDAO (0.025%), CHAPS (0.04%), or a mixture of sodium deoxycholate (0.2%) and sodium cholate (0.03%). Contamination with H was more pronounced in Triton X-100 (0.05%) or in octyl β-D-glucopyranoside (1.0%). The highest yields of LM (>95%) were obtained in LDAO. Lower, variable yields (30–90%) were obtained in other detergents. Typical yields obtained in the presence of cholate or deoxycholate were 50–80%.

Optical Absorption Spectrum. The optical absorption spectrum of LM resembled that of RCs at both room and cryogenic temperatures (Figure 1), with the following differences: (1) The absorption at 280 nm relative to that at 802 nm was diminished (A_{280}/A_{802} = 1.04 ± 0.03 for LM at 295 K, compared to 1.19 ± 0.03 for RCs). (2) The long-wavelength shoulder of the Soret band was less pronounced at 295 K and not resolved as a separate peak at 77 K, whereas in RCs a separate peak is resolved at 387 nm at 77 K. (3) The

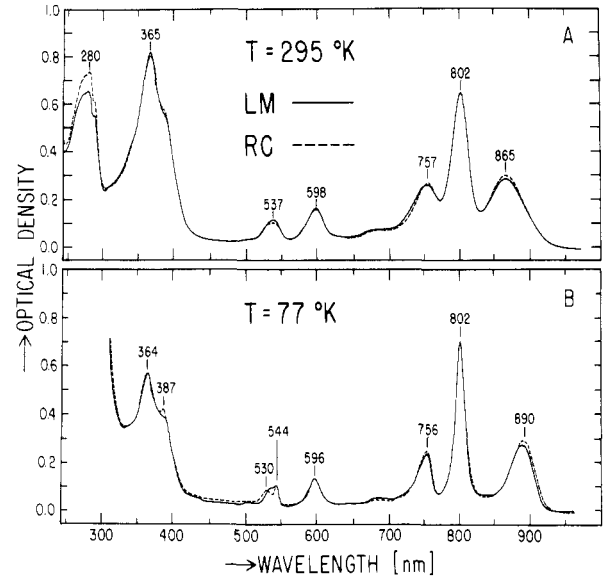


FIGURE 1: Optical absorption spectrum of LM (—) and RC (---) at 295 (A) and 77 K (B). Conditions for (A): 2.3 μM LM or RC containing ~1.9 Q-10 per LM or RC, 10 mM Tris-HCl, 0.025% sodium cholate, 0.2% sodium deoxycholate, and 0.1 mM EDTA, pH 8. Conditions for (B): LM or RC (35 μM), in the same buffer as in (A) and containing the same amount of Q-10, was mixed with an equal volume of glycerol and frozen in liquid nitrogen in optical cells with a path length of 1 mM. The Pyrex windows of the optical Dewar precluded the measurement of wavelengths <330 nm.

absorption band near 537 nm narrowed and increased in amplitude at 295 K. At 77 K, an asymmetric band with a partially resolved shoulder was observed. In contrast, RCs show well-resolved absorptions at 530 and 544. (4) The amplitude of the long-wavelength absorption (865 nm at 295 K and (890 nm at 77 K) relative to that at 802 nm was diminished by 5–10%. In many detergents (0.025% sodium cholate, 0.025% LDAO, 0.05% Triton X-100, 0.04% CHAPS, and 1.0% octyl β-D-glucopyranoside) the maximum of the long-wavelength absorption at 295 K appeared between 855 and 860 nm in LM prepared from RCs containing ~1.9 Q-10 per RC (data not shown). In general, the position of this absorption in both LM and RC depended on Q-10 content and detergent (Figure 2). In the presence of sodium deoxycholate (0.025–0.2%), sodium cholate (0.1%), or excess Q-10, the absorption maximum appeared at 865 nm in both LM and RC. A similar variation in the position of the long-wavelength absorption was observed at cryogenic temperatures (data not shown). The position of the long-wavelength absorption had no effect on the kinetics of charge recombination, k_{AD}, at 77 K (see Electron Transfer between D and Q_A).

Activity and Stability. Activity was measured by the extent of reversible bleaching of the absorption at 865 nm in response to a saturating flash of light at room or cryogenic temperatures (see Electron Transfer between D and Q_A). The activity of LM isolated at 25 °C in sodium cholate, CHAPS, or a mixture of sodium deoxycholate and sodium cholate was 95 ± 3% compared to RCs. In sodium deoxycholate (0.2%), LM could be stored for at least 1 day at room temperature or for a week at 4 °C without loss of activity. In sodium cholate (0.025%) or CHAPS (0.04–1.0%), slight losses (5–10%) in activity were observed under these conditions. LM was routinely frozen in liquid nitrogen and stored at −70 °C up to a year without detectable effects.

LM prepared in LDAO (0.025%) (Feher & Okamura, 1978) was less active and less stable than LM prepared in the detergents described above. Such LM could be stored for at

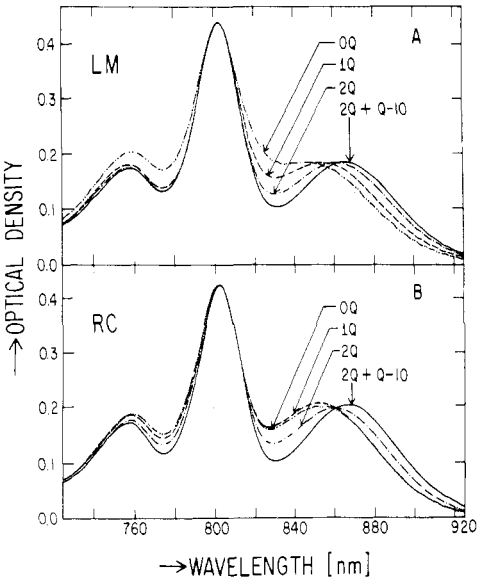


FIGURE 2: Dependence of the position of the long wavelength absorption (near 865 nm) on the Q-10 content of LM (A) and RC (B) at 295 K. 0Q, 1Q, and 2Q RC denote RCs containing 0.12 ± 0.03, 0.95 ± 0.03, and 1.9 ± 0.1 Q-10 per RC, respectively. 0Q, 1Q, and 2Q LM denote LM prepared from 0Q, 1Q, and 2Q RCs, respectively. Conditions: 1.5 μM LM or RC, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 8. Exogenous Q-10 (6 μM) was present as noted.

most 1 day at 4 °C or for a few hours at room temperature before appreciable loss of activity was observed. Frequently, an increase of A_{760} with concomitant decrease of A_{800} and A_{865} was also observed. These changes, attributed to denaturation of the LM, were substantially accelerated at higher concentrations of LDAO. LM prepared or stored in Triton X-100 (0.05%) also exhibited poor activity and stability. LM stored in octyl β-D-glucopyranoside (1.0%) lost activity rapidly, even at 4 °C. A possible explanation of the loss of activity is extraction of Q_A by detergent.

In all detergents tested LM showed optimal activity and stability between pH 6.5 and pH 8.0. The sensitivity of LM to light and heat was considerably greater than that of RCs.

Iron Content and EPR Spectra. LM prepared in sodium cholate or in a mixture of sodium cholate and sodium deoxycholate contained 0.94 ± 0.04 Fe/LM as determined by atomic absorption spectroscopy. The light-induced EPR spectrum of LM at cryogenic temperatures (T < 80 K) has been reported previously (Okamura et al., 1982b; Butler et al., 1984). It shows a broad signal at $g = 1.8$ that resembles the signal of $Q_A^-Fe^{2+}$ in RCs. The Fe atom may be removed from LM by including 1 mM *o*-phenanthroline in the incubation mixture during preparation of LM. The resulting LM contains <0.05 Fe/LM and retains 60–70% of the primary photochemical activity. Reduction of this LM with dithionite results in a single, structureless EPR signal at $g = 2.0046$ identified as the signal of a semiquinone anion radical. EPR and ENDOR specta of this signal have been reported (Okamura et al., 1980, 1982b).

H Subunit

H isolated after centrifugation of RCs into $LiClO_4$ was at least 95% pure with respect to contamination by LM, as judged by electrophoresis in polyacrylamide gels in the presence of $NaDodSO_4$. The H contained some pigment (~0.1 tetrapyrroles per H, as judged from the optical absorption ratios A_{360}/A_{280} and A_{760}/A_{280}) and Q-10 (~0.5 Q-10 per H). We believe this to be nonspecific contamination from detergent micelles that accumulate at the top of the centrifuge tubes during centrifugation (Okamura et al., 1974; Vadeboncoeur et al., 1979; Agalidis & Reiss-Husson, 1983) and are coisolated with H.

We define the "activity" of the isolated H in terms of its ability to reassociate with LM to form intact RCs with functional Q_B (see below). H could be dialyzed overnight against sodium cholate (0.025%) with only ~10% loss in the degree of reconstitution (the reconstituted samples contained ~10% unreassociated LM). In contrast, dialysis overnight against LDAO (0.1%) resulted in less reassociation of LM and H, with the reconstituted samples exhibiting anomalous electrophoretic mobilities in agarose. H was frozen in liquid nitrogen and stored up to a year at −70 °C (even in the presence of $LiClO_4$ and LDAO) without significant loss of ability to reassociate.

Localization of the Q_B Site with ^{14}C-Labeled Q-10

To localize the binding site for Q_B, isolated H and LM were assayed for ^{14}C-labeled Q-10. When LM was prepared from RCs containing approximately two ^{14}C-labeled Q-10 per RC, 92 ± 2% of the ^{14}C counts remained with LM in the supernatant and only 6 ± 2% coprecipitated with H. Approximately 98% of the counts in the supernatant, corresponding to ~1.9 Q-10/LM, were precipitated by anti-LM IgG, showing that the Q-10 was bound to LM. No increase in the counts per LM was observed upon precipitation by IgG in the presence of excess ^{14}C-labeled Q-10. This is consistent with specific binding of Q-10 to LM. Similar amounts of ^{14}C-labeled Q-10/RC were precipitated by anti-LM IgG in experiments with RCs. The antibody did not precipitate Q-10 in the absence of LM or RC.

Reconstitution of RCs from LM and H

Reconstitution of RCs from LM and H was assayed by gel electrophoresis, performed at 4 °C under nondenaturing conditions. Figure 3 shows stained electrophoretograms of RC, LM, H, and two samples of reconstituted RC formed with different proportions of LM and H. LM (Figure 3B) showed a 15% lower mobility than did RCs (Figure 3A). H (Figure 3C) showed an even lower mobility. Electrophoretograms resulting from two reconstituted samples are shown in Figure 3D,E. When the proportion of H/LM was 0.5, bands corresponding to both LM and RC were observed (Figure 3D). A nearly stoichiometric proportion of H and LM resulted in fully reconstituted RCs (Figure 3E); this material showed a complete absence of LM (compare parts A and E of Figure 3).

The small band observed at ~4.8 cm in the gels of both native and reconstituted RCs results from a population of RCs (~10%) that exhibits a higher mobility than that of the predominant population (Okamura et al., 1974). The two types of RCs could be separated by ion-exchange chromatography with DEAE-cellulose. (The major component was eluted with ~0.12 M NaCl and the minor component with ~0.2 M NaCl.) The two types of RCs showed the same optical absorption spectrum, subunit composition, Fe content, and kinetics of electron transfer (M. Y. Okamura, R. J. Debus, D. Kleinfeld, and G. Feher, unpublished results). Preliminary experiments indicate a difference in isoelectric points of ~0.3 pH unit between the two species (R. Boggs and T. Hazlett, unpublished results). Attempts to interconvert the two purified species [incubation under conditions designed to promote deamination [e.g., 1 week at pH 10.4 at 23 °C; see Flatmark (1966)], incubation with reducing agents, extraction and re-

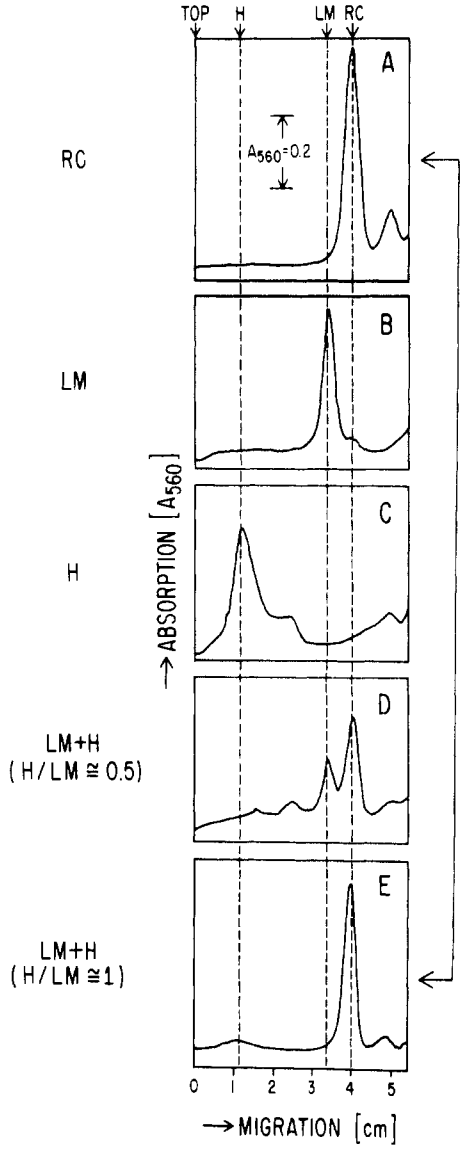


FIGURE 3: Agarose gel electrophoretograms (stained with Coomassie Brilliant Blue R250) of native RC (A), LM (B), H(C), and RCs reconstituted by using two different ratios of H/LM (D and E). The small band at ~4.8 cm in the RC and LM + H gels correspond to a faster migrating form of RCs (see text). Note that the electrophoretogram of fully reconstituted RCs (E) is the same as that of native RCs (A).

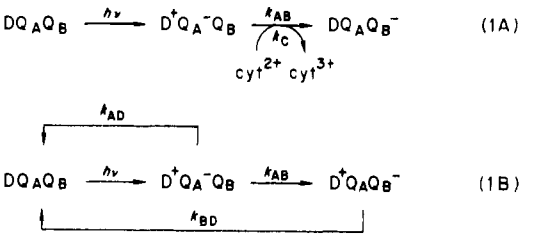
constitution of Q_A and Q_B, or prolonged storage at 23 °C] were unsuccessful. The heterogeneity of RCs was preserved in the absence of H: LM isolated from the more slowly migrating RCs migrated ~15% more slowly than LM isolated from the faster migrating RCs (not shown).

The optical absorption spectrum of reconstituted RCs was the same as that of native RCs, even at cryogenic temperatures: well-resolved absorptions at 387, 530, and 544 nm were observed at 77 K (not shown).

Electron Transfer in LM and RC

To determine the effect of removal of H on the kinetics of electron transfer, the rates of various electron transfer reactions in LM were measured and compared with those in RCs. The reactions with and without exogenous cytochrome *c* are schematically illustrated by eq 1A and 1B, respectively.

Reaction with Cytochrome c. To investigate whether dissociation of H affects the binding site for cytochrome *c*, the rate of electron transfer from reduced horse heart cytochrome



c (cyt^{2+}) to D^+ was determined by measuring the recovery of the absorption of $(BChl)_2$ at 865 nm following a saturating flash. The rates of oxidation of cyt^{2+} by RC and LM in the presence of 0.025% sodium cholate and excess cyt^{2+} were virtually identical (Figure 4). The kinetics could be fitted well with single exponentials. At an ionic strength of ~11 mM, these fits yielded first-order rate constants of $k_c = (1.3 \pm 0.1) \times 10^4\ s^{-1}$ for RC (Figure 4A) and $k_c = (1.7 \pm 0.1) \times 10^4\ s^{-1}$ for LM (Figure 4B). These kinetics contrast with those obtained for RCs in LDAO or Triton X-100 (data not shown; Ke et al., 1970; Overfield et al., 1979; Rosen et al., 1979), where the kinetics of oxidation were clearly biphasic. Presumably the difference is a consequence of the use of the negatively charged cholate as detergent. For both RC and LM the kinetics varied with ionic strength in accordance with simple Debye–Hückel theory, as previously demonstrated for RCs in LDAO (Prince et al., 1974; Rosen et al., 1979). The data, shown in Figure 4C, show that the rate of reduction of D^+ by cyt^{2+} was the same for RC and LM within a factor of 2 at any ionic strength up to 250 mM.

Electron Transfer between D and Q_A. Primary photochemical activity, defined as the extent of reversible light-induced charge separation between D and Q_A (McElroy et al., 1974; Feher & Okamura, 1978), was assayed by light-induced absorption changes in RC and LM at cryogenic temperatures ($T < 80$ K). Bleaching and recovery of the absorption at 890 nm (Figure 5) indicate formation and decay of the charge-separated state $D^+ Q_A^-$. The amplitude of the absorption change ($\Delta A_{890}/A_{890}$) in LM was 95 ± 3% of that observed in RCs, indicating that LM retained 95 ± 3% of the primary photochemical activity in the absence of H. From the recovery of the absorption at 890 nm, the first-order rate constant of charge recombination (k_{AD}) was obtained. The values were 37 ± 3 s^{-1} for native RCs, 19 ± 3 s^{-1} for LM, and 36 ± 3 s^{-1} for reconstituted RCs. Thus, the rate of charge recombination, k_{AD}, was reduced by a factor of ~2 when the H subunit was removed but was restored to the rate seen in native RCs when the H subunit was replaced.

Electron Transfer from Q_A^- to Q_B. To investigate the rate of electron transfer from Q_A^- to Q_B (k_{AB}), several procedures were employed. The first involved measuring transient optical absorption changes near 750 and 770 nm induced by an actinic flash (Vermeglio & Clayton, 1977). These changes, due to electron transfer from Q_A^- to Q_B, were observed in both native and reconstituted RCs (data not shown). They yielded $k_{AB} = (4.4 \pm 0.3) \times 10^3\ s^{-1}$ for native RCs and $k_{AB} = (5.0 \pm 0.3) \times 10^3\ s^{-1}$ for reconstituted RCs at 17 °C, in agreement with previously reported data (Vermeglio & Clayton, 1977; Wraight, 1979). However, in LM no corresponding rapid absorption changes were observed at either wavelength (data not shown). Because this assay is sensitive to absorption changes corresponding to k_{AB} between 10^4 and $10^2\ s^{-1}$, these observations suggest that in LM k_{AB} is slower than $10^2\ s^{-1}$.

To extend the measurement of k_{AB} to slower rates, the amount of cyt^{2+} oxidized on the second of two closely spaced actinic flashes was monitored (Parson, 1969; Halsey & Parson, 1974). Following one flash, RCs rapidly oxidize 1 cyt^{2+}/RC

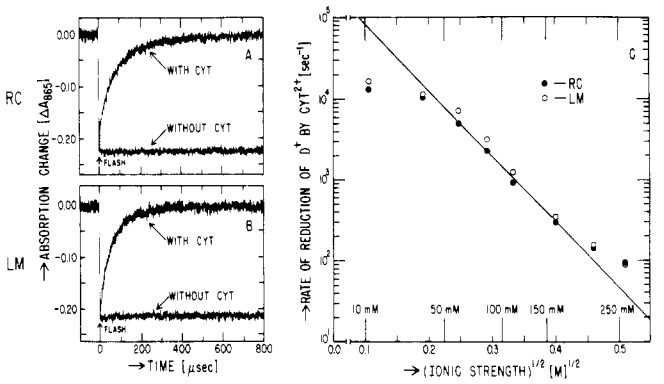


FIGURE 4: Kinetics of reduction of D^+ by cyt^{2+} in LM and RC in response to an actinic flash as measured at 865 nm. The much slower reduction of D^+ in the absence of cyt^{2+} is also shown. (A) Kinetics of RCs at an ionic strength of $I \sim 11$ mM. (B) Same as (A) for LM. (C) The rate of reduction of D^+ by cyt^{2+} in RC (●) and LM (○) as a function of ionic strength. Conditions: 2 μM LM or RC, 35 μM cyt *c* (horse heart), 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, 21 °C, pH 8. The ionic strength in (C) was varied with NaCl. The straight line in (C) represents a fit through the central part of the data.

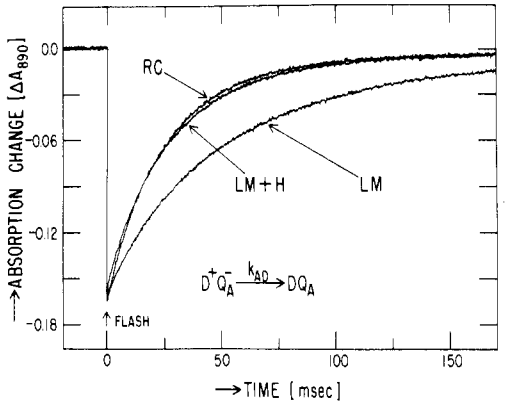


FIGURE 5: Kinetics of charge recombination between Q_A^- and D^+ (k_{AD}) at cryogenic temperature (77 K). The formation and decay of the charge-separated state $D^+Q_A^-$ was monitored at 890 nm following an actinic flash. Samples [17 μM native RC, LM, or reconstituted RC (LM + H), 10 mM Tris-HCl, 0.025% sodium cholate, 0.1 mM EDTA, and 170 μM Q-10, pH 8] were mixed with an equal volume of glycerol and frozen in liquid nitrogen in optical cells with a path length of 1 mm. Measurements were performed at 77 K.

to form DQ_A^-. The rate of regeneration of DQ_A (e.g., by electron transfer to Q_B) was measured optically from the amount of cyt^{2+} per RC oxidized in response to a second flash as the time between flashes was varied. The measured rate [which we denote k_{AB}(apparent)] represents an upper limit for k_{AB} because oxidation of Q_A^- by any exogenous agent would be indistinguishable in this assay from an electron transfer from Q_A^- to Q_B.

Parts A–C of Figure 6 show the oxidation of cyt^{2+} by native and reconstituted RCs and LM resulting from two actinic flashes spaced 4 s apart. Both native and reconstituted RCs (parts A and C of Figure 6, respectively) oxidized 1 cyt^{2+}/RC in response to each flash. LM (Figure 6B) oxidized 1 cyt^{2+}/LM in response to the first flash, but less in response to the second, reflecting a slower k_{AB} than in RCs. In contrast to either native or reconstituted RCs, LM also reduced cyt^{3+} in the dark period following each flash, with both the rate and extent of reduction increasing with the concentration of exogenous Q-10 (data not shown).

The ratio of the absorption change of cyt (ΔA_{550}) resulting from the second flash to that from the first was plotted as a function of time between flashes (Figure 6D). Cyt c_2 (*Rp. sphaeroides*) was used in place of cyt *c* (horse heart) for these measurements because cyt c_2 reduces D^+ faster than does cyt *c* (Ke et al., 1970; Overfield et al., 1979; Rosen et al., 1979). We took k_{AB}^{-1}(apparent) to be the time between flashes required for the ΔA_{550} ratio to reach 0.63 (i.e., $1-1/e$) of its maximum value. For native and reconstituted RCs at 4 °C k_{AB}(apparent) = $(3.3 \pm 0.5) \times 10^3$ s^{-1} and $(2.9 \pm 0.4) \times 10^3$ s^{-1}, respectively. These values are comparable to the values of k_{AB} determined at 17 °C discussed previously. For LM under these conditions k_{AB}(apparent) ~ 1 s^{-1}; this rate varied with the concentration of Q-10 and with the type and concentration of detergent present. The maximum rates were observed in the presence of 0.2% sodium deoxycholate at 21 °C. Under these conditions the rate varied from 0.22 ± 0.02 s^{-1} in LM prepared from RCs depleted of Q_B (1Q LM) to a maximum of 13 ± 1 s^{-1} in LM in the presence of excess Q-10 (Figure 6E). RCs depleted of Q_B (1Q RC) are shown for comparison.

The data of Figure 6D,E show that k_{AB} decreased by a factor of 10^2–10^3 in the absence of the H subunit. Reconstitution restored k_{AB} to the rate seen in the native protein. The slowness of k_{AB} in LM accounts for the absence of the transient optical absorption changes at 750 and 770 nm discussed above.

The rate of electron transfer from Q_A^- to Q_B in LM was also estimated from the oxidation of cyt^{2+} in response to continuous actinic illumination (Clayton et al., 1972a,b; Okamura et al., 1982a). Figure 7 shows the oxidation of cyt^{2+} by native and reconstituted RCs and LM illuminated in the presence of excess Q-10. Native RCs (Figure 7A) oxidized cyt^{2+} at two rates, with $k_{fast} \sim 200$ s^{-1} and $k_{slow} \sim 0.25$ s^{-1}. (These values were obtained by suitably changing the time scale of Figure 7A.) The faster rate was limited by the intensity of the actinic illumination, showing that charge separation $DQ_A \rightarrow D^+ Q_A^-$ was rate limiting. In sodium cholate or sodium deoxycholate, 10 or 20 cyt^{2+}/RC were routinely oxidized with k_{fast}. In LDAO (0.025%), under the same conditions, only 4 or 5 cyt^{2+}/RC were oxidized with k_{fast} (data not shown). These data suggest that electron transfer to the mobile pool of exogenous Q-10 is quite rapid and that the size of the pool available to the RC is less in LDAO than in the other detergents. In general, the number of cyt^{2+}/RC oxidized with k_{fast} appeared to be governed by depletion of either cyt^{2+} or the accessible Q-10 pool. In the presence of *o*-phenanthroline (Figure 7D), a known inhibitor of electron transfer from Q_A^- to Q_B (Parson & Case, 1970; Clayton et al., 1972b; Halsey & Parson, 1974), only 1 cyt^{2+}/RC was oxidized with k_{fast}.

LM also oxidized cyt^{2+} at two rates (Figure 7B) but was not appreciably affected by *o*-phenanthroline (Figure 7E). The faster rate was the same as in RCs, but only 1 cyt^{2+}/LM was oxidized at this rate, even in the presence of high concentrations of Q-10 (Figure 7G). The slower rate (k_{slow}, determined from the initial slope) varied from 5 ± 3 s^{-1} in LM prepared from RCs depleted of Q_B (1Q LM) to a maximum of 43 ± 5 s^{-1} in LM at high concentrations of Q-10 (Figure 7G). This rate increased with the intensity of actinic illumination, suggesting that electron transfer involving acceptors intermediate between D and Q_A may contribute to k_{slow} in LM. This rate, therefore, represents an upper limit for k_{AB} in LM. The data of Figure 7 demonstrate that k_{AB} decreased in the absence of the H subunit by at least a factor of $\sim 10^2$.

The slow rate of electron transfer from Q_A^- to Q_B in LM is also reflected in the kinetics of charge recombination in LM in response to an actinic flash at room temperature (Figure

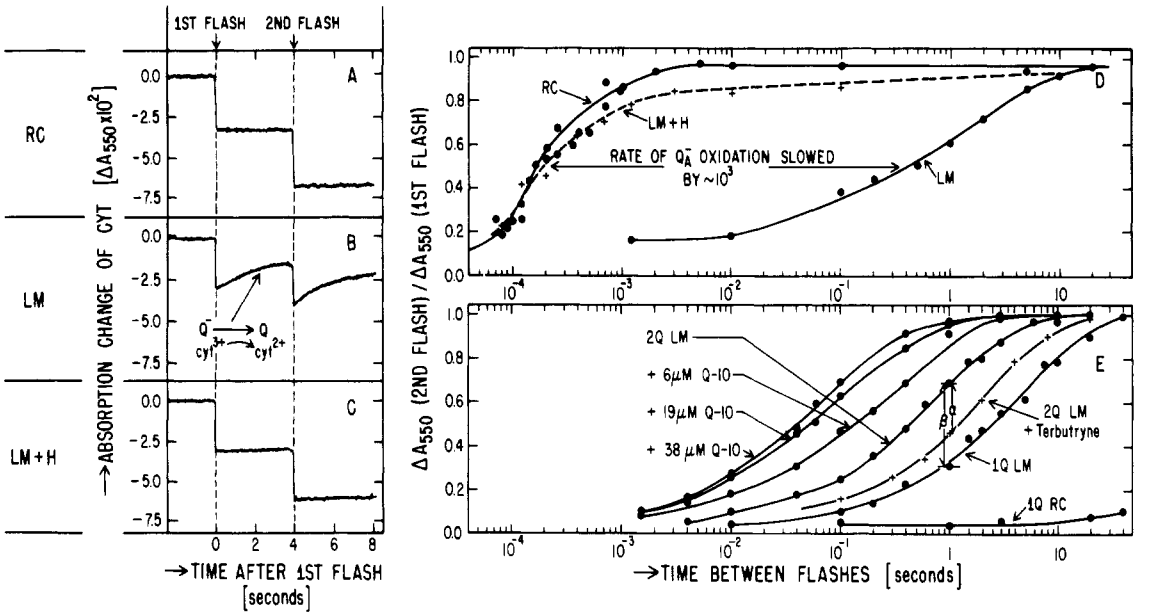


FIGURE 6: Estimates of k_{AB} in LM and RC from the amount of cyt^{2+} oxidized on the second of two actinic flashes. (A–C) Oxidation of cyt^{2+} by native RC, LM, and reconstituted RC (LM + H), resulting from two actinic flashes spaced 4 s apart [2 μM RC, LM, or LM + H, 30 μM cyt *c* (horse heart), 10 μM Q-10, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, 4 °C]. (D) Absorption change of cyt (ΔA_{550}) following the second flash, normalized to ΔA_{550} after the first flash, as a function of time between flashes [conditions the same as in (A–C), except that cyt c_2 (*Rp. sphaeroides*) was substituted for cyt *c*]. (E) Same as (D), but for LM in the presence of different amounts of Q-10. RCs lacking Q_B are shown for comparison. The affect of 0.7 mM terbutryn on k_{AB} in 2Q LM is shown. The percent inhibition at a fixed time interval between flashes was defined as $\% I = \alpha/\beta \times 100$, with the value of the ΔA_{550} ratio in 1Q LM corresponding to 100% inhibition (see Figure 11) [conditions the same as in (A–C), except that 0.2% sodium deoxycholate was present and the measurements were performed at 21 °C].

8). The rate of charge recombination between Q_B^- and D^+ (k_{BD}) in native RCs at 4 °C was determined to be 0.48 ± 0.02 s^{-1} (Figure 8A). The faster rate of recombination between Q_A^- and D^+ (k_{AD} = 10.0 ± 0.2 s^{-1} at 4 °C) was measured in RCs having electron transfer to Q_B blocked by *o*-phenanthroline (Figure 8D). In contrast to RCs, LM exhibited biphasic kinetics of charge recombination (Figure 8B) and was relatively insensitive to *o*-phenanthroline (Figure 8E). The faster component (k = 2.9 ± 0.1 s^{-1} at 21 °C) presumably represents k_{AD} since it was observed in LM prepared from RCs depleted of Q_B (1Q LM in Figure 8G) and in LM in the presence of $NaBH_4$ (not shown). $NaBH_4$ reduces Q_B to Q_B^{2-} in LM and RC but does not appear to reduce Q_A. (This is presumably a consequence of $NaBH_4$ being a two-electron reductant and Q_A a one-electron acceptor.) The faster component in LM was slower than k_{AD} in RCs by a factor of ~2.8 at 21 °C, resembling the observations made at cryogenic temperatures (Figure 5). The slower component of the biphasic kinetics in LM ($k \sim 10^{-2}$ s^{-1}) presumably represents k_{BD}, although the pathway of charge recombination between Q_B^- and D^+ in LM may differ from that in RCs. The rates of both components were independent of the concentration of Q-10 (Figure 8G). The rate of the slower component varied from ~5 × 10^{-3} s^{-1} at pH 6 to ~9 × 10^{-2} s^{-1} at pH 10 at 21 °C. The rate of the faster component increased by only ~30% over this range of pH. Polyphasic kinetics of charge recombination have been observed previously in LM isolated with $NaDodSO_4$ (Agalidis & Reiss-Husson, 1983).

Thus, the relative amount of the slower component of charge recombination in LM (percent slow) varied with the concentration of Q-10, ranging from ~8% in LM prepared from RCs depleted of Q_B (1Q LM) to a maximum of ~60% in LM at high concentrations of Q-10 at 21 °C (Figure 7G). Less slow component was observed at lower temperatures. Similar results were obtained in different detergents (LDAO, Triton X-100, CHAPS, octyl β-D-glucopyranoside). The inability to achieve 100% slow kinetics in LM, even at saturating concentrations of Q-10, suggests that k_{AD} competes favorably with k_{AB} in LM. Estimates for k_{AB} can be obtained from the relationship (Chamorovsky et al., 1976):

$$\% \text{ slow} = \frac{k_{AB}}{k_{AB} + k_{AD}}$$

This analysis assumes that all Q_B sites are fully functional. RCs isolated in the usual manner (Feher & Okamura, 1978) contain 10–20% nonfunctional Q_B sites (Okamura et al., 1982a; Kleinfeld et al., 1984a). Correcting for this fraction, and with k_{AD} = 2.9 ± 0.1 s^{-1}, we estimated a maximum rate of k_{AB} = 7 ± 2 s^{-1} at 21 °C in LM. This is in reasonable agreement with the maximum rate measured in Figure 6E. Thus, the data of Figure 8 are an independent indication that k_{AB} decreased by a factor of ~10^3 in the absence of the H subunit.

Stability of the Semiquinones Q_A^- and Q_B^- in LM and RC

The data of Figure 6 suggest that the semiquinones Q_A^- and Q_B^- are less stable to oxidation in LM than in RC. Figure 6B shows that the cyt^{2+} oxidized in response to an actinic flash was subsequently reduced in the dark by LM, presumably by electrons leaking off Q_A^- or Q_B^-. No such reduction of the cyt^{3+} formed in the light was observed for native or reconstituted RCs (Figure 6A,C). Furthermore, Figure 6E shows that k_{AB}(apparent) is much greater for 1Q LM than for 1Q RC, suggesting that Q_A^- is less stable to oxidation in LM than in RCs. To investigate further the stability of Q_A^- and Q_B^- in LM and RC, we monitored the optical absorption of anionic semiquinone at 450 nm (Land et al., 1971) resulting from an actinic flash (ΔA_{450}). Figure 9 shows the formation and decay of ΔA_{450} following an actinic flash in native and reconstituted RCs and LM. The observed rates of decay were nonexponential. We took k_{decay}^{-1} to be the time required for ΔA_{450} to decay to 0.37 (i.e., $1/e$) of its maximum value. Diamino-

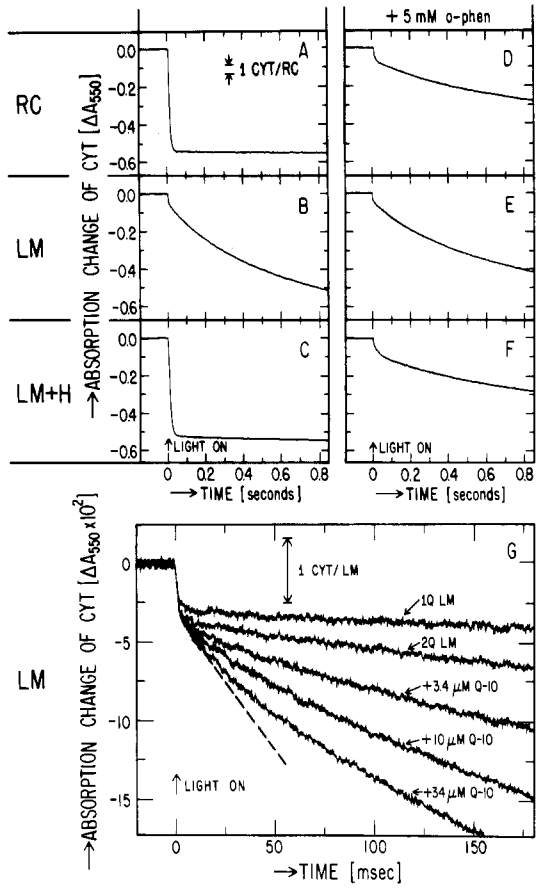


FIGURE 7: Oxidation of cyt^{2+} by native RC (A), LM (B), and reconstituted RC (LM + H) (C), in response to continuous actinic illumination [2 μM RC, LM, or LM + H, 34 μM cyt *c* (horse heart), 10 μM Q-10, 10 mM Tris-HCl, 0.025% sodium cholate, 0.2% sodium deoxycholate, and 0.1 mM EDTA, pH 7.7, 21 °C, light intensity = 1.05 W/cm^2]. The effect of 5 mM *o*-phenanthroline on native RC (D), LM (E), and reconstituted RC (F) is also shown. (G) Variation of the slow component of the rate of oxidation of cyt^{2+} by LM (k_{slow}) with the amount of Q-10 present [conditions the same as in (B)].

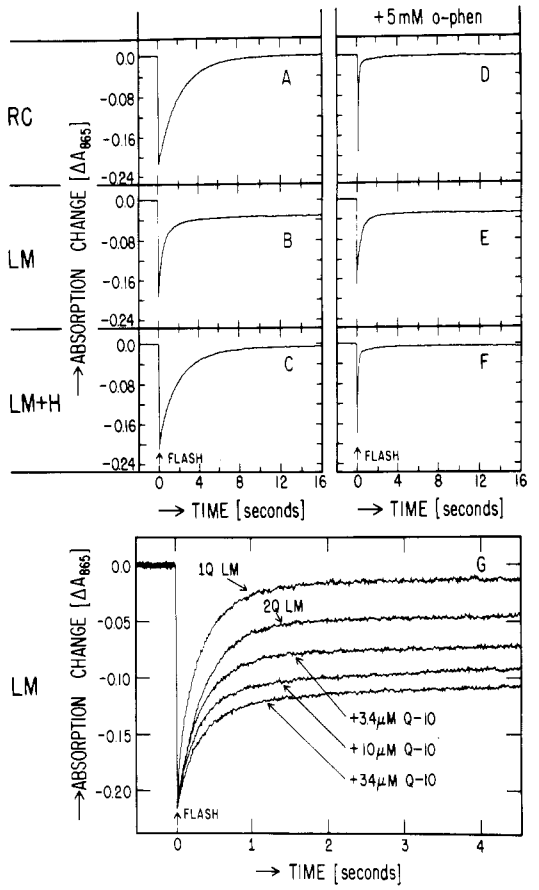


FIGURE 8: Kinetics of charge recombination in native RCs (A), LM (B), and reconstituted RCs (LM + H) (C) at 4 °C. The formation and decay of the charge-separated states $D^+ Q_A^-$ or $D^+ Q_B^-$ were monitored at 865 nm following an actinic flash. Conditions: 2 μM RC, LM, or LM + H, 10 μM Q-10, 10 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7, 4 °C. The effect of 5 mM *o*-phenanthroline on native RCs (D), LM (E), and reconstituted RCs (F) is also shown. (G) Kinetics of charge recombination in LM as a function of the amount of Q-10 present [conditions the same as in (B), except that 0.2% sodium deoxycholate was present and the measurements were performed at 21 °C].

durene and sodium ascorbate were present to rapidly reduce D^+. (The rate of reduction was 71 ± 2 s^{-1} under the conditions used.)

RCs containing 0.95 ± 0.03 Q-10 per RC (Figure 9A) exhibited $k_{decay} = (4 \pm 1) \times 10^{-3}$ s^{-1}. LM prepared from these RCs (Figure 9B) exhibited $k_{decay} = (8.4 \pm 0.7) \times 10^{-2}$ s^{-1}. RCs reconstituted with this LM (Figure 9C) showed approximately the same k_{decay} as native RCs, indicating that the lifetime of the semiquinone anion Q_A^- decreased by a factor of ~20 in the absence of the H subunit.

RCs containing ~2 Q-10 per RC (Figure 9D) also exhibited $k_{decay} = (4 \pm 1) \times 10^{-3}$ s^{-1}. LM prepared from these RCs (2Q LM in Figure 9E) exhibited $k_{decay} = 0.66 \pm 0.03$ s^{-1}, but the rate of decay increased in the presence of added Q-10, saturating with $k_{decay} = 3.1 \pm 0.2$ s^{-1} at high concentrations of Q-10 (Figure 9E). Reconstituted RCs (Figure 9F) showed approximately the same k_{decay} as native RCs, indicating that the lifetime of the semiquinone anion Q_B^- decreased by a factor of ~10^3 in the absence of H.

RCs exhibit oscillations with a periodicity of two in the optical absorption of semiquinone (A_{450}) in response to a series of actinic flashes in the presence of exogenous electron donors and quinone acceptors (Vermeglio, 1977; Wraight, 1977; Vermeglio & Clayton, 1977; Kleinfeld et al., 1984a). These oscillations are characteristic of the function of Q_B as a two-electron "gate" between the single electron process of light-induced charge separation and the two-electron process of electron pair donation to the exogenous quinone pool. Native and reconstituted RCs and LM were subjected to a series of actinic flashes in the presence of excess Q-10 (with diaminodurene and ascorbate present as exogenous electron donors). Both native RCs (Figure 10A) and reconstituted RCs (Figure 10C) exhibited oscillations characteristics of functional Q_B "gating". No such oscillations were observed in LM with any amount of Q-10 present (e.g., see Figure 10B). Thus, the H subunit is required for Q_B to function as a two-electron gate. This is presumably a consequence of the reduced stability of Q_B^- to oxidation and the lower value of k_{AB} in the absence of H.

Effect of Inhibitors on Electron Transfer from Q_A^- and Q_B in LM and RC

The data of Figures 7 and 8 show that *o*-phenanthroline has little effect on LM at the concentrations normally used with RCs. To determine whether any inhibition could be observed in LM, we examined the effect of higher concentrations of *o*-phenanthroline and various herbicides on k_{AB}(apparent) in LM as measured in Figure 6E. Values of k_{AB}(apparent) for LM and RC in the presence and absence of various inhibitors are presented in Table I. No excess Q-10 was present in these measurements. Since k_{AB} is much smaller and Q_A^- is much less stable to oxidation in LM, inhibition is manifested as a

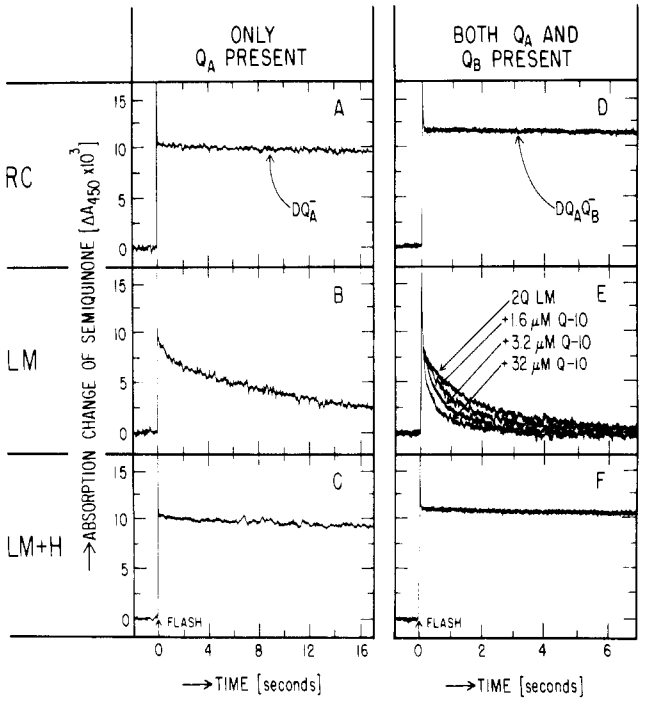


FIGURE 9: Stability of the semiquinone anions Q_A^- and Q_B^- in native RC, LM, and reconstituted RC (LM + H), as measured optically at 450 nm. The formation of semiquinone was induced by an actinic flash in the presence of diaminodurene, which rapidly reduced D^+. To examine the stability of Q_A^-, the absorption change ΔA_{450} was monitored in RCs lacking Q_B (A), in LM prepared from these RCs (B), and in RCs reconstituted with this LM (C). Similarly, the stability of Q_B^- was monitored in RCs containing Q_B (D), in LM prepared from these RCs (2Q LM) (E), and in RCs reconstituted with this LM (F). The effect of adding exogenous Q-10 to this LM is shown in (E). Conditions: 2 μM RC, LM, or LM + H, 0.5 mM diaminodurene, 10 mM sodium ascorbate, 10 mM Tris-HCl, 0.025% sodium cholate, 0.2% sodium deoxycholate, and 0.1 mM EDTA, pH 7.7, 21 °C. The diaminodurene was added from a stock of 50 mM diaminodurene in ethanol.

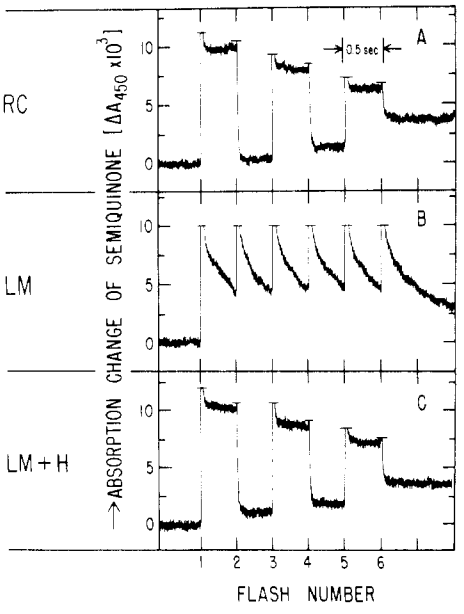


FIGURE 10: Changes in the optical absorption (A_{450}) of semiquinone anion in response to a series of actinic flashes (conditions the same as in Figure 8, but with 3.2 μM Q-10 present]. (A) Native RC, (B) LM, and (C) reconstituted RC (LM + H). The actinic flashes were spaced 0.5 s apart. Note the absence of oscillations in LM.

much smaller change in k_{AB} in LM than in RCs. The value of k_{AB}(apparent) in 1Q LM was taken to define 100% inhibition in LM. The percent inhibition (% I) at a fixed time

Table I: Inhibition of Electron Transfer in LM and RC

inhibitor	K_I (μM)[a] RC	K_I (μM)[a] LM	k_{AB}(apparent)[b] (s^{-1}) RC	k_{AB}(apparent)[b] (s^{-1}) LM
no inhibitor			4000	1.3 ± 0.1
terbutryn	1.9 ± 0.5	300 ± 50	5.9 ± 0.4	0.48 ± 0.02
ametryn	4.0 ± 0.5	1100 ± 100	ND	0.59 ± 0.04
prometryn	8.6 ± 1.1	500 ± 50	ND	0.48 ± 0.02
atrazine	62 ± 10	>400	ND	
o-phenanthroline	115 ± 15	>5000	0.71 ± 0.05	0.89 ± 0.09
DCMU	700 ± 100	500 ± 100	ND	0.59 ± 0.04
bromacil	>1000	>1000		
removal of Q_B[c]			<0.1	0.22 ± 0.02

[a] K_I is the apparent inhibitor dissociation constant obtained from inhibition curves like those shown in Figure 11. Conditions: 2 μM LM or RC containing ~1.9 Q-10/LM or RC, 35 μM cyt *c*, 10 mM Tris-HCl, 0.025% sodium cholate, 0.2% sodium deoxycholate, and 0.1 mM EDTA, pH 8, 21 °C. [b] Determined from the amount of cyt^{2+} oxidized on the second of two actinic flashes as a function of the time between flashes (see Figure 6). Concentrations of inhibitors: 0.7 mM terbutryn, 1.0 mM ametryn, 0.7 mM prometryn, 5 mM *o*-phenanthroline, and 0.5 mM DCMU for LM and 0.01 mM terbutryn and 5 mM *o*-phenantroline for RCs. [c] Measurements were performed with 1Q RC and 1Q LM.

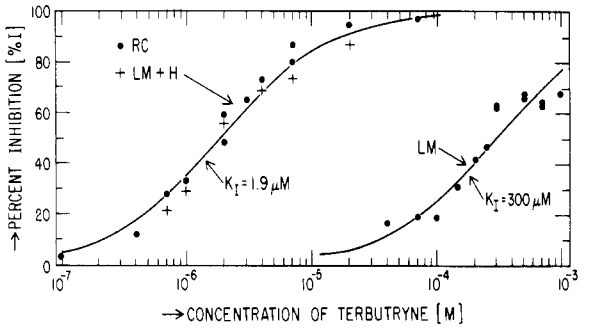


FIGURE 11: Inhibition of electron transfer from Q_A^- to Q_B by terbutryn in native and reconstituted RCs and LM. The percent inhibition (% I) is defined in the text and in the legend to Figure 6 (conditions the same as in Figure 6E, except that all samples contained 1% ethanol). The solid lines represent theoretical one-site binding isotherms with K_I as an adjustable parameter.

interval between flashes was defined as the difference between the numbers of cyt^{2+} per LM oxidized on the second flash in the absence and presence of inhibitor, normalized to the difference between the numbers of cyt^{2+} per LM oxidized on the second flash by 2Q LM and 1Q LM in the absence of inhibitor (% $I = \alpha/\beta \times 100$ in Figure 6E). To maximize β, a time of 1 s between flashes was chosen for LM. A time of 10 ms was chosen for RCs. Inhibition curves for the action of terbutryn on native and reconstituted RCs and LM are shown in Figure 11. Similar curves were obtained for ametryn, prometryn, atrazine, DCMU, and *o*-phenanthroline (data not shown). The curves could be fitted by simple one-site binding isotherms:

$$\% I = \frac{1}{1 + K_I/C} \times 100$$

where C is the concentration of inhibitor and K_I is its dissociation constant. Values of K_I extracted from data such as those of Figure 11 are presented in Table I. Similar values of K_I have been obtained for RCs using different methods and conditions (Okamura, 1984; Stein et al., 1984). For most inhibitors K_I increased by a factor of $\sim 10^2$ in the absence of the H subunit. Reassociation of H restored the potency of the inhibitors, as demonstrated for terbutryn in Figure 11.

SUMMARY AND DISCUSSION

In this work we prepared and characterized an active and stable LM complex containing ~1 Fe/LM. A comparison

Table II: Comparison of Characteristics of LM and RC

	RC	LM	reference
Spectral Characteristics			
optical absorption ratio A_{280}/A_{802}	1.19 ± 0.03	1.04 ± 0.03	Figure 1A
Soret band absorption maxima at 77 K (nm)	365, 387	365	Figure 1B
pheophytin absorption maxima at 77 K (nm)	530, 544	544	Figure 1B
EPR spectrum			
g value of D^+	2.0026	2.0026	Okamura et al. (1982b)
g value of $Q_A^-Fe^{2+}$	1.8	1.8	Okamura et al. (1982b)
ΔH of $Q_A^-Fe^{2+}$ in the absence of *o*-phenanthroline (G)	580	480	Butler et al. (1984)
ΔH of $Q_A^-Fe^{2+}$ in the presence of *o*-phenanthroline (G)	390	475	Butler et al. (1984)
photochemical activity (bleaching of 890-nm absorption at 77 K) (%)	100	95 ± 3	Figure 5
Chemical Characteristics			
iron content	0.97 ± 0.03	0.94 ± 0.03	
ubiquinone content	2.0 ± 0.2	1.9 ± 0.2	
K_D, dissociation constant of Q-10 for the Q_B site (μM)	2 ± 1[b]	15 ● 3, 10 ± 3	Figures 6E and 7G
K_I, dissociation constants for inhibitors (μM)			
triazine herbicides	2–10	300–1000	Table I
o-phenanthroline	115 ± 15	>5000	Table I
Electron Transfer Characteristics			
k_C, rate of reduction of D^+ by cyt^{2+} (11 mM ionic strength) (s^{-1}) (21 °C)	(1.3 ± 0.1) × 10^4	(1.7 ± 0.1) × 10^4	Figure 4A,B
k_{AD}, charge recombination between Q_A^- and D^+ (s^{-1})			
77 K	37 ± 3	19 ± 3	Figure 5
294 K	8.2 ± 0.3[a]	2.9 ± 0.1	Figure 8
k_{AB}, electron transfer from Q_A^- to secondary acceptors (e.g., Q_B) (s^{-1}) (21 °C)	4 × 10^3	13 ± 1	Figure 6E
k_{BD}, charge recombination between Q_B^- and D^+ (s^{-1}) (21 °C)	0.72 ± 0.02	~10^{-2}	Figure 8
Q_A^- decay rate (s^{-1}) (21 °C)	(4 ± 1) × 10^{-3}	(8.4 ± 0.7) × 10^{-2}	Figure 9
Q_B^- decay rate (s^{-1}) (21 °C)	(4 ± 1) × 10^{-3}	3.1 ± 0.2	Figure 9
does Q_B function as a two-electron "gate"?	yes	no	Figure 10

[a] Measured in RCs depleted of Q_B in the absence of *o*-phenanthroline. In the presence of *o*-phenanthroline k_{AD} = 10.0 ± 0.2 s^{-1} [see Kleinfeld et al. (1984b)]. [b] Measured as in Okamura et al. (1982a) but with the conditions used in Figure 8G.

of the properties of LM and RC investigated in this work is presented in Table II. The LM was obtained by dissociating the H subunit from RCs of *Rhodopseudomonas sphaeroides* R-26 with the chaotropic agent $LiClO_4$ in the presence of the mild detergents sodium cholate or sodium deoxycholate. Intact RCs were reconstituted by reassociating H with LM. Native and reconstituted RCs behaved identically in all assays performed, indicating that neither H nor LM was damaged during dissociation. We conclude that the observed differences between RC and LM result from the absence of the H subunit and not from denaturation accompanying its removal.

The optical absorption spectrum of LM resembled that of RCs at both room and cryogenic temperatures (Figure 1). Some differences were observed, most notably near 537 nm and on the shoulder of the Soret band, particularly at cryogenic temperatures (Figure 1B). Similar changes near 537 nm have been reported previously for LM isolated with $NaDodSO_4$ (Okamura et al., 1974; Agalidis & Reiss-Husson, 1983; Agalidis et al., 1984). These changes suggest that the environment of one of the BPh pigments is sensitive to the presence of H. Similar changes near 537 nm have also been reported for RCs treated with $NaDodSO_4$ or chaotropic agents (Clayton et al., 1972; Dutton et al., 1978; Blankenship & Parson, 1979), suggesting that these treatments may have dissociated the H subunit.

The position of the long-wavelength absorption in both LM and RC depended on Q-10 content and detergent (Figure 2). This suggests that the environment of the primary donor, $(BChl)_2$, is sensitive to the overall conformation or aggregation state of the LM or RC. Extraction of Q-10 may account for the variation in peak position previously reported in LM (Okamura et al., 1974; Agalidis & Reiss-Husson, 1983; Agalidis et al., 1984) and in RCs treated with $NaDodSO_4$, chaotropic agents, or elevated concentrations of LDAO or isolated with urea (Clayton & Straley, 1972; Clayton & Yau, 1972; Lin & Thornber, 1975; Romijn & Amesz, 1977; Dutton, et al., 1978; Blankenship & Parson, 1979).

EPR spectra of LM prepared by the procedure described in this work have been reported previously (Okamura et al., 1982b; Butler et al., 1984). The light-induced spectrum exhibited a narrow signal at g = 2.0026 and a broad signal at g = 1.8. These signals resemble those of D^+ and $Q_A^-Fe^{2+}$ in RCs, except that in LM the signal at g = 1.8 was ~30% narrower than in RCs. The addition of *o*-phenanthroline did not significantly affect this signal in LM. The signal at g = 1.8 could also be generated by reduction with sodium dithionite. The EPR spectra show that the binding sites for Q_A and Fe^{2+} are located on LM and that the magnetic interaction between Q_A^- and Fe^{2+} is not disrupted by removal of H. The binding site of Q_A was previously localized to the M subunit by experiments with a photoaffinity-labeled quinone analogue (Marinetti et al., 1979).

The rate of charge recombination between Q_A^- and D^+ (k_{AD}) in RCs is believed to be critically sensitive to changes in the distance between D and Q_A (McElroy et al., 1974; Hopfield, 1974; Jortner, 1976). Since k_{AD} changes only by a factor of ~2 in the absence of H (Figure 5), we conclude that the relative configuration of D and Q_A is affected only to a small extent by removal of H. However, this change in k_{AD} may reflect alterations in the environment of either D or Q_A.

The rapid oxidation of cyt^{2+} by LM and the dependence of the rate of oxidation on ionic strength (Figure 4) show that the binding site for cyt is located on LM and is not affected by removal of H. These data independently verify that the binding site for cyt does not involve the H subunit, in agreement with studies involving chemical cross-linking and inhibition by antibodies (Rosen et al., 1983) and protein modification reagents (Okamura & Feher, 1983).

The difference in the rate of electron transfer from Q_A^- to Q_B in LM and RC (Figures 6–8) suggests either that the environment of one or both quinones is substantially altered in the absence of H or that the binding site for Q_B is on H. The latter possibility is ruled out by the experiments with

^{14}C-labeled Q-10 and by independent studies indicating that Q_B binds to M or L (Debus et al., 1982; de Vitry & Diner, 1984; Brown et al., 1984). Since the parameters associated with Q_A are relatively unaffected by removal of H (see Table II), we suggest that mainly the Q_B site is altered on LM.

The rate of electron transfer from Q_A^- to Q_B (k_{AB}) in LM was measured by several methods. The following estimates for k_{AB} were obtained: 13 ± 1 s^{-1} (Figure 6E), 43 ± 5 s^{-1} (Figure 7G), and 7 ± 2 s^{-1} (Figure 8G). The value of 43 ± 5 s^{-1} is undoubtedly high since electron transfer from intermediate acceptors located between D and Q_A may have contributed to the measured rate in this assay. In addition, if there is a heterogeneity of k_{AB}'s in LM (suggested by the nonexponential shape of the curves in Figures 6D and 6E), the faster rates would dominate the measurements of Figure 7 because of the repeated excitation caused by continuous actinic illumination. Although the estimates differ quantitatively, all three estimates of k_{AB} in LM show that k_{AB} decreased by 2–3 orders of magnitude in the absence of the H subunit. Thus, the presence of H appears to be required for rapid electron transfer from Q_A^- to Q_B.

The dependence of k_{AB}(apparent) and k_{slow} in LM on the concentration of Q-10 (Figures 6E and 7G) suggests that the affinity of Q-10 for the Q_B site is less in LM than in RC. Dissociation constants were estimated from the concentrations of Q-10 required to give half the maximum rates in Figures 6E and 7G, respectively. The two sets of data yielded $K_D = 15 \pm 3$ μM and 10 ± 3 μM, respectively. For RCs an estimate of $K_D = 2 \pm 1$ μM was obtained from the kinetics of charge recombination as a function of the concentration of Q-10 [after Okamura et al. (1982a)]. In view of the low solubility of Q-10 it is not clear if the estimated K_D's are equilibrium dissociation constants; the low solubility of Q-10 may have resulted in estimates for K_D that are artificially low. Nevertheless, the data suggest that binding of Q-10 to the Q_B site is weaker by an order of magnitude in the absence of H. The presence of H is apparently required for tight association between Q-10 and the Q_B site.

Inhibition of electron transfer from Q_A^- to Q_B was achieved in LM with high concentrations of *o*-phenanthroline and triazine herbicides (Table I). This suggests that these inhibitors bind to LM, in agreement with studies involving the photoaffinity labeling of RCs with azidoatrazine (de Vitry & Diner, 1984; Brown et al., 1984). These studies indicate that the binding site of this inhibitor is on the L subunit. Much higher concentrations ($\sim 10^2$-fold) of the inhibitors were required to achieve the same degree of inhibition in LM as in RCs (Figure 11 and Table I). This indicates that the high-affinity inhibitor binding site(s) of RCs was (were) lost or substantially modified by removal of H. Weaker binding of inhibitors to LM agrees with studies involving equilibrium dialysis with ^{14}C-labeled atrazine: binding to the isolated H and LM units was weaker than to native RCs by an order of magnitude (Okamura, 1984). Weaker binding of inhibitors to LM may account for the lack of effect of *o*-phenanthroline on the EPR signal of $Q_A^-Fe^{2+}$ in LM (Butler et al., 1984). The potency of the inhibitors was the same in native and reconstituted RCs, showing that the H subunit is required for strong binding.

The data of Figure 6 suggest that the semiquinones Q_A^- and Q_B^- are less stable against oxidation in LM than in RCs. A direct measure of the stability of the semiquinone anions is provided by the ΔA_{450} data of Figure 9. The data of Figure 9A–C show that the lifetime of the semiquinone anion Q_A^- decreased by a factor of ~ 20 in the absence of H. Instability of Q_A^- has been previously reported in LM isolated with $NaDodSO_4$ (Agalidis & Reiss-Husson, 1983). The variation of the rate of decay of the semiquinone anion with the concentration of Q-10 (Figure 9E) suggests that Q-10 binds weakly to the Q_B site and that Q_B^- is much less stable than Q_A^- (compare part E with B of Figure 9). Weak binding to the Q_B site is consistent with the data of Figures 6E and 7G used to estimate K_D in LM. At high concentrations of Q-10 this site should be fully occupied and the rate of decay should saturate, as was observed (Figure 9E). The maximum rate of decay ($k_{decay} = 3.1 \pm 0.2$ s^{-1}) should correspond to the lifetime of the semiquinone anion Q_B^- in LM. The data of Figure 9E show, therefore, that the lifetime of Q_B^- decreased by a factor of $\sim 10^3$ in the absence of the H subunit.

The semiquinone Q_A^- appears more stable than Q_B^- in LM by a factor of ~ 40. This suggests that the binding site of Q_A is located in a much more inaccessible region of the protein than that of Q_B, such as in the interior of the protein. This conclusion is consistent with the apparent inaccessibility of the Q_A site to antibody fragments (Debus et al., 1982).

The loss of stability of both semiquinones in the absence of H suggests that they are both more exposed to exogenous agents in LM than in RCs or that stabilizing groups on the RC are absent in LM. Thus, protection of Q_A^- and Q_B^- against oxidation apparently requires the presence of the H subunit.

The slowness of k_{AB} and the instability of Q_B^- in LM account for the inability of Q_B to function as a two-electron gate in LM. Both native and reconstituted RCs showed oscillations in the optical absorption of semiquinone (A_{450}) in response to a series of actinic flashes (Figure 10). The oscillations are characteristic of gating: semiquinone (Q_B^-) is formed on odd-numbered flashes and annihilated on even-numbered flashes by the electron leaving Q_A^- to form Q_B^{2-}. No oscillations were observed in LM (Figure 10). Thus, the H subunit is required for Q_B to function in its characteristic role as a two-electron gate between Q_A^- and exogenous Q-10.

The H subunit dramatically affects electron transfer involving Q_B. This suggests that H either forms part of the Q_B site or affects the conformation of the region of LM involved in binding Q_B. Stabilization of Q_B^- may result from the influence of H on the position of a nearby charge. Both Q_A^- and Q_B^- interact with a nearby proton (Wraight, 1979, 1982; Kleinfeld et al., 1984b). This proton energetically stabilizes the state $Q_AQ_B^-$ relative to $Q_A^-Q_B$ (Kleinfeld et al., 1984b). Rapid electron transfer from Q_A^- to Q_B in RCs may result from the influence of H on the site of protonation.

To test the generality of the conclusions reached in this study, it would be of interest to characterize LM from other photosynthetic bacteria more completely. The H subunit appears easily dissociable from RCs of *Rhodospirillum rubrum* (Vadeboncoeur et al., 1979; Snozzi & Bachofen, 1979), and active LM has been isolated from *Rhodopseudomonas capsulata* (Nieth et al., 1975). Active RCs containing only two subunits have been isolated from *Rhodopseudomonas gelatinosa* (Clayton & Clayton, 1978; Prince et al., 1978) and *Chloroflexus aurantiacus* (Pierson & Thornber, 1983; Pierson et al., 1983). Although the presence of a third subunit may not be required for proper functioning of Q_B in these RCs, it is possible that a third subunit, corresponding to H, may have been lost during purification. The RCs from *Rp. gelatinosa* are labile and contain little Fe, resembling LM from *Rp. sphaeroides* prepared with $NaDodSO_4$ (Okamura et al., 1974; Agalidis & Reiss-Husson, 1983). The development of more stable preparations similar to the one obtained in the present study (e.g., through the use of milder detergents) may facilitate

more complete characterization of LM from other bacteria.

Isolation of H subunits from photosynthetic bacteria other than *Rp. sphaeroides* would also be of interest. Reconstitution of hybrid RCs from LM and H isolated from two different bacteria may yield insights into the mechanism of electron flow. We are at present reconstituting hybrid RCs from LM and H isolated from normal and herbicide-resistant mutants of *Rp. sphaeroides* to characterize the mechanism of herbicide action and to determine the site(s) of mutation (M. Y. Okamura, E. C. Abresch, and R. J. Debus, unpublished results).

Procedures developed to remove Fe from RCs (Loach & Hall, 1972; Feher et al., 1972; Dutton et al., 1978; Feher & Okamura, 1978; Blankenship & Parson, 1979; Tiede & Dutton, 1981) are similar to those developed to dissociate the H subunit. Results obtained by using these procedures may, therefore, be more characteristic of LM than RCs. Indeed, such preparations have frequently been reported to exhibit optical absorption changes resembling LM (Dutton et al., 1978; Blankenship & Parson 1979). One such study showed a correlation between Fe content and the amount of functional Q_B in RCs treated with $LiClO_4$ (Blankenship & Parson, 1979). However, it is not clear whether the loss of functional Q_B resulted from removal of Fe or of H. We are currently attempting to reassess the role of Fe^{2+} by examining RCs reconstituted from H subunits and LM depleted of Fe (Debus et al., 1984).

There are striking similarities between the RCs of PS II of green plants and the RCs of purple non-sulfur bacteria. Their respective electron acceptors are similar and function analogously [for reviews, see Vermaas & Govindjee (1981), Okamura et al. (1982b), Kaplan & Arntzen (1982), and Crofts & Wraight (1983)]. In addition, there are significant homologies between the primary structures of a ~32 kDa herbicide-binding polypeptide associated with PS II and the M and L subunits of *Rp. sphaeroides* (Williams et al., 1983, 1984) and *Rp. capsulata* (Youvan et al., 1984). One or more 30–34-kDa polypeptides influence the properties of Q_B in PS II and "shield" Q_A^- against exogenous redox agents (Renger, 1976; Mattoo et al., 1981; Arntzen et al., 1982; Kyle et al., 1983). These polypeptides may be structurally and functionally homologous to the H subunit.

Added in Proof

We have recently developed a simpler procedure for isolating H subunits. RCs ($A_{802}^{1cm} = 10$) were incubated for 1 h at 4 °C in 1.0 M $LiClO_4$, 0.1% LDAO, and 50 mM Tris-HCl, pH 7.7, and then passed (at 4 °C) through a column of organomercurial agarose (Affi-Gel 501 from Bio-Rad Laboratories) that had been equilibrated with 1.0 M $LiClO_4$, 0.1% LDAO, and 50 mM Tris-HCl, pH 7.7. LM passed through the column without binding. Bound H subunits were eluted with 50 mM cysteine, 0.1% LDAO, and 100 mM Tris-HCl, pH 7.8.

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