Characterization of Reaction Center-B875 Complex of *Rhodocyclus gelatinosus:* Q_B Site Properties Derived from Reconstitution Experiments

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Purified reaction center-B875 pigment-protein complex isolated from *Rc. gelatinosus* (I. Agalidis, E. Rivas, and F. Reiss-Husson, Photosynth. Res. **23**, 249−255 (1990)) was further characterized. In the chromatophores, the quinone content was shown to be 6 menaquinones 8 and 16 ubiquinones 8 per reaction center, indicating that the pool contained both quinone types. Besides the primary (MK_8) and secondary (UQ_8) electron acceptors of the reaction center, the complex contains residual quinones from the membrane pool (about $3\ MK_8$ and $5\ UQ_8$) probably associated with the phospholipids. Apparent particle weight of the complex including bound detergent was 520 ± 46 kDa.

The secondary quinone Q_B was partially removed from the RC by treatment with 2−3% octaethyleneglycol dodecyl ether and 3−4 mM orthophenanthroline. Reconstitution experiments showed that UQ_6, UQ_9 and UQ_{10} could replace Q_B but that MK_8 and MK_9 could not. It was concluded that Q_B site has a clear specificity towards ubiquinone binding.

Introduction

In a precedent paper [1] we have characterized a photochemically active reaction center-B875 pigment-protein complex solubilized with octyl-β-D-thioglucopyranoside from *Rhodocyclus gelatinosus* membranes. In this preparation, the RC is strongly attached to 20−30 light harvesting B875 complexes and to a tetraheme cytochrome *c;* the RC is composed of three polypeptides L (23 kDa), M (28 kDa), H (33 kDa). These features are similar with those of *Rc. gelatinosus* quantosomes preparations described by Fukushima *et al.* [2]. Moreover we identified the primary and secondary electron acceptors as MK_8 and UQ_8 respectively.

Up to now, purified RC preparations from *Rc. gelatinosus* have not been isolated in a native, functional state; published procedures resulted in preparations devoid of the H subunit and of the cytochrome [2, 3]. Therefore we ought to characterize the RC in a complex where it is still bound to B875. Here we will show that the purified complex contains phospholipids and several molecules of

MK_8 and UQ_8 probably left over from the membrane quinone pool. We focused our attention on the properties of Q_B site of the RC by partly removing UQ_8 from the complex and by carrying out reconstitution assays of Q_B activity with various quinones.

Materials and Methods

Biochemical methods

RC-B875 complexes from *Rc. gelatinosus* were prepared as previously described [1]. Protein was measured by the method of Peterson [4]; phospholipids were estimated from P analysis [5] assuming a P/phospholipid ratio of $1:25$ (w:w). The amount of Bchl in the complex was estimated taking $\varepsilon(875\ \text{nm}) = 128\ \text{mM}^{-1}\ \text{cm}^{-1}$ [6]. In chromatophores, Bchl was determined by methanol extraction as described in [7].

Quinones were extracted from various preparations as described [8]. After drying, the extracts were dissolved in a mixture of tetrahydrofurane/acetonitrile (7/3, v:v) and 20 μl aliquots were injected on a Ultrapack HPLC column (4.6 × 250 mm) (Merck). The mobile phase consisted of the same solvent mixture. The column was eluted at room temperature at a flow rate of 0.5 ml/min. The apparatus was a Beckman Model 332 liquid chromatograph equipped with two flow pumps (model 114 M) and a detector (model 160). Ab-

sorbance was measured at 254 nm on a recorder. The various elution peaks were identified in control experiments done on extracts from chromatophores by their visible and UV absorption spectra.

For a qualitative and quantitative estimation of quinones known amounts of commercially available quinones (Q_6 and Q_{10} (Sigma), Q_9, MK_8, MK_9 (Hoffman La Roche) were run with the same conditions as the sample and peak areas were compared. The amount of quinone in standard solutions was determined optically using extinction coefficients $\varepsilon(248\ nm) = 18,900\ \text{M}^{-1}\ cm^{-1}$ for MK [9] and $\varepsilon(275\ nm) = 14,700\ \text{M}^{-1}\ cm^{-1}$ for UQ [10].

Estimation of apparent MW of RC-B 875 complex

Gel chromatography was used to estimate the apparent MW of the purest fraction of the complex. Calibration was done with water soluble proteins (myoglobin, thyroglobulin, ovalbumin, transferrin, aldolase, and β-galactosidase); Blue Dextran and dithiothreitol served as markers of void volume V_o and total volume V_T respectively. K_d values were calculated as $K_d = (V_e - V_o/(V_T - V_o))$. Sepharose CL-6 B and CL-4 B columns (1.5 × 90 cm) were equilibrated with 0.1 M Tris-HCl, 1 mM EDTA pH 8 containing 0.1% $C_{12}E_8$. The sample (2–3 ml, $A_{875\ nm} = 5$–10) was adsorbed onto the gel and eluted at a flow rate of 10 ml/h. Alternatively, HPLC of the complex was carried out on a gel of Superose 8 (preparative grade, Pharmacia) (1 × 30 cm) with a flow rate of 0.5 ml/h, in the same buffer.

Preparation of RC-B 875 partly depleted of secondary quinone

Purified RC-B 875 (20–50 ml, $A_{875\ nm} = 2$) was incubated for 3 h in presence of 2.5% $C_{12}E_8$ and 3–4 mM orthophenanthroline in 10 mM Tris-HCl, 1 mM EDTA pH 8 buffer at 23 °C in the dark. Then the sample was adsorbed onto a DEAE-Sepharose column (5–10 ml bed volume), preequilibrated with 10 mM Tris-HCl, 1 mM EDTA, $C_{12}E_8$ 0.03%, pH 8. The column was extensively washed with the starting buffer in order to remove degraded antenna pigments and orthophenanthroline. The complex was desorbed from the column with this Tris buffer containing 0.25 M NaCl and subsequently dialysed to remove NaCl and traces of her-

bicide. Occasionnally, RC-B 875 was concentrated on a Amicon XM 100 membrane.

Reconstitution of quinone depleted RC-B 875 with various quinones

UQ_0 is easily soluble in ethanol or in aqueous solutions of $C_{12}E_8$ whereas ubiquinones with long isoprenyl side chains (UQ_6, UQ_9, UQ_{10}, MK_8, MK_9 and VitK 1) were poorly soluble in water. Therefore we used a method of solubilization which resembles that described for preparation of large lipid vesicles [11]. A quinone solution (5–10 mM) in chloroform was vigorously vortexed with an equal volume of isopropyl-ether (1 ml), until a milky mixture was formed. 0.5 ml of 10% $C_{12}E_8$ in Tris buffer was then added and the organic solvent phases were removed under N_2 stream. After removal of organic solvents quinone was dispersed in the aqueous phase of $C_{12}E_8$ as a clear solution of mixed detergent-quinone micelles. The concentration of quinone in these stock solutions was measured by absolute absorption spectra in ethanol (*cf.* above).

Quinone depleted RC-B 875 samples (0.6 ml, $A_{875\ nm} = 5$–7) were incubated with 50–100 μM quinone in 10 mM Tris-HCl pH 8, $C_{12}E_8$ 0.1%. Most of the samples were incubated in the dark at 4 °C for 20 h.

Spectrophotometric measurements

Absolute and difference absorption spectra were recorded on a Cary 2300. Photochemically active RCs were estimated in chromatophores or in RC-B 875 by light-induced absorbance changes at 870 nm or 600 nm assuming: $\Delta A_{870\ nm} = 113\ \text{mM}^{-1}\ cm^{-1}$ [12] and $\Delta A_{600\ nm} = 20\ \text{mM}^{-1}\ cm^{-1}$ [13]. Photooxidized cyt *c* was estimated by absorbance changes between 555–550 nm using $\Delta A_{553\ nm} = 19\ \text{mM}^{-1}\ cm^{-1}$ [13]. This value was checked by single flash experiments in presence of ascorbate taking for granted the above $\Delta \varepsilon_{600\ nm}$. Flash induced absorbance changes in IR and visible region and analyses of kinetics were done as in [1].

Results

Composition and size of RC-B 875 complex

In the precedent paper we have shown that during ion-exchange purification, RC-B 875 complexes were separated in two fractions, the purest

Table I. Relative amounts of BChl and phospholipids *versus* protein in chromatophores, and RC-B875 complex.

Sample	BChl/protein [μmol/g]	PL/protein [g/g]
Chromatophores	37.5 ± 4.3 ($n = 5$)	0.65 ± 0.09 ($n = 4$)
RC-B875	70.4 ± 6.8 ($n = 4$)	0.22 ± 0.02 ($n = 3$)

being eluted at 0.2 M NaCl. Therefore we focused on further characterization of this fraction. We measured the phospholipid contents of several preparations of the purified complex in comparison with those of chromatophores, on a protein basis (see Table I). It can be seen that the quantities of bound phospholipids are lower in RC-B875 then in chromatophores; nevertheless they are sufficient to account for the good stability of the preparations.

Analysis of the quinones present in RC-B875 by reverse phase HPLC was done (Fig. 1A) in parallel with chromatophores extracts. In chromatophores we found 6.5 ± 0.4 ($n = 4$) MK_8 per RC and 16 ± 1 ($n = 4$) UQ_8/RC *i.e.* ~ 2.4 UQ_8/MK_8. Thus MK_8 seems to be an important constituent of the quinone pool. In the RC-B875 complex the absolute amount of MK_8 and UQ_8 varied from one preparation to another but it was consistently

Fig. 1. Chromatogram of quinones from RC-B875 complex (A) and from a quinone depleted preparation (B). Elution times of 13.6 and 18.8 min corresponded to UQ_8 and MK_8 respectively. Fractions eluted earlier than 10 min (not shown) contained BChl and carotenoid pigments.

higher than 3 MK_8 and 5 UQ_8, when expressed as molar ratio relative to the initial RC concentration of the sample. The molar ratio $UQ_8 : MK_8$ was found to be $1.8 : 1$ (± 0.1, $n = 3$). These results indicate a) a slight enrichment in MK_8 versus UQ_8 in the complex, likely due to the increased contribution of the primary acceptor (MK_8), b) the presence of residual MK_8 and UQ_8 from the pool besides Q_A (MK_8) and Q_B (UQ_8).

Several preparations of RC-B875 were chromatographed on Sepharose CL-6B, CL-4B and on Superose and their apparent particle weights were determined by comparison with soluble proteins. A value of 520 ± 46 kDa ($n = 6$) was found. This particle weight includes the proteic part, the lipids and the bound detergent. One must keep in mind that calibration with water soluble proteins is not strictly applicable for this determination, because of anomalous migration on these gels of detergent solubilized membrane proteins [14]. However the apparent particle weight is low enough to indicate that only one RC with its bound cytochrome and associated antennas is present per particle. Thus the RC-B875 complex is solubilized in a monomeric state by octyl-β-D-thioglucopyranoside and $C_{12}E_8$.

Characterization of the Q_B site

Previously we have shown that in the photochemically active RC-B875 complex the flash induced $P^+Q_AQ_B^-$ state relaxes within 1 s to PQ_AQ_B state [1]. Meanwhile we have noticed that the charge recombination time (measured at 785 and 600 nm) was sensitive to the intensity of analytical light and reached a value of ≥ 2 s when the intensity of light was decreased. This actinic effect has already been studied in detail by Macino *et al.* on purified R26 *Rb. sphaeroides* RCs [15]. These kinetics indicate that in these RC-B875 preparations Q_B is present in at least 90% of the RCs. In presence of herbicides the electron transfer between Q_A^- and Q_B is blocked and the electron returns from
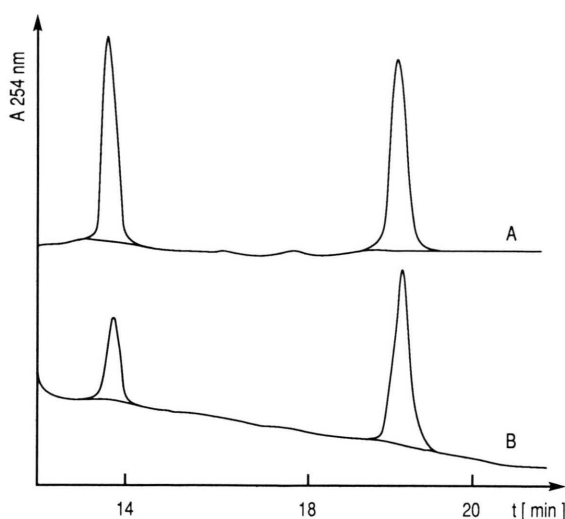
Q_A^- to P^+ in about 10 ms [1]. In order to get further insight on Q_B site properties we have tried to remove the secondary quinone by incubation of the complex with high concentrations of $C_{12}E_8$ and orthophenanthroline (see methods). Quinone analysis was performed after this treatment by HPLC of extracts, as described above. MK_8 and UQ_8 were found to be still present, but in a modified molar ratio indicating a preferential removal of UQ_8 ($UQ_8 : MK_8 = 0.8 : 1$) (Fig. 1 B). The total molar ratio ($UQ_8 + MK_8$) per RC was decreased by 50 to 70%.

Measurement of the charge recombination kinetics indicated that 50 to 70% of Q_B was removed by this treatment. Fig. 2 displays the dark rereduction of P^+ in a typical sample after Q_B depletion. The middle trace represents the biphasic relaxation kinetics of P^+ after removal of 60% of Q_B. The rapid phase corresponds to the fraction of RCs in which Q_B is absent and the slow phase to RCs still containing Q_B. By comparison, the lower trace represents P^+ rereduction in presence of orthophenanthroline.

The procedure we adopted for Q_B removal from RC-B 875 is similar to that used by Okamura *et al.*
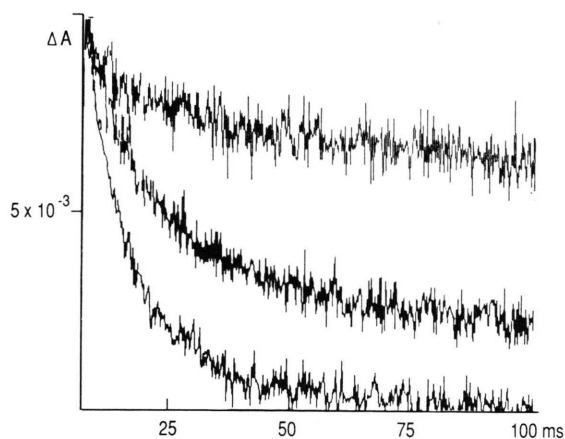
for purified R 26 *Rb. sphaeroides* RCs [16] but using $C_{12}E_8$ instead of lauryldimethyl amine oxide. In *Rb. sphaeroides* Q_B could be solubilized from all RCs. The fact that in *Rc. gelatinosus* Q_B cannot be detached so efficiently can be due to the different nature of detergent but also to the fact that the RC is still attached to an important mass of lipoprotein which could prevent a good accessibility of detergent micelles to the acceptor site.

Using these Q_B depleted samples, we have carried out reconstitution experiments by adding back an excess of either UQ_6, UQ_9, UQ_{10} or UQ_0. All these quinones but Q_0 reconstitute well Q_B activity as shown in the upper trace of Fig. 2 where only the slow phase is detectable ($\tau = 1.6$ s). However we should point out that 90–100% reconstitution of Q_B requires that these quinones are thoroughly solubilized in the stock solution before addition (see Methods). Addition of a large excess of Q_0 does not bring about reconstitution of Q_B activity (not shown); the high solubility in water of this small tailless quinone may preclude its binding to the Q_B site and this quinone remains preferentially trapped in the micelles of detergent.

As the primary quinone in *Rc. gelatinosus* RC is of 1,4-naphtoquinone type (MK_8), we also tried reconstitution experiments with MK_8, MK_9 and VitK 1. No change in the decay kinetics of P^+ was observed in presence of these quinones in Q_B depleted RC-B 875 samples (see Fig. 3).

Fig. 2. Exponential decay kinetics measured at 785 nm in Q_B depleted RC-B 875 complex (0.4 μM RC), in different conditions after a Xenon flash. RC-B 875 were suspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8 buffer containing 0.04% $C_{12}E_8$. Analytical light was low enough to avoid actinic effect. Middle trace: without addition. The computed meantimes are $\tau_1 = 0.012$ s (62%), $\tau_2 = 1.6$ s (38%). Lower trace: addition of 4 mM orthophenanthroline. $\tau = 0.012$ s. Upper trace: after incubation with 20 μM UQ_{10} (see Methods). $\tau_1 = 0.25$ s (13%), $\tau_2 = 1.6$ s (87%). Each trace represents the average of 10 experiments.
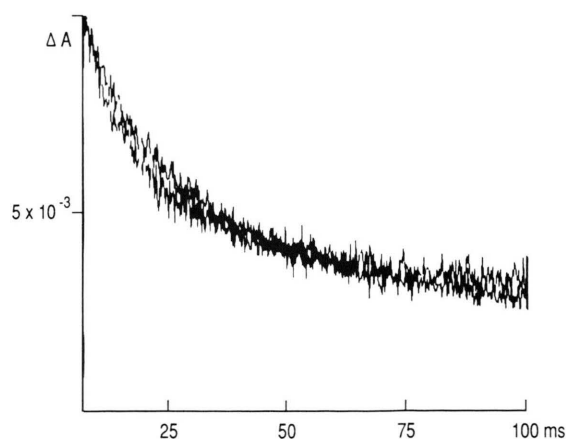

Fig. 3. Exponential decay kinetics measured at 785 nm in Q_B depleted RC-B 875 (0.4 μM RC); other conditions as in Fig. 2. Lower trace: without addition. $\tau_1 = 0.012$ s (49%), $\tau_2 = 1.5$ s (51%). Upper trace: incubation with 30 μM MK_8. Each trace represents the average of 30 experiments.

Photooxidation of bound cytochrome c

The treatment of intact RC-B875 preparations with high concentrations of $C_{12}E_8$ and ortho-phenanthroline did not split off the multiheme cytochrome c. This fact was demonstrated by di-thionite reduced-minus-oxidized absorption difference spectra (not shown) of two different quinone depleted preparations which gave a molar ratio close to 4 hemes per RC. About two of these hemes are reducible by 1 mM sodium ascorbate.

Flash experiments on RCs have shown that after a single turnover flash Q_B reoxidizes Q_A^- and one heme c is photooxidized. Therefore a second flash can photooxidize another reduced heme [20]. Similarly we considered the amount of photooxidized cytochrome c in continuous light as an indication of the presence of Q_B.

Continuous IR actinic illumination of intact RC-B875 in presence of 1 mM sodium ascorbate induced at 553 nm a negative absorbance change corresponding to 2.2–2.3 hemes c photooxidized per RC. In the presence of orthophenanthroline only one heme was photooxidized per RC (1 ± 0.2, $n = 6$). In the same manner in a preparation where about 70% Q_B was removed, 1.15–1.38 heme c per RC was photooxidized (Fig. 4, upper trace), a fact which is consistent with the absence of Q_B. As a control, we checked on the same Q_B depleted RC-B875 the quantity of heme reduced by ascorbate, by a difference absorption spectrum "reduced minus oxidized"; we obtained 2 hemes per RC complex (not shown).
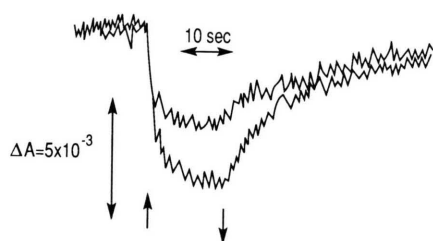

Fig. 4. Continuous light-induced photooxidation of cytochrome c measured at 553 nm in presence of 1 mM sodium ascorbate in Q_B depleted RC-B875 complex (0.27 μM RC); other conditions as in Fig. 3. Upper trace: no addition. $\Delta A_{553\,nm}$ corresponds to 1.14 heme c photooxidized/RC. Lower trace: after addition of 20 μM UQ_6. $\Delta A_{553\,nm}$ corresponds to 1.9 heme c photooxidized/RC.

When the Q_B-depleted preparation was incubated with an excess of UQ_6, it was able to photooxidize almost two hemes (Fig. 4, lower trace). This result confirms the binding of UQ_6 as functional Q_B otherwise seen by the reappearance of the slow kinetics of $P^+Q_B^-$ recombination (see above).

Discussion

In the present work we have tried to better characterize the electron acceptors of *Rc. gelatinosus* RC using a solubilized preparation where the RC is still not resolved from B875 antenna nor from lipids. Functional analysis of the quinone acceptors was carried out. Quinone composition of the B875-RC complex was determined before and after partial Q_B removal and compared to quinone content in chromatophores.

In several strains of *Rc. gelatinosus* the presence of noticeable amount of MK_8 besides UQ_8 has been observed [21] with a molar ratio UQ/MK = 3–4. To our knowledge, only one quantitative measurement of the pool size has been reported, indicating 29 UQ_8 and 9 MK_8 (if one assumes 1 RC per 100 Bchl) [22]. We found a slightly smaller pool size in strain 52; but the MK_8/UQ_8 ratio is closed to the values announced [21, 22].

We point out that other photosynthetic bacteria contain both menaquinones and UQ in the pool [21]. However, the precise role of the MKs pool is still not clear [23, 24].

In *Rc. gelatinosus* reaction center charge recombination kinetics support the presence of MK at Q_A site and of UQ at Q_B site [1]. Yet in the B875-RC complex, both quinone contents are higher than 1. This can be explained by the presence of pool quinones within residual lipids, which have not been split from the complex.

Detergent treatment of the complex resulted in partial loss of Q_B from the RC. Surprisingly, the quinone analysis of the Q_B depleted preparation indicated that residual pool quinones were not completely removed. However, there was a preferential loss of UQ_8 relative to MK_8 consistent with the assignment of UQ_8 as Q_B. The "extra" quinones may originate from the complexes still containing Q_A and Q_B (which represent 30–40% of the whole). Restoration of Q_B activity was observed only when excess of long chain ubiquinones were added. Residual pool ubiquinones seemed

unable to replace Q_B. For this observation two possible explanations may be proposed. First, the amount of endogeneous UQ_8 may be too low for binding at Q_B site. On the other hand ubiquinones may be localized in intact complexes and therefore cannot diffuse toward Q_B depleted complexes.

Up to date an extensive characterization of the Q_A and Q_B sites has been performed only in purified R26 *Rb. sphaeroides* RCs. It was essentially done by extraction of both quinones or only of Q_B followed by reconstitution experiments with a wide variety of quinonic molecular species [16, 19, 25, 26]. Recently extraction and reconstitution of Q_A have been performed on *Rp. viridis* RC [27]. Giangiacomo *et al.* [26] have shown that an important requirement for a quinone to function as Q_B is the free energy difference for electron transfer between quinones occupying the Q_A and Q_B sites. In *Rc. gelatinosus* RC the midpoint potential of Q_A is close to -150 mV [13]. The redox span between Q_A^-/Q_A (MK_8) and Q_B^-/Q_B (UQ_8) couples is thus large enough (>150 mV) to allow functional occupancy of Q_B site with UQ_{6-10} prenylogues.

Although the equilibrium constant of the electron transfer rate between Q_A and Q_B should be very high (Keq = 100–200) a rather slow phase (300 µs) was detected for this transfer at pH 8

(Vermeglio and Agalidis, unpublished results). In *Rps. viridis* and *Chromatium* RCs, which also contain MK and UQ as Q_A and Q_B respectively [28, 29], much faster rates (25 and 80 µs respectively) at pH 8 have been observed [20, 30].

Menaquinones cannot function as Q_B in *Rc. gelatinosus* RCs presumably because their interaction with the Q_B site does not rise enough their midpoint potential to allow a rapid electron transfer from Q_A^- to Q_B overcoming $P^+Q_A^-$ recombination. Another possibility is that the Q_B site cannot accommodate the head group of naphtoquinone type since even in presence of a large excess of MK_8 or MK_9 residual UQ_8 is not exchanged for these quinones. This assumption is confirmed by the weak affinity of MK_8 for the Q_B site measured by Shopes and Wraight [27] on *Rps. viridis* purified RC. As an unique example in *Chloroflexus aurantiacus* RC both the primary and secondary acceptors are MKs [31, 32].

Acknowledgements

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