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SEVERAL PROPERTIES OF THE LM UNIT EXTRACTED WITH SODIUM DODECYL SULFATE FROM *RHODOPSEUDOMONAS SPHAEROIDES* PURIFIED REACTION CENTERS

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We describe several characteristics of an LM unit extracted from purified wild-type *Rhodopseudomonas sphaeroides* reaction centers after treatment with SDS and lauryldimethylamine *N*-oxide. We also studied another preparation called RC-SDS in which after a similar detergent treatment the H-chain was not separated from the LM unit. The spectral properties of both preparations were similar to those of intact reaction centers; the main differences were a blue shift of the P-865 Q_y band and a narrowing of the bacteriopheophytin Q_x band. Studies of absorbance changes after steady light or flash illumination showed that LM was depleted of the primary electron acceptor Q_I and of the transition metal (Mn), but was still able to react with quinones, with a partial restoration of the photochemistry. The primary acceptor Q_{10} was still partly present in RC-SDS and formed in the light a very unstable semiquinone anion. In both preparations, in the presence of added quinones, the flash-induced P-865$^+$ decayed in the dark with multiexponential kinetics, the apparent half-times ranging between 60 ms and 100 s. This recovery was accelerated either by the absence of O_2 or (in the case of LM only) by *o*-phenanthroline, which also decreased the amplitude of the P-865$^+$ signal. From these experiments it was concluded that in these preparations the site of binding of the secondary quinone Q_{II} was damaged or absent; the altered properties of this LM unit were considered to result from the denaturing action of the detergents, rather than from the absence of the H polypeptide.

Introduction

Considerable advances have recently been made in the characterization of *Rhodopseudomonas sphaeroides* reaction centers, concerning their composition and the reactions they perform (for a review, see Ref. 1). However, the relative position of the pigment molecules and the cofactors within the three polypeptide chains is not known precisely, and the functional role of each of the polypeptide chains is still under study. One approach has been to resolve further the pigment-protein complex. Thus, Feher and co-workers [2] succeeded in splitting from the purified *Rps. sphaeroides* R-26 reaction center a fraction in which the L and M polypeptides remained associated with the BChls and BPhs; this was done by treating the reaction center either with a mixture SDS and laurydimethylamine *N*-oxide or with the chaotropic agent $LiClO_4$ [1]. The spectral properties of this LM unit were similar to those of the intact reaction center except for the position of the Q_y band of the P-865 donor (which was blue shifted) and for a sharpening of the Q_x band of BPh [2]. However, in these preparations only partial photochemistry took place; the mode of splitting of the LM unit from the H polypeptide appeared therefore to be crucial for the retention of photochemical activity.

Abbreviations: DQ, duroquinone; Q, ubiquinone; BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

Later, Debus et al. [3] isolated an LM unit which retained fully reversible photochemical activity at low temperature. Reconstitution with the H-chain was required both for restoring the same rate of recombination of charges at 77 K as in intact reaction centers and for allowing the electron transfer from the primary quinone Q_I to the secondary Q_{II} to occur. Interestingly, the M-subunit has been implicated in the binding of the primary [4] and secondary [5] quinones. In the purified *Rps. sphaeroides* reaction center the H unit is tightly bound to the LM unit, a feature which is not shared by the isolated reaction center of *Rhodospirillum rubrum* [6–8]. For the latter, results, quite contradictory to those summarized above have been reported recently by Gimenez-Gallego et al. [9] who concluded that the L-chain alone is involved in the binding of the pigments, the cofactors and the photochemical activity.

In the present paper we describe the characteristics of an LM unit we isolated after treatment of purified wild-type *Rps. sphaeroides* reaction centers with SDS in the presence of lauryldimethylamine *N*-oxide, a procedure adapted from those of Okamura et al. [2] and Vadeboncoeur et al. [7]. Our purpose was to investigate the effects of the loss of iron and of the splitting of the H-chain which are known to occur as a consequence of SDS treatment [2,10,11]. We will show that this LM preparation is practically devoid of the primary acceptor Q_I but still able to react with some quinones added externally. These quinones could partially play the role of the primary acceptor Q_I but did not restore the secondary acceptor Q_{II}. The study of this LM unit led us to conclude that its altered properties are the consequence of the combined action of SDS and lauryldimethylamine *N*-oxide rather than of the absence of Fe and of the H polypeptide.

Methods

Purified reaction centers from *Rps. sphaeroides* strain Y, prepared as described in Ref. 12, with an additional DEAE-cellulose chromatography step, were incubated for 1 h at 20°C at $A_{800nm} = 8–9$ in 50 mM Tris-HCl, 1 mM EDTA buffer, pH 8.0, containing 1% SDS and 0.03% lauryldimethylamine *N*-oxide (Onyx Chemical).

For isolating LM the incubated reaction centers (0.5 ml) were layered on top of a linear sucrose gradient (12 ml, 5–20%) in 50 mM Tris-HCl, 0.1% lauryldimethylamine *N*-oxide, pH 8.0, and centrifuged at 200 000 × *g* for 22 h at 15°C. The tubes were pierced and 0.35-ml fractions were collected and analyzed. In Fig. 1 is illustrated the separation of the H-chain and LM unit, which were identified by SDS gel electrophoresis and by their absorption spectra; these indicated that the H fraction comigrated with a low amount of degraded pigments. The peak fractions of LM were combined and dialyzed against 50 mM Tris-HCl, 1 mM EDTA, 0.03% lauryldimethylamine *N*-oxide buffer, pH 8.0, for 24 h at 4°C, then concentrated on an Amicon PM 30 membrane. Under these conditions the samples were stable for several days if kept in the dark at 4°C. We stress that if the dialysis was carried out at higher concentrations of lauryldimethylamine *N*-oxide (0.05–0.1%) an increased degradation of BChl would take place.

For preparing RC-SDS, which may be considered as a preparation intermediate between intact

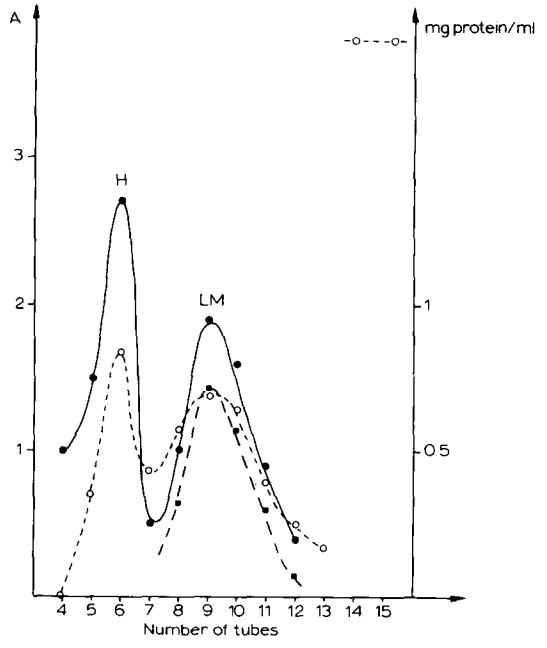


Fig. 1. Separation of the LM and H units, after incubation of reaction centers in 1% SDS and 0.03% lauryldimethylamine *N*-oxide, followed by centrifugation on a linear sucrose gradient (see Methods). (●———●) A_{280nm}, (■— — —■) A_{800nm}, (O— — —O) protein content.

reaction center and LM, reaction centers incubated as above in Tris-HCl buffer containing 1% SDS and 0.03% lauryldimethylamine *N*-oxide were dialyzed against the same buffer at 15°C but without SDS. This preparation was checked by sucrose gradient centrifugation as above; only one protein band was observed at a position close to LM, plus a minor and lighter band containing degraded pigments but no protein (not shown).

Two methods were used for incubating LM with various quinones: DQ menadione, Q_6 and Q_{10} (all from Sigma). The first consisted of mere addition of quinones in ethanolic solution (up to 500 μM) to an LM sample (2–50 μM) in Tris-HCl buffer containing 0.03% lauryldimethylamine *N*-oxide. Final ethanol content was under 5%. The mixture was incubated for 1 h at 20°C; prolonging the incubation overnight at 10°C did not change the results. For low-temperature experiments only, where the LM concentration was high (17–50 μM), we added the quinones as a dispersion (200–500 μM) in the nonionic detergent Brij 35. This was used specially for Q_6 and Q_{10} which are poorly soluble in water and polar solvents. A method similar to that described for preparing large lipid vesicles [13] was used, starting from a quinone solution in chloroform and an aqueous phase containing 5% Brij 35. The incubation with LM was done as described above, with a final concentration of Brij 35 between 0.5 and 1%. Identical results were obtained with both methods.

An LM sample incubated (see above) with a molar excess of Q_6 (Q_6/LM = 10–20 : 1) was applied on a Sephacryl S-300 column (0.9 × 27 cm) equilibrated at 4°C. The excess free Q_6 was eluted first (probably in an aggregated form) in a fraction well separated although close to that containing the complex (LM-Q_6). The elution profile was followed at 280 nm and the protein content of the fractions measured.

Absolute absorption spectra and 'light minus dark' difference spectra were recorded on a Cary 14R spectrophotometer equipped for cross-illumination; saturating infrared actinic light was provided by a tungsten iodide lamp and a Wratten 89 B filter, the photomultiplier being protected by a 10% $CuSO_4$ solution (1 cm optical path). For recording the spectra at 77 K, metallic cuvettes with plastic windows (0.1 or 0.2 cm optical path) were used, and immersed in a transparent Dewar containing liquid nitrogen. Usually the samples contained 58% glycerol in order to obtain a transparent glass. Light-induced absorbance changes were recorded in the dual-wavelength mode on an Aminco DW2A spectrophotometer equipped for cross-illumination. The intensity of light output at the sample cuvette was 10–50 $mW \cdot cm^{-2}$, as measured with a YSI radiometer, and was varied with neutral filters.

For following flash-induced absorbance changes in the time range greater than 5 ms we used either two Strobotac or an Applied Photophysics double-flash equipment. The flashes were triggered with the help of a pulse generator and their duration at half-width was 10–15 μs. The energy output of the flashes was varied with neutral filters. The signals were displayed on a Tektronix oscilloscope connected to the Aminco spectrometer, operating in the dual-wavelength mode. Slow absorbance changes were followed both on the oscilloscope and on the recorder. In the infrared, the excitation light was filtered through a Corning 4-96 filter and photomultiplier was protected by a Corning 7-69 filter. The absorbance changes in the range 430–498 nm were measured by inverting the preceding filters. The oscilloscope traces were photographed and subsequently analyzed graphically.

For experiments under anaerobiosis, Thurnberg cuvettes were evacuated under vacuum, then filled with nitrogen several times. Alternatively, oxygen was consumed by adding 12–24 U glucose oxidase to the sample containing 30 mM glucose, with a layer of paraffin oil on top. However, this could not be used when the sample contained DQ or menadione, as we observed that these compounds migrated from the aqueous solution into the paraffin phase.

Protein was measured by the method of Schaffner and Weissmann [14]. Quinone was determined as described in Ref. 15. The amounts of Fe and Mn bound to reaction center preparations were measured by atomic absorption spectroscopy; standards were run containing the same amount of detergent. Concentrations of reaction center, RC-SDS and LM solutions were measured adopting the same millimolar extinction coefficient, $\epsilon_{800\ nm} = 288$ [16].

Results

In preliminary experiments we tried to separate LM from the H-subunit by chromatography of SDS-treated reaction centers on various media: DEAE-cellulose, hydroxyapatite, Sephacryl S-300. These assays were all negative, so we turned like previous workers [1,2,7] to equilibrium centrifugation on a sucrose gradient. For splitting the protein into LM and H, we incubated the reaction center with SDS and lauryldimethylamine *N*-oxide, then ran it on a sucrose gradient containing only lauryldimethylamine *N*-oxide and no SDS (see Methods and Fig. 1). This prevented the formation during centrifugation of BPh or desvinylacetyl Chl *a* at the expense of BChl. We also studied another preparation intermediate between intact reaction center and LM, which will be called hereafter RC-SDS. This was obtained by incubating reaction center with SDS and lauryldimethylamine *N*-oxide as for LM, but then removing SDS by dialysis. We observed that in RC-SDS the H-subunit was still bound to LM (see Methods).

The absolute absorption spectrum of LM (Fig. 2) is roughly similar to that of intact reaction center, but several spectral differences were observed which are detailed elsewhere (unpublished data). (1) The Q_y band of the donor is blue shifted to 850 nm (867 nm in reaction center). (2) The Q_x band of the two BPhs is sharpened; this is due to the overlapping of the two components compared to the well split bands of the reaction center. These spectral differences are quite obvious at 77 K. (3) The maxima of the vibrational components of the visible absorption bands of spheroidene are slightly blue shifted in LM by about 1–2 nm. (4) The ratio of the absorbances at 280 and 800 nm diminishes from 1.33 for the intact reaction center to 1.2–1.23 for LM. In RC-SDS features 1–3 were also observed, except that the Q_y band was found at 860 nm.

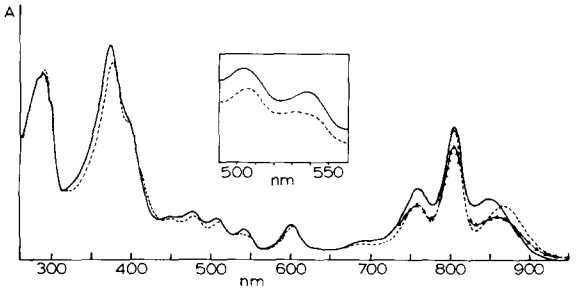


Fig. 2. Absolute absorption spectra at 25°C of: (———) LM, (▲———▲) LM incubated with excess Q_6, (— — —) reaction center. Inset: higher magnification showing the narrowing of the BPh Q_x band in LM (———) as compared to reaction center (— — —).

Metal content of reaction center and RC-SDS

It is now well established that in purified reaction centers from several photosynthetic bacteria, including those from *Rps. sphaeroides* strain R-26, about one Fe atom is bound per protein [1]. In the strain Y reaction center the Fe content is lower and Fe appears to have been substituted by Mn. Indeed, in several reaction center preparations we found about 0.4 Fe atom and 0.6 Mn atom per reaction center. In RC-SDS Mn is absent but Fe is not completely removed (Table I).

Photochemical activity at 77 K and the effect of various quinones

The LM fraction, as isolated from the gradient, could be totally bleached by continuous illumination at 25°C; in contrast, only 10–30% of the 890 nm absorption band was bleached at 77 K. This indicated that the major part of the LM subunits had lost the primary acceptor Q_I, and was confirmed by quinone analysis on one LM preparation, which contained at most 0.15 quinone per P-865 (as compared with 2.0 quinones per P-865 in

TABLE I

THE AMOUNT OF Fe AND Mn PRESENT IN SEVERAL REACTION CENTER AND RC-SDS PREPARATIONS

n.m., not measured.

Sample	No. of atoms	
	Fe	Mn
Reaction center	0.45	n.m.
Reaction center	0.46	n.m.
Reaction center	0.42	n.m.
Reaction center	0.4	0.61
Reaction center	0.54	0.77
RC-SDS	0.24	0
RC-SDS	0.2	0

the reaction center sample before SDS treatment). This loss of activity occurred at least partly during the initial SDS treatment; indeed, the RC-SDS preparation exhibited only 50% bleaching of the 890 nm band at 77 K.

We tried, therefore, to reconstitute the first stable electron acceptor in these preparations by incubating them with an excess of various quinones (see Methods). For RC-SDS excess DQ restored almost completely (approx. 90%) the photochemical activity at 77 K. For LM-Q_6 and LM-DQ mixtures a partial recovery (50 and 70%, respectively) was observed (Fig. 3). Q_{10} gave results similar to those of Q_6, but because of its low solubility we did not use it routinely. A mixture of LM and menadione did not show after incubation any improvement in photochemical activity at 77 K with respect to LM alone.

In order to determine whether tight binding of the quinones did occur, we tested the photochemical activity of the LM complex by following the steady-state light-induced absorbance changes at 20°C (see below) after its separation by gel chromatography from excess quinones. The activity of the LM complex freed from excess Q_6 was 80% of that of the initial mixture: thus, most of Q_6 was bound in an irreversible way. We checked also that point by measuring the photochemical activity at 77 K. On the other hand, DQ and menadione did not exhibit such binding, presumably because these smaller and less hydrophobic molecules readily exchanged during chromatography between the protein and the detergent micelles.

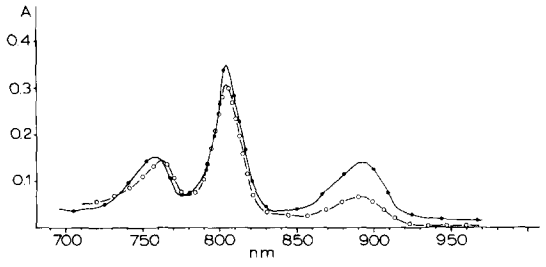


Fig. 3. Infrared absorption spectra recorded at 77 K with a Cary 14 R spectrophotometer of 10 μM LM incubated with 166 μM Q_6 and suspended in 16 mM Tris-HCl, 0.3 mM EDTA, 0.03% lauryldimethylamine *N*-oxide, 0.16% Brij 35 and 58% glycerol; (●———●) dark adapted (infrared 1 mode), (○———○) under strong white illumination (infrared 2 mode).

We noticed that the $(BChl)_2$ Q_y band was affected by the presence of Q_6: after incubation of LM with Q_6 as well as after separation of LM from excess Q_6, this band shifted from 850 to 860 nm (see Fig. 2), indicating the same wavelength as in RC-SDS. In contrast, the presence of DQ or menadione had no effect.

Light-induced steady-state absorbance changes

In intact reaction center and in LM or RC-SDS, infrared saturating illumination induced bleaching at 600 nm, indicating the formation of P-865$^+$. Whereas this change was rapid and monophasic in intact reaction center it was very slow and small in LM but became strongly enhanced in the isolated LM-Q_6 complex (Fig. 4) or in the presence of an excess of various quinones. However, it still differed from that of intact reaction center in its biphasic nature and slower dark decay. Thus, at the onset of illumination its amplitude amounted only to 80% of that of intact reaction center after 10 s illumination, and the dark recovery lasted for several minutes. In the absence of O_2 the dark decay of the 600 nm signal became 2–3-times faster for LM + quinone mixtures than in the presence of O_2, whereas O_2 did not affect the kinetics in intact reaction center. The 600 nm bleaching

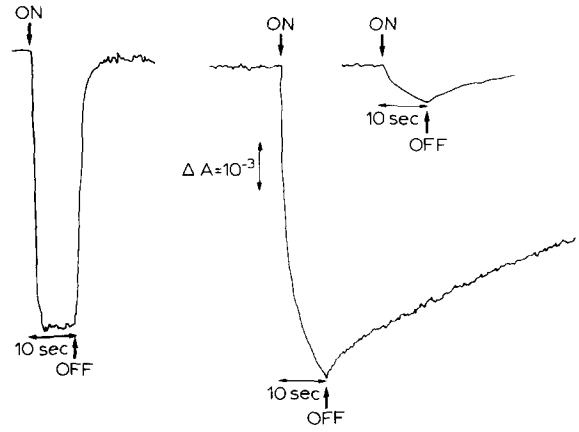


Fig. 4. Steady light-induced absorbance changes of the complex LM-Q_6 and reaction center, suspended in 50 mM Tris-HCl buffer, pH 8, 1 mM EDTA, 0.03% lauryldimethylamine *N*-oxide. Left trace: 0.4 μM reaction center, photobleaching measured as ΔA(602 − 585 nm). Upper right trace: 0.48 μM LM; ΔA(598 − 580 nm). Lower right trace: 0.48 μM LM-Q_6 complex freed from excess Q_6 by chromatography.

was also modified by the presence of 5 mM *o*-phenanthroline in two respects: its amplitude was diminished (by 50% for LM alone, by 20–30% for LM + Q) and its dark decay was accelerated. The effects of O_2 and *o*-phenanthroline were studied in more detail after flash excitation (see below).

Kinetics of P-865$^+$ dark decay in LM and RC-SDS after a saturating flash

The initial purpose of our flash experiments was the detection of the P-865$^+$ signal, both in RC-SDS and LM, the measure of its dark decay in comparison with intact reaction center, and also the detection of the semiquinone anion radical. Later, we extended this study to RC-SDS and LM preparations to which various quinones were added, in the presence or absence of O_2 and *o*-phenanthroline.

Following a short saturating flash on RC-SDS we could observe the formation and decay of the P-865$^+$ signal but not its rise, due to the slow response of our apparatus (approx. 5 ms). Under these conditions, the P-865$^+$ signal in RC-SDS was 2-fold weaker than that observed in intact reaction center. The decay of P-865$^+$ in RC-SDS was polyphasic, involving three kinetic phases, apparently first order; the slower one disappeared under anaerobiosis. The addition of excess Q_6 affected neither the amplitude of the P-865$^+$ signal nor its dark decay, which was also insensitive to *o*-phenanthroline. These properties suggest that the first electron acceptor Q_I is present in a fraction of the RC-SDS, but that the secondary one Q_{II} cannot be reconstituted.

Under the same experimental conditions, P-865$^+$ was either not detectable in LM (two preparations) or gave a very weak signal (in one preparation, 10-fold weaker than in intact reaction center). It could be observed in the presence of excess Q_6, with the same amplitude as in RC-SDS; the amount of Q_6 required to produce half the maximum signal in LM was 5 μM. The P-865$^+$

TABLE II

CHARACTERISTICS OF THE DARK RECOVERY OF THE FLASH-INDUCED P-865$^+$ SIGNAL IN RC-SDS AND LM, MEASURED AT 865 nm VERSUS 930 nm UNDER DIFFERENT CONDITIONS AT 25°C

Each sample (2 μM RC-SDS or LM) was suspended in 50 mM Tris-HCl buffer (pH 8), 0.03% lauryldimethylamine *N*-oxide. The amplitude of the P-865$^+$ signal was 0.05–0.06 in RC-SDS and LM + Q_6, as compared to 0.125 in intact reaction center (2 μM). Quinones and *o*-phenanthroline were added as ethanolic solutions (final concentrations: Q_6, 20–40 μM; DQ and menadione, 100–150 μM; *o*-phenanthroline, 5 mM). $t^i_{1/2}$, apparent half-times attributed to the multiexponential decay; when more than three phases were present only the extreme $t^i_{1/2}$ values are given. The percentage of the slow phases (defined as those with $t^i_{1/2} > 1$ s) in the decay was calculated with respect to the initial amplitude of P-865$^+$. n.m., not measured.

Material	Addition	Aerobic conditions				Anaerobic conditions			
		$t^i_{1/2}$			% slow phase	$t^i_{1/2}$			% slow phase
RC-SDS	$\pm Q_6$	65 ms,	250 ms,	1 s	15	60 ms,	150 ms		0
	$\pm Q_6$, *o*-phenanthroline	65 ms,	300 ms,	1.4 s	15	n.m.			n.m.
	+ DQ	170 ms,	...	45 s	50	63 ms,	...	18 s	27
	+ DQ, *o*-phenanthroline	53 ms,	...	20 s	23	65 ms,	...	10 s	22
	+ menadione	1 s,	...	150 s	100	40–100 ms,	...	40 s	65
	+ menadione, *o*-phenanthroline	n.m.			n.m.	n.m.,	...	24 s	70
LM	+ Q_6	100–200 ms,	...	32 s	50	50 ms,	...	n.m.	15
	+ Q_6, *o*-phenanthroline	50 ms,	...	25 s	30	47 ms,	...	n.m.	20
	+ DQ	1–2 s,	...	117 s	100	41 ms,	...	43 s	30
	+ DQ, *o*-phenanthroline	4 s, 15 s			100	n.m.			n.m.
	+ vitamin K-3	800 ms,	...	11–30 s	90	2 s, 5 s			100
	+ vitamin K-3, *o*-phenanthroline	870 ms,	1–5 s		90	n.m.			n.m.

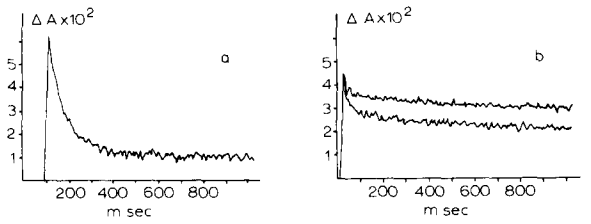


Fig. 5. Oscilloscope traces of flash-induced absorbance changes of 2 μM RC-SDS suspended in the same buffer as in Fig. 4, measured as ΔA(865 − 930 nm): (a) without addition, (b) 100 μM menadione. Upper trace, aerobic sample; bottom trace, anaerobic sample.

decay in LM + Q_6 involved more than three components; a semilogarithmic plot of the decay was indicative of a polyphasic phenomenon. Therefore, the values given in Table II for the apparent half-times of the fastest phase(s) and the slowest one are mere indications of the time range involved in P-865$^+$ decay, especially useful for comparing the effect of various conditions.

When we tried to reconstitute LM with DQ or menadione a very different kinetic behavior was observed as compared with an LM + Q_6 sample. When DQ or menadione (100–160 μM) was added to an aerobic sample of LM (2 μM) the P-865$^+$ signal amounted only to 30–60% of that obtained with LM + Q_6. This large excess of quinones was needed to reach saturation of the phenomenon. At

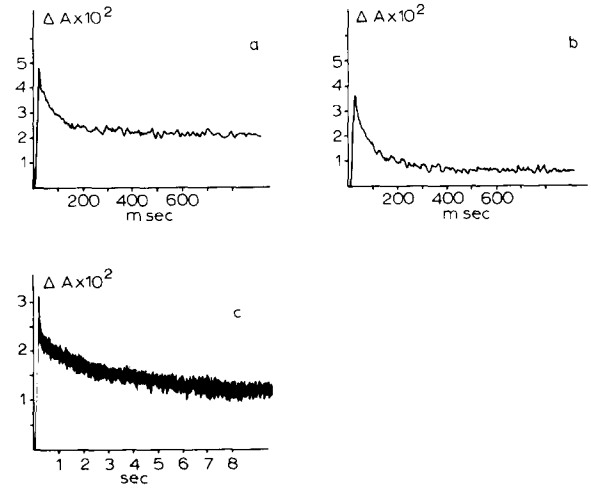


Fig. 6. Oscilloscope traces of flash-induced absorbances changes of 2 μM LM measured as ΔA(865 − 930 nm): (a) aerobic, 40 μM Q_6 added; (b) as in a but anaerobic; (c) aerobic, 150 μM DQ added.

the same time, the polyphasic dark relaxation became even slower, involving half-times between 1 s and 2 min (Table II and Fig. 6c). These observations would indicate that these quinones have a lower affinity than Q_6 for LM. This supposition is strengthened by the fact that the weak P-865$^+$ signal observed for LM in their presence increased back to its maximal amplitude when 20–40 μM Q_6 was added; furthermore, in that case the relaxation of P-865$^+$ involved again the fast component ($t1/2 \approx 60$–100 ms) besides the slow phases which were still present. A similar behavior was observed for RC-SDS to which DQ or menadione was added: besides the rapid phases, slow ones (in the time range 1–150 s) developed. (Table II and Fig. 5b).

It should be remarked that the amplitude of the P-865$^+$ signal was maximal at the same flash intensity in reaction center and in RC-SDS; it should be further increased by 30% to saturate the signal in LM + Q.

Effect of anaerobiosis

The absence of O_2 modified the decay kinetics of P-865$^+$ and sometimes the photochemical efficiency in the various SDS-treated samples. In anaerobic LM-Q_6 and LM + menadione samples a net acceleration of the slow phases involved in the relaxation of P-865$^+$ was observed (Table II and Fig. 6b). For LM + DQ mixtures the absence of O_2 drastically affected both the flash-induced P-865$^+$ signal and its relaxation in the dark. The signal amplitude was increased by a factor of 2.5 and its decay became faster and similar to that observed for the LM + Q_6 anaerobic sample. These effects of O_2 could indicate that an electron transfer from the semi-reduced quinones to O_2 took place, in competition with the reduction of P-865$^+$ (see Discussion).

Effect of o-phenanthroline

It is well established that the main effect of *o*-phenanthroline on intact reaction center is an inhibition of electron transport from the first electron acceptor Q_I to the secondary acceptor Q_{II} [17,18]. A different action was observed on SDS-treated preparations. When added to RC-SDS (together with Q_6 or not) *o*-phenanthroline inhibited appreciably the photochemistry (Table III) while

TABLE III

EFFECT OF *o*-PHENANTHROLINE ON THE AMPLITUDE OF THE FLASH-INDUCED P-865$^+$ SIGNAL IN RC-SDS AND LM

Conditions: Same concentrations as in Table II. In each case the amplitude of the P-865$^+$ signal (ΔA(865 − 930 nm)) observed in the presence of 5 mM *o*-phenanthroline is expressed relative to a control sample without *o*-phenanthroline.

Conditions		Relative amplitude
RC-SDS − Q_6	+ O_2	0.04–0.5
RC-SDS − DQ	+ O_2	0.60
RC-SDS − DQ	− O_2	0.75–1.00
RC-SDS + menadione	− O_2	0.60
LM + Q_6	+ O_2	0.90
LM + Q_6	− O_2	0.85
LM + DQ	+ O_2	0.60
LM + menadione	+ O_2	0.60

the decay kinetics were unchanged; in the presence of DQ or menadione inhibition was also observed, but the decay kintics were now accelerated (Table II). In LM samples to which quinones were added both effects of the inhibitor on the kinetics and on the P-865$^+$ signal were observed; however, when Q_6 was present the diminution of the P-865$^+$ signal was smaller.

The radical anion Q_I^-

In intact reaction center a short saturating flash induces the rapid formation of P-865$^+$ and of the anion radical Q_I^-; subsequently, in a lapse of 200 μs the negative charge migrates on to the secondary acceptor Q_{II} [19]. Under appropriate redox conditions where P-865$^+$ is rapidly reduced, e.g., by the DAD-ascorbate couple, the anion radical Q_{II}^- is very stable and can be detected even 5–10 min after the flash (Ref. 23 and this work). In contrast, Q_I^- is less stable (in the presence of the same redox couple); in our hands its half-time in intact reaction center (detected by its absorbance at 450 nm) was about 20 s. We tried to detect the Q_{10} radical in RC-SDS under the same experimental conditions as for intact reaction center and observed a weak absorbance change at 450 nm (measured with 495 nm as isobestic point) with a half-time of about 350–550 ms. Under anaerobiosis this signal was more stable ($t_{1/2} \approx 900$ ms). We checked that this signal was maximal at 450 nm, and tentatively attributed it to the anion radical Q_{10}^-. We have not been able to detect reproducibly such a signal in LM-Q_6. The instability of the semiquinone species was confirmed by steady light minus dark difference spectra of LM and RC-SDS samples, which did not display any absorbance change in the 400–450 nm region that could be ascribed to a quinone radical (QH$^\cdot$ or $Q^{\cdot-}$) whereas under similar experimental conditions reaction center displayed a positive absorption band at 450 nm [21,22]. These results are in agreement with those of Clayton and Straley [21] who did not detect by absorbance measurements any semi-reduced quinone formation in reaction centers treated with SDS.

Discussion

The LM and RC-SDS prepared as described above have some common features with several reaction center preparations (derived from the R-26 or wild-type strain), which have been treated with SDS, urea or chaotropic agents or high concentration of lauryldimethylamine *N*-oxide [2,10, 11,22,21,24–27]. The first one consists of spectral modifications, as the blue shift of the P-865$^+$ Q_y band and the narrowing of the BPh Q_x band [2,10,11,22,24,27], denoting an alteration in the pigment environment. The second is the depletion in the transition metal [2,10,11,24,25]. We should stress here that in the purified reaction center from wild-type *Rps. sphaeroides* (strain Y) Mn represents the major transition metal and not Fe, despite the fact that the growth medium was neither depleted in Fe nor enriched in Mn. After treatment with SDS this reaction center lost all the Mn but not all of the Fe. This suggests that in this reaction center Mn has been substituted naturally for Fe at its specific site in the quinone complex [28].

The partial loss of photochemical activity in RC-SDS and LM has been observed by other authors for similarly treated preparations [2,10,11]. In our case RC-SDS retained only 50% of the maximal photochemical activity induced either by continuous illumination at 77 K, or by a flash at 25°C. The LM preparation was more deficient in photochemical activity but this could be partly restored by adding various quinones. Blankenship

and Parson [11] have shown that reaction centers from *Rps. sphaeroides* R-26 treated with increasing concentrations of SDS progressively lost their photochemical activity in parallel with increased formation of the triplet state of the dimer, P^R. The possibility of a similar formation of the P^R state or of the triplet state of spheroidene [29] in our RC-SDS and LM samples cannot be excluded.

We monitored the photochemical activity at 25°C in reconstituted LM-Q preparations first by following the absorbance changes induced by continuous light. LM (without addition) displayed at the onset of illumination a weak P-865$^+$ formation which increased very slowly during illumination. This showed that almost all the first acceptor had been removed, which was confirmed by the lack of P-865$^+$ signal after a flash. When quinone was added, LM displayed in steady light illumination enhanced absorbance changes with a rapid rise as in intact reaction centers and a slower one, which is possibly due to a fraction of the preparation in which charge separation occurred with a low quantum efficiency. A similar reduction of the rapid absorbance change with a concomitant development of a slower phase was observed by Romijn and Amesz [26] with *Rps. sphaeroides* AUT particles treated with increasing concentrations of thiocyanate.

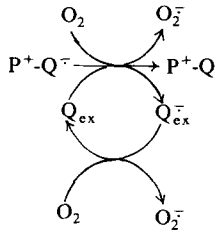
In this work the best reconstitutions of photochemical activity were obtained with Q_6 and DQ, which are benzoquinones as is Q_{10}. DQ seemed to be more effective at 77 K; perhaps the particular conditions chosen for carrying out assays at 77 K (large excess of LM protein + DQ versus detergent) favored the binding of DQ and (or) its accessibility to the binding site. The fact that the binding of only Q_6 or Q_{10} induced a red shift of the 850 nm band of the dimer to 860 nm, and not that of DQ or menadione, might be explained by a hydrophobic interaction between the BChl dimer and the isoprenoid side chain of Q. Only Q_6 could form an irreversible complex with LM, while DQ and menadione could not. Intact reaction centers from *Rps. sphaeroides* R-26 depleted of the first quinone acceptor under nondenaturing conditions [30] were efficiently reconstituted with a wide variety of quinones [30–33], including those we have used here. The dissociation constants (K_d) for Q_6 and Q_{10} were estimated to be less than 10^{-6} M [33]; for DQ various K_d values were reported, viz., 10^{-7} M [32] or 10^{-5} M [31]. In this work, the K_d value of Q_6 for LM seems to be in the 10^{-6} M range, which might indicate a modification of the binding site.

With our experimental conditions, we have been able to detect after a flash the formation of a semi-reduced Q^- radical in RC-SDS, but with a much shorter lifetime than in intact reaction centers. Other authors who treated purified *Rps. sphaeroides* reaction centers with chaotropic agents [11,24,34] or SDS [10,11,21] or alternatively extracted reaction centers (from *Rps. viridis*) with SDS [35] have observed that Fe was no longer interacting with the quinones, and that the photoinduced Q^- free radical was less stable than in untreated samples. According to them this instability precluded detection of the radical by chemical titration, which in this particular case proceeded by a two-electron transfer. In contrast, in *R. rubrum* AUT particles depleted of Fe the Q^- ESR signal was very stable and detectable even at room temperature [25,36,37]. Therefore, one may conclude that the instability of the semiquinone radical in reaction centers after treatment with SDS or chaotropic agents is not due to disruption of the ferroquinone complex, but rather to alterations in the pigment-protein complex which induced also loss of Fe and partial loss of photochemistry.

The dark decay of the flash-induced P-865$^+$ signal in LM or RC-SDS in the presence of various quinones differed from that of intact reaction center in several ways. It was polyphasic, involving pseudo-first-order kinetics with half-times ranging from 60 ms to 2 min. Similarly, a slow phase in the P-865$^+$ decay pattern seemed to arise at 25°C in reaction centers extensively dialyzed against SDS [11].

For our RC-SDS preparation, the insensitivity of the decay kinetics towards addition of Q_6 or *o*-phenanthroline (see Results) led us to conclude that the binding site of Q_{II} was damaged or absent. Therefore, we ascribe the fastest phase of the decay ($t_{1/2} \approx 60$ ms) to the direct recombination of charge between P-865$^+$ and Q_{I10}^-. As a matter of fact, this rate is close to that of the monophasic decay in intact reaction center when the transfer of electrons from the first quinone (Q_I) to the second

(Q_{II}) was blocked by the addition of *o*-phenanthroline or by the absence of Q_{II} [11,22,38,39]. The second major phase ($t_{1/2} \approx$ 150–300 ms) might be attributed to another fraction of RC-SDS in which the distance P-865–Q_I was altered by the SDS-lauryldimethylamine *N*-oxide treatment. The slow phases ($t_{1/2} > 1$ s) which were quite important when DQ or menadione were present and which diminished under anaerobiosis could originate from indirect recombinations between P-865$^+$ and semi-reduced radical species of quinones Q_{ex} moving freely in solution. The rates of these reactions were sensitive to O_2 and *o*-phenanthroline. We suppose, therefore, that in a fraction of the preparation the decay of P-865$^+$ could proceed indirectly, the reactants being semiquinone anions Q_{ex}^- as well as superoxide radical O_2^- according to a hypothetical scheme:



(1)

$$P^+Q + Q_{ex}^- \rightarrow P\text{-}Q \qquad (2)$$

$$P^+\text{-}Q + O_2^- \rightarrow P\text{-}Q \qquad (3)$$

According to this scheme, only those Q_{ex}^- anions which did not disproportionate might interact with photoinduced P-865$^+$ by a diffusion-controlled reaction. An electron transfer between semiquinone and quinone was already proposed to occur in a liposomal Chl *a*-quinone system [40] in order to explain the presence of a second slow phase in the decay of the photoinduced radical. On the other hand, the reactivity of O_2 towards semi-reduced quinone species in solution has been described elsewhere [41–43].

For LM preparations the kinetics of the recovery at 25°C were also complicated, and we cannot explain in detail the reasons for this heterogeneity. One of the reasons could be the fact that quinones which combine with LM did not all bind exactly in the same way [30,33]. Despite this heterogeneity, the fastest phases of the decay probably originate from back electron transfer from Q_I^- to P-865$^+$, whereas indirect recombination processes described above might mainly govern the slower part ($t_{1/2} > 1$ s) of the decay. Our observations that the recovery of the photobleached donor induced by steady-state illumination in LM + Q was slowed down by O_2 is similar to those of Boucher and Gingras [44] on purified *R. rubrum* raction centers. According to them the slower decay was due to the photogeneration of O_2^- originating from the transfer of an electron from Q_I^- to O_2. We would suggest that the relatively weak association between LM and H-subunits in reaction centers purified from *R. rubrum* [6–8] might be the reason for their greater reactivity toward O_2. Therefore, the sensitivity to O_2 of our RC-SDS and LM preparations might be due both to the absence of the H-subunit (in the case of LM) and to denaturation.

The particular case of the O_2 sensitivity of LM + DQ seems puzzling. The absence of O_2 not only greatly modified the decay kinetics after a flash, which became similar to those observed with Q_6, but also seemed to improve the quantum efficiency. Thus, the absence of O_2 seemed to favor the DQ binding at the Q_I site. Experiments with a better time resolution would be required to explain these results.

A detailed model for the action of *o*-phenanthroline has been recently proposed by Wraight [45]. It consists of a competitive displacement of the secondary quinone; when this is absent, the first quinone itself can be displaced by the inhibitor, resulting in the loss of photochemical activity. We have seen that *o*-phenanthroline added to a sample of RC-SDS in the presence or absence of exogenous Q_6 did not change the decay of the flash-induced P-865$^+$ signal but decreased its extent. This observation confirmed pur interpretation that after SDS treatment the reaction centers have lost the secondary acceptor $(Q_{10})_{II}$. Moreover, the fact that the addition of Q_6 to RC-SDS did not slow down the decay of P-865$^+$ indicated that the secondary quinone site was also irreversibly denatured by SDS, as in *Rps. sphaeroides* R-26 reaction centers treated with $LiClO_4$ or SDS [11]. In contrast, in the LM preparation the addition of Q_6 protected against *o*-phenanthroline inhibition of photochemistry; the

weaker protection afforded by DQ and menadione could be due to their loose binding to LM.

Another effect of *o*-phenanthroline consisted of the acceleration of the reduction of P-865$^+$ in LM + Q and RC-SDS + Q. This accelaration may be due to the direct action of this inhibitor on the quinones in solution, preventing them from reacting with each other. Clayton et al. [18] have shown that such an effect can exist in vitro. Their system consisted of a detergent solution containing the redox couple phenazine methosulfate-Q_6 in which the transfer of electrons was photosensitized by BChl, but inhibited by *o*-phenanthroline. Such reactions could take place between the inhibitor and the excess of quinone present in solution in our experiments on RC-SDS and LM. From these results altogether one can infer that the mode of action of *o*-phenanthroline on reaction centers is independent of the presence of the transition metal complexed with the quinones, but is directed toward the quinones themselves, a conclusion reached more directly from other experiments [46,47].

In conclusion, the LM unit obtained after SDS treatment was depleted of the primary acceptor Q_I, but was still able to react with quinones added in solution, with partial restoration of the primary photochemical activity. This result was in good agreement with the experiments of Feher and coworkers [4] which indicated that the site of binding of Q_I was near or at the M-subunit. However, the instability of the semiquinone anion species and the slow polyphasic relaxation of the photoinduced P-865$^+$ dimer, the rate of which depended on the presence of O_2, suggested that the binding site of Q_I in LM was partially denatured and probably open to the aqueous solvent, as a result of SDS action.

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