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The effect of cytochrome *c*, hexammineruthenium and ubiquinone-10 on the kinetics of photoelectric responses of *Rhodospirillum rubrum* reaction centres

L.A. Drachev [a], O.P. Kaminskaya [a], A.A. Konstantinov [a], E.A. Kotova [a], M.D. Mamedov [a], V.D. Samuilov [b], A.Yu. Semenov [a] and V.P. Skulachev [a,*]

[a] *A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry and* [b] *Department of Microbiology, Moscow State University, Moscow W-234, 119899 (U.S.S.R.)*

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Rhodospirillum rubrum **reaction centre (RC) complexes with or without antenna bacteriochlorophyll have been incorporated into liposomes; the latter were associated with a phospholipid-impregnated collodion film, and flash-induced electric responses were measured. It is shown that in the antenna-free RC-proteoliposomes a very rapid phase of charge separation (less than 200 ns) due to P-870 → Q_A electron transfer has a polarity (negative inside) opposite to that of the intact chromatophores or of the antenna-containing RC-proteoliposomes. In the antenna-free RC-proteoliposomes (but not in the chromatophores), P-870 is readily accessible to membrane-impermeable cationic electron donors like cytochrome *c* and $Ru(NH_3)_6^{2+}$, which indicates localization of P-870 near the outer surface of the membrane. Oxidation of externally added cytochrome c^{2+} by $P\text{-}870^+$ in these proteoliposomes initiates an additional slower phase of $\Delta\psi$ generation comprising about 25% of the total photoelectric response. The dependence of the slower phase rate on the cytochrome *c* concentration is characteristic of a saturating reaction with K_m of 0.9 μM and k_{max} of approx. $6 \cdot 10^3$ s^{-1} at low ionic strength. A similar electrogenic phase can be observed without cytochrome *c* in the presence of sufficiently high concentrations of artificial redox dyes, e.g., 2 mM TMPD or more than approx. 50 μM PMS. The new phase of $\Delta\psi$ generation is tentatively ascribed to e^- transfer within reaction centre molecule from the protein surface to the porphyrin ring of $P\text{-}870^+$.**

Introduction

An electron flow via the cyclic electron-transfer chain of photosynthetic bacteria is coupled to generation of the transmembrane electric potential difference (for a review, see Refs. 1 and 2). Rapid kinetic studies of the electrogenic events could provide a clue to the mechanism(s) of the coupling processes.

When laser-flashed, the reaction centre bacteriochlorophyll dimer (P-870) is photooxidized and the electron is transferred via bacteriochlorophyll monomer and bacteriopheophytin to the so-called primary quinone acceptor Q_A (a tightly bound ubiquinone-10). The electron transfer from P-870 to Q_A takes about 0.2 ns. The reduced Q_A is oxidized by the secondary quinone acceptor Q_B (a less tightly bound CoA_{10}) with a $\tau_{1/2}$ of approx. 0.2 ms. The photooxidized P-870 is re-reduced by

* To whom correspondence should be addressed.

Abbreviations: RC, reaction centre; $\Delta\psi$, transmembrane electric potential difference; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; PMS, phenazine methosulfate; P-870, reaction centre bacteriochlorophyll dimer; DCIP, 2,6-dichlorophenolindophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; Chl, chlorophyll.

a *c*-type cytochrome; in reaction centres of non-sulphur purple bacteria like *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides*, a soluble cytochrome c_2 similar to mitochondrial cytochrome *c* serves as an immediate electron donor to P-870$^+$ [2]. It is well established that the P-870 $\rightarrow Q_A$ electron transfer is coupled to a $\Delta\psi$ generation, as demonstrated by both the carotenoid absorption band shift method [3,4] and a direct electrometric assay [5–8]. That other electron-transfer steps in the reaction centre, and in particular the $c_2 \rightarrow$ P-870 reaction, can be electrogenic was demonstrated by several independent experimental approaches (Refs. 4–6 and 8; see also Results). However, these additional electrogenic events have not been studied in detail.

Isolated reaction centre complexes (RC) reconstituted in proteoliposomes proved a useful model for the studies of the generation by these pigment-protein complexes [7,9,10]. In particular, we showed earlier that the polarity of a membrane potential built up in the continuous light across the membrane of proteoliposomes with RC from *R. rubrum* depends on the presence of antenna bacteriochlorophyll in the RC preparation: $\Delta\psi$ positive inside is observed with the antenna-containing complexes (as well as with the intact chromatophores), whereas $\Delta\psi$ negative inside is formed by the antenna-depleted RC proteoliposomes [10]. This interesting phenomenon has been proved specific for *R. rubrum* preparations, and was tentatively ascribed to a different orientation of the RC complexes in the proteoliposomal membrane [10].

Here we describe rapid kinetic studies of the $\Delta\psi$ generation and decay in collodion film-associated proteoliposomes inlaid by the two kinds of RC preparation from *R. rubrum*. The availability of proteoliposomes with P-870 exposed at the outer side of the membrane allowed us to investigate reduction of the photooxidized RC bacteriochlorophyll by the externally added mammalian cytochrome *c*. We show that this rapid reaction ($\tau \approx$ 150–200 μs *) is electrogenic and can contribute as much as 25% to the total $\Delta\psi$ across the membrane of proteoliposomes generated upon a laser flash.

* $\tau_{1/e}$ values are used throughout the paper if not indicated otherwise.

Materials and Methods

Cytochrome *c* type III from horse heart and ubiquinone-10 were purchased from Sigma. Cholic acid (Sigma) was recrystallized from 70% ethanol. We used also LDAO (Fluka), Hepes (Serva), $Ru(NH_3)_6Cl_3$ (Alfa Products, Denver, CO), *o*-phenanthroline (Chemapol, Czechoslovakia), ascorbic acid (Koch-Light). Other reagents were commerical products of the highest purity available.

Chromatophores from *R. rubrum* were prepared by the ultrasonic treatment procedure described previously [11], and RC with and without BChl-antenna complexes were isolated from the chromatophores, as in Refs. 10 and 12, respectively. Proteoliposomes were prepared by a cholate dialysis method [13] as follows: a mixture containing asolectin (80 mg/ml) and cholate (2%) in 100 mM phosphate buffer (pH 7.5) was sonicated in an ultrasonic desintegrator UZDN-2T in 15–20 s outbursts (22 kHz, 50 mA) up to a total sonication time of 2–3 min. The clear solution was mixed with an equal volume of the RC preparation (A_{870} = 1.5) in 10 mM phosphate buffer (pH 7.0) containing 1.5% cholate and sonicated for an additional 20 s. The solution was then dialyzed against 50 mM phosphate buffer (pH 7.5) overnight. The proteoliposomes were sedimented by centrifugation (165 000 $\times$ *g* for 60 min), and resuspended in 50 mM phosphate buffer (pH 7.5).

The photoelectric activity of proteoliposomes was measured as previously [5,6] (see also Refs. 10 and 14). The proteoliposome suspension was added to one of the two compartments of a teflon cell containing 20 mM Hepes buffer (pH 7.5) insulated by a phospholipid-impregnated collodion film and incubated for 4–5 h in the presence of 30 mM $CaCl_2$. Thereupon the solution in both compartments was replaced with the aid of peristaltic pump by a 20 mM Hepes buffer (pH = 7.5) (free of $CaCl_2$ and proteoliposomes). A laser flash (λ = 694 nm, 20 ns half-width, 20 mJ output) was used to activate electron transfer in the collodion film-associated proteoliposomes. Their photoelectric activity was monitored electrometrically as

electric potential difference across the collodion film with a pair of light-shielded Ag|AgCl electrodes connected via an operational amplifier (Burr Brown 3554 BM) with a transient recorder (Datalab DL-922) and a NOVA 3D minicomputer. The resolution time of the system was 0.2 μs, as limited by the DL 922 parameters.

Results

Fig. 1 shows the typical laser flash-induced electrical responses as observed with *R. rubrum* chromatophores (a) and two types of RC-containing proteoliposomes (b and c) associated with the collodion film. In both the chromatophores and the antenna-containing RC-liposomes the flash generates $\Delta\psi$ of a sign which corresponds to a positive charging of the interior of the vesicles. In contrast, the photoelectric response observed with the antenna-free RC-liposomes (c) has an opposite polarity, i.e., negative inside the proteoliposomes. In each case $\Delta\psi$ was built up in less than 200 ns after the flash and, in the absence of other additions, decayed according to essentially single-exponential kinetics with $\tau \approx 70$ ms (a 'rapid decay' phase [5]) characteristic of recombination of the primary dipole P-870$^+ \cdot Q_A^-$ → P-870 · Q_A [15]. This

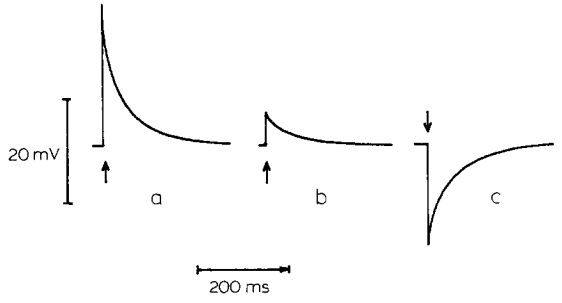


Fig. 1. Typical laser flash-induced electrical responses of *R. rubrum* reaction centre complexes in various types of membrane vesicle associated with a phospholipid-impregnated collodion film. (a) Intact chromatophores; (b) proteoliposomes with isolated antenna bacteriochlorophyll-containing reaction centre complexes; (c) proteoliposomes with isolated antenna-free reaction centre complexes. The incubation medium, 20 mM Hepes (pH = 7.5). Here and below, the vertical arrows indicate laser flash, and upward deflection of the traces corresponds to generation of $\Delta\psi$ negative on the side of the collodion film to which the vesicles are adhered.

means that $Q_A \rightarrow Q_B$ electron transfer is not operative under these conditions.

The different polarity of the photoelectric responses observed in the two types of RC-proteoliposomes on a submicrosecond scale corroborates our earlier data obtained in continuous light experiments [10] and strongly supports the hypothesis that it is the orientation of RCs in the membrane that determines the sign of $\Delta\psi$ because of the vectorial topography of the primary dipole P-870-Q_A and that depends on the presence of antenna bacteriochlorophyll. Further evidence for different orientation of reaction centres in the two types of proteoliposomes has been obtained in experiments with membrane-impermeable electron donors to P-870$^+$. According to the photoelectric response sign, P-870 has to be localized at (or near to) the inner face of the membrane of the chromatophores and the antenna-containing RC-liposomes, where it should be accessible to hydrophilic reductants. The results given in Fig. 2 show that this is indeed the case. We used ascorbate-reduced horse-heart cytochrome *c* or an inorganic complex hexammineruthenium as impermeable electron donors. Notably, both compounds are cationic; according to our observations, anionic compounds (e.g., ascorbate itself) virtually do not reduce P-870$^+$ on a fast time scale [16,17]. That cytochrome *c* does not permeate phospholipid membranes is well established [18]. As to hexammineruthenium, control experiments were performed as described below.

Our earlier studies established that in the presence of ascorbate as electron donor and vitamin K_3 (or some other naphtho- or benzoquinone of an appropriate midpoint potential) as electron acceptor, the collodion film- or teflon filter-associated chromatophores from *R. rubrum* can generate a steady-state $\Delta\psi$ in the continuous light only provided a permeable mediator like TMPD, DAD or DCIP, functioning between ascorbate and P-870, has been added [10,16,17]. We have found now that hexammineruthenium fails to replace TMPD, DAD or DCIP in such experiments with chromatophores, but can substitute for those compounds when antenna-free RC-liposomes are studied (data not shown). That hexammineruthenium does not penetrate the mitochondrial membrane was shown recently (see Ref. 19

and see also Tsofina, L.M., personal communication).

As seen in Fig. 2, in the antenna-free RC-proteoliposomes, addition of 100 μM hexammineruthenium causes a significant deceleration of the photoelectric response decay (about 50% of the $\Delta\psi$ is now dissipated with τ of approx. 1 s). This means that hexammineruthenium competes effectively with Q_A^- for re-reduction of P-870$^+$ and thus prevents a rapid discharge of the primary dipole (Fig. 2A, trace b). Horse-heart cytochrome *c* proved even more efficient (Fig. 2A, trace c) so that in the presence of only 2 μM of this hemoprotein, which is a nearly saturating concentration (see Fig. 5), there is virtually no rapid phase of the $\Delta\psi$ decay. At the same time, neither hexammineruthenium nor cytochrome *c* exerts any significant effect on the $\Delta\psi$ decay kinetics in the antenna-containing RC-proteoliposomes (Fig. 2B) or in the chromatophores (data not shown). These findings clearly show that P-870$^+$ is located near the side of the proteoliposome membrane which is positively charged upon illumination, and directly confirm a different orientation of the reaction centres in the two types of proteoliposome.

Our earlier experiments enabled us to conclude that upon association of the chromatophores with the artificial membrane, an extraction of the CoQ pool and of Q_B into the hydrophobic volume of the phospholipid-impregnated collodion film or teflon filter took place [10,16]. Recently, we found that the secondary acceptor function in chromatophores of *R. rubrum* and *Rps. sphaeroides* associated with the collodion film can be retained by adding CoQ_{10} to the decane solution of phospholipid used for impregnation of the film (Ref. 20; see also Drachev et al., unpublished data). Here we attempted to reconstitute the $Q_A \rightarrow Q_B$ electron-transfer step in RC-proteoliposomes by the same method. As one can see from Fig. 3, CoQ_{10} incorporation in the collodion film results in an apperance of a slow phase of $\Delta\psi$ decay (curve 2), although the effect is weaker than in the chromatophores, where 70–75% of Q_B can be reconstituted [20] (cf. Ref. 8). This slow phase is completely abolished by the inhibitor of $Q_A \rightarrow Q_B$ electron transfer *o*-phenanthroline (not included in Fig. 3; cf. Fig. 4) and can be assigned to $Q_B^- \rightarrow$ P-870$^+$ recombination in part of the reaction centres. Subsequent addition of cytochrome *c* causes further deceleration of $\Delta\psi$ decay (curve 3) which is

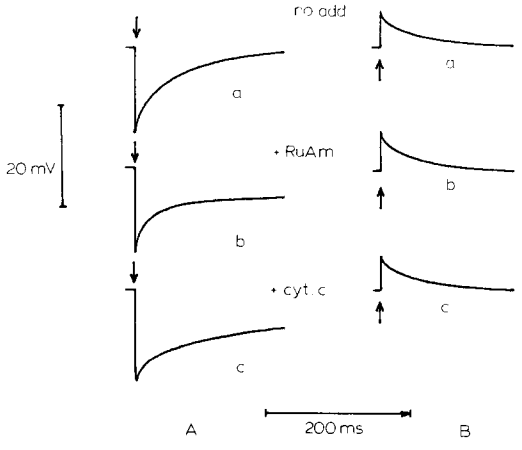


Fig. 2. Effect of cytochrome *c* and hexammineruthenium on the kinetics of $\Delta\psi$ decay in reaction centre-proteoliposomes with (B) and without (A) antenna bacteriochlorophyll. Basic incubation medium was supplemented with 2 mm ascorbate (a) and also 100 μM hexammineruthenium (RuAm) (b) or 2 μM horse heart cytochrome *c* (c).

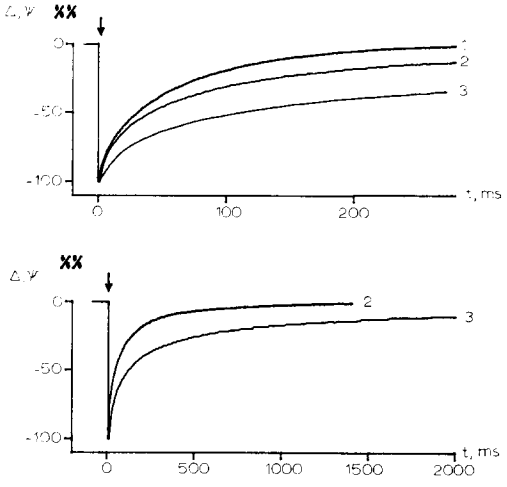


Fig. 3. Kinetics of $\Delta\psi$ decay in the antenna-free RC-proteoliposomes supplemented with ubiquinone and cytochrome *c*. Trace 1, without additions; trace 2, 20 mg/ml of CoQ_{10} added to the decane solution of phospholipids used to impregnate the collodion film; trace 3, as 2+2 mM ascorbate and 2 μM cytochrome *c*. The traces were normalized with respect to the amplitude of the response to facilitate comparison of the decay kinetics. The lower panel gives the same traces 2 and 3 as the upper one, but on a slower time scale to document more clearly the appearance of a very slow phase of $\Delta\psi$ decay ($\tau > 1$ s) in the presence of both CoQ_{10} and cytochrome *c*.

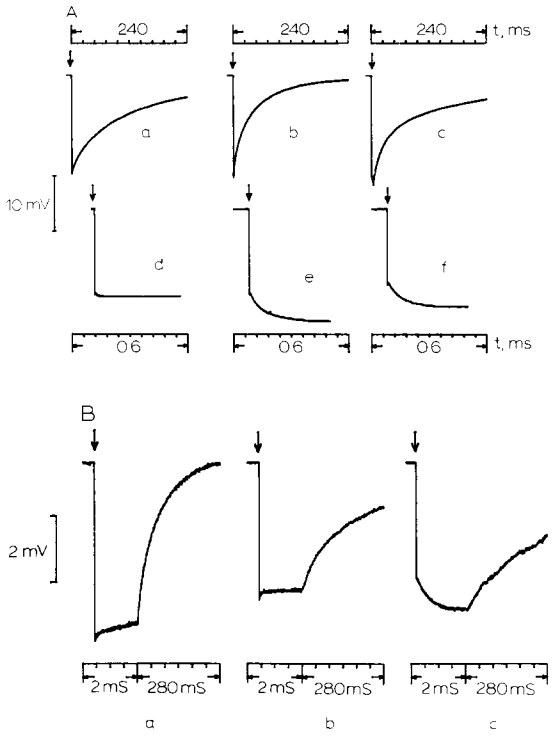


Fig. 4. Cytochrome *c*-induced slow phase of $\Delta\psi$ generation in the antenna-free RC-proteoliposomes. (A) CoQ_{10} (30 mg/ml) was added to the decane solution of phospholipids used to impregnate the collodion film. (a, d), No other additions; (b), +1 mM *o*-phenanthroline; (c, e), +2 mM ascorbate and 2 μM cytochrome *c*; (f), as (e), +1 mM *o*-phenanthroline. (B) Experiments in the absence of added CoQ_{10}. Hepes concentration was reduced to 1 mM (pH 7.5) to decrease ionic strength. (a), No other additions; (b), +2 mM ascorbate, 10 μM PMS and 100 μM menadion (2-methyl-1,4-naphthoquinone); (c), as (b) +0.1 μM cytochrome *c*. Menadion was included in the medium in this series of experiments to promote regeneration of the oxidized form of Q_A. Control experiments showed that 10^{-4} M menadion does not affect the kinetics of $\Delta\psi$ generation or decay, since Q_A^- reoxidation requires tens of seconds under these conditions. Different amplitudes of the photoelectric responses in A and B are due to different preparations of proteoliposomes used in the two experiments.

due not only to an increase in the slow phase contribution, but also to a significant increase in the τ of the slow phase (up to approx. 2s). Evidently, cytochrome c^{2+} rapidly reduces P-870$^+$ and hence prevents a discharge of both the primary and the secondary dipoles; under these conditions, the kinetics of $\Delta\psi$ decay is likely to be determined by a time constant of passive discharge of the proteoliposome membrane [10], which is typically 1–2 s for the protoliposome/collodion film system.

Our interpretation of the effects of cytochrome *c* and CoQ_{10} on the kinetics of $\Delta\psi$ decay in the RC-containing proteoliposomes is further substantiated by the data given in Fig. 4A.

One can see that the slow phase of $\Delta\psi$ decay caused by CoQ_{10} addition to the collodion-film impregnating lipid (trace a) is abolished by *o*-phenanthroline, a classical inhibitor of $Q_A \rightarrow Q_B$ electron transfer (trace b) [21,22]. At the same time, addition of cytochrome *c* after *o*-phenanthroline results in a strong deceleration of $\Delta\psi$ decay, bringing back a slow phase of membrane potential dissipation (trace c).

Expanded recordings of the photoelectric response kinetics in Fig. 4 (traces d, e and f) show that cytochrome *c* affects not only the $\Delta\psi$ decay, but also the kinetics of the $\Delta\psi$ generation. In addition to the very fast (less than 200 ns) stage of the photoelectric response (trace a), a slower phase appears on the sub-millisecond time-scale (trace e). Oxidized cytochrome *c* did not elicit such an effect (not shown). This new phase of $\Delta\psi$ generation is not sensitive to *o*-phenanthroline (trace f) and can be observed in the absence of added CoQ_{10} (data not included, see also Fig. 4B). Hence it can be assigned to $c^{2+} \rightarrow$ P-870$^+$ electron transfer. The electrogenic nature of this electron-transfer step was reported earlier in Refs. 4, 8, 23 and 24 for *Rps. sphaeroides* reaction centres and in Ref. 25 for *Chromatium vinosum*. Computer analysis of the kinetics of photoelectric responses shows that the amplitude of the additional phase of $\Delta\psi$ generation comprises up to approx. $\frac{1}{4}$ of the total amplitude of the response. A similar observation on the electrogenic cytochrome *c*-dependent phase of $\Delta\psi$ generation in chromatophores of *Ectothiorhodospira shaposhnikovii* (recently identified as *Ectothiorhodospira mobilis* [26]) and *Chromatium minutissimum* was recently made in this laboratory [6,27].

Redox mediators like PMS, TMPD or DAD at moderately high concentrations were capable of rapid re-reduction of photooxidized P-870$^+$, as evidenced by their retarding effect on the decay of $\Delta\psi$ (e.g., compare traces a and b in Fig. 4B, and see Refs. 20 and 28 for detailed quantitative stud-

ies), but did not give rise to a measurable additional electrogenic phase * unless cytochrome *c* was added (Fig. 4B, trace c). The rapid phase of $\Delta\psi$ decay abolished by redox-mediators allows for a better resolution of the slower electrogenic phase at low concentrations of cytochrome *c*. Titrations of the photoelectric response with cytochrome *c* were then carried out in the presence of either 10 μM phenazine methosulfate (Fig. 5) or 0.5 mM TMPD (data not shown). Very similar results were obtained in both cases. The amplitude of the slow phase changed only slightly throughout the cytochrome *c* concentration range studied (e.g., it comprised 16% of the total response at $c = 0.3$ μM and 22% at $c = 10$ μM for the experiment given in Fig. 5; as a matter of fact, even this 30% difference is most likely apparent, since the computer analysis tends to underestimate the amplitude of the phase as the latter strongly decelerates at cytochrome *c* concentrations below approx. 0.5 μM). At the same time, the rate of the slow phase was found to be strongly dependent on cytochrome *c* concentration. A typical titration curve of the first-order rate constant of the slow phase is given in Fig. 5 by the upper curve and shows a saturation behaviour.

Linearization of the data allows to approximate the cytochrome *c* concentration dependence of the slow phase rate by a saturating process with apparent K_M of 0.9 μM and k_{max} of $6 \cdot 10^3$ s^{-1}. The former value is close to K_d of horse heart cytochrome *c* interaction with *Rps. sphaeroides* reaction centres at low ionic strength in solution [29] and in liposomes [30], as measured spectrophotometrically. Also the rate constant values of the slow electrogenic phase in the [*c*] range studied are in reasonable agreement with these for horse heart ferrocytochrome *c* oxidation by photogenerated P-870$^+$ in solution [31–34]. At given concentration of cytochrome *c*, the rate of the slow phase greatly decreased upon ionic strength increase (e.g., see Fig. 5) or upon addition of divalent cations (not shown). These effects could be reversed by excess cytochrome *c*.

At 2 μM cytochrome *c*, the rate constant of the slow phase was found to decrease approx. 3-fold as the temperature was lowered from 21°C to 6°C. Preliminary estimates give a value of 54 kJ/mol for activation energy of the reaction, which is in accordance with the value of 50 kJ/mol as measured in Ref. 35 for the reaction of horse heart cytochrome c^{2+} with solubilized RC from *Rps. sphaeroides*.

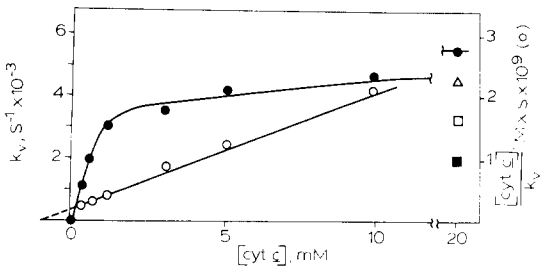


Fig. 5. Effect of cytochrome *c* concentration on the first-order rate constant of the slow phase of photoelectric response of antenna-free RC-proteoliposomes. The medium contained 1 mM Hepes (pH 7.5)/0.5 mM ascorbate/10 mM PMS/50 μM menadion and increasing concentrations of cytochrome *c* (●, ○). NaCl was added (symbols △, □ and ■) at concentration of 10, 20 and 40 mM, respectively. Closed symbols give k_v values (the left-hand ordinate); open circles show linearization of the data (●) (the right-hand ordinate).

All these data are in good agreement with the slow phase of $\Delta\psi$ generation being due to a second-order reaction between the soluble cytochrome c^{2+} and P-870$^+$. The 5–20 μs reaction typical of the oxidation of cytochromes *c* and c_2 pre-bound to RC [35–38] has not so far been observed under our experimental conditions.

Discussion

Earlier investigations carried out in our group have established unequivocally that the very rapid phase of laser flash-induced membrane potential generation by the photosynthetic electron-transfer chain which takes less than 200 ns (the rise-time of the apparatus), is due to electron transfer from P-870 to Q_A [5,6]. The observation on the opposite polarity of this phase in the chromatophores and the antenna-containing RC-proteoliposomes on the one hand (positive inside the vesicles) and in the antenna-free RC-proteoliposomes on the other (negative inside the vesicles) strongly indicates that the vectorially oriented primary dipole P-870$^+$. Q_A^-

* Actually, the mediators do induce the additional electrogenic phase (Ref. 28, and see also below Fig. 6), but this phase is too slow and difficult to resolve at [TMPD] $\lesssim$ 1 mM or [PMS] $\lesssim$ 20 μM.

is oriented upside down in the antenna-free RC-proteoliposomes as compared to the other two types of vesicle. This conclusion is corroborated by the evidence to the effect that in the antenna-free RC-proteoliposomes, photooxidized P-870$^+$ is reduced very rapidly by membrane-impermeable positively charged electron donors like hexammineruthenium and cytochrome c^{2+}, whereas in the chromatophores and the antenna-containing RC-proteoliposomes, reaction centre bacteriochlorophyll is not accessible to these reductants.

Remarkably, ascorbate, which is thermodynamically a stronger reductant than either hexammineruthenium or cytochrome *c*, reacts very slowly with the externally localized P-870$^+$. That there is a negative charge in the vicinity of P-870, hindering its reactivity towards anionic electron donors, or neutral reductants which yield negatively charged intermediates upon oxidation (e.g., semiquinone anions in case of quinols) was suggested earlier on the basis of the studies on the interaction of *R. rubrum* chromatophores with artificial electron donors and acceptors [16,17]. The same conclusion was inferred from experiments on the kinetics of cytochrome *c* oxidation by isolated *R. rubrum* recent centres [33]. The difference in the activities of anionic ascorbate and cationic hexammineruthenium as electron donors to P-870$^+$ in the antenna-free RC-proteoliposomes is in agreement with this suggestion. As proposed in Ref. 16, the charge-gating mechanism might serve to prevent direct oxidation of ubiquinol by reaction centres, which would otherwise short-circuit the Q-cycle.

The accessibility of P-870 from the outer aqueous phase makes the antenna-free RC-proteoliposomes an ideal model for the studies of electrogenic events coupled to RC interaction with cytochrome *c*. In particular, this reaction is difficult to investigate in the chromatophores of *R. rubrum* which loose most of the endogenous cytochrome c_2 during the isolation procedure [39].

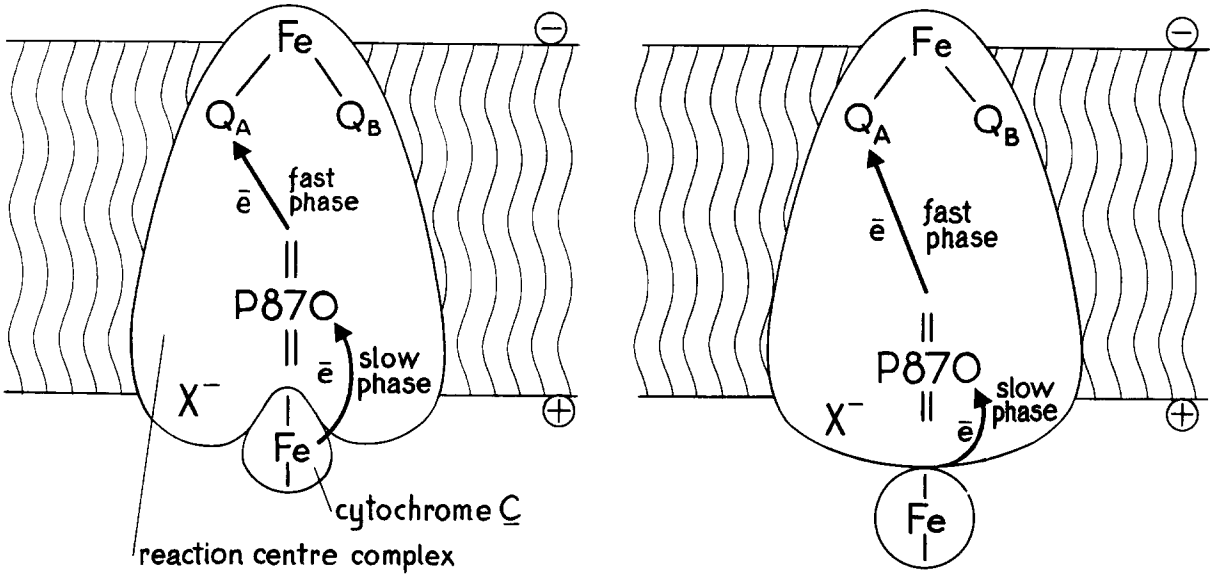
It is important that the excellent signal-to-noise ratio of the direct electrometric $\Delta\psi$ assay allows to monitor single turnover kinetics of the $\Delta\psi$ generation and decay without need for averaging data [5,6,20]. As described above, by using this method we were able to resolve and characterize in some detail a slow electrogenic phase, originating upon horse-heart cytochrome *c* addition to the inside-out RC-proteoliposomes in the presence of ascorbate. There is a substantial body of evidence that this phase observed earlier in Ref. 8 is coupled to P-870$^+$ reduction by cytochrome c^{2+}, rather than to any e^- or H^+-transfer events on the acceptor side of the photoreaction. Indeed (i) oxidized cytochrome *c* did not induce the effect; (ii) the slow phase could be observed under the conditions in which $Q_A \rightarrow Q_B$ electron transfer was not operative (i.e., in the collodion film-associated RC-proteoliposomes in the absence of added CoQ or in the CoQ-replenished system inhibited by *o*-phenanthroline); and (iii) the slow-phase kinetics is similar to those characteristic of the reaction between cytochrome c^{2+} and P-870$^+$. It has to be pointed out that according to the present observations, the contribution of the $c^{2+} \rightarrow$ P-870$^+$ electron transfer to $\Delta\psi$ generation is only approx. 1/3 that of the P-870 $\rightarrow Q_A$ step, which is much less than suggested in Refs. 8 and 24, where P-870 was assumed to be localized 'electrically' about midway from c_2 to Q_A.

The dependence of the slow phase rate rather than of its amplitude on ferrocytochrome *c* concentration indicates that it is a second-order reaction of P-870$^+$ with the soluble or, probably, with the proteoliposome membrane-adsorbed (cf. Ref. 37) cytochrome that initiates the electrogenic step. No evidence for the very rapid first order reaction between c^{2+} and P-870$^+$ expected for the electron transfer within the preformed complex c^{2+}-P-870 [35–38] could be obtained in the cytochrome *c* concentration range studied.

However, formation of a transient complex c^{2+}-P-870$^+$ in the bimolecular reaction pathway can be envisaged. An interesting question then arises (considered also in Ref. 8), whether: (i) the charge separation displayed by the slow phase is confined to electron transfer from the electrolyte-accessible cytochrome *c*-reactive site of RC-surface to $BChl_2$ (Scheme IB); or (ii) cytochrome *c* bound to RC forms an integral electrically isolated system with the P-870 protein environment so that the charge separation takes place across the whole distance between the central Fe atom of the heme and the Mg-porphyrin ring of P-870 and, hence, resides partially in cytochrome *c* itself (Scheme IA). In the latter case, dissociation of the cytochrome *c* molecule oxidized by P-870$^+$ from the binding site

would partially discharge $\Delta\psi$. Unfortunately, we have no data at hand on the ferric horse heart cytochrome *c* binding to *R. rubrum* reaction centres and it is difficult to evaluate the life-time of this complex.

Finally, is the slow phase of a $\Delta\psi$ generation specific for cytochrome c^{2+} as an electron donor to P-870$^+$? Before answering this question, it has to be noted that cytochrome *c* at concentrations as low as 1 μM reacts with P-870$^+$ still much more rapidly than sub-millimolar concentrations of artificial redox dyes like TMPD or DAD (cf. Refs. 20 and 28). This can form the basis for the apparent specificity of the electrogenic reaction, since upon deceleration of the slow phase it tends to be obscured by $\Delta\psi$-dissipation processes (as, for instance, it has been observed in this work at very low cytochrome-*c* concentrations or in high ionic strength experiments (data not shown). Indeed, in the preliminary experiments we were able to observe an additional electrogenic phase with τ of several ms even in the absence of added cytochrome *c* when very high concentrations of redox mediators were used, e.g., at least 2 mM TMPD or at least 50 μM PMS (Fig. 6). This observation strongly favours localization of the electron-transfer process underlying the electrogenic slow phase solely within the reaction centre molecule (Scheme IB) rather than within the cytochrome *c*-P-870 complex (Scheme IA) as also suggested in Ref. 8.



Scheme I. Two possible mechanisms of the cytochrome *c*-dependent slow phase of $\Delta\psi$ generation. In A, cytochrome c^{2+} when bound to RC, is integrated into the solvent-inaccessible dielectric phase of the P-870 environment. Consequently, the entire process of e^- transfer from the heme iron to P-870$^+$ is electrogenic, and at least part of this electrogenic phase can be envisaged to dissipate upon dissociation of cytochrome c^{3+} from the complex. In B, cytochrome c^{2+} serves merely as an electron donor, and the zone of redox contact between the hemoprotein and RC complex is not shielded from electrolyte. The slow electrogenic phase should then be confined to electron transfer within RC-complex molecule from its surface to the protein-embedded bacteriochlorophyll Mg-porphyrin ring. Version B of the scheme implies that the slow phase of $\Delta\psi$ generation may not be absolutely specific for cytochrome *c* as electron donor and, under appropriate conditions, would be observed with artificial redox dyes as well, which is indeed the case (see the text). A fixed negatively charged group is drawn tentatively near P-870 in order to account for the low reactivity of the latter with electron donors which are negatively charged or form negatively charged intermediates upon oxidation (see the text and see also Refs. 16 and 17).

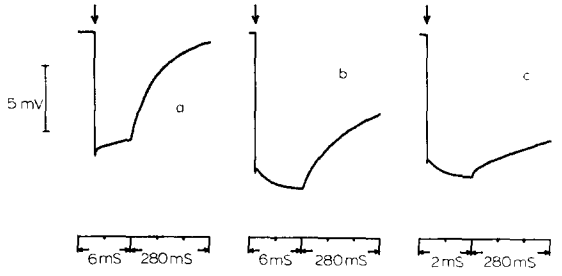


Fig. 6. Induction of the slow-phase of $\Delta\psi$ generation in reaction centre-proteoliposomes by *N,N,N',N'*-tetramethyl-*p* phenylenediamine and phenazine methosulfate. Incubation medium contained 20 mM Hepes (pH 7.5). (a), No additions; (b), +10 mM ascorbate, 2 mM TMPD and 100 μM menadion; (c), +2 mM ascorbate, 150 μM PMS and 100 μM menadion.

A detailed account on the electrogenic reduction of P-870$^+$ by artificial redox dyes will be given presently in a separate publication [28]. Here, we would note that the precise localization of P-870 in the *R. rubrum* RC complex has not been established. However, from the crystallographic data on the RC of *Rps. viridis* [40] and preliminary results on the topography of RC from *rubrum* [41] and *Chromatium vinosum* [42] it follows that the pigment Mg-porphyrin rings can be well below the protein surface (e.g., within approx. 1.0–1.5 nm from the aqueous phase charging positive upon illumination). Therefore, it is likely that electron transfer from exogenous donors to $BChl_2$ has to be electrogenic regardless of the nature of the donor and of the reaction mechanism which could be physical tunnelling or protein-mediated conduction. Conceivably, much more experiments is required to establish whether the cytochrome *c*- and artificial redox dye-dependent electrogenic phases are equivalent.

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