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**Demonstration of a collisional interaction of ubiquinol  
with the ubiquinol-cytochrome  $c_2$  oxidoreductase complex in chromatophores  
from *Rhodobacter sphaeroides***

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(1) Ubiquinone-10 can be extracted from lyophilized chromatophores of *Rhodobacter sphaeroides* (previously called *Rhodopseudomonas sphaeroides*) without significant losses in other components of the electron-transfer chain or irreversible damages in the membrane structure. The pool of ubiquinone can be restored with exogenous UQ-10 to sizes larger than the ones in unextracted membranes. (2) The decrease in the pool size has marked effects on the kinetics of reduction of cytochrome  $b$ -561 induced by a single flash of light and measured in the presence of antimycin. The initial rate of reduction, which in unextracted preparations increases on reduction of the suspension over the  $E_h$  range between 170 and 100 mV (pH 7), is also stimulated in partially UQ-depleted membranes, although at more negative  $E_h$ 's. When the UQ pool is completely extracted the rate of cytochrome (Cyt)  $b$ -561 reduction is low and unaffected by the redox potential. (3) In membranes enriched in UQ-10 above the physiological level the titration curve of the rate of Cyt  $b$ -561 reduction is displaced to  $E_h$  values more positive than in controls. This effect is saturated when the size of the UQ pool is about 2–3-times larger than the native one. (4) The reduction of Cyt  $b$ -561 always occurs a short time after the flash is fired; also the duration of this lag is dependent on  $E_h$  and on the size of the UQ pool. A decrease or an increase in the pool size causes a displacement of the titration curve of the lag to more negative or to more positive  $E_h$ 's, respectively. Similarly, the lag becomes  $E_h$  independent and markedly longer than in controls when the pool is completely extracted. (5) These results demonstrate that the rate of turnover of the ubiquinol oxidizing site in the  $b$ - $c_1$  complex depends on the actual concentration of ubiquinol present in the membrane and that ubiquinol from the pool is oxidized at this site with a collisional mechanism. Kinetic analysis of the data indicates that this reaction obeys a Michaelis–Menten type equation, with a  $K_m$  of 3–5 ubiquinol molecules per reaction center.

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Abbreviations: BChl, bacteriochlorophyll; Cyt, cytochrome; DAD, 2,3,5,6-tetramethyl- $p$ -phenylenediamine; Mops, 4-mor-

pholineethanesulphonic acid; RC, reaction center; UQ, ubiquinone; UQH<sub>2</sub>, ubiquinol.

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## Introduction

Chromatophores from the photosynthetic bacterium *Rhodobacter sphaeroides* (formerly called *Rhodospseudomonas sphaeroides*) contain a cyclic electron-transfer system formed by a reaction center complex and a ubiquinol-cytochrome  $c_2$  oxidoreductase ( $b$ - $c_1$  complex) [1,2]. The redox interaction between these two functional multi-enzyme systems is mediated by a pool of ubiquinone (30–60 mol per mole  $b$ - $c_1$  complex) and by cytochrome  $c_2$  [3–6]. Although the role of ubiquinone in electron transfer has been discussed mainly in terms of ubiquinone bound to specific sites, and therefore distinct from the pool and catalyzing specific steps in the photosynthetic chain, a more recent model has proposed a collisional mechanism (a modified Q-cycle) for the interaction of ubiquinone molecules of the pool with the  $b$ - $c_1$  complex [7].

Several experimental approaches have been already utilized to test the validity of the modified Q-cycle model. The concentration of  $\text{UQH}_2$  in the pool has been controlled by poisoning the ambient redox potential, and its effect on the reaction rate at the oxidase site of the  $b$ - $c_1$  complex (measured as the rate of reduction of Cyt  $b$ -561 induced by a single turnover flash in the presence of antimycin) has been studied. At low concentrations of quinol the reaction rate has been shown to follow a second-order kinetics with respect to the oxidized complex and to the concentration of ubiquinol present in the membrane immediately after the flash; the rate saturates when a portion of the UQ pool is pre-reduced before the flash [7,8].

The rate of rereduction of RC and Cyt ( $c_1 + c_2$ ) was found to be decreased as a consequence of fusion of chromatophores with liposomes [9]. A fast electron transfer through the  $bc_1$  complex could be restored when the concentration of the UQ pool within the lipid bilayer was reconstituted by additions of exogenous ubiquinone.

The kinetics of Cyt  $b$ -561 reduction responded to the varying UQ concentration in the way predicted by the model in phospholipid enriched membranes obtained (a) through fusion of chromatophores with liposomes and (b) utilizing synchronized cells where the total UQ/lipid ratio changes by a factor of two during the cell cycle. In

the latter case a quantitative demonstration of the model was reached [8].

A possible third approach for a quantitative test of the model is to modulate the size of the UQ pool by selectively extracting quinones from dehydrated chromatophores with isooctane. Bacterial chromatophores have been proved to withstand lyophilization and extraction without losing irreversibly their capability in electron transfer, energy conservation and photophosphorylation [6, 10–14]. The pool size could be reduced to a few UQ/RC without markedly decreasing the rate of reduction of photooxidized Cyt  $c$  and of the electrogenic events associated to the secondary electron transport. This was taken as one of the lines of evidence for the presence of a tightly bound quinone at the quinol oxidizing site of the  $b$ - $c_1$  complex ( $Q_Z$ ) [15]. In the same kind of preparations, we observed that when the pool was extensively extracted Cyt  $b$ -561 was still photoreduced in the presence of antimycin. The binary pattern of reduction as a function of flash number showed that the quinol available to the  $b$ - $c_1$  complex at high redox potential was delivered by the reaction center, but the rate of reduction failed to increase on lowering the ambient redox potential, as it is otherwise in controls [14]. This behaviour would be consistent with the expectations of the collisional model after complete extraction of the UQ pool, and would not require any bound quinone.

In this paper the technique of lyophilization, UQ extraction, reconstitution and enrichment has been extensively utilized for a detailed study of the kinetics of reduction of Cyt  $b$ -561 as a function of the size of the UQ pool. The results obtained are largely consistent with the collisional mechanism proposed.

## Materials and Methods

### Cell culturing and preparation of chromatophores

*Rhodobacter sphaeroides*, strain Ga, was grown at 30°C with the medium described in Ref. 16, in Roux bottles of 1 l, at a light intensity of approx. 20 W · m<sup>2</sup>. Cells were harvested after 36–48 h, at  $A_{660\text{ nm}} = 3$ –4, and were washed with 50 mM Mops (pH 7.0). Chromatophores were prepared by disrupting the cells in a French press at 98 MPa and by differential centrifugation as described in Ref.

17. Occasionally, in order to obtain a more homogeneous culture condition, the cells were grown in a 12 l fermentor (Microferm, New Brunswick Sci. Co., NJ), under a slight nitrogen pressure. It was illuminated by three reflector 150 W incandescent lamps placed at 10 cm from the vessel surface. These conditions of culture, harvesting at 1.4 A at 660 nm, affected the UQ content of the chromatophores (16 UQ per reaction center as compared to the 24–25 in chromatophores prepared from normal batch culture).

*Lyophilization, extraction of ubiquinone and reconstitution*

The chromatophores to be lyophilized were suspended in 50 mM Mops (pH 7.0), frozen in liquid nitrogen, lyophilized for 12–18 h at  $-10^{\circ}\text{C}$  and stored at  $-20^{\circ}\text{C}$  in a desiccator in the vacuum.

For extraction of the ubiquinone, the lyophilized powder was finely subdivided and suspended in isooctane at a BChl concentration of 0.3 mg/ml. The suspension was stirred at  $4^{\circ}\text{C}$  for a set of times and the extracted chromatophores collected by low-speed centrifugation. The residual solvent in the pellet was thoroughly evaporated with a stream of nitrogen.

For reconstituting (or enriching) with exogenous ubiquinone-10, an amount of dehydrated extracted (or native) chromatophores (0.5–2 mg BChl) was mixed with 0.1–0.5 ml of an isooctane solution containing an amount of pure ubiquinone-10, equivalent to 3–100-fold the UQ complement of the total mass of chromatophores to be treated. The mixture was incubated for 5–20 min at  $4^{\circ}\text{C}$  and the solvent evaporated with a nitrogen stream. At least 1 h before the spectrophotometric measurements, an aliquot of dehydrated membranes was gently homogenized in a small volume of  $\text{N}_2$ -gassed 50 mM Mops/100 mM KCl (pH 7). The suspension was centrifuged for 2 min at  $600 \times g$  to remove poorly resuspended material, and the supernatant utilized as such for spectrophotometric measurements. In the case of UQ enrichment the excess unabsorbed UQ was carefully removed by a centrifugation at  $200\,000 \times g$  through a sucrose cushion (20% w/v) in Mops/KCl buffer.

*Spectrophotometric measurements*

Redox-controlled spectrophotometric measure-

ments were performed essentially as described in Ref. 18. The platinum electrode was carefully cleaned before each titration by a thoroughly polishing with a cotton pad soaked with 32% ammonia. The assay mixture was the same as in Ref. 8. The spectrophotometric signals were recorded using a single beam kinetic spectrophotometer (time resolution, 0.1  $\mu\text{s}$ ; bandwidth, less than 2 nm) interfaced with a DataLab (DL905) transient recorder and with a Tektronix 4051 computer. During averaging 60 s were allowed between each measurement for complete recovery of light-induced redox changes. Actinic flashes of light, 15  $\mu\text{s}$  halfwidth, were provided by a xenon lamp (3.2 J, discharge energy) screened through two layers of Wratten 88A gelatin filter; the photomultiplier was protected by a Corning 4/96 glass filter. The reaction center concentration was adjusted to give a flash saturation of 90%.

Cyt *b*-561 and Cyt ( $c_1 + c_2$ ) were measured at 561–569 nm and 551–542 nm, respectively, using an extinction coefficient of  $19.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  for both of them [7,18,19]. The total concentration of photooxidizable reaction centers was measured from the change of absorbance at 542 nm, induced by a train of eight flashes, 20 ms apart, at  $E_h = 160 \text{ mV}$  using an extinction coefficient of  $10.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  [18,19]. Total photooxidizable Cyt ( $c_1 + c_2$ ) and photoreducible Cyt *b*-561 were measured following an analogous procedure. A test for the presence of ubiquinone able to act as secondary acceptor ( $\text{Q}_B$ ) in the reaction center complex, was routinely performed in UQ extracted preparations by analyzing the kinetics of charge recombination after a single flash, in the presence of antimycin, at  $E_h = 420 \text{ mV}$ , i.e., when no electron transfer from Cyt  $c_2$  to the oxidized RC could occur [20,14].

*Analytical techniques*

The total membrane UQ content was determined by exhaustive extraction and reversed phase HPLC analysis, essentially as described in Ref. 21. An aliquot of chromatophores (0.5 mg BChl or less) suspended in 0.5 ml of Mops/KCl buffer, was extracted three times with a mixture of methanol-petroleum ether (6 ml/4 ml). The ether phases were pooled, evaporated to dryness and the residue dissolved in HPLC grade ethanol. The

HPLC elution was monitored at 280 nm. The mobile phase contained 995 ml HPLC grade methanol/1.2 ml 60%  $\text{HClO}_4$ /5 ml  $\text{H}_2\text{O}$ /7 g  $\text{NaClO}_4 \cdot \text{H}_2\text{O}$ /0.21 g citric acid. By this method oxidized UQ-10 could be clearly separated from the other components present in the extracts (Fig. 1A and 1B). The peak heights of pure UQ-10 elution profile appeared to vary linearly with concentration up to 3 nmol UQ-10 per sample (10  $\mu\text{l}$  effective injection in the HPLC column) and have been used to calibrate the chromatograms of the extracts. The sensitivity of the method allowed the detection of values as low as 0.5 UQ/RC.

Bacteriochlorophyll content was determined in acetone-methanol extracts according to Clayton [22]. The neurosporene and chloroxanthin carotenoids present in this strain were estimated spectrophotometrically at 438 nm in *n*-hexane ( $E = 157 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  [23,24]).

In order to evaluate the total lipid content of chromatophores, the extraction procedure de-

scribed in Ref. 25 was utilized. The water-methanol phase was extracted two times at room temperature; the chlorophorm phases were pooled and washed with one volume of 1% KCl solution. Thin layer chromatography (TLC) of the total lipid extracts and of the total residue of the isooctane supernatant from UQ extraction was performed at room temperature on silica gel plates, Merk N.11.845. Chlorophorm/methanol/water, 65:25:4 (v/v) was used as the developing solvent for polar lipids [26] and petroleum ether (40–70°C)/diethyl ether/acetic acid, 70:30:2 (v/v) for the non polar components [27]. The different lipid fractions in the TLC plates were detected with potassium dichromate in sulfuric acid, iodine vapor, ammonium molybdate-perchloric acid and ninhydrin [27].

For the gas chromatography determination of the fatty acids associated with the phospholipids, approx. 1 mg of the residue of the total lipid and isooctane extracts was transmethylated according to Metcalfe et al. [28] by treatment with methanolic  $\text{BF}_3$ . Methyl-*n*-heneicosanoate ( $\text{C}_{21}$ ) was used as internal standard before saponification.

## Results

### *Characterization of ubiquinone-extracted and enriched chromatophores*

In Fig. 2A the time course of UQ extraction by isooctane in a suspension of lyophilized chromatophores is compared with that of carotenoid depletion. The amount of total carotenoid in this chromatophore preparation was 0.48 mol per mol BChl (cf. Ref. 29). About 85% of the total UQ complement appears to be extracted with a half-time of approx. 5 min, while the remaining appears to be bound more tightly and is extracted with a half-time of approx. 170 min. The loss in carotenoids is much more limited and never exceeds about 30% of the original content even after one hour of extraction.

Polar lipids [26] were not detectable on TLC plates when isooctane extracts were compared with methanol-chloroform extracts containing the total lipid complement. The detection limit of the analysis was estimated to be less than 0.5% of each of the components of the total polar lipid complement. The amount of polar lipid extracted by

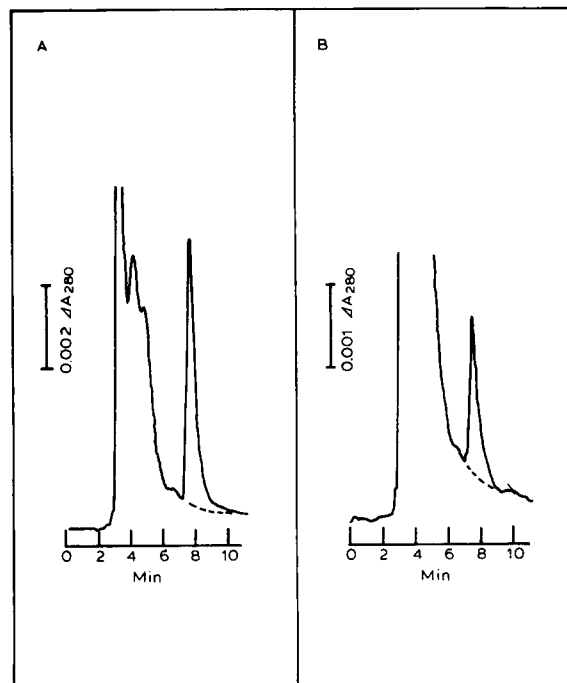


Fig. 1. Determination of the UQ-10 content of chromatophores by HPLC. (A) Chromatogram of the extract from lyophilized control chromatophores (24 UQ/RC). (B) Same from partially UQ-extracted vesicles (7 UQ/RC).

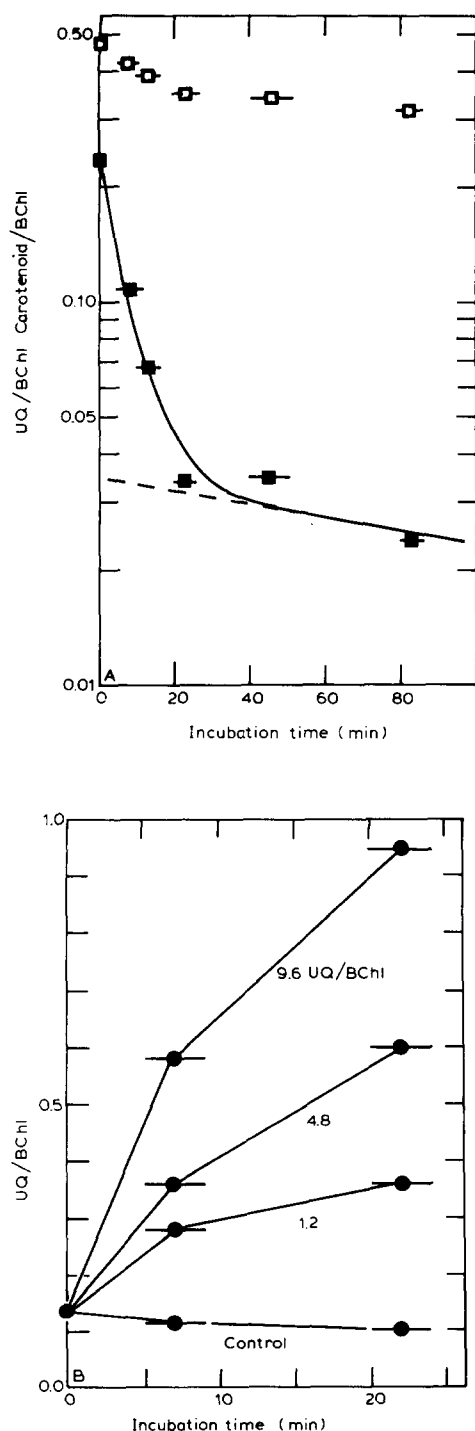


Fig. 2. (A) Time-course of UQ (■) and carotenoid (□) extraction by isooctane in lyophilized chromatophores (0.3 mg BChl/ml). Carotenoids were estimated spectrophotometrically in the extracts utilized for the HPLC analysis of UQ, kept in the dark and using *n*-hexane as solvent. Horizontal bars indi-

isooctane is therefore negligible. This conclusion is supported by the result of the analysis of fatty acid methyl esters, which indicated that the isooctane extracts contained less than 1% of the total content in *cis*-vaccenic acid, the predominant acyl residue of the chromatophore polar lipids [30].

The only apolar component migrating differently from carotenoids, ubiquinol and ubiquinone, detected in the isooctane extracts was characterized by  $R_f = 0.48$ . The staining intensity of this component suggests, however, that it is present at low concentration in the membrane. This component is partially extracted already after 10 min of incubation with isooctane and is almost completely removed after 60 min of incubation. Both the nature and the possible relevance of the extraction of apolar components different from ubiquinone remains to be established.

Less than 0.01% of the total BChl is removed by isooctane extraction after 60 min of incubation. This was also reflected in the constant size of the photosynthetic unit in differently extracted preparations, which was found always to be approximately 120 BChl/RC. Likewise constant was the amount of the cytochrome components of the electron-transfer chain, measured on the basis of their photoinduced redox changes. The amount of Cyt ( $c_1 + c_2$ ) was generally between 0.7 and 0.9 mol per mol RC being only marginally affected by lyophilization and extraction. The total amount of Cyt *b*-561 reduced by a train of eight flashes in the presence of antimycin, was always between 0.6 and 0.5 mol per mol RC. This cytochrome titrated out with a midpoint potential of  $55 \pm 10$  mV at pH 7, in line with previously reported data [31],

cate the time elapsed between the end of incubation with isooctane and the final separation of the supernatant after centrifugation. Experimental points for UQ extraction are fitted by the sum of two exponential decays. (B) Time-course of the incorporation of UQ-10 into lyophilized unextracted chromatophores. The UQ/BChl indicated for each curve corresponds to the amount added as isooctane solution (150  $\mu$ l) to lyophilized chromatophores equivalent to 0.55  $\mu$ mol BChl. The ordinate gives the amount of UQ/BChl retained in the membrane after centrifugation on a sucrose cushion. As the control, chromatophores were suspended in an equal volume of pure isooctane. Horizontal bars correspond to the time required for a thorough evaporation of the solvent under nitrogen stream. The chromatophore preparation is the same as in the experiments of Fig. 6.

both in lyophilized, extracted or UQ-enriched vesicles, as a proof of the lack of any functional damage to this component of the  $bc_1$  complex. The maximal extent of Cyt ( $c_1 + c_2$ ) photo-oxidized and of Cyt  $b$ -561 photoreduced by a train of flashes was found to be decreased only in drastically extracted particles where  $Q_B$  was partially removed. In summary these data confirm that a normal  $bc_1$  complex is generally present in the extracted preparations, in a molar ratio approaching 1:2 with respect to the reaction center, and functions normally unless  $Q_B$  is extracted.

About 6-fold more UQ-10 than the normal endogenous content can be incorporated in dehydrated unextracted chromatophores when they are incubated with a large excess of UQ-10 dissolved in isooctane. This is a time- and concentration-dependent process (Fig. 2B). A further support of an actual UQ-10 incorporation is given by the results of isopicnic centrifugation on a linear sucrose gradient (26–38% w/w), which indicated that chromatophores, containing an amount of ubiquinone 3-times larger than the endogenous one, were characterized by a density of 1.1425 g/cm<sup>3</sup>, lower than the density of control chromatophores, i.e. native, lyophilized and treated with pure isooctane as described in the legend of Fig. 2B (1.1475 g/cm<sup>3</sup>, cf. Ref. 32).

#### *The effect of the size of the ubiquinone pool on the kinetics of Cyt $b$ -561 reduction*

In Fig. 3 typical traces for Cyt  $b$ -561 redox changes induced by a single 15- $\mu$ s long flash in the presence of antimycin are shown. According to a Q-cycle scheme, this process corresponds to the transfer to Cyt  $b$ -561 of one reducing equivalent from ubiquinol oxidized at the  $Q_Z$  site by the high and low potential chains of the  $bc_1$  complex in a concerted reaction [7]. In the presence of antimycin, Cyt  $b$ -561 becomes reduced after a flash following a short lag. Both the initial reduction rate and the duration of this lag are affected by the ambient redox potential. The effects of the size of the quinone pool on the titration of the rate and of the lag will be discussed separately in the following sections.

#### *Effects on the initial rate*

Fig. 3A shows the rate of reduction of Cyt

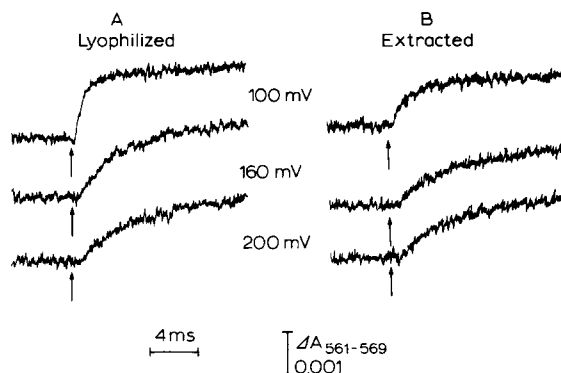


Fig. 3. Kinetic traces of Cyt  $b$ -561 reduction induced by a single turnover flash at three different ambient redox potentials (200, 160 and 100 mV). (A) Control lyophilized chromatophores, characterized by 24 UQ/RC. (B) Partially UQ-extracted chromatophores (8 residual UQ/RC). The assay conditions are described under Materials and Methods. Photo-oxidized reaction center concentration was 0.28  $\mu$ M for control and 0.25  $\mu$ M in partially UQ-extracted chromatophores. Traces are the average of eight measurements, with a sweep of 20 ms and a time constant of 20  $\mu$ s.

$b$ -561 in control lyophilized chromatophores (24 UQ/RC). The traces exhibit the kinetic characteristics previously described in fresh chromatophores which had not undergone the process of lyophilization [33,7]: at  $E_h = 200$  mV, when the quinone pool is completely oxidized before the flash, the initial rate of reduction is slow; at  $E_h = 160$  mV, when the pool is reduced by 0.5%, a slight stimulation in the rate is observed; at  $E_h = 100$  mV the rate of reduction is maximum and no further increase can be detected at lower redox potentials (see below). In partially extracted chromatophores, whereas the kinetics is not affected at  $E_h = 200$  mV, the redox dependent stimulation is small and observed only at relatively lower  $E_h$ 's (Fig. 3B).

The increase in rate observed upon lowering the redox potential has been interpreted as due to the presence in the membrane of pre-reduced quinone from the pool, which interacts collisionally with the oxidizing site ( $Q_Z$  site) [7]. The data of Fig. 3 demonstrate that, as expected, the size of the UQ pool has an effect on the rate, when measured at the appropriate redox poise (cf. Ref. 15).

A full titration of this phenomenon is presented

in Figs. 4 and 5 for different membrane preparations. In membranes containing 24–25 UQ/RC the rate measured at  $E_h = 200$  mV is stimulated 6–8-fold when the  $E_h$  is decreased to 100 mV; the onset of this stimulation is already detectable at  $E_h = 170$  mV. A comparison between untreated and lyophilized chromatophores, indicated that lyophilization alone had no significant effect on this kinetics (Fig. 4, curve a). On the other hand, partial extraction of the UQ pool markedly displaced the titration curve. When the size of the pool is approximately halved (14 Q per RC, Fig. 4, curve b) the stimulation of the rate upon decreasing the redox potential can be observed only at  $E_h$  lower than 150 mV; the maximal observed rate is reached around 90 mV and is slightly decreased

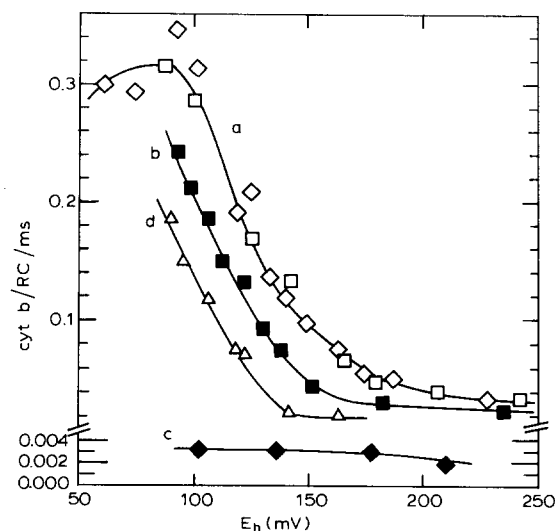


Fig. 4. Titration curves of the rate of Cyt *b*-561 reduction vs.  $E_h$ . The values of the initial reduction rate were taken from traces similar to those in Fig. 3.  $\square$ , native chromatophores (25 UQ/RC, 0.87 total photooxidizable Cyt ( $c_1 + c_2$ )/RC, 0.57 total photoreducible Cyt *b*-561/RC, 119 BChl/RC);  $\diamond$ , lyophilized chromatophores (25 UQ/RC, 0.80 Cyt ( $c_1 + c_2$ )/RC, 0.58 Cyt *b*-561/RC, 113 BChl/RC);  $\blacksquare$ , UQ-extracted chromatophores (14 UQ/RC, 0.80 Cyt ( $c_1 + c_2$ )/RC, 0.58 Cyt *b*-561/RC, 119 BChl/RC);  $\blacklozenge$ , UQ-extracted chromatophores (3 UQ/RC, 0.62 Cyt ( $c_1 + c_2$ )/RC, 0.37 Cyt *b*-561/RC, 103 BChl/RC);  $\blacktriangle$ , UQ-10 reconstituted chromatophores (29 UQ/RC, 0.74 Cyt ( $c_1 + c_2$ )/RC, 0.41 Cyt *b*-561/RC, 122 BChl/RC). This reconstituted preparation was obtained by reincorporating exogenous UQ-10 in the UQ-extracted preparation characterized by 3 UQ/RC ( $\blacklozenge$ ), as described under Materials and Methods.

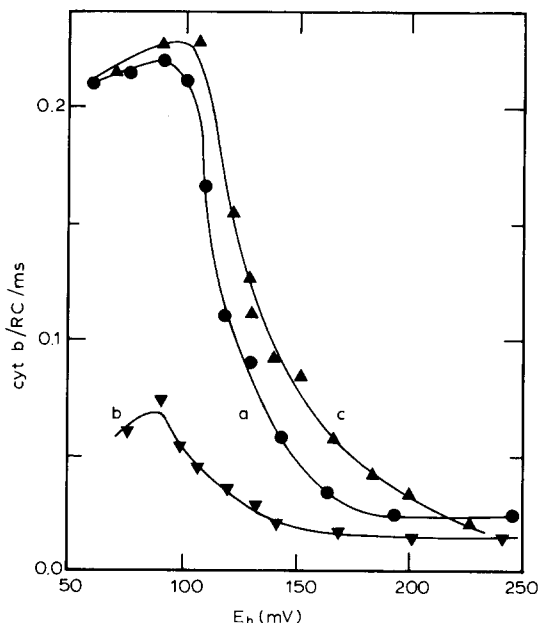


Fig. 5. Titration curves of the initial reduction rate of Cyt *b*-561 in UQ-extracted and reconstituted/enriched chromatophores.  $\bullet$ , control lyophilized chromatophores (24 UQ/RC, 0.78 Cyt ( $c_1 + c_2$ )/RC, 0.58 Cyt *b*-561/RC, 118 BChl/RC);  $\blacktriangledown$ , partially UQ-extracted chromatophores (8 UQ/RC, 0.76 Cyt ( $c_1 + c_2$ )/RC, 0.56 Cyt *b*-561/RC, 114 BChl/RC);  $\blacktriangle$ , UQ-reconstituted/enriched chromatophores (53 UQ/RC, 0.74 Cyt ( $c_1 + c_2$ )/RC, 0.59 Cyt *b*-561/RC, 109 BChl/RC) obtained by reincorporation of UQ-10 in the extracted preparation ( $\blacktriangledown$ ) characterized by 8 UQ/RC.

with respect to controls. A further decrease in the size of the pool (8 UQ per RC) enhances these effects (Fig. 5, curve b).

A complete extraction of the pool (3 UQ per RC, Fig. 4, curve c), results in the total disappearance of the effect of the redox potential on the rate of Cyt *b*-561 reduction (cf. Ref. 14), which remains constant between 220 and 100 mV. However, the rate was always much lower than that measured at  $E_h = 200$  mV when at least a part of the UQ pool was present. The reduction of Cyt *b*-561 observed in this and in other preparations even more drastically extracted, is completely inhibited by 15  $\mu$ M myxothiazol (not shown). The kinetic test for the presence of ubiquinone able to act as secondary acceptor ( $Q_B$ ) in the reaction center complex, indicated that  $Q_B$  was available to all reaction centers in all prepara-



tions, except for the most extracted one (Fig. 4, curve c) for which 50% of  $Q_B$  had been removed. In general  $Q_B$  was not removed from the membrane as long as the total amount of UQ left in the membrane was higher than 5–6 UQ/RC; for more drastic extractions a progressive depletion of  $Q_B$  was paralleled by a significant decrease in the rate of Cyt *b*-561 reduction observed in the range of  $E_h$  around 200 mV, which was not affected by lowering the redox potential.

The kinetic effects of the extraction with iso-octane could be largely reversed by the reincorporation into dehydrated membranes of pure UQ-10, Fig. 4 (curve d) and Fig. 5 (curve c). The pool was reconstituted to a size of 29 UQ/RC in membranes in which previous extraction had reduced the amount of UQ to 3 UQ per RC (and reduced consequently the amount of  $Q_B$ ). In this preparation the  $Q_B$  function and the response of the rate of Cyt *b*-561 reduction to a decrease in the  $E_h$  value, were restored, although the rates of Cyt *b*-561 reduction were not completely overlapping with controls. The absolute rate, observed when the UQ pool was oxidized before the flash ( $E_h = 200$  mV) was almost completely recovered, but the threshold for observing stimulation was about 40 mV less positive than in control preparations, and, at given  $E_h$ 's, the rates were systematically lower than in controls. In these drastically extracted preparations therefore the reincorporated quinone does not appear to be completely kinetically competent; this might reflect either an irreversible damage or a heterogeneity in the chromatophore population with respect to the UQ distribution, generated during the extraction and/or the reconstitution procedures. These secondary effects seemed less important if some residual UQ were left in the extracted membranes before reconstitution. In the experiment illustrated in Fig. 5, curve c, an amount of quinone 2-fold larger (53 UQ per RC) than the natural pool was incorporated in the extracted chromatophores utilized for the experiment of curve b (8 UQ per RC). In this case the recovery of the absolute rates of Cyt *b*-561 reduction was complete and, at fixed values of  $E_h$  below 200 mV, an actual increase over the rates in the control was observed. The threshold for measuring stimulation in the rate of reduction was also displaced to more positive redox potentials

(approx. 200 mV). It appears therefore that, at appropriate redox potentials, a kinetic effect of ubiquinone added at concentrations larger than the physiological ones can be observed.

Similarly, lyophilized, unextracted chromatophores showing a relatively low endogenous UQ content (16 UQ/RC) were enriched in UQ by factors of 2.6 and 5.8 (Fig. 6). Again a displacement of the threshold  $E_h$  value for the stimulation of Cyt *b*-561 reduction was found; the maximal rate of reduction at optimal  $E_h$  was, however, not further stimulated. Interestingly, the rates for both enriched preparations were coincident, suggesting that incorporation of quinone in the lipid bilayer in a kinetically competent state reached saturation. It is not clear at present whether this effect is reflecting a phase segregation of UQ in the membrane lipid matrix or in the chromatophore lumen.

#### Effects on the lag

The effects described above on the initial rate of reduction of Cyt *b*-561 are paralleled by the behaviour of the duration of the lag observed between the time of the flash and the onset of the reaction. As described previously this lag becomes

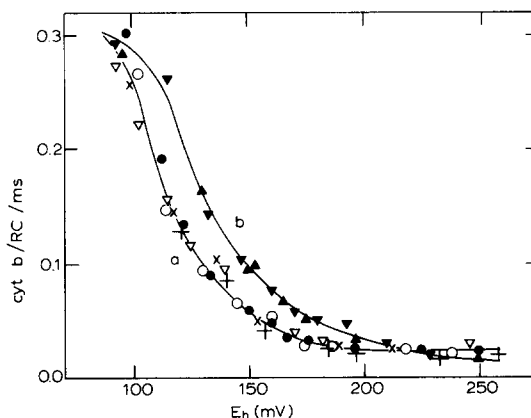


Fig. 6. Effect of UQ enrichment on the titration curve of the rate of Cyt *b*-561 in unextracted chromatophores. ●, native chromatophores (16 UQ/RC); ○, ×, ▽, lyophilized chromatophores (16 UQ/RC); the different symbols refer to the results of three independent sets of measurements, performed on the same preparation; ▲, ▼, UQ-enriched chromatophores (42 UQ/RC and 92 UQ/RC, respectively); +, control lyophilized chromatophores treated with iso-octane as ▲, ▼, but in the absence of exogenous UQ-10.

shorter upon lowering the redox potential [7,8]. In chromatophores containing 25 UQ/RC the lag progressively decreases from 800  $\mu$ s at 200 mV to about 100  $\mu$ s at  $E_h < 100$  mV with an apparent  $E_m$  around 165 mV (Fig. 7A, curve a) (cf. Ref. 7 and 8). However, both in chromatophores containing a smaller native pool (Fig. 7B, curve a) and in partially UQ-extracted ones (Fig. 7A, curve b) a displacement of the apparent  $E_m$  toward less positive  $E_h$ 's (150 mV and 140 mV, respectively) is

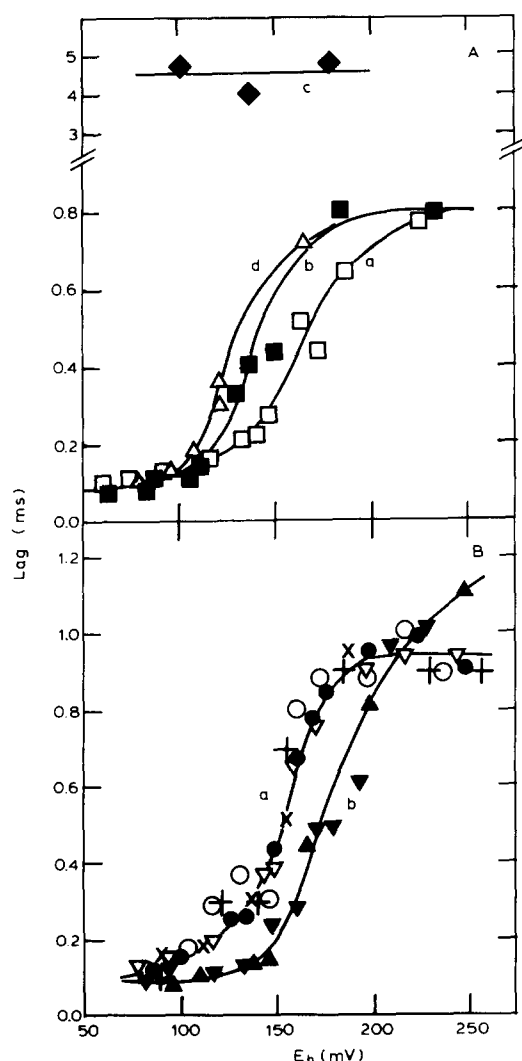


Fig. 7. Titration curves of the lag observed between the time of the flash and the onset of Cyt *b*-561 reduction in the UQ-extracted, reconstituted and enriched chromatophore preparations utilized for the measurements illustrated in Fig. 4 and Fig. 6. (A) Symbols as in Fig. 4. (B) Symbols as in Fig. 6.

found, although the upper and lower values of the lag are not affected. When the extraction is so drastic as to affect the function of the  $Q_B$  site (Fig. 7A, curve c), the lag is, however, dramatically increased to a few ms and becomes totally insensitive to the redox poise. This parallels the behaviour of the initial rate of the reaction. Readition of exogenous ubiquinone to this type of very extracted preparations restores both the upper value of the lag to 800  $\mu$ s and the sensitivity to  $E_h$  changes as expected from the effect on the corresponding values of the initial rate of Cyt *b*-561 reduction (Fig. 7A, curve d). However, the titration curve of the lag does not completely correspond to that of the control, the apparent  $E_m$  being at a potential significantly less positive (130 mV) than expected for the amount of quinone measured in the preparation.

The enrichment of the quinone content above the native amount also affects the titration of the lag. For the two enriched preparations described in Fig. 7B (curve b) a displacement of the apparent  $E_m$  to an  $E_h$  value more positive by about 20 mV is observed, with respect to that of unextracted membranes. This drastic enrichment does not change the lag value at low redox potential but slightly prolongs it at high  $E_h$ . This is reminiscent of the effect of phospholipid enrichment described in Ref. 8.

## Discussion

The present work confirms that UQ can be selectively extracted from lyophilized chromatophores without impairing irreversibly the electron-transfer chain. The extraction is accompanied by a relatively small loss of carotenoids, being the lipid content mostly unaffected. The resulting material offers therefore a suitable system for specific studies on the role of quinones in electron transfer. The additional possibility of increasing the quinone content above the physiological level has allowed us to vary further the size of the UQ pool covering a change from a 10-fold decrease to a 6-fold increase.

On the basis of the modified Q-cycle model proposed in Ref. 7, a change in the size of the UQ pool is expected to have a number of kinetic effects on the reduction of Cyt *b*<sub>561</sub>. In order to

discuss quantitatively these expectations, redox titrations of the initial rate of reduction of Cyt *b*-561 have been simulated for different sizes of the UQ pool. It has been assumed that the initial reduction rate obeys a Michaelis–Menten type equation (cf. Ref. 8 and see Fig. 9 below) of the form:

$$v(E_h, Q_p) = \frac{f(E_h) V_{\max} s(E_h, Q_p)}{K_m + s(E_h, Q_p)} \quad (1)$$

where  $v(E_h, Q_p)$  is the initial rate (Cyt *b* per RC per ms), and  $V_{\max}$  and  $K_m$  ( $QH_2/RC$ ) have the usual meaning. The substrate  $s(E_h, Q_p)$ , i.e., the concentration of ubiquinol ( $QH_2/RC$ ) available at a given  $E_h$  and for a given size of the UQ pool ( $Q_p$ ), plus the flash-generated quinol ( $0.5 UQH_2/RC$ ), is given by:

$$s(E_h, Q_p) = 0.5 + \frac{Q_p}{1 + \exp \frac{E_h - E_{m,Q}}{RT/2F}} \quad (2)$$

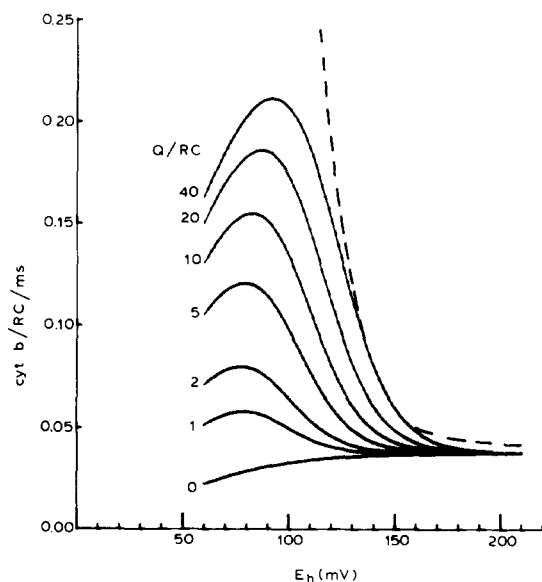


Fig. 8. Theoretical titration curves of Cyt *b*-561 reduction rate as a function of the size of the UQ pool, calculated according to Eqns. 1, 2 and 3. A Michaelis–Menten type kinetics has been assumed for ubiquinol oxidation at the  $Q_z$  site. The values used for  $V_{\max}$  and  $K_m$  ( $0.3$  Cyt *b*-561 per RC per ms and  $3.5$   $UQH_2$  per RC, respectively) have been obtained from the data of Fig. 5 (cf. Fig. 9, below). The dotted line represents a titration curve calculated assuming a second-order kinetics, first-order in ubiquinol, for a pool of  $20$   $UQ/RC$ . The pseudo-first order rate constant has been obtained from the  $V_{\max}$  and  $K_m$  values.

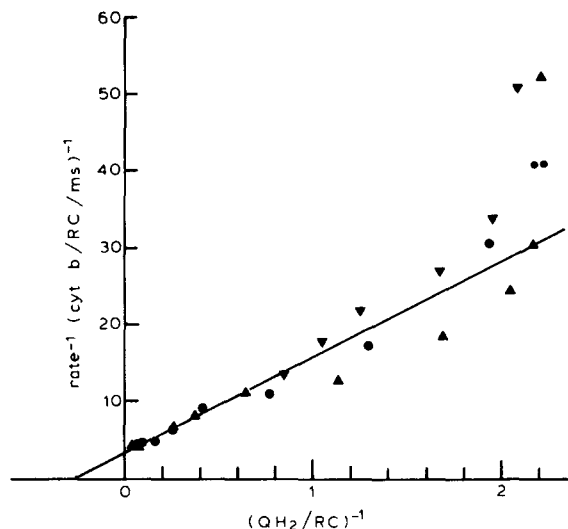


Fig. 9. Double reciprocal plot of the rate of Cyt *b*-561 reduction as a function of ubiquinol concentration in control, UQ-extracted and enriched chromatophores. The reciprocal values of the initial rates were calculated from the data of Fig. 5. Symbols as in Fig. 5. The reciprocal values of the quinol concentrations in the membrane were calculated from the midpoint potential of the pool, the ambient redox potential and the total quinone concentration, assuming that 20% of the native UQ complement determined by HPLC accounts for bound quinone, is not kinetically competent and is not affected by extraction or enrichment. It has been considered that  $0.5$   $UQH_2/RC$  generated by the flash at the reaction center contribute to the substrate concentration.

where  $E_{m,Q}$  is the midpoint potential of the UQ pool ( $90$  mV at pH 7 [6]). The factor  $f(E_h)$ , which modulates  $v$ , takes into account the fraction of Cyt *b*-561 oxidized before the flash, according to the relation:

$$f(E_h) = \frac{\exp \frac{E_h - E_{r,b}}{RT/F}}{1 + \exp \frac{E_h - E_{m,b}}{RT/F}} \quad (3)$$

where  $E_{m,b}$  is the midpoint potential of Cyt *b*-561 ( $50$  mV at pH 7).

The results of this simulation, utilizing the kinetic parameters obtained in this study (cf. Fig. 9 below) are shown in Fig. 8. It is apparent that a lowering of the  $E_h$ , causing an increase in the quinol concentration, corresponds to a stimulation of the reduction rate. In control chromatophores,

containing a physiological amount of UQ (20 UQ/RC), this stimulation becomes significant around 160 mV; when the  $E_h$  is further lowered, the rate increases, but a progressive deviation from a simple second-order kinetics (dotted curve) is found, due to the saturation kinetics of the enzymatic reaction. The rate reaches a maximum at approx. 90 mV: this is caused by the approach to the maximal velocity, and by the reduction of Cyt *b*-561 before the flash at  $E_h < 110$  mV.

A decrease of the pool size produces a displacement towards more negative values of the threshold for the stimulation of the reduction rate, since a higher degree of reduction of the pool is necessary to obtain an equivalent concentration of ubiquinol. Additional effects are also predicted on the rates expected at lower potential, which are progressively reduced, and, less evident, on the  $E_h$  corresponding to the maximal rates, which is also shifted to lower redox potentials. Symmetrical phenomena, namely displacements of the threshold at more positive potential and further stimulation of the reaction rate, are expected when the pool is enlarged above its physiological size.

An inspection of the experimental results illustrated in Figs. 4–6, demonstrates that the expectations of this simulation are essentially met. This kinetic pattern is quite different from that expected if, at the  $Q_Z$  site, a bound ubiquinone molecule ( $UQ_Z$ ) were present, characterized by a midpoint potential (150 mV at pH 7 [15]) different from that of the UQ pool, and dissociable from its binding site only following drastic extractions, i.e., when the pool is nearly exhausted. A similar situation would be simulated by a family of redox titrations all characterized by the same midpoint potential, whatever the size of the UQ pool, and only reduced in absolute rate when the hypothetical  $UQ_Z$  starts to be extracted. In this case, in fact, the redox response of the rate would be interpretable as a true Nernst titration of the bound  $UQ_Z$  species. Obviously in this case no effect would be expected upon increasing the size of the UQ pool above the physiological one. The general agreement of the experimental data with the former of these two models can be considered as a clear demonstration of a collisional interaction of ubiquinol at the  $Q_Z$  site. In this view the apparent midpoint potential for the  $E_h$  depen-

dence of the reaction rate has a kinetic origin and becomes a function of many parameters, including the pool size.

An analysis of the reaction rates as a function of the concentrations of ubiquinol available to the  $Q_Z$  site, calculated according to Eqn. 2, is presented in the reciprocal plot of Fig. 9. The actual size of the UQ pool has been estimated from the HPLC data, assuming that a fraction of the UQ native complement (20% of the total) is present in the membrane as a bound species and is not kinetically relevant [6]. The Lineweaver–Burk representation confirms that in control chromatophores, as well as in extracted or enriched membranes, the rates obey with a sufficient accuracy a unique Michaelis–Menten behaviour with a  $K_m$  equal to 3.5 UQ/RC (corresponding to approx. 7 mM in the membrane phase, when a concentration of RC in the lipids in the range of 2 mM is assumed [8]) and a  $V_{max}$  of 0.3 Cyt *b* per RC per ms. A deviation from the simple Michaelis–Menten pattern towards smaller rate values is systematically observed at very low quinol concentrations. This could suggest that, when the quinol concentration is very low, other electron acceptors (e.g., the respiratory ubiquinol oxidase complex or even an artificial redox mediator present in the assay) might compete with the  $Q_Z$  site for  $UQH_2$  oxidation. The deviation observed at high redox poise is more evident in preparations which have been treated with isooctane, either for UQ extraction or enrichment (cf. also Fig. 4, curve b, d and Fig. 6). Although the Michaelis–Menten behaviour is generally obeyed, in other lyophilized and extracted preparations the  $V_{max}$  and  $K_m$  values did not always correspond to those of Fig. 9. No simple interpretation of this variability can be offered at present. The  $K_m$  values varied, however, between 3 and 5 UQ/RC, in good agreement with Ref. 8. It has to be mentioned that, since the enzyme ( $Q_Z$  site) and the substrate ( $UQH_2$ ) are nearly in the same range of concentrations, a better equation to fit the velocity–substrate dependence should be a more general one valid for all values of substrate and enzyme concentration [34]. The use of this equation, however, did not improve markedly the fit to our data, and, in any case, did not account for the deviation observed at high redox poise.

The variation of the UQ pool size has also remarkable effects on the lag observed between the flash and the onset of the reduction of Cyt *b*-561. It has been suggested that the minimum lag (100  $\mu$ s) observed at low redox poise reflects the time needed to oxidize the Rieske FeS center [7,33]. This interpretation is supported by the observation that as long as the reduction rate of Cyt *b*-561 responds to a lowering in  $E_h$  in extracted chromatophores, the value of the short lag is not altered by UQ depletion, although it is reached at increasingly negative  $E_h$  values. The maximum lag is observed under conditions in which the only reductant of Cyt *b*-561 is the ubiquinol produced by the photoactivity. This situation occurs at  $E_h$  positive enough to keep the UQ pool completely oxidized, or, at all redox potentials, in UQ-depleted preparations in which the UQ pool is completely absent. In addition to the reaction step within the  $bc_1$  complex, therefore, this longer lag should include also the time needed for the dissociation of ubiquinol from the  $Q_B$  site and/or for its diffusion to the  $Q_Z$  site of the  $bc_1$  complex and/or the time for the translocation of the quinol head group across the membrane [7,8].

The apparent midpoint potential for the redox response of the lag depends on the size of the UQ pool and corresponds to redox potentials at which only a very small fraction of the UQ pool is reduced before the flash. This would indicate that the lag titrates out as soon as pre-reduced UQ begins to be present in the membrane. In contrast with the univocal dependence of the initial rate on the  $UQH_2$  concentration, a similar correspondence for the titration of the lag could not be found.

We have identified two conditions in which the value of the maximum lag is affected by extraction or reconstitution. The first is obtained when chromatophores are massively enriched with exogenous ubiquinone. In such preparations the slight increase detected in the lag at  $E_h > 200$  mV (see Fig. 7B) is presumably due to an increase in the average distance of the RC and  $bc_1$  complexes. This effect can be considered to support the idea that a diffusion step is included in the lag [8]. The second condition is obtained when the extraction is so drastic as to affect the function of the  $Q_B$  site. A tentative interpretation of the very marked

increase observed in the lag for such preparations, is that, when the UQ complement is decreased below 5–4 UQ/RC, part of the  $Q_B$  sites which are not occupied by UQ and/or other binding sites liberated by the extraction, can capture and temporarily bind the quinol diffusing from the reaction center. This could also account for the dramatic decrease observed in these preparations in the initial rate of reduction of Cyt *b*. It is noteworthy, in any case, that this effect is fully reversed by the reincorporation of UQ to the extracted membranes, demonstrating that these very long lags do not depend on an irreversible denaturation caused by a drastic extraction.

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