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THE FUNCTION AND LOCALIZATION OF UBIQUINONE IN THE NADH AND SUCCINATE OXIDASE SYSTEMS OF *RHODOPSEUDOMONAS PALUSTRIS*

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SUMMARY

1. The functions of ubiquinone in the respiratory chain were studied with membrane fractions from aerobically grown *Rhodopseudomonas palustris*.

2. Ubiquinone-10 was found to be the only quinone present (5.70 nmoles/mg protein).

3. The extent of reduction of ubiquinone was independent of the respiratory rates of the substrates. 80% of the total amount of ubiquinone was equally accessible to the redox equivalents provided by NADH and succinate.

4. Extraction of the membranes with *n*-pentane, or irradiation with ultraviolet light, greatly lowered the activities of both the NADH and succinate oxidase systems. On reincorporation of ubiquinone into the ubiquinone-depleted membranes, both the NADH and succinate oxidase systems were restored. The restoration of the succinate oxidase system was found to be independent of the length of the isoprenoid side chain of ubiquinone, whereas only the higher homologues of ubiquinone were active in the NADH oxidase system. Phospholipid was essential for the effect of ubiquinone homologues in restoring the NADH oxidase system, whereas no effect was found for restoration of the succinate oxidase system.

5. The ubiquinone-depleted membranes exhibited no, or very small, cytochrome reduction with NADH or succinate. The cytochromes of normal, lyophilized, and ubiquinone-incorporated membranes were all equally reduced by succinate, NADH and dithionite.

6. Neither extraction nor irradiation of endogenous ubiquinone had any effect on the NADH dehydrogenase of the membranes as measured with 2,6-dichlorophenolindophenol as the electron acceptor, whereas the succinate dehydrogenase activity measured with phenazine methosulphate–2,6-dichlorophenolindophenol as the electron acceptor was diminished. However, this latter activity was fully restored when ubiquinone was reincorporated into the membranes.

7. It is concluded that ubiquinone not only serves as a redox carrier between the dehydrogenases of NADH and succinate, respectively, and the cytochromes, but may also exert an effect on the succinate dehydrogenase. It was further concluded

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate; Q, ubiquinone; MK, menaquinone.

that the sites for ubiquinone are structurally different in the two oxidase systems. The site for ubiquinone in the NADH oxidase system has, in contrast to the site in the succinate oxidase system, a specific requirement for the high hydrophobicity of an isoprenoid side chain and for phospholipid. However, these two sites interact with each other and exchange electrons.

INTRODUCTION

The function of ubiquinone (Q) in the electron transport chain of mitochondria has been intensively studied, and numerous, though partially contradictory, views as to their role have been proposed. For example, it has been suggested that Q is not involved directly in the pathway of electron transfer but merely constitutes a side branch of the respiratory chain[1,2] and serves as an inter-chain mediator which links together the individual chains of the electron carriers[3]; or that Q functions on the main electron transport pathway between cytochromes *b* and c_1 (ref. 4) or between the flavoproteins and the cytochromes[5–7]. Strong evidence for the latter viewpoint has been provided by Ernster *et al.*[8] using the method of extraction and reincorporation of Q.

Furthermore, Lenaz *et al.*[9] established that there are two structurally different sites for Q in the mitochondrial electron transport chain having different receptivities for the isoprenoid side chains of Q.

Bacterial membranes, unlike mitochondria, may contain Q or menaquinones (MK) or both. Most of the aerobic Gram-positive bacteria contain only MK, whereas the aerobic Gram-negative bacteria generally contain only Q[10]. A few microorganisms, including enteric bacteria[10] and photosynthetic bacteria[11] contain both Q and MK.

It is widely accepted that the quinone is localized in the membrane fraction and is involved in the electron transport systems of bacteria[12].

The most common quinone in the photosynthetic, non-sulphur purple bacteria (*Athiorhodaceae*) is Q. There is strong evidence that Q is a part of the photosynthetic reaction centre complex[13–16] and interacts as an electron acceptor with reaction centre bacteriochlorophyll[14,17,18]. There is some evidence that Q does not function as a primary electron acceptor in the photosynthetic system[13,19,20]. The function and the localization of Q in the oxidative electron transport system of aerobically grown *Athiorhodaceae* and the interaction of Q between the oxidative and photosynthetic electron transport systems is far from clear, although in *Rhodospirillum rubrum* a coupling between the oxidative and photochemical systems through Q has been suggested[13]. The possibility that the Q of the two systems are separate in nature is indicated by the fact that the difference spectrum obtained for the reduction of endogenous Q by light differs from that obtained by chemical reduction with succinate[21].

The present communication describes the function of Q in the oxidative electron transport system of *Rhodopseudomonas palustris*. In addition, the localization of the Q in relation to the NADH and succinate oxidase systems was studied.

MATERIALS AND METHODS

Growth of organism

Rhodopseudomonas palustris, strain 11/1, was cultivated aerobically in the dark in the culture medium RH[22] with 0.25% sodium acetate as the carbon source. Growth proceeded at 30 °C under vigorous stirring and aeration in a fermentor (Microferm MF 14, New Brunswick) at oxygen pressures between 140 and 150 mm Hg.

The cells were harvested in the late exponential phase. They were washed twice in 0.05 M potassium phosphate buffer (pH 7.5) and stored at −85 °C. Under these conditions, enzyme activities in the electron-transfer system remained stable for 30 days.

Preparation of the membrane fraction

All of the following procedures were performed at 4 °C. The frozen cells were thawed and resuspended in 0.05 M phosphate buffer containing 0.01 M $MgCl_2$, and were then disrupted by two passages through the French pressure cell at 19000 lb/inch^2. Whole cells and debris were removed from the crude extract by centrifugation at $17300 \times g$ for 20 min. The supernatant was centrifuged at $144000 \times g$ for 90 min. The sediment was washed once and used in the experiments as the membrane fraction.

Extraction, detection, and identification of quinones

Three different procedures were used for extraction of quinones from the membranes.

(1) Direct extraction of membranes with acetone–methanol as described by Okayama *et al.*[23].

(2) Direct extraction of lyophilized membranes with *n*-pentane by the procedure of Szarkowska[24].

(3) Saponification of membranes with methanolic alkali in the presence of pyrogallol as described by Bishop *et al.*[10], with the following modification: The light petroleum (b.p. 40–60 °C) phase was shaken three times with an equal volume of 95% methanol and dried over anhydrous Na_2SO_4. The solvent was removed by evaporation at 4 °C in a stream of N_2.

The extracts were dissolved in small volumes of isooctane, applied to silica gel thin-layer plates. Thin-layer chromatography was run by a mixture of chloroform and benzene (1:1, v/v). Q fractions were visualized under ultraviolet light as fluorescent brown spots (MK: fluorescent greenish spot) or under ultraviolet light after spraying with Rhodamine B (0.25% (w/v) in ethanol) as fluorescent violet spots[25]. The Q spots were quickly scraped off into acetone, and the silica gel was removed by centrifugation. The acetone was evaporated under N_2 and the residues were resuspended in ethanol. These solutions were used to obtain oxidized and reduced spectra with a Cary Model 14R recording spectrophotometer. The quinones were reduced by adding $NaBH_4$.

Further identification of the length of the isoprenoid side chain of the isolated Q was achieved by means of reversed-phase thin-layer chromatography with authentic Q as references on paraffin-treated silica gel G plates with acetone–water (19:1, v/v) as the mobile phase[26].

Determination of redox state of Q

The degree of reduction of Q in the steady state was measured after determination of total Q and oxidized Q by the rapid solvent extraction technique as described by Crane and Barr[27].

Preparations of Q-depleted and Q-incorporated membranes

The membranes, suspended in 0.05 M phosphate buffer (pH 7.5) at a concentration of about 7 mg protein/ml, were quickly frozen at −85 °C and were lyophilized for 30 h at −35 °C. The lyophilized membranes were kept over P_2O_5 in vacuum.

Q was extracted from lyophilized membranes in essentially the same manner as described for mitochondria by Szarkowska[24]. Lyophilized membranes (approx. 20 mg protein) were suspended in 10 ml *n*-pentane by gentle homogenization for 5 min in an ice bath, and the suspension was shaken for 10 min at 4 °C. The pentane extract was removed by centrifugation. The extraction was repeated 4–6 times until ultraviolet-absorbing material (320–220 nm) was no longer detectable in the extract The Q-depleted membranes were dried under N_2 at 0 °C.

The preparation of Q-incorporated membranes was carried out according to the method of Ernster *et al.*[8] described for submitochondrial particles. The Q-depleted membranes were gently homogenized for 10 min in a small volume of *n*-pentane containing either Q (100 nmoles/mg membrane protein) or Q with phospholipid (mg/mg protein). [Phospholipid (soybean lecithin) was first dissolved in pentane, and the insoluble materials were removed by centrifugation.] The suspension was shaken in ice for 20 min. The Q-reincorporated membranes were collected by centrifugation and were dried under N_2 at 0 °C. The same method was employed to incorporate pentane extracts into Q-depleted membranes.

The reincorporated membranes contain approx. 6 nmoles Q/mg protein, which is about the same concentration as in the untreated membranes.

Preparation of phospholipid micelles

Phospholipid (soybean lecithin) micelles were prepared as described by Fleischer and Fleischer[28].

Measurement of difference spectra

The reduced-*minus*-oxidized difference spectra of the membrane cytochromes were obtained with a Cary Model 14R recording spectrophotometer supplied with 0 to 0.1 absorbance slide wire, using 1-cm light path cuvettes, at room temperature.

Enzyme assays

Both NADH and succinate oxidase activities were measured polarographically at 25 °C using a Beckman oxygen macroelectrode (Beckman Instruments, Inc., Palo Alto, Calif.) connected to a Varian model linear recorder. The reaction mixtures contained 0.05 M potassium phosphate (pH 7.5), appropriate quantities of the membranes, and either 10 mM succinate or 1 mM NADH in a final volume of 3.4 ml. The initial dissolved oxygen concentration in equilibrium with atmospheric oxygen was assumed to be 240 μM.

The following enzyme activities were measured spectrophotometrically (d= 1 cm; t=25 °C) with an Eppendorf photometer connected to a linear recorder in

a final volume of 1 ml. Specific activities were calculated by using millimolar extinction coefficients of 20.6 $mM^{-1} \cdot cm^{-1}$ for DCIP at 578 nm, and of 21 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c* at 546 nm.

NADH:cytochrome *c* reductase (EC 1.6.99.3) and succinate:cytochrome *c* reductase (EC 1.3.99.1) were assayed by measuring the increase in absorbance at 546 nm resulting from the reduction of horse heart cytochrome *c*. The reaction mixtures contained the following: 40 mM phosphate buffer (pH 7.5), 2 mM KCN, 1 mg cytochrome *c*, and 0.2 mM NADH or 6 mM sodium succinate as substrate.

NADH dehydrogenase (EC 1.6.99.3) and succinate:2,6-dichlorophenolindophenol (DCIP) reductase (EC 1.3.99.1) were assayed by following the rate of reduction of DCIP at 578 nm. The reaction mixtures contained 40 mM phosphate buffer (pH 7.5), 2 mM KCN, 0.06 mM DCIP, and 0.15 mM NADH or 6 mM sodium succinate.

Succinate dehydrogenase (EC 1.3.99.1) activity was measured by the phenazine methosulphate (PMS)-mediated reduction of DCIP assay of Arrigoni and Singer[29], except that a fixed PMS concentration was used. The assay mixture contained 40 mM phosphate buffer (pH 7.5), 2 mM KCN, 0.06 mM DCIP, 0.4 mM PMS, and 6 mM sodium succinate.

Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured by following the decrease in absorbance at 550 nm in the Cary spectrophotometer using the chemically reduced cytochrome *c* at room temperature[30]. The reaction mixture contained 100 mM phosphate buffer (pH 7.5), 2 mg ferrocytochrome *c*, membranes, and water to 3 ml. The millimolar extinction coefficient for horse heart ferrocytochrome *c* is 19 $mM^{-1} \cdot cm^{-1}$ at 550 nm. Ferrocytochrome *c* was prepared by reducing cytochrome *c* with a small amount of ascorbate, and the reduced cytochrome *c* was freed from reductant and oxidized ascorbate products either by dialysis or by Sephadex G-25 chromatography.

Protein was determined by the method of Lowry *et al.*[31] with crystalline bovine serum albumin as the standard.

Chemicals

Cytochrome *c* (horse heart), phenazine methosulphate, and Q-10 were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Antimycin A was obtained from Serva Entwicklungslabor, Heidelberg, Germany; NADH from C. F. Boehringer and Söhne, Mannheim, Germany, and rotenone from British Drug Houses Ltd, Poole, England. All other chemicals were of the finest or spectroscopically pure grade.

RESULTS

Nature of the quinone in R. palustris

It has been reported that only Q-10 is present in *R. palustris* grown under both aerobic oxidative and anaerobic photosynthetical conditions[32]. However, another report[13] showed that a vitamine K-like substance is also present in this organism in addition to Q. Our investigations with the membranes of aerobically grown *R. palustris* strain 11/1 was consistent with the first finding that Q-10 is the only quinone present (Fig. 1). The amount of Q-10 present in the membrane fraction was about 5.70 nmoles/mg protein.

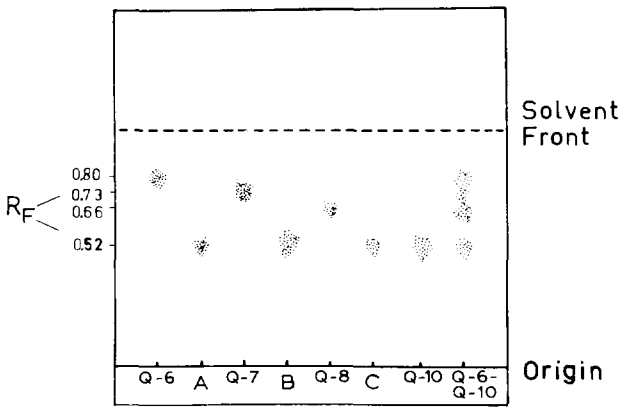

Fig. 1. Reversed-phase thin-layer chromatography of Q homologues and the Q isolated from the membranes of *R. palustris* by three different extraction procedures involving (A) extraction after hydrolysis with alkali (saponification), (B) direct extraction with acetone–methanol, and (C) direct extraction with pentane. The plate was developed with the solvent system acetone – water (19:1, v/v), which was saturated with liquid paraffin at room temperature. The Q fractions were detected under ultraviolet light after spraying with Rhodamine B (0.25% (w/v) in ethanol) as fluorescent violet spots.

Redox reactions of Q

The degrees of reduction of Q under a variety of conditions, as determined after chemical extraction, are given in Table I. If no substrate was added and the membrane vigorously aerated, the extracted Q was predominantly in the oxidized form. Aerobic incubations of membranes with either NADH or succinate resulted in reduction of the endogenous Q to steady-state concentrations of 36 and 32% of

TABLE I

STEADY-STATE OXIDATION–REDUCTION LEVELS OF Q IN THE MEMBRANE FRACTION OF *R. PALUSTRIS*

Membrane fractions (1 ml, about 10 mg of protein) in 0.05 M Tris buffer, pH 7.5, were incubated under constant and vigorous shaking at 25 °C with substrate. The reduction state of Q was measured as described under Materials and Methods. KCN (5 mM) was preincubated with the membrane suspension for 8 min before adding succinate (40 mM) or NADH (10 mM). The figures are the averages of several experiments.

Substrate	*Respiratory activity (nmoles $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$)*	*Cyanide*	*% reduction of total Q*
—		−	16
		+	47
Succinate	47	−	32
		+	80
NADH	131	−	36
		+	80

the total Q, respectively. The inhibition of the electron flow at the level of the cytochrome oxidase by KCN caused an increase in the percentage of reduced Q. The extent of reduction of Q appeared to be independent of the respiratory activities with different substrates, since 80% of the total Q present in the membrane was reduced enzymatically in the presence of KCN with either succinate or NADH.

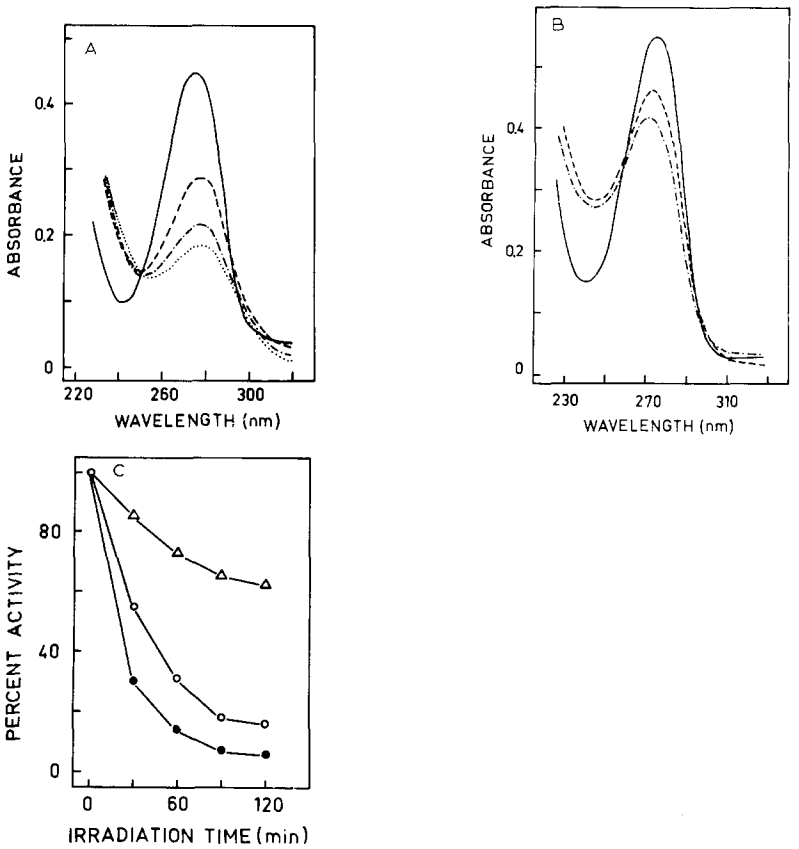

Fig. 2. (A) Effect of time of irradiation on Q-10 purified from the membranes of *R. palustris*. Q, purified as described in the Materials and Methods section, was dissolved in 95% ethanol and was irradiated with ultraviolet light at 350 nm for the periods indicated with a Camag Universal ultraviolet lamp TL-900 (Camag, Muttenz, Switzerland) at 0 °C from a distance of 6 cm. The absorption spectra were recorded in the Cary 14R spectrophotometer. ———, – – –, —·— and ····· indicate spectra of unirradiated Q, Q after 60 min, after 120 min and after 180 min of irradiation, respectively. (B) Absorbance spectra of Q-10 extracted from untreated and irradiated membranes. Membrane suspensions in 0.05 M phosphate buffer (pH 7.5), containing about 15 mg of protein per ml, were irradiated with ultraviolet light for different periods of time as described in A. Q was then extracted as described in Materials and Methods. ———, – – – and —·— indicate the spectra of Q extracted from untreated, 60 min and 120 min irradiated membranes, respectively. (C) Effect of increasing exposure to ultraviolet light on NADH, succinate and cytochrome *c* oxidase activities of membrane fractions. Membrane suspensions in 0.05 M phosphate buffer (pH 7.5), containing about 2 mg of protein per ml, were irradiated with ultraviolet light for different periods of time as described in A. The assays of the enzymatic activities were performed as described in Materials and Methods. ●—●, NADH oxidase; ○—○, succinate oxidase; △—△, cytochrome *c* oxidase.

TABLE II

RESTORATION OF THE NADH AND SUCCINATE OXIDASE SYSTEMS IN IRRADIATED MEMBRANES BY Q-10 REINCORPORATION

Membrane suspensions (10 ml) in 0.05 M phosphate buffer (pH 7.5), containing about 20 mg protein, were irradiated with ultraviolet light of 350 nm for 90 min as described in Fig. 2A. The lyophilization* of irradiated membranes, the extraction and reincorporation of Q-10 and the assays of the enzymatic activities were performed as described in Materials and Methods and in Table IV.

Membrane preparation	*% activity*							
	NADH oxidase	*NADH dehydrogenase*	*NADH: cyt. c reductase*	*Succinate oxidase*	*Succinate: DCIP reductase*	*Succinate dehydrogenase*	*Succinate: cyt. c reductase*	*Cyt. c oxidase*
Normal	100	100	100	100	100	100	100	100
Irradiated	7	96	25	18	43	60	40	65
Irradiated, lyophilized*, extracted and Q-10 incorporated	15	90	35	60	75	80	80	60

* Lyophilization caused no change in activities of both oxidase systems.

Effect of ultraviolet irradiation on Q and on oxidase systems in the membrane

Exposure of the Q purified from the membranes of *R. palustris* to ultraviolet light appeared to cause a structural modification of the Q (as shown by the alteration in spectral properties, Fig. 2A). Similar results were also observed after irradiation of the membrane fractions (Fig. 2B). Besides the spectral alteration in the Q, irradiation of the membranes resulted in a concomitant inhibition of the NADH and succinate oxidase systems, as shown in Fig. 2C and Table II. The NADH oxidase system was more labile to irradiation than the pathway linked to succinate. It is interesting to note that in contrast to NADH dehydrogenase, which was not affected by ultraviolet light, succinate dehydrogenase activity was significantly inhibited (Table II), although phenazine methosulphate is known to be the primary acceptor of electrons from succinate dehydrogenase, and to pass these electrons directly to DCIP[29]. The cytochrome *c* oxidase, in which electrons are directly transferred to the cytochrome region of the electron transport chain, was also affected by ultraviolet light (Fig. 2C and Table II).

In order to examine whether the modification of endogenous Q was the cause of the inhibition of the NADH and succinate oxidase systems, the irradiated membranes were lyophilized and extracted with pentane, followed by reincorporation of authentic Q-10 into the depleted membranes (see Materials and Methods). Table II

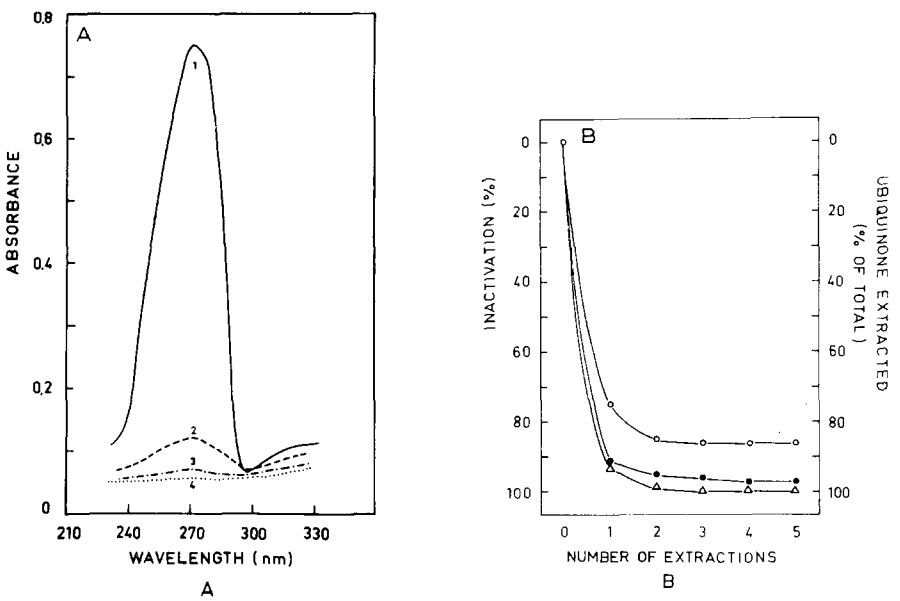

Fig. 3. (A) Difference spectra of the successive pentane extracts from the lyophilized membranes of *R. palustris*. Each extract was reextracted twice with one-fourth volume of 95% methanol, and was evaporated to dryness under nitrogen. Each residue was dissolved in 95% ethanol and the difference spectrum of oxidized *minus* $NaBH_4$-reduced Q was recorded in the Cary 14R spectrophotometer. The number at each difference spectrum indicates the order of extractions. (B) Relationship between the extraction of Q and the succinate oxidase and NADH oxidase activities. The oxidase activities of the extracted membranes were measured polarographically as described in Materials and Methods. △—△, Q extracted; ●—●, NADH oxidase; ○—○, succinate oxidase.

shows that reincorporation of Q-10 into irradiated–extracted membranes resulted in a partial restoration of both oxidase systems. The lack of complete restoration is apparently due to the partial destruction of the terminal oxidase in the case of the succinate oxidase system, however in the case of the poor restoration of the NADH oxidase system it must be assumed that additional components have been destroyed by the ultraviolet treatment. However, the restored NADH and succinate oxidase systems appeared to be physiological, in as much as antimycin A (50 μg/mg membrane protein) inhibited the restored NADH and succinate oxidase activities by 68 and 40%, respectively.

These results indicate that the modification of endogenous Q is one of the causes of the inhibition of oxidase systems caused by ultraviolet irradiation.

Effect of pentane extraction and restoration of succinate and NADH oxidase activities by direct addition of Q-10

Fig. 3 shows that successive extraction with pentane efficiently removed Q from the lyophilized membranes. NADH oxidase was more sensitive to pentane extraction as well as to irradiation with ultraviolet light (Table II) than the succinate oxidase.

By the direct addition of authentic Q-10 to the pentane-extracted membranes, only succinate oxidase activity could be largely restored. The NADH oxidase activity was restored to 10% of the original activity by the simultaneous addition of Q and

TABLE III

RESTORATION OF NADH OXIDASE AND SUCCINATE OXIDASE ACTIVITIES BY DIRECT ADDITION OF Q-10 AND OTHER COMPOUNDS TO THE AQUEOUS SUSPENSIONS OF THE Q-DEPLETED MEMBRANES

Oxidase activity was measured polarographically using either 1 mM NADH or 10 mM succinate as a substrate. Final volume, 3.4 ml; temperature, 25 °C. The amounts of lyophilized, and Q-depleted membranes corresponded to 0.8–1.0 and 1.0–1.3 mg protein, respectively. The additions were as follows: Q-10, 100 nmoles; phospholipid micelles (P), 2 mg; cytochrome *c* (cyt. *c*), 1 mg; Triton X-100, 0.3 mg; Tween-80, 0.3 mg. Q-10 was added as a solution in absolute ethanol (15 μl/ml reaction mixture). All figures represent the average of several experiments.

Membrane preparation	*Additions*	*% activity*	
		NADH oxidase	*Succinate oxidase*
Lyophilized	—	100	100
	Q-10	99	96
Q depleted	—	3	14
	Ethanol	3	16
	Q-10	4	66
	Cyt. *c*	3	14
	P	4	12
	P, cyt. *c*	4	13
	Q-10, P	10	67
	Q-10, P, cyt. *c*	10	69
	Tween-80, ethanol	2	8
	Tween-80, Q-10	2	35
	Triton X-100, ethanol	1	6
	Triton X-100, Q-10	1	12

phospholipid, whereas the succinate oxidase activity was not influenced by the phospholipid (Table III). In the absence of added Q-10, neither phospholipid nor cytochrome *c*, alone or in combination, had any influence on the rate of oxidation of either substrate (Table III).

In contrast to the findings of Crane and Ehrlich[33] in mitochondria, the nonionic detergents Tween-80 and Triton X-100 not only did not replace phospholipid in restoring either of the oxidase activities of pentane-extracted membranes of *R. palustris*, but, in fact inhibited the oxidase activities (Table III).

Effects of extraction and reincorporation of Q-10 on the NADH and succinate oxidase systems

As shown in Table IV, contrary to other reports[8,34], lyophilization caused no change in activities of both oxidase systems. The lyophilized preparation was therefore taken as the control for the effects of extraction and reincorporation of Q.

The reincorporation of authentic Q into pentane-extracted membranes according to the method of Ernster *et al.*[8] is more effective than the direct addition procedure, since the Q is reincorporated by a procedure comparable to that of the depletion, and probably results in an insertion of the Q into the original site of the membrane[12]. This is supported by the finding that only amounts of the Q equal to the original content are required for restoration (see Materials and Methods and ref. 8). The activities of the succinate dehydrogenase and succinate:DCIP reductase were significantly inactivated by extraction of the membranes with pentane, as well as by irradiation with ultraviolet light; however, the NADH dehydrogenase remained intact. Reincorporation of Q-10 almost completely restored the succinate-linked enzyme systems, whereas the activities of NADH oxidase and NADH:cytochrome *c* reductase were restored to only 34 and 47% of the controls, respectively. Cytochrome *c* oxidase activities were the same in all four types of membranes (Table IV).

On the simultaneous reincorporation of Q-10 and phospholipid, the NADH oxidase activity was restored to 45% of the control, while the NADH:cytochrome *c* reductase was restored nearly to 100% of the original activity. As shown in Table III, phospholipid had no effect on the succinate-linked enzyme systems (Table IV).

Neither the NADH nor the succinate oxidase systems were fully restored by reincorporation of the pentane extract into Q-depleted membranes (Table IV). The restored activities were lower than the activities measured after reincorporation of authentic Q-10 alone, and it is remarkable that the addition of Q-10 to the pentane extract did not, upon reincorporation, increase the activities of either oxidase system (Table IV).

Effect of respiratory chain inhibitors on the NADH and succinate oxidase systems

The sensitivity of the NADH and succinate oxidase systems to KCN, rotenone, and antimycin A was the same in untreated membranes as in pentane-extracted–reincorporated membranes (Table V); these data indicate that the restored activities are not due to a bypass of electrons out of the electron transfer chain. However, the restored NADH:cytochrome *c* reductase was less sensitive to antimycin A (Table V). This antimycin insensitivity may be related in some way to the additional restoration of NADH:cytochrome *c* reductase compared to NADH oxidase (Tables IV and VI).

TABLE IV

ENZYME ACTIVITIES OF NADH AND SUCCINATE OXIDASE SYSTEMS OF MEMBRANE FRAGMENTS AFTER VARIOUS TREATMENTS

The preparation of the various membranes and the assays of the enzymatic activities were performed as described in Materials and Methods. All values represent determinations performed on three to four different preparations. Specific enzyme activities of lyophilized membranes in nmoles/min per mg protein served as control (100%): NADH oxidase, 105–133; succinate oxidase, 45–56; NADH dehydrogenase, 110–150; NADH:cytochrome *c* reductase, 52–62; succinate:DCIP reductase, 27–39; succinate dehydrogenase, 83–117; succinate:cytochrome *c* reductase, 35–44; and cytochrome *c* oxidase, 240.

Membrane preparation	*% activity*							
	NADH oxidase	*NADH dehydro-genase*	*NADH: cyt. c reductase*	*Succinate oxidase*	*Succinate: DCIP reductase*	*Succinate dehydro-genase*	*Succinate: cyt. c reductase*	*Cyt. c oxidase*
Normal	103	101	94	104	94	100	105	100
Lyophilized	100	100	100	100	100	100	100	100
Qdepleted	2–3	90–100	6–8	11–14	20–22	42–48	14–17	100–110
Q-10 incorporated	29–34	95–103	40–47	86–95	83–93	94–99	97–105	105
Q-10 incorporated + phospholipid	43–45	90–104	92–98	90–93	83–100	88–104	93–110	105
Q depleted + pentane extract	7–10	94–96	16	51–54	59–62	68–76	69–80	
Q depleted + pentane extract + Q-10	7–12	92–103	10–14	49–56	60–66	70–88	61	

TABLE V

INFLUENCE OF RESPIRATORY CHAIN INHIBITORS ON THE UNTREATED AND RESTORED NADH AND SUCCINATE OXIDASE SYSTEMS

The experimental conditions were those described in Materials and Methods section. Absolute ethanol was used as a solvent for rotenone and antimycin A; its final concentration in the reaction mixture was 2%.

Membrane preparation	*Additions*	*% inhibition*			
		NADH oxidase	*NADH: cyt. c reductase*	*Succinate oxidase*	*Succinate: cyt. c reductase*
Normal	KCN, 1 mM	70		90	
	Rotenone, 10 μM	83			
	Antimycin A, 50 μg/mg protein	78	70	40	35
Lyophilized, extracted, Q and phospholipid reincorporated	KCN, 1 mM	70		100	
	Rotenone, 10 μM	80			
	Antimycin A, 50 μg/mg protein	72	54	35	47

TABLE VI

EFFECT OF Q HOMOLOGUES AND PHOSPHOLIPID ON THE RESTORATION OF THE NADH OXIDASE SYSTEM IN Q-DEPLETED MEMBRANES

The experimental conditions were described in Table IV and in Materials and Methods. P = phospholipid.

Membrane preparation	*% activity*					
	NADH oxidase		*NADH dehydrogenase*		*NADH: cyt. c reductase*	
	−P	*+P*	*−P*	*+P*	*−P*	*+P*
Lyophilized	100	100	100	100	100	100
Q depleted	2–3	2–3	90–100	90–100	6–8	6–8
Reincorporated						
Q-10	29–34	43–45	95–104	90–104	40–47	92–98
Q-8	20–26	33–40	94–102	85–96	39–44	67–82
Q-6	11–15	24–26	95–100	92–93	24–27	36–41
Q-4	12	15	96–98	89–96	23	20–25

Effects of extraction and reincorporation of Q on redox patterns of cytochromes b and c types

The difference spectra of normal, lyophilized, Q-depleted, and Q-incorporated membranes at room temperature are shown in Fig. 4. Lyophilization caused no change in the enzymatically reducible cytochrome contents in comparison with the normal membranes. The non-enzymatically (dithionite) reducible amounts of cytochromes were the same in all four types of membranes. Q-extracted membranes

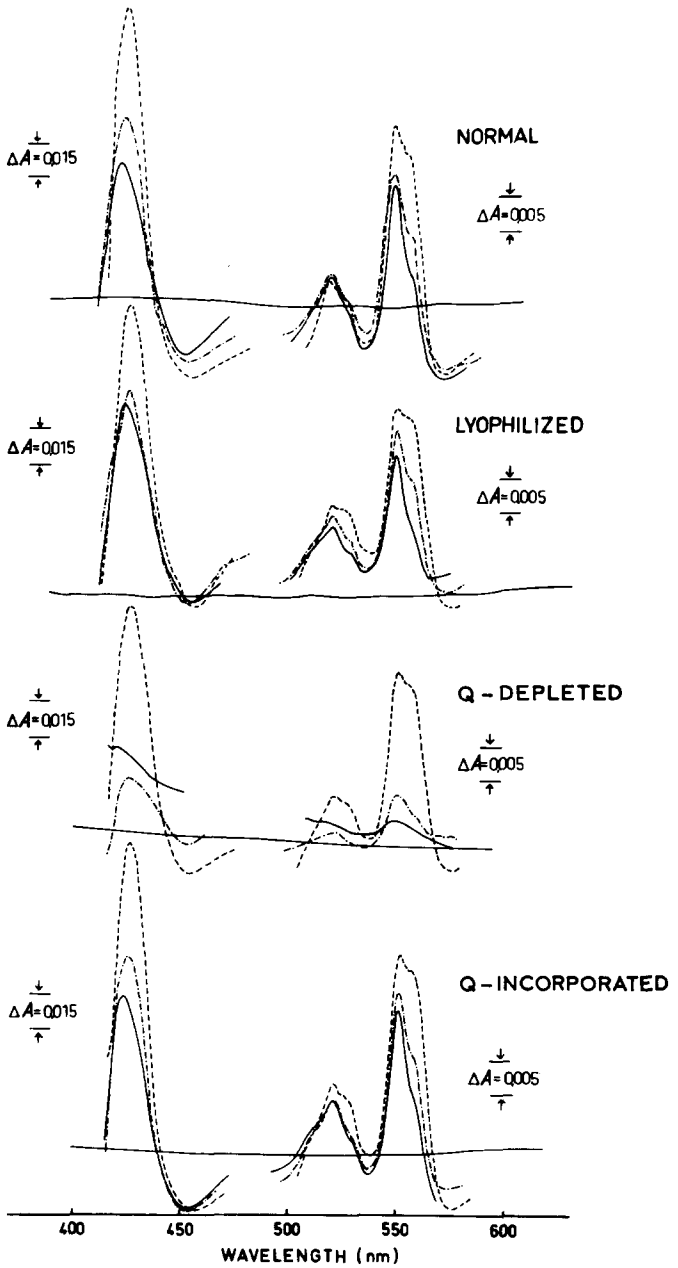

Fig. 4. Difference spectra of reduced-*minus*-oxidized cytochromes of the membrane fraction of *R. palustris* after various treatments. The normal, lyophilized, Q-depleted and Q-reincorporated membranes were suspended in 0.05 M potassium phosphate buffer, pH 7.5, at final concentration of 2.42, 1.80, 1.86 and 2.48 mg protein/ml, respectively. All spectra were monitored with the Cary 14R spectrophotometer at room temperature. ———, —·— and – – – indicate difference spectra of partially reduced (with 3 mM NADH or 10 mM succinate) and of fully reduced (with dithionite) *minus* oxidized cytochrome, respectively.

TABLE VII

EFFECT OF Q HOMOLOGUES AND PHOSPHOLIPID ON THE RESTORATION OF THE SUCCINATE OXIDASE SYSTEM IN Q-DEPLETED MEMBRANES

The experimental conditions were described in Table IV and in Materials and Methods. P = phospholipid.

Membrane preparation	*Succinate oxidase*		*Succinate: DCIP reductase*		*Succinate dehydrogenase*		*Succinate: cyt. c reductase*	
	−P	*+P*	*−P*	*+P*	*−P*	*+P*	*−P*	*+P*
Lyophilized	100	100	100	100	100	100	100	100
Q depleted	11–14	11–14	20–22	20–22	42–48	42–48	14–17	14–17
Reincorporated								
Q-10	86–95	90–93	83–93	83–100	94–99	88–104	97–105	93–110
Q-8	81–91	85–94	76–90	79–92	90–94	93–98	96–110	98–116
Q-6	78–82	81–85	89–90	80–85	87–102	92	94–105	90–100
Q-4	85	90–95	103	88–96	99–109	90–104	82–89	90

showed only slight cytochrome reduction with succinate and no cytochrome reduction with NADH, whereas Q-reincorporated membranes revealed cytochrome redox patterns identical to those found for the normal and lyophilized membranes.

Effects of Q homologues and phospholipid on the restoration of the NADH oxidase system

As shown in Table VI, the various Q homologues were increasingly effective with increasing length of the isoprenoid side chain in restoring the NADH oxidase system of Q-depleted membranes. Q-8 showed about two-thirds the activity of Q-10, while Q-6 and Q-4 showed about one-half the activity of Q-10. With the exception of Q-4, phospholipid increased the efficiency of the Q homologues in restoring the NADH oxidase system (Table VI). The increased effectiveness of the longer isoprenoid side chains was also reflected in the phospholipid-stimulated activity (Table VI).

Effects of Q homologues and phospholipid on the restoration of the succinate oxidase system

In contrast to the results obtained for the NADH oxidase system, all the Q homologues tested were equally effective in restoring the succinate-linked enzyme systems of pentane-extracted membranes (Table VII). No effect of phospholipid was observed in the succinate oxidase system.

DISCUSSION

The data presented show that Q-10 is the only quinone present in membranes of aerobically grown *R. palustris*. The approximately 10-fold molar ratio of the Q-10 to the cytochrome *b*-type (King, M. T. and Drews, G., unpublished) suggests a similar function for the Q in *R. palustris* and in mitochondria.

The enzymatic reduction of the endogenous Q by NADH or succinate as well as the photoinactivation and extraction–reincorporation experiments indicate the interaction of Q in the electron transport system of *R. palustris* (Tables I, II and IV). 80% of the Q is reducible by either NADH or succinate. This suggests that a pool of Q exists, which is functionally linked to each of the dehydrogenases in the respiratory chain.

Irradiation of the membranes with ultraviolet light (350 nm) or extraction of the membrane with pentane results in a loss of all the examined enzymatic activities with the exception of NADH dehydrogenase. After reincorporation of Q, all examined enzyme systems are restored (Tables II and IV). In addition, the Q-depleted membranes exhibit no, or very small, cytochrome reduction with NADH or succinate. The cytochromes of normal, lyophilized, and Q-incorporated membranes were all equally reduced by succinate, NADH and dithionite (Fig. 4). These results indicate that Q is present as a pool between the dehydrogenases and cytochromes and mediates the electron flow from the dehydrogenases to the cytochromes.

Photoinactivation or extraction of Q leads to a decrease in the activity of succinate dehydrogenase. The activity is fully restored when Q is reincorporated into the Q-depleted membranes (Tables IV and VII). These data indicate that Q is needed for full activity of the succinate dehydrogenase. Therefore, Q not only serves as a redox catalyst in the respiratory chain of *R. palustris*, but may also exert an effect

on the succinate dehydrogenase. A similar function of Q was found in submitochondrial particles by Rossi *et al.*[35], who showed that on removal of Q from these particles the kinetic properties of succinate dehydrogenase change to those characteristic of the soluble enzyme, and that on reincorporation of Q the original properties of the particle-bound enzyme reappear.

The observation that the succinate dehydrogenase can be restored by all examined Q homologues (Table VII) leads to the conclusion that the effect of Q on succinate dehydrogenase is independent on the length of isoprenoid side chain of Q.

Rossi *et al.*[35] have suggested that extraction of Q causes a modification of the catalytic properties of the non-haem iron moiety of succinate dehydrogenase, and consequently of the overall catalytic properties of succinate dehydrogenase. A chelate formation between the non-haem iron and Q has been proposed by Moore and Folkers[36] for organic chemical reasons and by Blumberg and Peisach[37] on the basis of electron paramagnetic resonance experiments. It is therefore very likely that the succinate dehydrogenase is fully functional only when its non-haem iron moiety is chelated with Q molecules.

By direct addition of authentic Q-10 into the aqueous suspension of Q-depleted membranes of *R. palustris*, only succinate oxidase activity can be restored. The restoration of NADH oxidase activity by Q shows an absolute requirement for phospholipid, whereas the phospholipid had no effect on the rate of oxidation of succinate (Table III). These observations suggest that there are two sites for Q in the respiratory chain of *R. palustris*; one which is associated with the succinate oxidase system, and the other with the NADH oxidase system. This concept is supported by the results of the extraction and reincorporation experiments (Tables VI and VII). The observation that all the Q homologues tested were equally effective in restoring the succinate-linked enzyme systems, suggests that there is no structural specificity with regards to the isoprenoid side-chain length in the succinate oxidase system. Conversely, the results with the NADH oxidase system show that isoprenoid side chain length is important in the restoration of this system. In addition, phospholipid is essential for the restoration of the NADH oxidase system, but is not required for restoration of the succinate oxidase system. These data support the suggestion that there are two sites for Q in these systems. The site for Q in the NADH oxidase system has a specific requirement for the high hydrophobicity of the isoprenoid side chain and for phospholipid. These lipoidal requirements are negligible in the site of the succinate oxidase system. On the other hand, equal degrees of reduction of Q are obtained with NADH and succinate, respectively (Table I). This demonstrates that the pool of Q is functionally linked to each of the dehydrogenases. A possible explanation for the relationship between these findings is that the two sites of Q interact with each other and exchange electrons. A similar result was found by Lenaz *et al.*[9] in yeast and beef heart mitochondria, which were studied by direct additions of Q homologues and phospholipids to the Q-depleted preparations. Their findings indicated that there are two sites for Q in yeast and beef heart mitochondria.

One explanation for the incomplete restoration of the NADH oxidase system (Tables IV and VI) is that the pentane treatment of the membranes released other components in addition to Q, and that these components are required for the full restoration of the NADH oxidase system.

On reincorporation of the pentane extract, as well as of the pentane extract

plus authentic Q-10, into Q-depleted membranes of *R. palustris*, both the NADH- and the succinate-oxidase systems are not fully restored (Table IV). The restored activities are lower than the activities obtained by reincorporation of authentic Q-10 alone. Q-10 has no stimulatory effect in this case. These observations suggest that some other component released by the extraction of the lyophilized membranes possibly blocks the site for Q and so prevents the insertion of Q to its original site in the electron transport system.

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