STUDIES WITH UBIQUINONE-DEPLETED SUBMITOCHONDRIAL PARTICLES

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1. Introduction

Evidence for the involvement of ubiquinone (UQ) in the respiratory chain has been provided by many workers on the basis of its oxidation-reduction changes parallel with those of other electron carriers during steady-state transitions [1–4] as well as of the requirement for UQ in reactivating the NADH and succinate oxidase systems in UQ-depleted mitochondrial preparations [5–7]. However, the kinetic capability of UQ in electron transfer has been questioned [8–16] since its rate of oxidoreduction in mitochondria and submitochondrial particles has been found to be slow as compared with the overall rate of electron flow through the respiratory chain. These conflicting observations have led to the proposal of various schemes for the composition of the respiratory chain, especially regarding the region between the NADH and succinate dehydrogenase flavoproteins and cytochrome c_1 [4,12,15–18].

In the work summarized in the present paper, we have attempted to localize the site of inactivation of the NADH and succinate oxidase systems that occurs when UQ is removed from lyophilized submitochondrial particles by pentane extraction as originally described by Szarkowska [7] and recently confirmed in this laboratory [19]. We have also elaborated a procedure which allows what appears to be a re-incorporation of UQ (ubiquinone-50) into the particles in an amount similar to the original UQ content. The results show that the site of inactivation caused by the extraction of UQ occurs in the region between the NADH and succinate dehydrogenases and cytochrome *b*, and that UQ is essential for electron transport between the two flavoproteins and the cytochrome system. Evidence is also presented which indicates that part or all of the cytochrome *b* can be modified to interact with succinate dehydrogenase, but not with NADH dehydrogenase, in the absence of UQ.

A full account of this work is being published elsewhere [20].

2. Experimental procedure

2.1. *Preparations*

Submitochondrial particles were prepared from heavy beef-heart mitochondria after sonication at pH 8.6 in the presence of 2 mM EDTA and 0.25 M sucrose as described by Lee and Ernster [21]. UQ-depleted particles were prepared according to the method of Szarkowska [7]. The particles were suspended in 0.15 M KCl at a concentration of 20 mg protein/ml, and lyophilized for more than 9 hr. The lyophilized particles were then extracted 5–6 times with pentane at 0°C. After the extractions, the UQ-depleted particles were dried in a vacuum evaporator to remove residual pentane.

"UQ-incorporated" particles were prepared by shaking UQ-depleted particles in 1 to 2 ml of pentane containing UQ (ubiquinone-50) at a concentration of 40 nmoles/mg protein for 30 min at 0°C. The excess UQ was removed by centrifugation, followed by one gentle rinse with 2 ml pentane. The "UQ-incorporated" particles were dried in a vacuum evaporator.

2.2. *Measurements*

Oxidase activities were measured polarographically with a Clark oxygen electrode. NADH oxidation by fumarate was followed fluorometrically with an

Eppendorf fluorometer. The redox kinetics of cytochromes were studied with a Phoenix dual-wavelength spectrophotometer. UQ was assayed at 275–290 nm with a Hitachi double-beam spectrophotometer. Particle protein was determined by the biuret method.

All biochemicals were obtained from Sigma Chem. Co., or Boehringer und Söhne Co. *n*-Pentane was purchased from Merck Chem. Co.

3. Results and discussion

Table 1 illustrates the NADH and succinate oxidase activites of normal, lyophilized, UQ-depleted, and "UQ-incorporated" particles. The UQ-depleted particles showed virtually no oxidase activities, which were restored upon the re-incorporation of UQ. No activation occurred with cytochrome *c*, showing that dislocation or structural damage of the respiratory chain by pentane treatment was not as severe as in the case of acetone extraction [22], where cytochrome *c* or UQ may act as electron carriers at sites where they do not normally act in the intact chain. Added cytochrome *c* had little or no effect on the NADH or succinate oxidase activities of normal particles. The same was true for the succinate oxidase activity of lyophilized and "UQ-incorporated" particles. On the other hand, added cytochrome *c* did stimulate the NADH oxidase activity of these preparations. These results suggest that, in normal particles, the concentration of cytochrome *c* is rate-limiting for NADH oxidase (but not for succinate oxidase, which is limited by succinate dehydrogenase [23]), but these particles may not be readily accessible to externally added cytochrome *c*.

Table 1
NADH and succinate oxidase activities of submitochondrial particles (ESP) after various treatments.

ESP	Additions	NADH (natoms O/mg protein/min)	succinate (natoms O/mg protein/min)
normal	none	560	146
	100 μg cyt. *c*	558	146
lyophilized	none	418	392
	100 μg cyt. *c*	612	392
UQ-depleted	none	0	0
	100 μg cyt. *c*	0	0
"UQ-incorporated"	none	402	382
	100 μg cyt. *c*	566	384

The oxidase activity was measured polarographically using either 0.93 mM NADH or 5 mM succinate as a substrate. The reaction mixture consisted of 167 mM sucrose, 50 mM Tris-acetate, pH 7.4, and particles. The amounts of normal, lyophilized, UQ-depleted and "UQ-incorporated" particles corresponded to 0.68, 0.95, 1.10 and 1.35 mg protein, respectively. Final volume, 3 ml; temperature, 24°C

The amount of UQ re-incorporated into the particles was of the same order of the magnitude (3–6 nmoles/mg protein) as that originally present in the particles, although the incorporated UQ was somewhat more easily extracted from the particles than the original UQ content (fig. 1, top). There was a close relationship between the inactivation of the NADH and succinate oxidase systems, caused by UQ extraction, and the amount of UQ extracted (fig. 1, bottom), indicating that all the UQ is necessary for maximal oxidase activities. It should be pointed out that, after one pentane-extraction, the remaining amount of UQ was still considerably greater, on the molar basis, than the amount of cytochromes. This finding is in line with the suggestion that UQ acts as a "homogeneous hydrogen-collecting pool" [4], operating, as expected, according to saturation kinetics rather than a mole-to-mole stoichiometry.

Spectrophotometric measurements of the reduction of cytochromes *b* (560–575 nm), c_1 (553–540 nm), and *a* (605–630 nm) in the normal, lyophilized, UQ-depleted, and "UQ-incorporated" particles (fig. 2) show that, in the UQ-containing particles, the reduction of all cytochromes by NADH was very rapid in the presence of cyanide, whereas that in the UQ-depleted particles was very slow and incomplete. Reduction by dithionite indicates that the total amounts of cytochromes were approximately the same in all four preparations.

Results similar to those shown in fig. 2 with NADH were obtained with succinate, with one exception (fig. 3): a fraction of the cytochrome *b* in the UQ-depleted particles, ca. 26% of the total cytochrome *b*, was rapidly reduced by succinate, and this rapid reduction occurred both in the presence and absence of cyanide. The fraction of cytochrome *b* so reduced was not reoxidized when succinate oxidation was inhibited by malonate. In other experiments (not shown) it was found that this fraction of cytochrome *b*

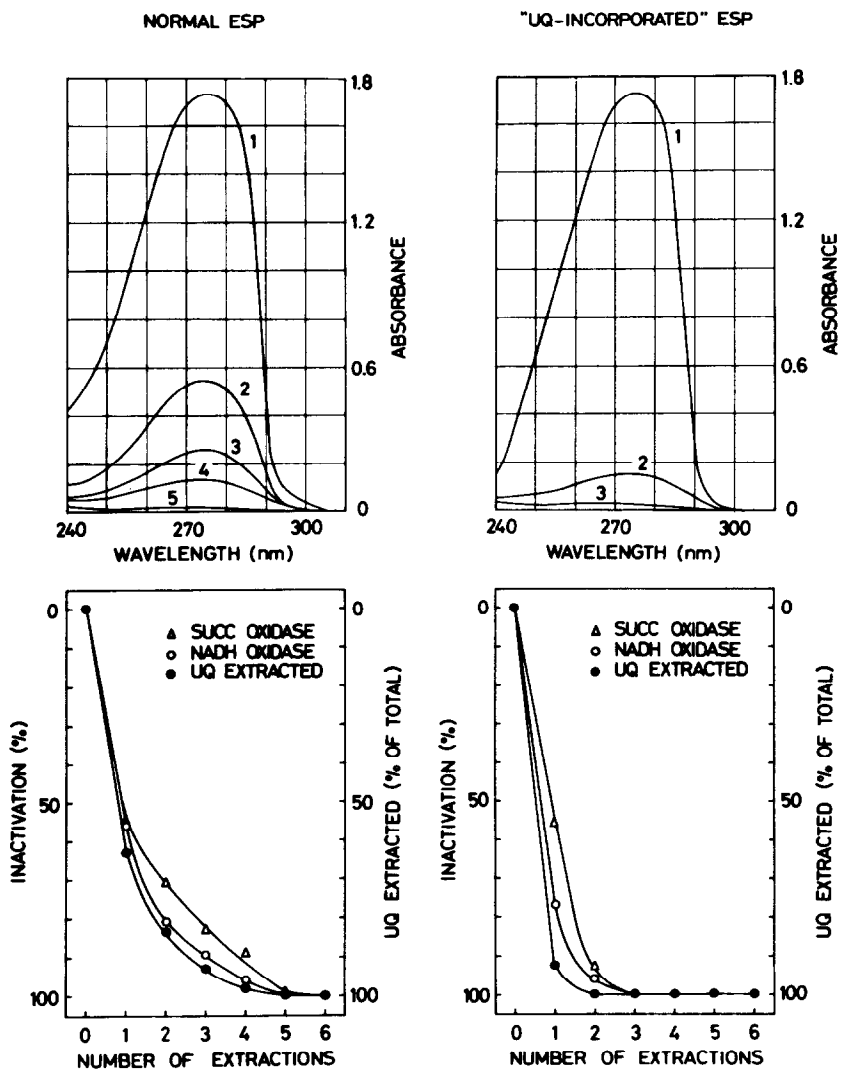

Fig. 1. (*upper*) Extraction patterns of UQ from normal and "UQ-incorporated" particles. The number at each difference spectrum of oxidized minus KBH_4-reduced UQ indicates the order of extractions. The amounts of normal and "UQ-incorporated" particles used were equivalent to 142 and 96.1 mg protein, respectively. (*lower*) Relationship between the extraction of UQ and the NADH and succinate oxidase activities. The experimental conditions were similar to those described in table 1.

was also present in the UQ-containing preparations, where it accounted for approximately 12, 24 and 29% of total cytochrome *b* in the normal, lyophilized, and "UQ-incorporated" particles, respectively. Whereas, in the UQ-depleted particles, this fraction of cytochrome *b* was only reduced by succinate, in the 3 types of UQ-containing particles it was also reduced by NADH.

Addition of antimycin A to the UQ-depleted particles caused a rapid and complete reduction of cytochrome *b* by succinate (fig. 3D), but not by NADH (fig. 3C). These experimental findings suggest that cytochrome *b* can be modified to interact with succinate dehydrogenase, but not with NADH dehydrogenase, in the absence of UQ.

As shown in table 2, extraction of UQ resulted in

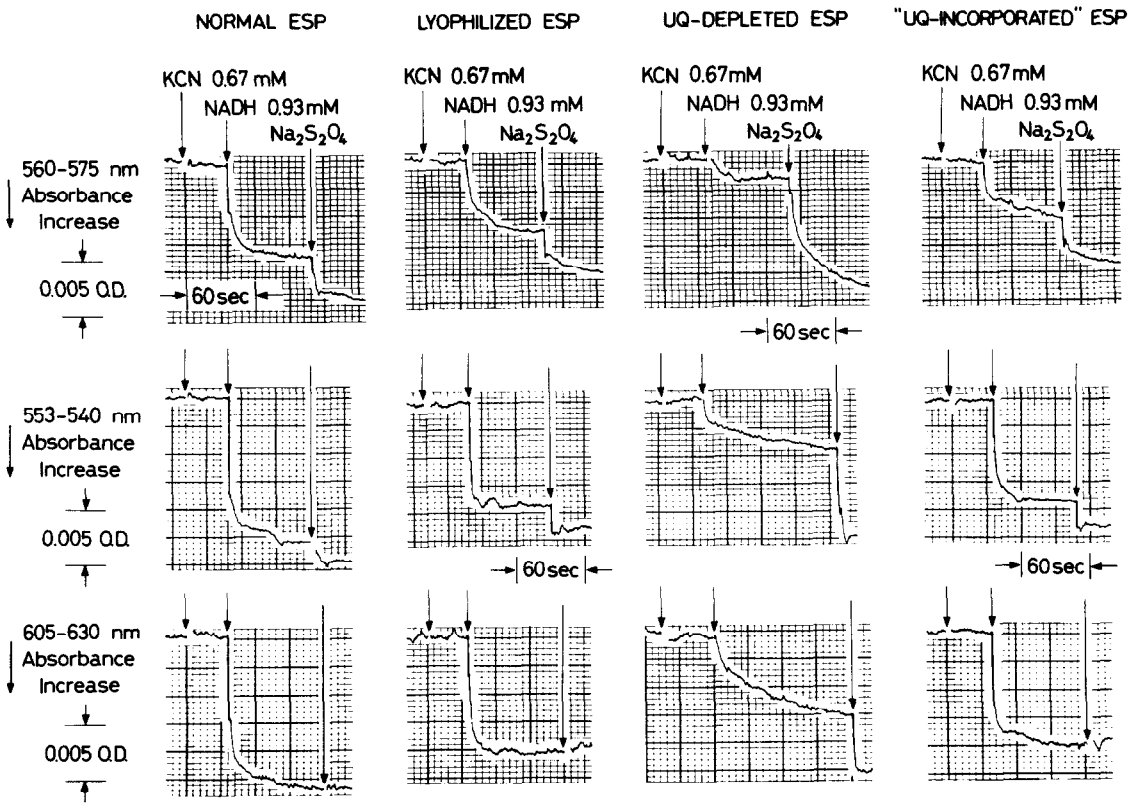

Fig. 2. Comparison of the kinetics of cytochrome *b* (560–575 nm), c_1 (553–540 nm), and *a* (605–630 nm) reduction by NADH in cyanide-pretreated submitochondrial particles (ESP) after various treatments. The normal, lyophilized, UQ-depleted, and "UQ-incorporated" particles were suspended in 167 mM sucrose, and 50 mM Tris-acetate, pH 7.4, at a final concentration of 1.14, 0.92, 1.10 and 0.90 mg protein/ml, respectively. Temperature 24°C.

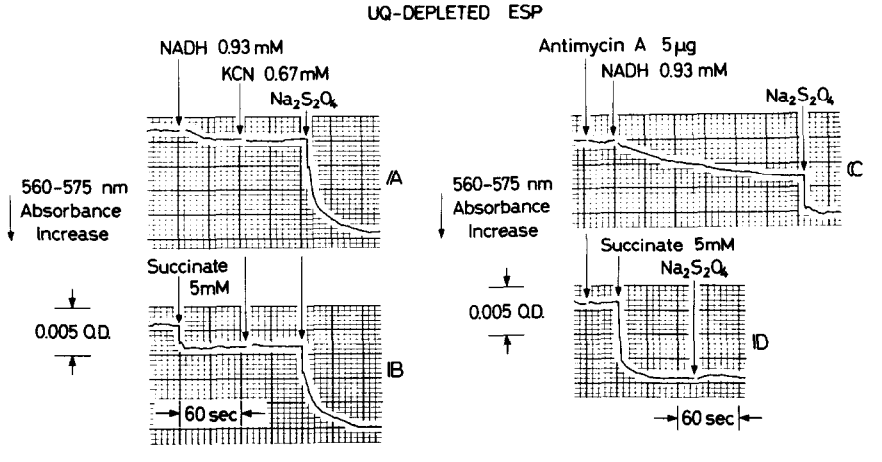

Fig. 3. Comparison of reduction kinetics of cytochrome *b* by NADH and succinate in UQ-depleted particles in the presence of cyanide (traces A and B) or antimycin A (traces C and D). $Na_2S_2O_4$ was added to measure the total amount of cytochrome *b*. The particles were suspended in 167 mM sucrose, and 50 mM Tris-acetate, pH 7.4, at final concentrations of 0.83 mg protein/ml in A and B, and 0.51 mg protein/ml in C and D. Temperature 24°C.

Table 2
Comparison of rates of oxidation of NADH by fumarate in submitochondrial particles (ESP) after various treatments.

ESP	− rotenone	+ rotenone (1.7 μM)
	(nmoles NADH/mg protein/min)	
normal	6.46	0
lyophilized	4.90	0
UQ-depleted	0	0
"UQ-incorporated"	3.11	0

The change in NADH concentration was measured fluorometrically. The reaction mixture consisted of 167 mM sucrose, 50 mM Tris-acetate, pH 7.4, 50 μM NADH, 1.7 mM KCN, 2 μg antimycin A, and particles. The reaction was started by the addition of 10 mM fumarate. The final concentrations of the normal, lyophilized, UQ-depleted, and "UQ-incorporated" particles were 0.26, 0.26, 0.24, and 0.28 mg protein/ml, respectively. Temperature, 30°C.

Table 3
Effect of *N, N, N', N'*-tetramethyl-*p*-phenylenediamine (TMPD) on the NADH and succinate oxidase activities of submitochondrial particles (ESP) after various treatments.

ESP	Additions	NADH	succinate
		(natoms O/mg protein/min)	
normal	none	524	130
	AA	0	0
	AA + TMPD	182	188
	AA + TMPD + rot.	82	
lyophilized	none	436	392
	AA	0	0
	AA + TMPD	258	222
	AA + TMPD + rot.	162	
UQ-depleted	none	0	0
	TMPD	232	146
	TMPD + AA	232	146
	TMPD + AA + rot.	204	
"UQ-incorporated"	none	402	362
	AA	0	0
	AA + TMPD	244	254
	AA + TMPD + rot.	176	

The oxidase activity was measured polarographically under the conditions described in table 1. The amount of normal, lyophilized, UQ–depleted, and "UQ-incorporated" particles corresponded to 0.69, 0.96, 1.10 and 1.35 mg protein, respectively. When indicated, 0.17 mM TMPD, 5 μg antimycin A (AA), and 3.3 μM rotenone (rot.) were added.

an abolition, and re-incorporation of UQ in a restoration, of the rotenone-sensitive oxidation of NADH by fumerate [24]. It is evident from these data that UQ is required for the interaction of the NADH and succinate dehydrogenases.

Both the NADH and succinate oxidase activities of the "UQ-incorporated" particles were sensitive to rotenone and antimycin A (table 3) just as those of the normal or lyophilized particles. Addition of TMPD partially restored the respiration in the presence of these inhibitors. The TMPD-restored NADH oxidase activity of the UQ-depleted particles was of the same order of magnitude of those of the lyophilized and "UQ-incorporated particles" in the presence of both antimycin A and rotenone, but was, in contrast to the former, virtually insensitive to rotenone. These results indicate that electron transfer between the NADH and succinate dehydrogenase flavoproteins and cytochrome c_1 or c through the TMPD shunt [25] remained active in the UQ-depleted particles.

When menadiol was used as a substrate [26], the rate of oxidation and its sensitivity to antimycin A in the UQ-depleted particles was virtually the same as in 3 types of UQ-containing particles. These data demonstrate that menadiol can reduce cytochrome *b* in the absence of UQ, and, furthermore, that the cytochrome $b \rightarrow O_2$ segment of the respiratory chain is functional in the UQ-depleted particles.

In summary, the results presented in this paper indicate that UQ is essential for the interaction of NADH dehydrogenase, succinate dehydrogenase and cytochrome *b*, and that this interaction is a requisite for the normal function of the respiratory chain. Direct interaction between succinate dehydrogenase and cytochrome *b*, without the mediation of UQ, can arise as a result of modifications of the structure of the respiratory chain, such as those caused by mechanical damage or antimycin A treatment. Such modifications, when properly understood and taken into consideration, may explain current kinetic difficulties in recognizing cytochrome *b* and UQ as obligatory components of the mitochondrial electron transport system.

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