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Direct Interaction between Yeast NADH-Ubiquinone Oxidoreductase, Succinate-Ubiquinone Oxidoreductase, and Ubiquinol-Cytochrome *c* Oxidoreductase in the Reduction of Exogenous Quinones*

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The reduction of the following exogenous quinones by succinate and NADH was studied in mitochondria isolated from both wild type and ubiquinone (Q)-deficient strains of yeast: ubiquinone-0 (Q_0), ubiquinone-1 (Q_1), ubiquinone-2 (Q_2), and its decyl analogue 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB), duroquinone (DQ), menadione (MQ), vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), the plastoquinone analogue 2,3,6-trimethyl-1,4-benzoquinone (PQ_{Oc1}), plastoquinone-2 (PQ_2), and its decyl analogue (2,3-dimethyl-6-decyl-1,4-benzoquinone). Reduction of the small quinones DQ, Q_0, Q_1, and PQ_{Oc1} by NADH occurred in both wild type and Q-deficient mitochondria in a reaction inhibited more than 50% by myxothiazol and less than 20% by antimycin. The reduction of these small quinones by succinate also occurred in wild type mitochondria in a reaction inhibited more than 50% by antimycin but did *not* occur in Q-deficient mitochondria suggesting that endogenous Q_6 is involved in their reduction. In addition, the inhibitory effects of antimycin and myxothiazol, specific inhibitors of the cytochrome *b*–c_1 complex, on the reduction of these small quinones suggest the involvement of this complex in the electron transfer reaction. By contrast, the reduction of Q_2 and DB by succinate was insensitive to inhibitors and by NADH was 20–30% inhibited by myxothiazol suggesting that these analogues are directly reduced by the primary dehydrogenases. The dependence of the sensitivity to the inhibitors on the substrate used suggests that succinate-ubiquinone oxidoreductase interacts specifically with center *i* (the antimycin-sensitive site) and NADH ubiquinone oxidoreductase preferentially with center *o* (the myxothiazol-sensitive site) of the cytochrome *b*–c_1 complex. The NADH dehydrogenase involved in the myxothiazol-sensitive quinone reduction faces the matrix side of the inner membrane suggesting that center *o* may be localized within the membrane at a similar depth as center *i*.

The mitochondrial respiratory chain is considered to be composed of discrete lipid-protein enzyme complexes, each of which catalyzes a distinct part of the overall oxidation reaction. Ubiquinone, by virtue of its stoichiometric abundance, its hydrophobicity, and small molecular weight, has been considered to act as a mobile electron carrier between NADH-ubiquinone (Q)[1] oxidoreductase (complex I), succinate-Q oxidoreductase (complex II), other primary dehydrogenases and ubiquinol (QH_2)-cytochrome *c* oxidoreductase (complex III). In support of this view, Kroger and Klingenberg (1) demonstrated that 80–90% of the ubiquinone (Q) pool is homogeneous such that each ubiquinone molecule rapidly reaches and interacts with many dehydrogenase and cytochrome *b*–c_1 complex molecules. Recent studies in Hackenbrock's laboratory (2, 3) have also provided convincing evidence that the respiratory chain complexes as well as ubiquinone are free to diffuse laterally and independently of each other in the plane of the mitochondrial membrane.

On the other hand, the idea that ubiquinone can be reduced through direct interaction between the primary dehydrogenases and the cytochrome *b*–c_1 complex has also been suggested. Such a direct interaction was implicated initially in the Q cycle in which succinate dehydrogenase was proposed to donate an electron at center *i* to form ubiquinol (4). Several types of experimental evidence have provided evidence for the direct interaction of the complexes. For example, the sensitivity to antimycin of duroquinone (DQ) reduction by succinate implies the possibility of a direct interaction between succinate-Q oxidoreductase and center *i* of the cytochrome *b*–c_1 complex (5, 6). Similarly, Ragan and Heron (7, 8) proposed that electron transfer can occur in a complex I–III "supercomplex" based on the kinetics of electron transfer from isolated complex I to complex III. Interestingly, pool kinetics can still be realized in this system if complex I and III can move freely around each other. From the dependence of the ubiquinol oxidation capacity (V_2) of the respiratory chain on the substrate used, Zhu *et al.* (9) concluded that both the free movement of ubiquinone molecules and the direct collision between ubiquinone-reducing and ubiquinol-oxidizing enzymes may play a role in electron transfer through the Q pool.

Further evidence for the direct interaction of the different complexes of the respiratory chain includes the isolation of I:III and II:III supercomplexes (10, 11), the presence of the two small subunits of succinate-ubiquinone oxidoreductase in the cytochrome *b*–c_1 complex after removal of succinate dehydrogenase by alkali (12), and the specific interaction between complexes II and III (13). In an earlier study (6), we

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[1] The abbreviations and trivial names used are: $Q(H_2)$, ubiquinol (reduced ubiquinone); *b*–c_1 complex, mitochondrial ubiquinol-cytochrome *c* oxidoreductase; Q_0, ubiquinone without a side chain; Q_1, Q_2, Q_6, ubiquinone with side chains containing 1, 2, and 6 isoprene units, respectively; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; DQ, duroquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone; MQ, menadione, 1,4-naphthoquinone, vitamin K_3; vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone; PQ_{Oc1}, 2,3,6-trimethyl-1,4-benzoquinone; PQ_{Oc10}, 2,3-dimethyl-6-decyl-1,4-benzoquinone; PQ_2, plastoquinone-2.

TABLE II

Reduction of exogenous quinones by succinate and the inhibition by antimycin and myxothiazol

Mitochondria (concentration of cytochrome c_1 0.016 μM) were incubated with quinones in the standard buffer for 5 min in the presence of 1.6 mM KCN and the reaction started by addition of 2 mM succinate. Antimycin (2 μM) and myxothiazol (2 μM), when used, were added to the standard buffer immediately after the mitochondria. In calculating the specific activities, it was arbitrarily assumed that the extinction coefficient in buffer at all wavelength pairs is 8 mM^{-1} cm^{-1}. This value will give approximate, but nevertheless, close values to the specific activities and will not affect the percentage of inhibition. The concentrations of the quinones were: DQ, Q_0, Q_2, DB, MQ, PQ_{Oc10}, and PQ_2, 40 μM; Q_1, 22 μM; PQ_{Oc1}, 112 μM. Stimulation of activity is expressed by a negative sign.

Quinone	Wavelength pairs	Activity (nmol Q min^{-1} mg^{-1})		Inhibition (%)					
				Antimycin		Myxothiazol		Antimycin + myxothiazol	
		M[a]	W	M	W	M	W	M	W
	nm								
DQ	266–290	1.5	7.0	–	65	–	0	–	75
Q_0	268–290	1.2	10.8	–	55	–	−9	–	77
Q_1	275–290	1.1	13.9	–	95	–	0	–	87
Q_2	275–290	16.4	18.8	0	0	−2	0	−1	0
DB	279–290	18.0	15.6	−9	−23	0	−23	−10	14
MQ	264–290	0	0	–	–	–	–	–	–
Vitamin K_1	264–290	0	0	–	–	–	–	–	–
PQ_{Oc1}	260–280	1.3	21.9	–	71	–	−6	–	73
PQ_{Oc10}	260–280	1.7	16.5	–	−36	–	22	–	93
PQ_2	260–280	0.9	14.8	–	−72	–	−30	–	97

[a] M and W refer to mutant (Q-deficient) and wild type mitochondria.

proposed an interaction between complexes II and III based on the antimycin-sensitivity of DQ and plastoquinone reduction by succinate in submitochondrial particles and isolated succinate-cytochrome *c* reductase.

The current study provides further evidence for the direct interaction between the primary dehydrogenases and the cytochrome b–c_1 complex in the reduction of several quinones and quinone analogues in both wild type and ubiquinone-deficient yeast mitochondria. In addition, the inhibition of quinone reduction by antimycin with succinate as substrate and by myxothiazol with NADH as substrate suggests that succinate-Q oxidoreductase specifically interacts with center *i* and NADH-Q oxidoreductase preferentially with center *o* of the cytochrome b–c_1 complex. Since the NADH dehydrogenase involved in quinone reduction has been shown to face the matrix side of the inner membrane, we also suggest that center *o* may actually be localized in the center rather than on the outer side of the membrane.

MATERIALS AND METHODS

RESULTS[2]

Reduction of Exogenous Quinones by Succinate—The rates of reduction of various exogenous quinones by succinate observed in mitochondria isolated from both wild-type and Q-deficient yeast cells and the sensitivity of the reactions to antimycin and myxothiazol are summarized in Table II.

The reduction by succinate of the "small" quinones: DQ, Q_0, Q_1, and PQ_{Oc1} in wild type mitochondria was significantly inhibited by antimycin, but was essentially insensitive to myxothiazol. Similar results were previously reported with DQ as substrate in pig heart mitochondria (6). The reduction of PQ_{Oc10} and PQ_2 by succinate in wild type mitochondria was also largely inhibited when both antimycin and myxothiazol were present, but antimycin or myxothiazol alone had little or no effect. Previously, we had reported that in pig heart mitochondria succinate could reduce PQ_2 in an antimycin-sensitive reaction and PQ_{Oc10} in a reaction sensitive to the combined effects of myxothiazol and antimycin (6). These results suggest that the pathway of electron transfer from succinate to these small quinones must involve the cytochrome b–c_1 complex where these inhibitors specifically act.

A much slower rate of reduction of all these quinones by succinate was detected in Q-deficient mitochondria suggesting that endogenous ubiquinone is required for the reduction by succinate of all plastoquinone-type quinones and the ubiquinone-type quinones without a side chain or with a side chain not longer than 5 carbons.[3]

By contrast, the reduction of DB and Q_2 occurred at a similar rate in both wild type and Q-deficient mitochondria, and was not sensitive to antimycin or myxothiazol or their combined use. Succinate dehydrogenase must thus directly reduce these compounds probably by the same pathway involved in the reduction of endogenous quinones.

Menadione and vitamin K_1, due to their low mid-point potentials, could not be reduced by succinate by either mitochondrial preparation.

Reduction of Exogenous Quinones by NADH—When NADH was used as the reductant instead of succinate, major differences were observed (Table III). 1) The quinone reduction rate was 10–20 times faster than that observed with succinate as electron acceptor. The observed faster rate of quinone reduction by NADH compared to succinate may reflect the presence of two NADH dehydrogenases in yeast mitochondria. It should be noted, however, that the rate of NADH-DQ oxidoreductase in beef heart mitochondria was 346 nmol of Q min^{-1} mg^{-1} (Fig. 2), a value comparable to that observed with yeast mitochondria. 2) The reduction of the small quinones: DQ, Q_0, Q_1, and PQ_{Oc1} was not or only slightly inhibited by antimycin, but was significantly inhibited by myxothiazol. The sensitivity to myxothiazol was greater with the Q-deficient (55–75%) than with the wild type mitochondria (40–50%). 3) The reduction of these quinones as well as PQ_{Oc10} and PQ_2 no longer required endogenous Q_6. When the

[2] Portions of this paper (including "Materials and Methods," part of "Results," Tables I and VI, and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[3] Zhu, Q. S., and Beattie, D. S., submitted for publication.

TABLE III

Reduction of exogenous quinones by NADH and the effect of antimycin and myxothiazol

The experimental conditions and the calculation of specific activities were the same as in Table II except that the cytochrome c_1 concentration was 0.004 μM and 50 μM NADH replaced succinate. When the specific activities were calculated from NADH oxidation monitored at 340–380 nm, an extinction coefficient of 5 mM^{-1} cm^{-1} was used.

Quinone	Wavelength pairs	Activity (nmol Q min^{-1} mg^{-1})		Inhibition (%)					
				Antimycin		Myxothiazol		Antimycin + myxothiazol	
		M[a]	W	M	W	M	W	M	W
DQ	340–380	270	169	0	8	69	43	69	63
Q_0	270–247	240	135	13	19	62	40	63	40
Q_1	270–247	301	171	14	17	82	78	42	100
Q_2	340–380	488	370	0	0	15	21	10	30
DB	340–380	360	165	0	19	20	32	12	36
MQ[b]	340–380	290	262	12	8	75	38	76	54
Vitamin K_1	340–380	0	0						
PQ_{Oc1}	340–380	300	173	0	10	53	34	53	52
PQ_{Oc10}	340–380	87	137	−3	0	0	37	0	40
PQ_2	340–380	340	355	−6	0	0	50	−6	51

[a] M and W refer to mutant (Q-deficient) and wild type mitochondria.
[b] In the wild type the wavelength pair 270–247 nm was used.

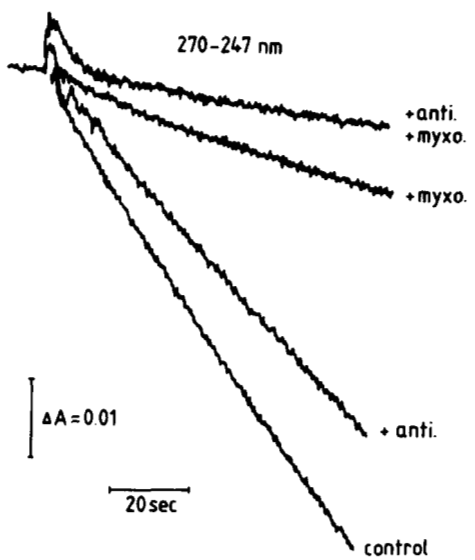

FIG. 2. **Reduction of DQ by NADH in beef heart mitochondria.** The experimental conditions were the same as in Table III, except that beef heart mitochondria (cytochrome c_1 concentration, 0.016 M) replaced yeast mitochondria.

small quinones were used as electron acceptors, the reduction rate was faster in the mitochondria from the Q-deficient mutant than in those from the wild type cells suggesting that the presence of Q_6 was actually inhibitory to the reduction of exogenous quinones. One explanation is that the diffusion of endogenous Q from the dehydrogenase to the b–c_1 complex is the rate-limiting step in this reaction. 4) Menadione was reduced at the similar rate as the other small quinones and with the same pattern of inhibition. However, vitamin K_1, a naphthoquinone with the same ring as menadione and a phytyl side chain similar in structure to that of Q_3, could not be reduced by NADH either in the wild type or Q-deficient mitochondria. 5) The reduction of PQ_{Oc10} and PQ_2 was 40–50% inhibited by myxothiazol in wild type mitochondria but lacked such sensitivity to myxothiazol in the Q mutant. It should be noted that the reduction of PQ_{Oc10} proceeded at a much slower rate than that of PQ_2. 6) The reduction of DB and Q_2 in both wild and Q mutant mitochondria was inhibited 20–30% by myxothiazol. It should be recalled that the reduction of these quinones by succinate was not inhibited by either antimycin or myxothiazol. These results indicate that the reduction of various analogues of ubiquinone by NADH also involves electron transfer through the cytochrome b–c_1 complex and that the sensitivity to antimycin and myxothiazol depends on the substrate used. We suggest that these results can be explained by direct protein-protein interactions between the primary dehydrogenases and the cytochrome b–c_1 complex.

The Sideness of the NADH Dehydrogenase Which Is Responsible for the Myxothiazol Sensitivity—In yeast, there is a single succinate-Q reductase located on the matrix side of the mitochondrial inner membrane. Two different kinds of NADH-Q reductase, however, are present in yeast mitochondria. One reductase is located at the matrix side while the other is located on the cytoplasmic side of the membrane; however, both reductases interact with the b–c_1 complex through the Q pool (21). Identification of the NADH-Q reductase which is responsible for the myxothiazol-sensitive reduction of small quinones may suggest a location in the membrane for center o, where myxothiazol has been shown to act (22). In the experiments described previously in this paper, mitochondria were obtained by breaking yeast cells with glass beads, and hence may have become fragmented. Indeed, significant succinate-Q reductase activity was observed after addition of the impermeable substrate, succinate. Therefore NADH added externally to such mitochondrial preparations would be accessible by both NADH-Q reductase complexes. To differentiate the two NADH-Q reductases, yeast mitochondria were prepared from spheroplasts obtained by incubating yeast cells with zymolyase (16). Mitochondria prepared after zymolyase treatment showed low succinate-ferricyanide and succinate-DQ activities indicating that succinate was not available to the enzyme and that the mitochondria were intact (Table IV). In the same mitochondria, however, there was a very high NADH-ferricyanide activity (950 nmol Fe^{3+}/min/mg), which can be attributed to the enzyme on the outer surface of the membrane. The rate of DQ reduction by NADH in the zymolyase-prepared, and thus intact mitochondria, was approximately half that observed with broken mitochondria, and was not inhibited by myxo-

thiazol (Table IV). In addition, no sensitivity to myxothiazol was observed when Q_0, PQ_{Oc1}, and menadione were used as electron acceptors. However, when the reduction of these Q analogues was studied in submitochondrial particles, prepared from the same mitochondrial sample, sensitivity to myxothiazol was apparent (Table IV). These results indicate that the NADH-Q reductase on the outer surface of the inner membrane can directly reduce DQ and the small analogues, but that the NADH-Q reductase localized on the matrix surface of the membrane reduces them indirectly by electron transfer through the cytochrome b–c_1 complex. Since these data suggest that center o is accessible to the enzyme located at the inside of the membrane, the postulated location of center o toward the outside of the membrane may need to be reevaluated.

Duroquinone Reduction by NADH in Beef Heart Mitochondria—Yeast lack site I of oxidative phosphorylation (23) suggesting that the two NADH dehydrogenases are possibly smaller, less complicated enzymes than their counterparts in mammalian mitochondria. Indeed, the NADH-Q reductase of yeast isolated by deVries (24) is composed of only one polypeptide. To see if the direct interaction with the b–c_1 complex is limited to small primary dehydrogenases such as succinate dehydrogenase and the NADH-Q reductase in yeast, the reduction of DQ by NADH was also studied in beef heart mitochondria. These mitochondria contain a 12 subunit, transmembrane NADH-Q reductase with a site for energy coupling. Furthermore, in beef heart mitochondria, the oxidation of NADH occurs strictly from the matrix side of the membrane. The mitochondria used in these studies were prepared by breaking beef heart muscle cells with a blender (19) and hence are sufficiently damaged for NADH to reach its reaction site on the enzyme. It can be seen from Fig. 2 that the reduction of DQ by NADH was highly sensitive (74%) to myxothiazol and somewhat sensitive (28%) to antimycin. The results with inhibitors indicate that the large mammalian enzyme complex also interacts directly with the cytochrome b–c_1 complex.

The Dependence of the Sensitivities to Antimycin and Myxothiazol on Temperature—The sensitivity of reduction of the small quinones by NADH and succinate to inhibitors of the b–c_1 complex suggests that the pathway of electron transfer must involve the reaction sites of this complex, where these inhibitors have been shown to interact. Furthermore, the dependence of the sensitivity on the substrate used, to antimycin when succinate was the reductant and to myxothiazol when NADH was used, suggests that the reduction of the small quinone molecules may occur through a specific and direct interaction between the dehydrogenases and the b–c_1 complex. For example, the NADH dehydrogenase on the matrix side of the membrane may interact primarily with center o, while succinate dehydrogenase may interact primarily with center i of the b–c_1 complex via protein-protein interactions between the complexes. Such interactions would result from the movement of enzyme complexes in the membrane and hence should be temperature dependent.

Table V shows that lowering the temperature from 30 to 14.5 °C greatly increased the antimycin inhibition of DQ and Q_0 reduction by NADH in the Q-deficient mutant; however, such an increased antimycin sensitivity was not observed in wild type mitochondria. By contrast, this temperature decrease had only a small effect on the degree of myxothiazol inhibition of NADH reduction of these quinones in Q-deficient mitochondria, but essentially abolished the myxothiazol

TABLE IV

Comparison of reactions and sensitivities to myxothiazol in different yeast mitochondrial preparations

The methods of preparation of mitochondria and submitochondria particles are described under "Materials and Methods." The experimental conditions were the same as in Table III.

	Mitochondrial preparation		
	Glass beads (broken)	Zymolyase digestion (intact)	Submitochondrial particles[a]
Succinate-ferricyanide[b]	119	21	
Succinate-DQ[c]	14	0.9	
NADH-DQ[c]	ND[d]	98	74
Sensitivity to myxothiazol (%)			
NADH-DQ	43	0	36
NADH-Q_0	40	0	28
NADH-MQ	54	0	34

[a] Prepared from mitochondria isolated from yeast cells digested with zymolyase.
[b] Nanomole of $Fe(CN)^{-3}$ reduced min^{-1} mg^{-1}.
[c] Nanomole of DQ reduced min^{-1} mg^{-1}.
[d] ND, not determined.

TABLE V

The effect of temperature on the sensitivities of the reduction of Q_0 and DQ to antimycin and myxothiazol

The experimental conditions were the same as in Tables II and III for succinate and NADH as substrate, respectively. The reduction of DQ and Q_0 was monitored at 270–247 nm.

Quinones	Temperature	Activity (nmol Q min^{-1} mg^{-1})		Sensitivity (%)					
				Antimycin		Myxothiazol		Antimycin + myxothiazol	
		M[a]	W	M	W	M	W	M	W
	°C								
NADH as substrate									
Q_0	14.5	204	100	47	7	69	0	79	46
	30.5	254	127	3	2	64	32	74	64
DQ	14.5	154	128	63	−3	92	1	93	30
	30.5	240	168	20	8	79	43	85	63
Succinate as substrate									
Q_0	14.5		2.6		84		−4		100
	30.5		8.4		55		−9		77
DQ	14.5		3.6		66		−3		100
	30.5		7.0		46		0		91

[a] M and W refer to mutant (Q-deficient) and wild type mitochondria.

sensitivity in wild type mitochondria. A surprising observation was that the large temperature decrease (15.5 °C) had only a slight effect (20–30% decrease) on the rate of electron transfer from NADH to DQ and Q_0 (Table V).

For the studies with succinate as the reductant, only the wild type mitochondria were used as the Q-deficient mitochondria cannot catalyze the reduction of DQ and Q_0 (Table II). The drop in temperature significantly increased the amount of antimycin inhibition, and the reaction was completely inhibited by the combined use of antimycin and myxothiazol. Moreover, the rate of reduction of DQ and Q_0 by succinate was lowered 2–3-fold by the temperature change.

DISCUSSION

The results of the present study suggest that protein-protein interactions between the primary dehydrogenases and the cytochrome b–c_1 complex are involved in the reduction of exogenous quinones. Furthermore, the myxothiazol sensitivity of quinone reduction by NADH suggests that NADH-Q oxidoreductase preferentially interacts with center *o*, while the antimycin sensitivity of the reduction by succinate suggests that succinate-Q oxidoreductase interacts preferentially with center *i*.

Theoretically, exogenous quinones could be reduced by the mitochondrial respiratory chain by any of the following possible mechanisms: 1) direct electron transfer from the ubiquinone reducing site on the primary dehydrogenases; 2) from endogenous Q_6H_2 formed by the primary dehydrogenases; 3) on center *i* and/or center *o* of the cytochrome b–c_1 complex with free Q_6H_2 as the electron donor; 4) on center *i* and/or center *o* of the cytochrome b–c_1 complex but with the electrons donated directly by the primary dehydrogenases through protein-protein interactions between these complexes.

The first mechanism represents the "classical" pathway of electron transfer and does occur as evidenced by the antimycin- and myxothiazol-insensitive rate of quinone reduction; however, this mechanism cannot explain the observation that quinone reduction is largely blocked by specific inhibitors of the cytochrome b–c_1 complex. The second mechanism requires direct quinol-quinone electron exchange without the involvement of protein which Rich (25) has shown to occur too slowly to explain the observed rates. Quinol-quinone reactions would also be insensitive to antimycin and myxothiazol. The third mechanism involving the reduction of exogenous quinones with free Q_6H_2 may occur to some extent; however, this mechanism fails to explain why quinone reduction is largely antimycin sensitive with succinate as electron donor and mainly myxothiazol sensitive with NADH as electron donor. It may be argued that the Q_6H_2 formed by NADH-Q oxidoreductase goes preferentially to center *o* and that formed by succinate-Q oxidoreductase to center *i*, but such a pathway is not consistent with the homogenous reduction kinetics of the Q pool observed in the presence of a single substrate.

The fourth mechanism involving specific interactions between the primary dehydrogenases and the cytochrome b–c_1 complex not only explains the substrate-dependent sensitivities to inhibitors of the b–c_1 complex, but also gives a possible explanation to the large differences in the rates of reduction of the small quinones with NADH and succinate as electron donors. The quinone-reducing site on NADH-Q oxidoreductase, possibly due to the more negative midpoint potential of NADH compared to succinate, may have a much greater reducing capacity to the quinones on the Q-reacting sites of the cytochrome b–c_1 complex.

Protein-protein interactions are also necessary to explain the reduction of small quinones in the Q-deficient mitochondria that lack endogenous Q_6. For example, the reduction of DQ by DQH_2 formed by the primary dehydrogenases will not create more DQH_2 molecules. Consequently, the reduction of DQ which is antimycin- and myxothiazol-sensitive must involve the transfer of electrons from the dehydrogenases directly to DQ on the cytochrome b–c_1 complex. Similar reasoning can explain the reduction of dichlorophenylindophenol by NADH in the mitochondria lacking ubiquinone.

In general, the sensitivity of Q_2 and DB reduction to antimycin and myxothiazol was low or not detectable suggesting that these quinones can interact directly with the primary dehydrogenases as do the endogenous quinones. The high concentrations of these analogues used in these experiments would also favor a direct interaction. Protein-protein interactions, however, may well play a role in their reduction as evidenced by the 20–30% inhibition by myxothiazol in both wild type and Q-deficient mitochondria when NADH was the substrate. As discussed previously (9), direct protein-protein interactions in electron transfer to quinones becomes important only when most of the quinone molecules are bound.

Protein-protein interactions may occur through diffusion-based collisons, as the diffusion rate of the various complexes in the mitochondrial membrane has been calculated to occur more rapidly than electron transfer (2, 3). Alternatively, the complexes may occur in aggregated form (7, 8). Our data cannot distinguish between these two models.

The preferential interaction of center *i* and *o* with succinate and NADH oxidoreductases, respectively, suggests that these centers must each have different chemical properties as well as physical identities composed of different amino acid residues on the same or different subunits of the complex. Moreover, each center must be located at different sites on the cytochrome b–c_1 complex. The specific interactions proposed in the current study may offer an important clue as to the actual location of the two centers, especially center *o*, in the inner membrane. The localization of succinate dehydrogenase at the matrix side of the inner mitochondrial membrane in both mammalian and yeast mitochondria is consistent with the localization of center *i* at this side of the membrane where succinate-Q oxidoreductase and the cytochrome b–c_1 complex might interact.

By contrast, the generally accepted localization of center *o* (*o* referring to outer) on the outer side of the inner membrane in close proximity to the iron-sulfur protein and cytochrome c_1 (26) is inconsistent with the interaction of this center with NADH-Q oxidoreductase. The substrate oxidizing site of mammalian NADH-Q oxidoreductase has been shown to face the matrix, while the results of the current paper demonstrate that the yeast NADH dehydrogenase facing the matrix is involved in the myxothiazol-sensitive (center *o*) reduction of small quinones. These results strongly suggest that center *o* may indeed be located far from the outer surface of the membrane, possibly near the center of the membrane. Placing center *o* at the center of the membrane is consistent with physical studies which have indicated that ubiquinone molecules are located at the central region of the phospholipid bilayer (27).

Furthermore, NADH-Q oxidoreductase can also interact with center *i* as indicated by the partial sensitivity to antimycin of the NADH reduction of the small quinones. The interaction of NADH-Q oxidoreductase with both centers *o* and *i* suggests that the two centers may be localized at a similar depth in the membrane especially if the movement of enzyme complexes occurs mainly in the plane of the membrane without much movement perpendicular to the membrane. Such a localization is also consistent with the semi-

quinone model for electron transport in the cytochrome b–c_1 complex which proposes a ubiquinone pocket localized between centers o and i (28). The suggestion that center o might be far from the outer surface of the membrane is also supported by previous results from our laboratory suggesting that subunit VII, the Q-binding protein, is involved in electron transport at center o but faces the matrix side of the membrane (29).

The effects of lowering the temperature on the rates of quinone reduction and on the degree of inhibition by the specific antibiotics suggest that endogenous quinone affects the interactions between the dehydrogenases and the cytochrome b–c_1 complex. NADH-Q appears to interact more tightly with the b–c_1 complex, especially with center i in Q-deficient compared to wild type mitochondria as evidenced by the increased antimycin sensitivity at the lower temperature. In the wild type mitochondria, the endogenous ubiquinone may hinder the interaction of NADH-Q reductase with center i as evidenced by the low antimycin sensitivity at either temperature and the decreased sensitivity to myxothiazol at the lower temperature. Succinate-Q oxidoreductase in the wild type mitochondria appears to interact more tightly with the cytochrome b–c_1 complex at lower temperatures as indicated by the increased sensitivity to antimycin and the 100% inhibition by antimycin plus myxothiazol at 14.5 °C. Hence, lowering the temperature may result in changes in the distance between the complexes or in the depth of their Q-reacting sites in the membrane.

Another important conclusion from this work is that each of the primary dehydrogenases have different structural requirements for their interactions with the different quinones and quinone analogues. Previously, we reported that the reduction of DQ by succinate requires endogenous ubiquinone or ubiquinone homologues with an alkyl side chain containing a minimum of two isoprene units.[3] The data of the current paper extend this observation and suggest that all quinones with a plastoquinone-type ring as well as ubiquinones with a side chain not more than 5 carbons long require endogenous quinone for their reduction by succinate dehydrogenase. In addition, the reduction of these analogues by succinate is highly sensitive to inhibitors of the cytochrome b–c_1 complex suggesting that even in the presence of endogenous quinone, these quinones do not react directly with succinate-ubiquinone oxidoreductase. It is still not clear which of the three Q-reacting sites, center o, center i, or that on succinate dehydrogenase requires endogenous ubiquinone.

NADH-Q oxidoreductase, on the other hand, appears to tolerate considerable changes in the side chain and ring structure of the quinones. The antimycin- and myxothiazol-insensitive rates of quinone reduction are high (as is the total reduction rate) indicating that this enzyme can directly reduce all of the quinones tested with the exception of vitamin K_1. Endogenous Q_6 is not required for the direct reduction of these quinones. Indeed, its presence is actually inhibitory indicating that the quinone reductions occur through the Q-reacting site on NADH dehydrogenase without the mediation of Q_6.

The results obtained with Q_1 were unexpected. The rate of Q_1 reduction by either succinate or NADH was greater than that of Q_0 or DQ and similar to that of Q_2 or DB suggesting that Q_1 may interact with the primary dehydrogenases in a similar manner as these analogues. However, the reduction of Q_1 by succinate required the presence of endogenous quinone and was inhibited 95% by antimycin while its reduction by NADH was inhibited 82% by myxothiazol suggesting that Q_1 interacts with the respiratory chain in the same manner as Q_0 or DQ. We thus conclude that Q_1 is a poor choice to use for a replacement of Q_6 when studying reaction mechanisms of ubiquinone in the electron transfer chain of yeast mitochondria.

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Supplementary material to: Direct Interaction Between Yeast NADH-Ubiquinone Oxidoreductase, Succinate-Ubiquinone Oxidoreductase and Ubiquinol-Cytochrome o Oxidoreductase in the Reduction of Exogenous Quinones. Qin-shi Zhu and Diana S. Beattie.

Materials and Methods

The prototrophic strain, D273-10B and the ubiquinone (Q)-deficient strain, E3-24 (14), were obtained from Dr. Alexander Tzagoloff. The cells were grown aerobically at 30° in semisynthetic medium (15) containing 3% galactose as carbon source and harvested at the late logarithmic phase of growth. Mitochondria were obtained by two methods: one involves breaking cells with glass beads in the Dynomill (Zhu and Beattie[a]). In the other method mitochondria were prepared from spheroplasts obtained by treating the cells with zymolyase as described by Gasses (16). The mitochondria prepared by the latter method were more intact and thus used for the determination of the sideness of the dehydrogenases. Submitochondrial particles were prepared in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA according to Clejan et al. (17). Mitochondria obtained from strain E3-24 were shown to have no detectable ubiquinone in spectra of ether-methanol extracts (18). Beef heart mitochondria were prepared according to Crane et al. (19).

Unless otherwise stated, all enzymatic reactions were performed in 50 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose and 1 mM EDTA using an Aminco-DW2C double beam dual wavelength spectrophotometer thermostated at 25°C. The wavelength pairs used for monitoring quinone reductions by different substrates will be reported in the Results section.

Antimycin, DQ, menadione (vitamin K_3) and 2-methyl-3-phytyl-1,4-naphthoquinone (Vitamin K_1) were purchased from Sigma and myxothiazol from Boehringer Mannheim. Q_1 and Q_2 (ubiquinone -1-2) were a generous gift from Hoffman LaRoche. DB was prepared according to Margolis (20). Q_0, PQ_{0c1}, PQ_{0c10} and PQ_2 (methyl and decyl analogue of plastoquinone) were synthesized in the laboratory of Dr. L.Q. Gu as previously reported (6). All these chemicals were added to the reaction mixture as ethanol solutions when mitochondria prepared with the Dynomill or submitochondrial particles were used. Both of these preparations lacked ethanol dehydrogenase activity. Mitochondria prepared from spheroplasts by the zymolyase method possessed high ethanol dehydrogenase activity. Therefore, the exogenous analogues were added as dimethyl sulfoxide solutions. Fumarate was purchased from British Drug House. All other chemicals were of the highest purity available.

Results

Measurement of reduction of exogenous quinones by succinate and NADH. Both succinate and NADH undergo spectral changes upon oxidation in the ultraviolet region where the redox changes of quinones are monitored. Hence, a detailed inspection of all the redox difference spectra and a careful selection of wavelength pairs for measuring different quinones in the presence of these substances was a prerequisite for these experiments.

Succinate does not absorb light in the range of the instrument used; however, the product of its oxidation, fumarate, has an asymmetrical absorption peak at 240 nm with an extinction coefficient of 1.8 mM^{-1} cm^{-1}. As long as the wavelengths used to monitor the quinone reduction are not close to 240 nm, the simultaneous formation of fumarate will lower slightly the sensitivity of the detection of quinone reduction but will not affect the kinetics of the reaction.

The other substrate used, NADH, has a broad positive absorption peak at 260 nm with an extinction coefficient of 4.0 mM^{-1} cm^{-1} as well as a negative absorption peak at 340 nm in the difference spectrum (oxidized minus reduced) (Fig 1). The sensitivity at the wavelength pairs generally used to measure quinone reduction will therefore be greatly decreased. The wavelength pair 270-247 nm, however, will not reflect any NADH oxidation but will remain sensitive to the oxidation of most of the quinones used in these experiments.

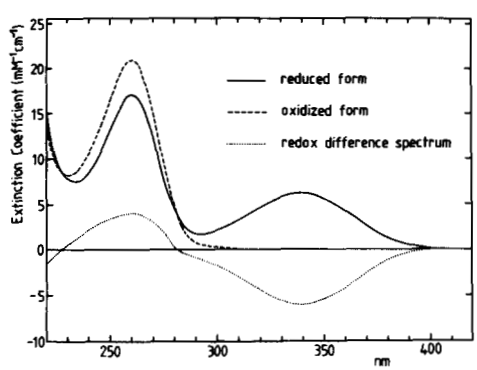

Figure 1. Absorption spectra of NADH. The absorption spectrum of NADH was measured by adding NADH solution (10 mM) to standard buffer to a concentration of 20 μM (————). For the measurement of the spectrum of NAD^+, wild type mitochondria were first added to the standard buffer to a cytochrome c_1 concentration of 0.002 μM in both sample and reference cuvettes. After the baseline was corrected 20 μM NADH was added to the sample cuvette. When the oxidation of NADH was complete, as judged by the disappearance of the 340 nm absorption peak, the spectrum was taken as that of NAD^+ (– – – –). The redox difference spectrum (· · · · · ·).

The reduction of the exogenous quinones by NADH was also measured by NADH oxidation monitored at the 340-380 nm wavelength pair in the presence of 1.6 mM cyanide which inhibited more than 90% of the NADH oxidase activity in our mitochondrial preparations. Similar rates of electron transfer from NADH to quinone were calculated from measurements of quinone reduction at the wavelength pair 270-247 nm and for NADH oxidation. Similar extents of inhibition by antimycin and myxothiazol were also obtained by using these two wavelength pairs.

Although menadione and Vitamin K_1 have more complicated absorption spectra in their oxidized form, the negative peaks in the redox difference spectra were similar to that of DQ and plastoquinone. Thus, the reduction of these two compounds was measured as other quinones. The negative peak position of various quinones in their redox difference spectra and the wavelength pair or pairs used for their reduction are listed in Table I.

Table I

Spectral Analysis of Quinones

The position of the negative peak of quinones in their redox difference spectra in ethanol and the wavelength pairs used for the detection of their reduction by succinate or NADH. In all the experiments 1.6 KCN was included to prevent oxidation of the reduced quinones by the respiratory chain.

Quinone	peak position (nm)	Wavelength pair (nm) succinate	NADH
Q_0	268	268-290	270-247, 340-380
Q_1, Q_2	273-275	275-290	270-247, 340-380
DB	279	279-290	275-240, 340-380
DQ	266, 273	266-290	270-247, 340-380
Menadione	257, 264	264-290	270-247, 340-380
Vit. K_1	261, 269	269-290	270-247, 340-380
PQ_{0c1}, PQ_{0c10}, PQ_2	254-256	260-280	270-247, 340-380

Inhibition of dichloroindophenol (DCIP) reduction in yeast mitochondria by antimycin and myxothiazol. DCIP, an artificial electron acceptor of the primary flavoprotein dehydrogenases, is believed to interact with the mitochondrial respiratory chain through bound quinone, i.e., through the ubiquinone-reacting centers. The reduction of DCIP by succinate in wild type yeast mitochondria was inhibited 74% by antimycin and 46% by myxothiazol (Table VI). Furthermore, the reduction of DCIP was almost completely inhibited in the presence of both antimycin and myxothiazol suggesting that the ubiquinol bound to succinate dehydrogenase in the membrane cannot transfer electrons to DCIP without involving both center i and center o of the cytochrome b-c_1 complex. As anticipated, mitochondria from the Q-deficient yeast could not reduce DCIP.

In the presence of NADH, DCIP was reduced by both wild type and Q-deficient mitochondria; however, the rate of reduction was almost double in the mitochondria lacking ubiquinone. In both wild-type and Q-deficient mitochondria, the rate of DCIP reduction was inhibited moderately by myxothiazol and by antimycin. These results suggest that NADH dehydrogenase can directly reduce DCIP in a reaction promoted by endogenous Q_6 and that the ubiquinol produced by NADH dehydrogenase can also transfer electrons to DCIP through the antimycin and myxothiazol sensitive sites of the cytochrome b-c_1 complex.

Table VI

Inhibition Of DCIP Reduction By Antimycin And Myxothiazol

Mitochondria (c_1 content, 0.008 μM and 0.016 μM for NADH reduction and succinate reduction respectively) were incubated with 50 μM DCIP in standard buffer for 5 min in the presence of 1.6 mM KCN. Then 2 mM succinate or 50 μM NADH was added to start the reaction. The reduction of DCIP was monitored using the wavelength pair 600-630 nm. An extinction coefficient of 0.9 $mM^{-1}cm^{-1}$ was used to calculate activity.

Substrate	Activity (nmol DCIP $min^{-1}mg^{-1}$)		Inhibition (%)					
			antimycin		myxothiazol		antimycin plus myxothiazol	
	M	W	M	W	M	W	M	W
NADH	498	245	6	17	34	23	49	28
succinate	-	25.84	-	74	-	46	-	97