The Role of Quinones in the Mitochondrial Electron Transport System

DANIEL M. ZIEGLER, PH.D.*

OF THE NATURALLY occurring quinones that have been considered as possible electron carriers in the mitochondrial electron transport system, only coenzyme Q (CoQ) has been unambiguously shown to function in this capacity and this report will be concerned primarily with the role of CoQ in mitochondria. Prior to the discovery of CoQ, reports by Nason and his co-workers[1,2] suggested that α-tocopherol might function as an electron carrier in the mitochondrial electron transport system. The following is a brief summary of our current views on the role of tocopherol in the mitochondrial electron transport system.

Hatefi and his associates in our laboratory have examined the tocopherol content of beef heart mitochondria and of submitochondrial particles and some of their data is given in Table I. In agreement with the earlier reports from the laboratories of Nason[2] and of Slater,[3] substantial quantities of α-tocopherol are present in mitochondria and its distribution would suggest that it is closely associated with the reduced diphosphopyridine nucleotide (DPNH) oxidase system. In contrast to total lipid and CoQ, the level of α-tocopherol is almost twice as high in the purified DPNH cytochrome *c* reductase preparation as in the original mitochondria and it is evident that concentration of tocopherol parallels concentration of DPNH cytochrome *c* reductase activity. Whether this has any physiologic significance or is merely a reflection of the methods used to isolate the particle remains to be determined. So far we have not been able to detect cyclic oxidation and reduction of tocopherol during oxidation of DPNH, and it appears unlikely that the quinone to which it gives rise functions as an electron transport compound in the DPNH oxidase system. If tocopherol has a role, other than that of a lipid antioxidant,[4,5] it has yet to be clearly demonstrated in isolated mitochondria, and at the present time we cannot assign a more specific function to tocopherol. However, when we fully understand the role of the other mitochondrial lipids in electron transport[6] we may be able to define more clearly the role of tocopherol.

In contrast to tocopherol, CoQ does undergo cyclic oxidation and reduction during the oxidation of succinate or DPNH.[7-9] The evidence that this quinone is an obligatory electron transport component of the terminal electron transport system has been well documented and is summarized in recent reviews by Green.[6,10] Since most researchers in this field are probably familiar with the earlier work on CoQ, I have omitted the details. Instead, I shall comment on some of our recent experiments dealing with the mechanism of CoQ reduction in heart mitochondria.

POSITION OF COENZYME Q IN THE ELECTRON TRANSPORT CHAIN

It is generally accepted that CoQ is positioned between the flavoproteins and cytochrome c_1 and is required to link the flavoproteins to the cytochromes.[11,12] Reduced CoQ is reoxidized by cytochrome c_1 and at least in nonphosphorylating systems, cytochrome *b* is not an obligatory electron carrier either in the reoxidation of CoQ or in the reduction of the quinone by substrate.[11] However, the relative positions of CoQ and cytochrome *b* in phosphorylating preparations remain to be

From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

* Established Investigator.

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TABLE I
Tocopherol Content of Heart Mitochondria and the DPNH-cytochrome *c* Reductase Particle*

Preparation	Total Lipid (mg./mg. protein)	CoQ mμM / mg. protein	Tocopherol mμM / mg. protein
Beef heart mitochondria	0.37	3.5	0.97
DPNH-cytochrome *c* reductase particle†	0.33	3.7	1.56

* Unpublished data of Hatefi, Y., Haavik, A. G. and Jurtshuk, P.

† This preparation was isolated by the method described by Hatefi et al.[23] The specific activity was 60 μM cytochrome *c* reduced per min. mg. protein at 38°c.

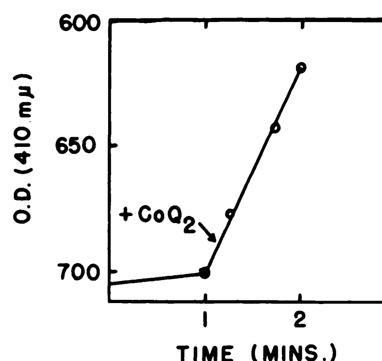

FIG. 1. Effect of CoQ_2 on the succinic ferricyanide reaction. The reaction medium contained 100 μM phosphate, pH 7.0, 0.7 μM potassium ferricyanide, 0.1 μM Versene and 3.0 μM succinate per ml. The reaction was started by adding enzyme and the rate of ferricyanide reduction was measured at 410 mμ. After one minute 1 μL. CoQ_2 (5 μM per ml. in ethanol) was added.

determined. The problem of the function of cytochrome *b* lies outside the scope of this report and I shall confine my comments to the mechanism of CoQ reduction in nonphosphorylating preparations. We cannot rule out the possibility that in mitochrondria, CoQ is reduced by an alternate pathway involving cytochrome *b*, but on the other hand, the evidence for such a pathway is far from convincing.

CoQ does not react directly with the flavoproteins since neither the primary succinic flavoprotein[13] nor the lipoflavoprotein diaphorase[14] is able to catalyze the reduction of CoQ. It is apparent that an additional component is required to link the flavoprotein to CoQ. By purifying the succinic CoQ reductase of heart mitochrondria we have been able to define the components required for the enzymatic reduction of CoQ, but before I comment on the properties of the isolated enzyme I would like to describe the methods we use to measure succinic CoQ reductase activity.

METHODS OF ESTIMATING SUCCINIC CoQ REDUCTASE ACTIVITY

Coenzyme Q_{10}, the naturally occurring quinone, is practically insoluble in water and as such cannot be used as a final electron acceptor. However, the effective concentration of CoQ_{10} in aqueous solution can be increased many fold by dispersing the quinone with small quantities of nonionic detergents. The amount of detergent required to disperse CoQ_{10} does not affect the succinic dehydrogenase, and an assay system for measurement of succinic CoQ_{10} reductase activity using CoQ_{10} emulsified with Triton® X-100 has been described.[15] The DPNH dehydrogenase is far more sensitive to detergents and the conditions necessary to obtain rapid reduction of substrate amounts of CoQ_{10} have not as yet been found.

While CoQ_{10} can be used as the final electron acceptor with the succinic dehydrogenase, the succinic CoQ reductase activity of mitochondrial preparations can be more readily estimated by measuring spectrophotometrically the reduction of either 2-6-dichloroindophenol or ferricyanide. Neither of these dyes is reducible by the succinic flavoprotein[16] at concentrations below 1 μM per ml. at pH 7.0, but they are rapidly reducible by reduced CoQ nonenzymatically. The earlier report of Crane et al.[17] suggested that CoQ may be required for the rapid reduction of ferricyanide and this has been confirmed by studying the effect of CoQ on the succinic-ferricyanide reaction catalyzed by a submitochondrial particle free of endogenous CoQ (Fig. 1). The quinone stimulates the rate of ferricyanide reduction about eightfold which indicates that it can act as an intermediate electron carrier between the flavoprotein and the dye.

The succinate-ferricyanide or the succinate-2,6-dichloroindophenol reactions provide a convenient and rapid spectrophotometric method for estimating the succinic CoQ reductase activity of mitochondrial preparations.

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TABLE II

The CoQ Reductase Activities and the Turnover Rate of the Succinic Flavoprotein in Mitochondria and the Two Soluble Preparations

Preparation	CoQ Reductase Activities ($\frac{\mu M\ CoQ\ reduced^*}{minute/mg.\ protein}$)	Turnover Rate† of Flavoprotein		
		Phenazine Methosulfate	CoQ	O_2
Beef heart mitochondria	1.1	9,800	10,000	9,100
Succinic CoQ reductase[19]	56.0	11,300	12,600	...
Primary succinic flavoprotein[13]	0.0	...	4,100	...

* 2-6-dichloroindophenol assay.
† The turnover rate is expressed as moles of succinate oxidized per minute per mole of succinic flavin with CoQ, phenazine methosulfate and oxygen as final electron acceptors.

In these assays the quinone is used in catalytic amounts and the water insolubility of CoQ_{10} is not a serious problem since 10 mμM of CoQ_{10} per ml. is usually sufficient to saturate the enzyme. This concentration of the quinone in water can be readily achieved by adding 20 to 60 μgm. of Triton X-100 per ml. to the assay medium. The detergent is not necessary when CoQ_2 is used instead of CoQ_{10}, since CoQ_2 is considerably more water soluble than the naturally occurring quinone.

Either 2,6-dichloroindophenol or ferricyanide can be used as the final electron acceptor in these assays. However, 2,6-dichloroindophenol is more suitable with crude enzyme preparations since it does not react directly with the primary succinic flavoprotein.[16] By contrast, ferricyanide, under certain conditions,[18] can react directly with the flavoprotein, and the reaction becomes CoQ independent.

PROPERTIES AND COMPOSITION OF THE PURIFIED SUCCINIC COENZYME Q REDUCTASE

With the aid of the spectrophotometric assay method a soluble form of the succinic dehydrogenase that contains all the components required to catalyze the rapid reduction of CoQ by succinate was isolated in our laboratory.[19] This enzyme will be referred to as the succinic CoQ reductase to distinguish it from the primary succinic flavoprotein isolated earlier by Singer et al.[13] In Table II is listed the relative dehydrogenating activities of the two forms of the dehydrogenase. Both forms of the flavoprotein catalyze the reduction of phenazine methosulfate, but only the reductase can react with CoQ, and the isolated enzyme is about fifty times more active than heart mitochondria in catalyzing the reduction of CoQ (Table II).

The turnover of the flavoprotein (Table II) in the succinic CoQ reductase is slightly greater than it is in the electron transport particle. This demonstrates that the components required to link the flavoprotein to CoQ are fully preserved in the isolated enzyme.

It is evident from the composition data (Table III) that the CoQ reductase is a more complex form of the succinic dehydrogenase than the primary flavoprotein. Both forms of the dehydrogenase contain 4.2 to 4.5 mμM flavin per mg. protein and in both, the flavin is released by acid only after the enzyme is treated with a proteolytic enzyme. In contrast to the primary succinic flavoprotein the CoQ reductase contains lipid and heme. The nonheme iron content of the reductase is twice that of the Singer flavoprotein. The heme component (cytochrome *b*) associated with CoQ reductase is not reduced by succinate (Fig. 1)

TABLE III

Composition of the Primary Succinic Flavoprotein and the Succinic CoQ Reductase

Preparation	Component			
	Flavin ($\frac{m\mu M}{mg.\ protein}$)	Nonheme Iron ($\frac{m\mu M}{mg.\ protein}$)	Heme ($\frac{m\mu M}{mg.\ protein}$)	Lipid ($\frac{mg.}{mg.\ protein}$)
Primary succinic flavoprotein*	4.2	18	0	0
Succinic CoQ reductase	4.3	35	4.6	0.18

* The flavoprotein isolated by the method of Singer et al.[13]

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TABLE IV
The Reduction of Nonheme Iron in Mitochondrial Subfractions*

Preparation	Total Nonheme Iron (mμM/mg. protein)	Per cent Total Iron Reduced	
		By DPNH	By Succinate
Succinic dehydrogenase[13]	18	0	0
Succinic CoQ reductase[19]	35	0	23
Succinic-cytochrome *c* reductase[22]	13	< 1	23
DPNH-cytochrome *c* reductase[23]	15	30	< 2
Electron transport particle[28]	9	39	27

* The reduction of the nonheme iron was followed by the method of Ziegler and Doeg.[27]

which makes its participation as an electron carrier between flavoprotein and CoQ most unlikely. Some of the other properties of the heme component will be discussed later but first let us consider another major difference between the two forms of the succinic dehydrogenase. The nonheme iron in the CoQ reductase is functionally intact, i.e., it is reduced by succinate (Table IV); whereas, as shown earlier by Massey,[20] the nonheme iron present in the primary succinic flavoprotein is not reduced by succinate. In the CoQ reductase the ratio of iron, rapidly reduced by succinate, to flavin is close to 3:1. Since the ratio of total nonheme iron to flavin is 8:1, apparently only part of the iron functions as an electron carrier between the flavoprotein and CoQ. The electron paramagnetic spectra of CoQ reductase published by Beinert and Sands[21] also indicate that the enzyme contains at least two forms of a paramagnetic species, only one of which is reduced by succinate and reoxidized by CoQ. The "nonfunctional" iron does not seem to be extraneous iron adsorbed by the enzyme during its isolation, since the ratio of total iron to flavin is quite constant from one preparation to another. This ratio, furthermore, remains unchanged after treating the enzyme with Dowex® A-1 chelating resin. However, half of the nonheme iron can be removed by prolonged aerobic dialysis against Versene,® but the CoQ reductase activity of the preparation is irreversibly destroyed by this procedure.

TABLE V
Effect of Inhibitors on the Reduction of Nonheme Iron in the Electron Transport Particle

Inhibitors	Concentration	Per cent of Total Iron Reduced	
		By DPNH	By Succinate
None	...	39	27
Amytal	1×10^{-3} M	0	59
Malonate	2×10^{-2} M	65	0

TABLE VI
Inhibition of Succinic-CoQ and Succinic Phenazine Methosulfate (PMS) Reductase Activity by 2-Thenoyltrifluoroacetone

Preparation	Per cent Inhibition at 10^{-4} M	
	CoQ	PMS
Primary succinic flavoprotein[13]	..	0.0
Succinic CoQ reductase[19]	98	17
Succinic cytochrome *c* reductase[22]	96	12

The nonheme iron undergoes cyclic oxidation and reduction in other preparations capable of catalyzing the reduction of CoQ (Table IV). Succinate, but not DPNH reduces significant amounts of iron in the succinic cytochrome *c* reductase particle prepared from heart mitochondria by the method of Green and Burkhard.[22] This preparation does not contain a functional DPNH chain and cannot catalyze the reduction of CoQ by DPNH; whereas, in the DPNH cytochrome *c* reductase particle[23] which is essentially free of the succinic dehydrogenase, only DPNH reduces the nonheme iron. In the electron transport particle (ETP) where both the DPNH and succinic chains are intact either substrate can reduce between 25 to 35 per cent of the iron; however, when both substrates are added approximately 70 per cent of the total nonheme iron is reduced.

Inhibitors which specifically block either the DPNH oxidase or succinoxidase activity of ETP (Table V) have a pronounced effect on the

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TABLE VII
Effect of 2-Thenoyltrifluoroacetone on the Reduction of Nonheme Iron by Succinate

Preparation	Per cent Inhibition at 10^{-3} M
Succinic CoQ reductase[19]	84
Succinic cytochrome *c* reductase[22]	91
Electron transport particle[28]	90

reduction of nonheme iron. Amytal,® a specific inhibitor of DPNH oxidase activity, completely blocks the reduction of iron by DPNH, but more iron is reduced by succinate in the presence of Amytal than by succinate alone (Table V). Malonate, a specific inhibitor of succinoxidase, prevents the reduction of iron by succinate, but increases the amount reduced by DPNH. It is difficult to interpret these phenomena with the limited amount of information available, but they do demonstrate that in the absence of inhibitors the nonheme iron compounds associated specifically with the DPNH and succinic flavoproteins do not freely intercommunicate.

The iron chelate compound, 2-thenoyltrifluoroacetone (TTA) is one of the best selective inhibitors of succinic CoQ reductase activity (Table VI).[24] The level of inhibitor required to block the reduction of CoQ has only a slight effect on the succinate-phenazine reaction catalyzed by the CoQ reductase and does not affect at all the phenazine reductase activity of the primary succinic flavoprotein. Since TTA blocks the reduction of CoQ, but not the reduction of phenazine it cannot be acting on the flavin and must act on a component between the flavin and CoQ. TTA also prevents the reduction of nonheme iron in the CoQ reductase (Table VII) and it is probable that TTA inhibits the over-all succinate to CoQ reaction by combining with the iron compound associated with the succinic dehydrogenase.

Spectra of the CoQ reductase (Figs. 2 and 3) also suggest that the enzyme contains a component in addition to the flavin that is reduced by succinate and reoxidized by CoQ. In addition to the band at 450 mμ, succinate also bleaches components that adsorb at about 480 and 415 mμ. Even at 450 mμ the changes in the spectrum produced by succinate or hydrosulfate cannot be entirely due to the flavin. Even if all of the flavin is reduced by succinate,

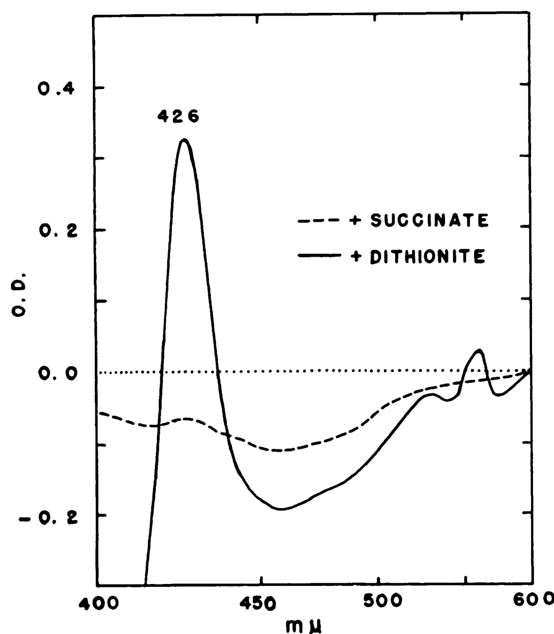

FIG. 2. Difference spectra of solutions of the succinic CoQ reductase recorded with a Beckman DK-2 spectrophotometer. The enzyme (1.8 mg./ml.) was dissolved in 0.1 M phosphate buffer, pH 7.0 and first reduced by succinate and then with dithionite.

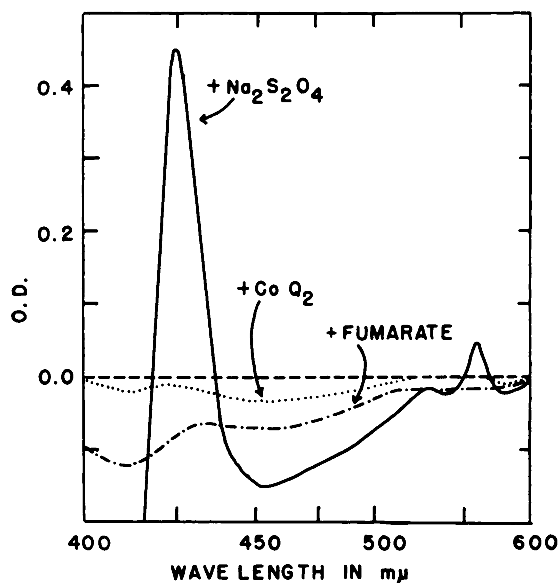

FIG. 3. Reoxidation of reduced F_s(b) with fumarate and CO_2. Difference spectra of solutions of the succinic CoQ reductase. The enzyme was first reduced by adding limiting amounts of dithionite and then reoxidized first by adding fumarate (final concentration .02 M) and CoQ_2 (final concentration 10^{-4} M).

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the decrease in optical density at 450 mμ is greater than could be attributed to this component alone.

Fumarate only partially reoxidizes the reduced enzyme, reduced with dithionite (compare with Fig. 3), and it is unlikely that any of the bands remaining after the addition of fumarate can be attributed to the flavin prosthetic group. The components of the enzyme that remain reduced in the presence of excess fumarate are, however, reoxidized by CoQ (Fig. 3).

The spectral changes in the transition from the reduced to oxidized forms of the enzyme which cannot be attributed to the flavin are probably due to the nonheme iron since the nonheme iron is the only component of the enzyme other than the flavin known to be reduced by succinate and reoxidized by CoQ.

The function of the heme associated with the flavoprotein is not known. It is not reduced by succinate so it is not an obligatory electron carrier between the flavin and CoQ, but as yet it has not been possible to remove the heme without removing at the same time the lipid and some of the nonheme iron and these latter two components appear to be necessary for CoQ reductase activity. Since the reduced heme is rapidly reoxidized by fumarate (Fig 3) a functional link still exists between the flavin and heme prosthetic groups. However, the potential of the heme must be considerably more negative than that of the flavoprotein since succinate even at a final concentration of 0.5 M. does not reduce the heme.

The CoQ reductase is not a simple mixture of a hemoprotein and a flavoprotein since separation of the two compounds cannot be achieved by the usual physical methods used to fractionate proteins. The molecular weight of the isolated enzyme as determined by measurement of sedimentation velocities corresponds very closely to the minimum molecular weight based on the flavin or heme content which suggests that the succinic dehydrogenase as a flavohemoprotein similar to the yeast lactic dehydrogenase.[25,26]

SUMMARY

Two quinones, α-tocopherol and coenzyme Q are present in high concentration in mitochondria isolated from mammalian tissues; however, only coenzyme Q has been shown to undergo oxidation and reduction during electron transport.

Coenzyme Q functions as an intermediate electron carrier between the flavoprotein and cytochrome regions of the terminal electron transport system. Coenzyme Q does not, however, react directly with the flavoproteins and evidence obtained from studies on the isolated succinic coenzyme Q reductase suggests that an additional redox component is required to mediate the transfer of electrons from the flavoprotein to coenzyme Q. This component has not been thoroughly characterized but the preliminary studies indicate that it is an iron-containing compound (nonheme) in which the iron undergoes cyclic oxidation and reduction during the oxidation of succinate by coenzyme Q.

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