Biochimica et Biophysica Acta 893 (1987) 161–169
Elsevier

BBA 42579

Reaction of electron-transfer flavoprotein ubiquinone oxidoreductase with the mitochondrial respiratory chain

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(Received 15 October 1986)
(Revised manuscript received 26 February 1987)

Key words: Electron-transfer flavoprotein; Ubiquinone oxidoreductase; Ubiquinone; bc_1 complex; Acyl CoA dehydrogenase

Submitochondrial particles catalyze the reduction of electron-transfer flavoprotein (ETF) by NADH and succinate under anaerobic conditions in reactions that are totally inhibited by rotenone and thenoyl trifluoroacetone, respectively. The particles also catalyze the ATP-dependent reduction of NAD^+ by enzymatically reduced ETF. The latter reaction is inhibited by rotenone and carbonyl cyanide chlorophenylhydrazone and all three reactions are inhibited by antibody to electrontransfer flavoprotein-ubiquinone oxidoreductase (ETF-QO). These observations indicated that ETF-QO reacts with the pool of ubiquinone that is reduced by NADH and succinic dehydrogenases. Consistent with this hypothesis, NADH- and succinic-ETF reductase activities are inhibited 99% in ubiquinone-depleted particles, and reincorporation of exogenous ubiquinone restores at least 90% of these activities. Reduction of the bc_1 complex by ETF and acyl CoA oxidase activity are also inhibited by antibody to ETF-QO. Myxothiazole and antimycin which inhibit the quinonol oxidation and quinone reduction sites, respectively, in the bc_1 complex also inhibit electron transport from ETF-QO through the complex according to current models of the Q-cycle (Rich, P.R. (1986) J. Bioenerg. Biomembranes 18, 145–156). The results show that ETF-QO is an obligatory component of the electron transport pathway between ETF and the ubiquinone pool and suggest a mechanism for the steady-state turnover of ETF-QO.

Introduction

Electron-transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) is an iron-sulfur flavoprotein that catalyzes the oxidation of electron-transfer flavoprotein (ETF) in the mitochondrial catabolism of fatty acids, branched chain amino acids, lysine, dimethylglycine and sarcosine [1–4]. Ruzicka and Beinert [1,2] initially proposed that this iron-sulfur flavoprotein functioned as the electron acceptor for ETF. This hypothesis was supported by kinetic studies [2–4] and by the demonstration that ETF-QO deficiency is a cause of glutaric acidemia type II, a human inborn error in which substrates of the ETF-linked flavoprotein dehydrogenases accumulate [5]. Electron transport through other segments of the respiratory chain are unaffected in this disease [6]. These investigations supported the specificity of ETF-QO as the

Abbreviations: ETF, electron-transfer flavoprotein; ETF-QO, electron-transfer flavoprotein ubiquinone oxidoreductase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Q, oxidized ubiquinone; $QH^{\cdot}$, ubisemiquinone; QH_2, ubisemiquinol; Q_1 and Q_2, ubiquinone analogs substituted with one or two isoprenyl groups, respectively; TTFA, thenoyltrifluoroacetone; CCCP, carbonylcyanide cholorophenylhydrazone; I_gG, immunoglobulin G.

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oxidant of ETF; however, relatively little is known about the suggested oxidative half-reaction of ETF-QO with mitochondrial ubiquinone. Before the discovery of ETF-QO, Lee and Beinert had shown that the cytochromes and ubiquinone is submitochondrial particles were reduced under anaerobic conditions, or in the presence of cyanide, by butyryl CoA, butyryl CoA dehydrogenase and ETF (unpublished data in Ref. 7); however, the rate of respiration by the particles was negligible in the absence of inhibitors. Purified ETF-QO can be reversibly oxidized and reduced by short-chain, water-soluble ubiquinone homologs [2,3,8,9]. However, these homologs can react nonspecifically with redox proteins, including the anion flavin semiquinone of ETF [7,9].

The EPR signal of the iron-sulfur cluster of ETF-QO was initially detected in submitochondrial particles reduced with NADH under anaerobic conditions and shown to be on the oxygen side of the rotenone inhibition site [10]. This EPR signal has also been observed in succinate-reduced submitochondrial particles [11] and in membrane preparations of *Paracoccus denitrificans* [12], which also contains an iron-sulfur flavoprotein with properties similar to mammalian ETF-QO [13]. Thus, electrons from NADH and succinate dehydrogenases can equilibrate with the iron-sulfur cluster of ETF-QO.

To consolidate the function of ETF-QO as a mediator of electron transport between ETF and the ubiquinone pool, NADH-ETF and succinate-ETF reductase activities were characterized in submitochondrial particles. These investigations show that ETF-QO reacts with ubiquinone that is accessible to NADH and succinate dehydrogenases. Antimycin and myxothiazole inhibit ETF oxidation via ETF-QO measured polarigraphically and inhibit reduction of the bc_1 complex according to the same pattern as that previously reported for reduction of the complex by NADH and succinate [14,15]. Thus, reduction of this segment of the respiratory chain by ETF-QO occurs by a mechanism that is similar to that proposed for other dehydrogenases [16,17].

Materials and Methods

Materials. Butyryl and octanoyl CoA were purchased from Pharmacia-PL Biochemicals. Antimycin, CCCP, rotenone, NAD, NADH, Coenzyme Q_{10} and ATP were obtained from Sigma. Myxothiazole was from Boehringer-Mannheim. The ubiquinone analog, Q_1, was a gift from Eisai Corporation, Japan. All other chemicals were from commercial sources and were of the best grade available. Inhibitors were dissolved in dimethylsulfoxide. The millimolar extinction coefficients used were 10.5 at 313 nm for myxothiazole and 4.8 at 320 nm for antimycin [14].

Mitochondria and submitochondrial particles. Submitochondrial particles were prepared by the method of Low and Vallin [18] from mitochondria isolated from bovine heart by the procedure of Crane et al. [19]. The particles were stored in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 at $-80\,^{\circ}C$ and were used within 7–10 days after preparation. The NADH-ETF and succinate-ETF reductase activities were stable when stored under these conditions for at least 2 weeks.

Enzyme Preparations. ETF was purified from pig liver mitochondria as described and quantitated using $\epsilon_{436\ nm} = 13.4\ mM^{-1}$ [20]. The general acyl CoA dehydrogenase was purified as previously described [21], except that a final step employing chromatography on Matrex Gel-A was included [22]; the dehydrogenase flavin concentration was quantitated using $\epsilon_{448\ nm} = 15.4\ mM^{-1}$ [22]. The IgG fraction of anti ETF-QO serum was that described previously by Frerman and Goodman [5].

Assays. NADH-ETF and succinate-ETF reductase activities were determined fluorometrically under anaerobic conditions by following the decrease of fluorescence of ETF flavin, essentially as described for the assays of the acyl CoA dehydrogenases [23], except that excitation was at 404 nm; emission was measured at 496 nm. Reaction mixtures contained 20 mM Tris-HCl (pH 8.0), 20 mM glucose, 6 units glucose oxidase, 20 units catalase, 1 μg antimycin, 1 μM CCCP, 70 μM NADH (or 2.5 mM succinate) and 1 μM ETF in 0.8 ml. The incubation mixture containing buffer, glucose, CCCP and antimycin were made anaerobic by evacuation and purging with argon. After injection of glucose oxidase and catalase and a 5 min preincubation at $25\,^{\circ}C$, anaerobic solutions of NADH or succinate and ETF were added; all reactions were initiated by the addition of

the enzyme preparations (2.5–10 μg protein) and were run at 25°C. Initial velocities were determined from analyses of fluorescence decay curves [23]. Inhibitors were added in dimethylsulfoxide, which never exceeded a concentration of 0.5% in the assays and was not inhibitory at this concentration. ATP-dependent reduction of NAD^+ by succinate or butyryl CoA were assayed at 25°C in reaction mixtures containing 50 mM Tris-acetate (pH 7.5), 0.2 M sucrose, 6 mM $MgSO_4$, 1.6 mM KCN and 10 mM succinate or, when butyryl CoA was the reductant, 1 μM ETF, 0.5 μM general acyl CoA dehydrogenase flavin and 350 μM butyryl CoA. Reactions were initiated by the addition of 0.3 mM ATP. Succinate-ubiquinone oxidoreductase activity was determined as described [24] but Q_1 was substituted for Q_2 and the reaction was carried out in the presence of 1 μg/ml antimycin at pH 8 in 20 mM Tris-HCl. One unit of activity is equal to 1 μmol of substrate consumed or product generated in all assays.

Acyl CoA, succinate and NADH oxidase activities were measured as described [25] at 25°C in a 0.36 ml glass water-jacketed chamber with a Clark electrode. The medium was 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4 or pH 8.0). Between 40 and 300 μg of submitochondrial particles were used in the assays. The substrates used were 0.7 mM NADH, 5 mM succinate, or 0.2 mM octanoyl CoA in the presence of 1 μM ETF and 1 μM general acyl CoA dehydrogenase flavin. All reactions were conducted in the presence of 1 μM CCCP. Protein was determined as previously described [26].

Difference spectra. Difference spectra were measured with an Aminco DW2-A spectrophotometer at 25°C using a 1 nm band pass. Submitochondrial particles were diluted in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 1 μM ETF, 0.5 μM general acyl CoA dehydrogenase flavin. The particles were then reduced with 1 mM octanoyl CoA. After determining the difference spectra of the enzymatically reduced cytochromes, the contents of the reference cuvette were reduced with $Na_2S_2O_4$ to determine which cytochromes remained oxidized after enzymatic reduction. Absorbance coefficients of cytochromes were as described in Ref. 27.

Ubiquinone depletion and reconstitution of submitochondrial particles. Lyophilized submitochondrial particles were depleted of ubiquinone by extraction with dry pentane using the procedure of Ernster et al. [25] with the following modifications. The particles were washed and lyophilized in 20 mM KCl containing 2 mM Hepes (K^+) (pH 7.5). When unbuffered 150 mM KCl was used, as in the original procedure, the recovery of NADH-ETF reductase activity was reduced to 5–10% of the activity of the submitochondrial particles. using the modified procedure, 50–60% of the initial activity was recovered after lyophilization. Also, after drying the particles on a vacuum evaporator, the ubiquinone-depleted and reconstituted preparations were resuspended by gentle homogenization in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol. Addition of dithiothreitol at this step increased the recovery of the reductase activities in reconstituted particles 2–3-fold. For chemical determinations, ubiquinone was extracted from the preparations as described by Kroger and Klingenberg [28] and quantitated spectrophotometrically ($\epsilon_{275-290\ nm} = 12.2\ mM^{-1}$).

Results

Fig. 1 illustrates a basic initial observation in this study. Submitochondrial particles exhibited an NADH-ETF reductase activity in the range of 100–130 nmol ETF reduced/min per mg protein. Activity was totally inhibited by 0.6 μM rotenone and by antibody to pig liver ETF-QO. Preimmune IgG had no effect on this activity. Thus, NADH-ETF reductase requires oxidation of the N-2 iron-sulfur cluster of Complex I, which remains reduced in the presence of rotenone. The N-2 cluster is the presumed reductant of ubiquinone [29]. The particles also catalyze the reduction of ETF by succinate. The latter activity was in the range of 90–120 nmol ETF reduced/min per mg protein. Fig. 2 shows the parallel inhibition of NADH-ETF and succinate-ETF reductase activities as a function of anti ETF-QO concentration. Thus, both ETF reductase activities are absolutely dependent on ETF-QO and there are not detectable alternate pathways for ETF reduction by NADH or succinate. Rotenone, 0.6 μM, completely inhibited NADH-ETF reductase, but had a negligible effect (4–8% inhibition) on succinate-ETF reductase ac-

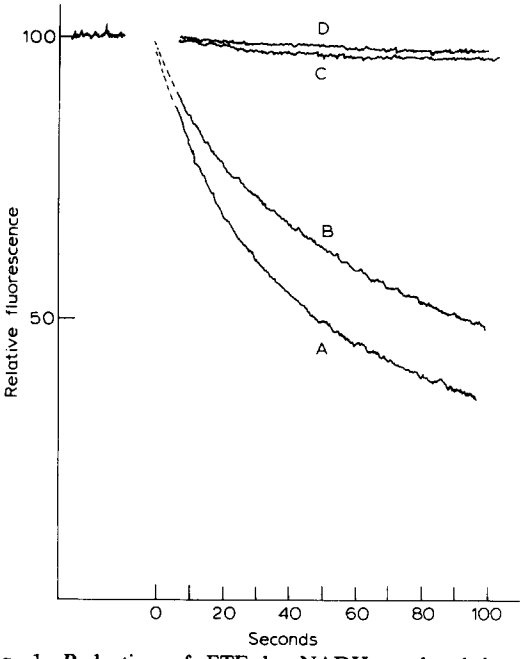

Fig. 1. Reduction of ETF by NADH catalyzed by submitochondrial particles. NADH-ETF reductase was assayed fluorometrically under anaerobic conditions as described in Materials and Methods. Reactions were initiated by the addition of submitochondrial particle protein. The traces indicate: the uninhibited reaction, with (A) 9 μg protein, (B) 6 g protein; (C) the reaction in the presence of 6 μg of protein and 0.6 μM rotenone; in (D), 200 μl of particles (1.8 mg/ml) were preincubated with 70 μg of the anti ETF-QO IgG preparation for 1 h at 4°C prior to assay with 6 μg protein. The specific activity of the preparation was 101 nmol ETF reduced/min per mg protein.

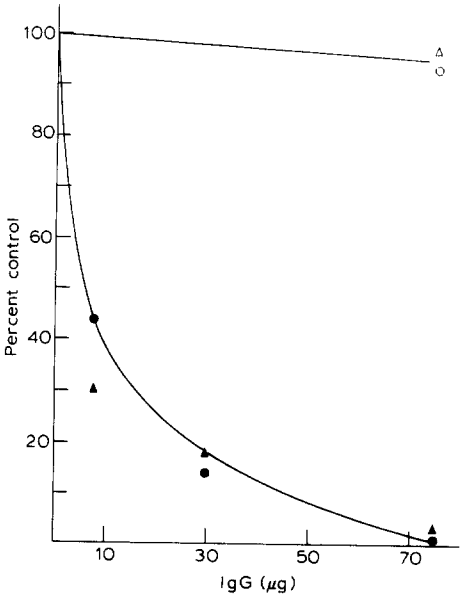

Fig. 2. Inhibition of NADH-ETF reductase and succinate-ETF reductase by anti ETF-QO. Submitochondrial particles (340 μg) were preincubated for 1 h at 4°C with the indicated amount of the anti ETF-QO IgG preparation (closed symbols) or a preimmune IgG (open symbols). The preparations were then assayed for NADH-ETF reductase activity [●, ○] and succinate-ETF reductase activity [▲, △]. The uninhibited activities were: NADH-ETF reductase, 130 nmol ETF reduced/min per mg; succinate-ETF reductase, 120 nmol ETF reduced/min per mg. The data are expressed as percent activity in the absence of the inhibitors.

tivity of the particles. Succinate-ETF reductase activity was inhibited by TTFA which had no effect on the initial velocity of NADH-ETF reductase. Succinate-ubiquinone oxidoreductase activity was considerably more sensitive to inhibition by TTFA ($K_i = 14 \pm 3$ μM) than succinate-ETF reductase activity ($K_i = 76 \pm 14$ μM). This difference is probably due to the fact that succinate-ubiquinone oxidoreductase activity is 20-fold greater than the ETF reductase activity which is thus rate-limiting.

Spectrophotometric evidence for the identity of the reduced ETF product of the succinate-ETF reductase reaction is shown in Fig. 3 and demonstrates that the reduced ETF product is the anion semiquinone [21]. Isosbestic points of the oxidized and anionic semiquinone are maintained at 476 nm and 404 nm, but lost at 342 nm due to absorption and scatter by the particle preparation. Identical results were obtained with NADH as the reductant (not shown). Also, when the reactions were run to completion, the residual fluorescence of the reduced ETF product was always in the range of 9% of the oxidized ETF flavin, which is characteristic of the anion semiquinone [23].

Coupled particles also catalyzed the ATP-dependent reduction of NAD^+ by enzymatically reduced ETF at about 40% of the rate of succinate reduction of NAD^+. Reduction of NAD^+ by ETF was inhibited by CCCP, rotenone and antibody to ETF-QO (Table I). Electron transfer between NADH, succinate and ETF-QO and the inhibition by rotenone and TTFA could be explained if ETF-QO reacts with ubiquinone that is also accessible to NADH and succinic dehydrogenases.

The results shown in Table II present evidence for the dependence of NADH- and succinate-ETF reductase activities on ubiquinone. Extraction of

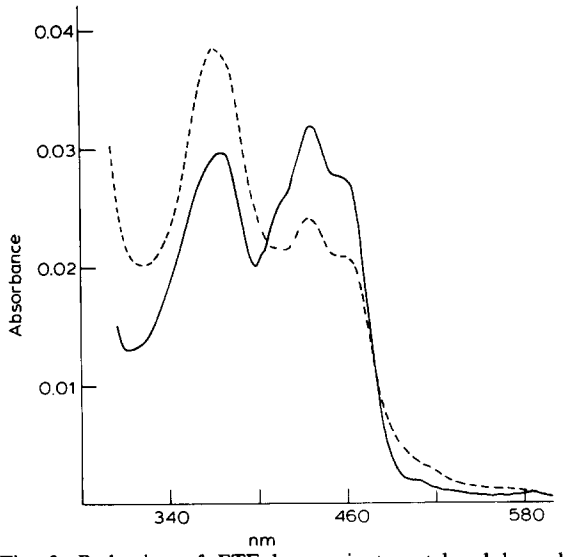

Fig. 3. Reduction of ETF by succinate catalyzed by submitochondrial particles. The absorption spectrum of ETF was determined under the conditions of the fluorimetric assay. The solid curve indicates the spectrum of oxidized ETF; the dashed curve indicates the spectrum of ETF recorded 4 min after the addition of 5 μg of submitochondrial particle protein. The specific activity of the succinate-ETF reductase was 108 nmol ETF reduced/min per mg determined in the fluorometric assay.

lyophilized submitochondrial particles with dry pentane results in the loss of chemically detectable ubiquinone and 99% loss of the ETF reductase activities. Reincorporation of ubiquinone restored about 90% of the ETF-reductase activities relative to the activities in the lyophilized particles. The reconstituted activities were inhibited by rotenone or TIFA and by antibody to ETF-QO (data not shown).

The reaction of reduced ETF with the bc_1 complex was investigated using the antibody to ETF-QO, antimycin and myxothiazole to compare reduction of this segment of the respiratory chain by ETF-QO with reduction by succinate and NADH [14,15]. The difference spectra shown in Fig. 4 illustrate the effects of these reagents on electron transport into the main respiratory chain from enzymatically reduced ETF. Fig. 4A shows the cytochrome in submitochondrial particles after reduction with 1.0 mM octanoyl CoA in the presence of 1.0 μM ETF and 0.5 μM general acyl CoA dehydrogenase flavin. Under these conditions, about 80% of cytochromes $c + c_1$, 60% of the b cytochrome and 90% of the $a + a_3$ cytochrome were reduced. These values were almost identical to the values obtained when the particles were reduced with 5 mM succinate under the same conditions with this preparation. Reduction of the cytochromes is absolutely dependent on ETF-QO, since 0.8 mg of anti ETF-QO added to the reaction mixture almost completely inhibited cytochrome reduction (Fig. 4B); 0.2 mg of the antibody preparation inhibited cytochromes $c + c_1$ and cytochrome b reduction by 70% (not shown). Ruzicka and Beinert previously reported a slow 70% reduction of cytochrome c_1 and the iron-sulfur protein in purified complex III by ETF, acyl CoA dehydrogenase and butyryl CoA in the absence of ETF-QO [1]; however, no evidence of this apparent bypass reaction is observed in Fig. 4B. Antimycin inhibited reduction of cytochromes $c + c_1$ by octanoyl CoA; 96% of the b cytochromes were reduced in the presence of antimycin (Fig.

TABLE I

Acyl-CoA-NAD$^+$ REDUCTASE ACTIVITY OF SUBMITOCHONDRIAL PARTICLES

Additions	mUnits/mg protein
None	22.7
− ATP	≤ 0.20
+ Rotenone (0.6 μM)	1.08
+ CCCP (1 μM)	≤ 0.30
+ anti ETF-QO (0.22 mg) [a]	0.91

[a] 0.49 mg of particles were preincubated with 0.22 mg of anti ETF-QO IgG for 15 min at 4° C prior to assay.

TABLE II

EFFECT OF UBIQUINONE DEPLETION AND RECONSTITUTION OF SUBMITOCHONDRIAL PARTICLES ON NADH-ETF AND SUCCINATRE-ETF REDUCTASE ACTIVITIES

Preparation	Reductase activities (nmol ETF reduced per mg protein)		Q content (nmol/mg protein)
	NADH-ETF	succinate-ETF	
Submitochondrial particles	104	94	3.7
Lyophilized particles	56	44	3.4
Pentane extracted particles	0.8	0.8	≤ 0.05
Q-reconstituted particles	48	42	2.6

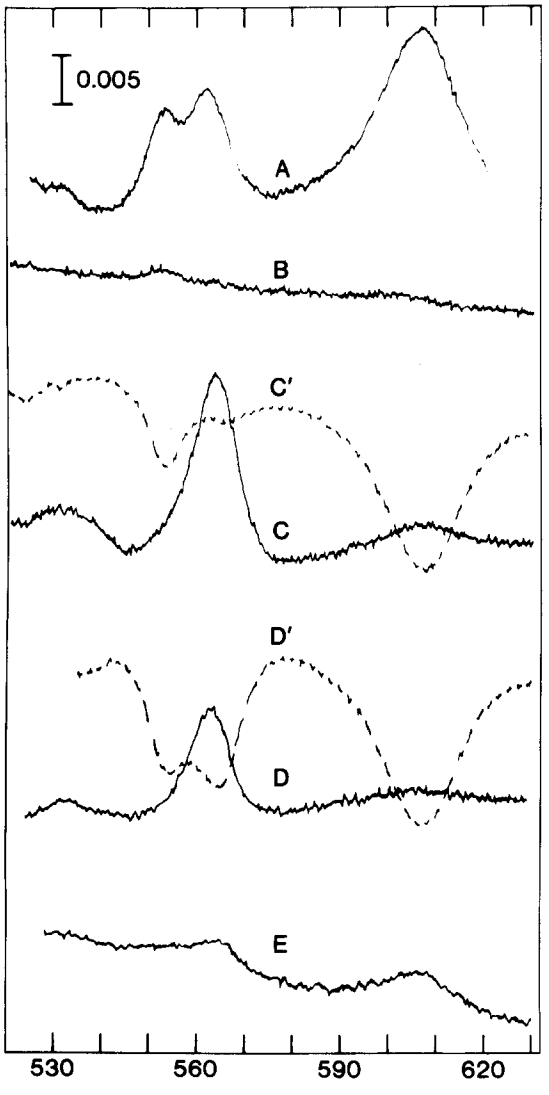

Fig. 4. Reduction of cytochromes in the presence of ETF, general acyl CoA dehydrogenase and octanoyl CoA. The difference spectra of the cytochromes were determined in incubations containing 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 0.5 μM ETF, 1.0 μM general acyl CoA dehydrogenase flavin, and 1.16 mg submitochondrial particle protein (0.83 μmol cytochrome *b*/mg protein); reduction was with 1 mM octanoyl CoA. The additions to the reaction mixtures were: (A) none; (B) 800 μg anti ETF-QO; (C) 5 μM antimycin; (D) 7.5 μM myxothiazole and (E) 5 μM antimycin plus 7.5 μM myxothiazole. The spectra indicated by the solid curves were determined after addition of acyl CoA substrate. The dashed, inverted spectra indicate cytochromes that remained oxidized after substrate reduction and were obtained after addition of dithionite to the reference cuvette.

4C) and reduction of the $a + a_3$ cytochromes is inhibited 90%.

Myxothiazole inhibited the reduction of cytochromes $c + c_1$ to about the same extent as antimycin (Fig. 4D). 50% of the total *b* cytochrome was reduced in the presence of myothiazole, which inhibited cytochrome *b*-566 reduction, since the *b* cytochrome reduced by the acyl CoA substrate had an absorption maximum at 562 nm and reduction of the reference showed that cytochrome *b*-566 remained oxidized in the presence of myxothiazole. There was minimal reduction of cytochrome oxidase by octanoyl CoA in the presence of myothiazole. When the cytochromes were reduced by octanoyl CoA in the presence of antimycin and myxothiazole, cytochrome reduction was negligible (Fig. 4E). These results are identical to those reported with NADH [14] and succinate [15] as reductants of the bc_1 complex. Neither antimycin nor myxothiazole inhibited NADH- or succinate-ETF reductase activities.

The rate of oxygen consumption in a system similar to that used to record the difference spectra was very low [7]. Also the time required for maximum reduction of the cytochromes by octanoyl CoA, about 8 min, was slow compared to reduction by succinate which was complete within 3 min. To investigate this point further, acyl CoA oxidase activity and its sensitivity to antimycin and myxothiazole were determined polarigraphically. Succinate and NADH oxidase activities were determined in parallel. Rates of oxygen consumption by submitochondrial particles in the presence of octanoyl CoA, general acyl CoA dehydrogenase and ETF were constant for about 2 min then progressively decreased, probably due to the accumulation of octenoyl CoA and ETF semiquinone, potent product inhibitors of the dehydrogenase [30,31]. Crotonyl CoA, 0.4 mM, inhibited the initial rate 60%. The initial rate of respiration with octanoyl CoA as the substrate, in the presence of general acyl CoA dehydrogenase and ETF, was 158 nmol/min per mg, and could be completely inhibited by antibody to ETF-QO (Table III). Inhibition of acyl CoA oxidase activity by antimycin and myxothiazole was comparable to inhibition of the NADH and succinate oxidases. The inhibition data indicate that the rates of

TABLE III

Acyl CoA, SUCCINATE AND NADH OXIDASE ACTIVITIES AND INHIBITION BY ANTIMYCIN AND MYXOTHIAZOLE IN SUBMITOCHONDRIAL PARTICLES

Substrate	Additions	Oxygen consumption (nmol/min per mg)	Inhibition (%)
Octanoyl CoA [a]	none	158	–
Octanoyl CoA	antimycin [b]	11	93
Octanoyl CoA	myxothiazole	13	92
Octanoyl CoA	anti ETF-QO [c]	≤1	100
Succinate	none	500	–
Succinate	antimycin	49	90
Succinate	myxothiazole	9	98
NADH	none	851	–
NADH	antimycin	104	88
NADH	myxothiazole	71	92

[a] Determined in the presence of 1 μM general acyl CoA dehydrogenase and 1 μM ETF.

[b] Reactions contained 1 μg/ml antimycin or 1.5 μg/ml myxothiazole, where indicated.

[c] 0.6 mg particles were preincubated with 0.18 mg ant ETF-QO IgG for 60 min at 4°C prior to assay.

oxygen uptake do not reflect turnover of the flavoproteins with oxygen.

Discussion

The results of these experiments confirm previous observations that the iron-sulfur cluster of ETF-QO in mitochondria and submitochondrial particle can be reduced by NADH and succinate [10,12]. Since ETF is reduced under these conditions, the data indicate that both redox centers of ETF-QO are functional in the ETF reductase reactions. Reduced ETF-QO has been assumed to reduce the bc_1 complex via ubiquinone, since short-chain, water-soluble homologs of ubiquinone oxidize both redox centers in the protein [2,3,9]. Because of the relative nonspecificity of water-soluble quinones as electron donors and acceptors in some cases [7,9], the reductive and oxidative reactions of ETF-QO with mitochondrial ubiquinone were investigated. The function of ubiquinone in the mitochondrial electron-transport chain was established in part by demonstration of the capacity of exogenous ubiquinone to reconstitute NADH and succinate oxidase activities in ubiquinone-depleted submitochondrial particles [25]. Reconstitution of acyl-CoA oxidase activity with this methodology would have little meaning because of the function of ubiquinone in the bc_1 complex [32]. Therefore, the ubiquinone dependence of ETF-QO activity in NADH-ETF reductase and succinate-ETF reductase activities was investigated to demonstrate how ETF-QO reacts with mitochondrial ubiquinone. Rotenone and TTFA inhibition of NADH-ETF and succinate-ETF reductase suggested a ubiquinone dependence for these reactions, since the N-2 cluster of NADH dehydrogenase [29] and the S-3 cluster of succinate dehydrogenase [33] remain reduced in the presence of the respective inhibitors and ubiquinone remains oxidized. Further, NAD^+ reduction by succinate is ubiquinone-dependent, indicating that the two dehydrogenases communicate via ubiquinone [25]. The capacity of exogenous ubiquinone to restore the two ETF reductase activities in ubiquinone-depleted particles constitutes direct evidence that ETF-QO reacts with the main respiratory chain via the ubiquinone pool.

ETF-QO can be chemically (2) or photochemically reduced (3) to a 3-electron reduced state; however, enzymatic reduction of ETF-QO by ETF proceeds only to the 2-electron-reduced state [3], with electrons partitioned between a flavin semiquinone and the reduced iron-sulfur cluster. It is not unreasonable to think that NADH and succinate also reduce ETF-QO to the 2-electron-reduced state in submitochondrial particles. Since the product of the NADH-ETF and succinate-ETF reductase reactions is the ETF flavin semiquinone, it follows that the ubiquinone reductant of ETF-QO is a 1-electron donor and that ETF-QO oscillates between 2- and 1-electron-reduced forms in the steady state. The product of the oxidative half-reaction of purified ETF-QO with short-chain ubiquinone analogs is the corresponding quinol [2,3,9]. That ubiquinol is the product of the oxidative half-reaction in the particles is suggested by the inhibitory activities of myxothiazole and antimycin on the reduction of the bc_1 complex by ETF-QO. The results of these experiments are consistent with the current model of the Q-cycle [17] originally proposed by Mitchell [16] in which the ubiquinol product of dehdyrogenase activities is the reductant of the complex. Thus, ETF-QO

must reduce ubiquinone in two 1-electron transfer steps and stabilize the ubisemiquinone intermediate. This is in contrast to succinic dehydrogenase which requires additional integral membrane protein to stabilize a semiquinone intermediate [34–36] and convert the dehydrogenase to a succinate-ubiquinone oxidoreductase which catalyzes formation of the ubiquinol reductant by the bc_1 complex [17].

The assumption has been made here that ETF-QO reduces ubiquinone directly and that no other protein(s) intervenes. This assumption is supported by several lines of evidence. First, purified ETF-QO can reduce ubiquinone homologs directly to the corresponding quinols [2,3,9]; no other proteins are required for ubiquinone reduction as in the case of succinic dehydrogenase [34–36]. Second, when submitochondrial particles are solubilized with Triton X-100 and the solubilized fraction treated with antibodies to ETF-QO, the only immunoprecipitated polypeptide observed was ETF-QO (Loehr, J. and Frerman, F.E., unpublished data). When similar experiments were carried out with antibodies directed against single proteins in complexes I, II or IV, all the associated proteins of the complexes were coprecipitated from the solubilized complexes or solubilized mitochondria [34,37,38]. Therefore, if another protein(s) is required for reduction of ubiquinone by ETF-QO system, it is not tightly bound to ETF-QO so as to survive detergent solubilization. Third, in the inherited metabolic disease, glutaric acidemia type II, in which electron transport from acyl-CoA dehydrogenases to the bc_1 complex is impaired, primary defects have been identified only in ETF or ETF-QO [5,39]. Although these constitute only negative evidence, they all suggest that no other protein(s) are required for ubiquinone reduction by ETF-QO.

Finally, these experiments also indicated that the intramitochondrial NADH/NAD^+ ratio may regulate fatty acid oxidation at a site other than at the level of the NAD-linked 3-hydroxyacyl CoA dehdyrogenase, since the reactions from the acyl CoA dehydrogenases to ubiquinone are reversible.

Acknowledgments

The technical assistance of Miss Mary DeVore is greatfully acknowledged. This research was supported by grant, AM 37749 and HDO 4024, from the National Institutes of Health.

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