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Coenzyme Q-Pool Function in Glycerol-3-Phosphate Oxidation in Hamster Brown Adipose Tissue Mitochondria

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We have investigated the role of the Coenzyme Q pool in glycerol-3-phosphate oxidation in hamster brown adipose tissue mitochondria. Antimycin A and myxothiazol inhibit glycerol-3-phosphate cytochrome *c* oxidoreductase in a sigmoidal fashion, indicating that CoQ behaves as a homogeneous pool between glycerol-3-phosphate dehydrogenase and complex III. The inhibition of ubiquinol cytochrome *c* reductase is linear at low concentrations of both inhibitors, indicating that sigmoidicity of antimycin A and myxothiazol inhibition is not a direct property of antimycin A and myxothiazol binding. Glycerol-3-phosphate cytochrome *c* oxidoreductase is strongly stimulated by added CoQ_3, indicating that endogenous CoQ is not saturating. Application of the pool equation for nonsaturating ubiquinone allows calculation of the K_m for endogenous CoQ of glycerol-3-phosphate dehydrogenase of 3.14 mM. The results of this investigations reveal that CoQ behaves as a homogeneous pool between glycerol-3-phosphate dehydrogenase and complex III in brown adipose tissue mitochondria; moreover, its concentration is far below saturation for maximal electron transfer activity in comparison with other branches of the respiratory chain connected with the CoQ pool. HPLC analysis revealed a lower amount of CoQ in brown adipose mitochondria (0.752 nmol/mg protein) in comparison with mitochondria from other tissues and the presence of both CoQ_9 and CoQ_{10}.

KEY WORDS: Glycerol-3-phosphate dehydrogenase; coenzyme Q-pool; ubiquinol cytochrome *c* reductase; brown adipose tissue mitochondria.

INTRODUCTION

Mitochondrial glycerol-3-phosphate dehydrogenase (EC 1.1.99.5), localized on the outer surface of the mitochondrial membrane, catalyzes the transfer of hydrogen from glycerol-3-phosphate to ubiquinone, thus forming one of the branches of the mitochondrial respiratory chain. Unlike other dehydrogenases of the respiratory chain (NADH dehydrogenase, succinate dehydrogenase), glycerol-3-phosphate dehydrogenase oxidizes its substrate on the cytoplasmic side of the mitochondrial membrane (Donnellan *et al.*, 1970; Klingenberg and Buchholz, 1970). Despite the localization of the enzyme on the C side, electron transfer from glycerol-3-phosphate to oxygen gives the same $H^+/2e^-$ ratio of 8 as from succinate (Alexandre and Lehninger, 1982).

In comparison with other mitochondrial dehydrogenases, its content varies in mitochondrial preparations from different tissues. The highest activity of this enzyme was found in insect flight muscle (Estabrook and Sacktor, 1958) and in brown adipose tissue (Chaffee *et al.*, 1964), being in stoichiometric proportions with cytosolic NAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.8.1). In these tissues both dehydrogenases operate as a glycerol-3-phosphate shuttle which transports hydrogen from the cytosolic NADH to the respiratory chain enzymes.

The mitochondrial enzyme from different sources (pig and rabbit brain, rabbit skeletal muscle, normal

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and hyperthyroid rat liver) was isolated, purified, and reconstituted (Ringler and Singer, 1961; Dawson and Thorne, 1969; Cole *et al.*, 1978; Cottingham and Ragan, 1980a, b; Garrib and McMurray, 1986; Beleznai and Jancsik, 1987).

Extensive studies of Cottingham and Ragan (1980b) on the partially purified enzyme reconstituted with isolated Complex III in the presence of excess of ubiquinone-10 (CoQ) indicated that in the glycerol-3-phosphate dehydrogenase branch of the mitochondrial respiratory chain a Q-binding protein such as postulated for NADH and succinate dehydrogenase (Yu *et al.*, 1978) is not present.

Cottingham and Ragan (1980b) also found that electron transfer between glycerol-3-phosphate dehydrogenase and Complex III obeys a classical "pool" behavior as previously indicated by Kröger and Klingenberg (1973a, b) for NADH and succinate oxidation.

The only report available on the role of Coenzyme Q (CoQ) in mitochondrial glycerol-3-phosphate dehydrogenase concerns a reconstituted system; on the other hand, the possibility that part of the respiratory chain is organized in the form of stoichiometric solid-state complexes has been advanced (Rich, 1984). For this reason, we have considered it of interest to investigate the role of the Q-pool in mitochondrial glycerol-3-phosphate dehydrogenase *in situ* in mitochondrial membranes.

In our experiments we have compared the effects of antimycin A and myxothiazol, specific inhibitors of ubiquinol cytochrome *c* reductase, and palmitoyl-CoA, a specific inhibitor of glycerol-3-phosphate dehydrogenase, on enzyme activity of both enzyme systems and in the integrated glycerol-3-phosphate cytochrome *c* reductase activity in brown adipose tissue mitochondria, and we present data supporting the idea that the Q-pool is the system through which reducing equivalents are transported from glycerol-3-phosphate dehydrogenase to the bc_1 segment of the respiratory chain.

METHODS AND MATERIALS

Chemicals

Cytochrome *c* (horse heart type III), palmitoyl-CoA, glycerol-3-phosphate, thenoyltrifluoroacetone, and salicylhydroxamic acid were obtained from Sigma Chemicals Co. (St. Louis, Missouri). Coenzyme Q homologs were kind gifts from Eisai Co., Tokyo. Other chemicals were of the highest purity commercially available.

Preparation of Mitochondria

The experiments were performed on mitochondria isolated from pooled interscapular, cervical, and axillary brown adipose tissue of adult golden hamster (*Mesocricetus auratus*) who had lived at least two weeks at 4°C with a 12-hour light/dark cycle. Tap water and food were provided *ad libitum*. Mitochondria were prepared by differential centrifugation in 250 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4, as described by Hittelmann *et al.* (1969).

The experiments were performed on fresh or frozen–thawed isolated mitochondria.

Determination of Enzyme Activity

Glycerol-3-phosphate cytochrome *c* oxidoreductase activity was measured, using an extinction coefficient of 19.1 $mM^{-1} cm^{-1}$, at 550 nm by means of a Perkin-Elmer 559 spectrophotometer in a reaction medium containing 100 mM K-phosphate, pH 7.6, 1 mM KCN, 50 μM cytochrome *c*, and 2.5 or 25 mM glycerol-3-phosphate as described by Sottocasa *et al.* (1967).

Succinate cytochrome *c* oxidoreductase activity was measured under the same conditions using 25 mM succinate.

Glycerol-3-phosphate dehydrogenase activity was assayed by measuring the rate of oxygen uptake using a Clark-type electrode, in a reaction coupled with phenazine methosulfate reduction and its subsequent reoxidation in the medium (final 1 ml) containing 250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, 1 mM KCN, 3 mM phenazine methosulfate, and 25 mM glycerol-3-phosphate.

Ubiquinol cytochrome *c* reductase activity was measured using a Sigma-Biochem dual-wavelength spectrophotometer equipped with a rapid mixing device, and following the reduction of cytochrome *c* at 550 minus 540 nm using an extinction coefficient of 19.1 $mM^{-1} cm^{-1}$. In our experiments ubiquinol cytochrome *c* reductase was assayed in 100 mM K-phosphate (pH 7.4) using a short-chain ubiquinol homolog, ubiquinol-2, which is partly water soluble and dissolves in the membrane by partitioning from the water phase before reaching the active site (Fato *et al.*, 1988). Ubiquinol-2 concentration never exceeded 15 μM according to Battino *et al.* (1986). Non-enzymatic reaction of cytochrome *c* with ubiquinol-2

was substracted from the total reaction rate; the extent of the nonenzymatic reaction was usually negligible. The assay temperature for all enzyme estimations was 25°C.

Coenzyme Q Determination

The mitochondria were assayed for ubiquinone content by reversed-phase HPLC analysis (Tsai *et al.*, 1985; Battino *et al.*, 1991a, b) after extraction with methanol and light petroleum using the method of Kröger (1978). Separations were performed using a Spherisorb S5 ODS I 25 × 0.46 cm column with a guard column containing the same material of the main column; the mobile phase was prepared by dissolving 7.0 g $NaClO_4 \cdot H_2O$ in 1000 ml ethanol/H_2O/70% $HClO_4$ (969:30:1). The flow rate was 1 ml/min. Measurements were performed at 25°C. The HPLC system was a Waters Data Module M730-Model 721 programmable system controller equipped with a Lambda-Max Model 481 LC spectrophotometer.

Protein Determination

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

RESULTS

Inhibition of Ubiquinol Cytochrome *c* Reductase by Antimycin A and Myxothiazol

Antimycin A and myxothiazol are excellent inhibitors of ubiquinol cytochrome *c* reductase (EC 1.10.2.2) operating at two different sites: at centre i (antimycin A) and at center o (myxothiazol) of the Q-cycle (Mitchell, 1975). In our experiments we showed that both have strong inhibitory effects on ubiquinol cytochrome *c* reductase of brown adipose tissue mitochondria.

Figure 1A shows the inhibition of ubiquinol cytochrome *c* reductase by antimycin A. The enzyme activity is considerably depressed even at inhibitor concentrations of 5 nM, and the shape of the inhibition curve is in its first phase linear. As we can see in Fig. 1B, the enzyme activity is also monotonically decreasing by myxothiazol. We may conclude that both antimycin A and myxothiazol have similar shapes of the inhibition curves. In both cases the maximal inhibitory effects are obtained at similar concentrations of inhibitors.

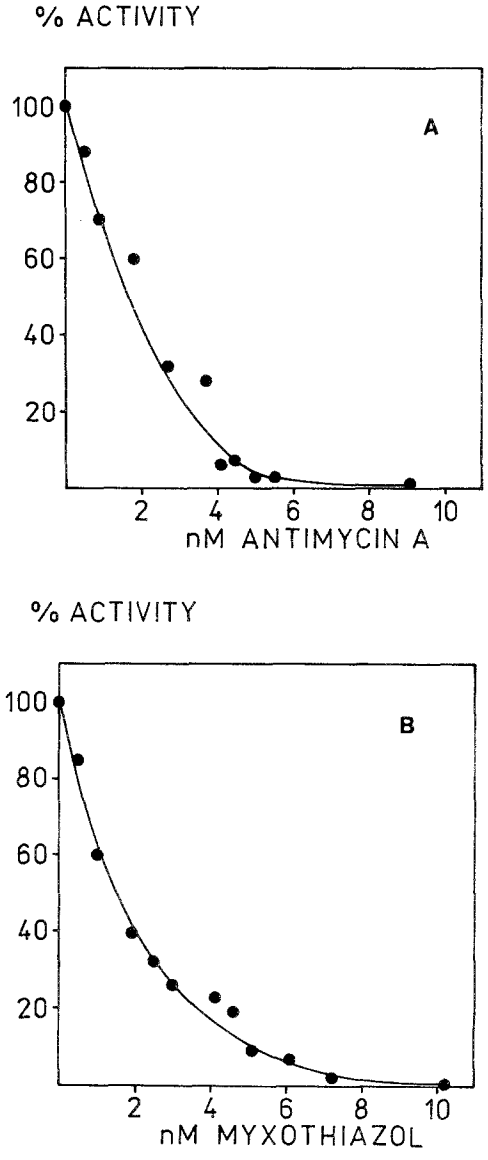

Fig. 1. Inhibition of ubiquinol-2-cytochrome *c* reductase activity with antimycin A (A) and with myxothiazol (B) in frozen-thawed brown adipose tissue mitochondria (0.0013 mg protein/ml). The enzyme activity was assayed using ubiquinol-2, as described under Materials and Methods.

Inhibition of Glycerol-3-phosphate Cytochrome *c* Oxidoreductase by Antimycin A and Myxothiazol

Figure 2A shows the titration curves of glycerol-3-phosphate cytochrome *c* oxidoreductase activity with antimycin A and myxothiazol. The inhibition curves have a sigmoidal shape with both inhibitors, and the inhibitor concentration required for full inhibition is higher than 30 nM. The sigmoidicity is

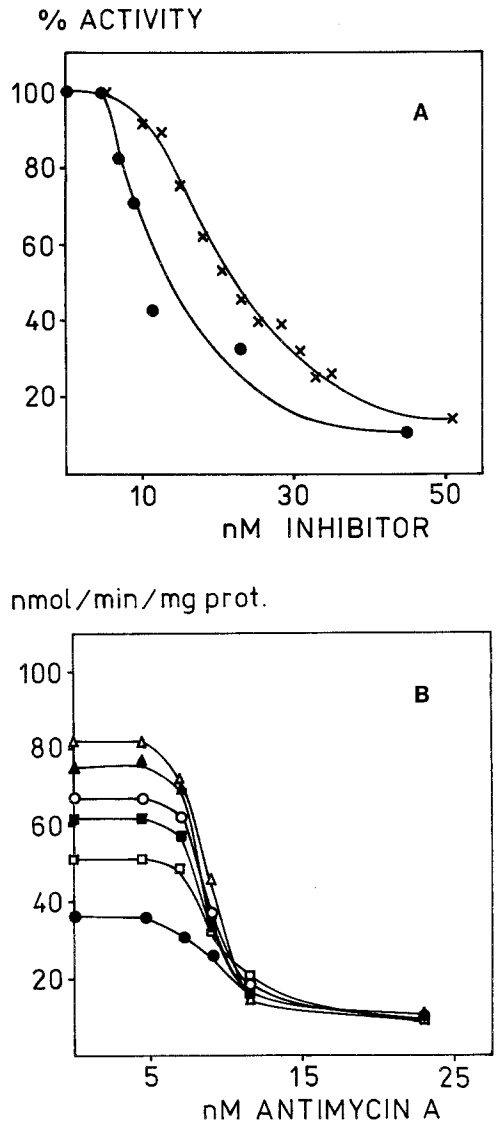

Fig. 2. Inhibition of glycerol-3-phosphate cytochrome *c* oxidoreductase by antimycin A (●) and myxothiazol (×) in the presence of 2.5 mM glycerol-3-phosphate (A) and by antimycin A at different concentration of glycerol-3-phosphate (B). The glycerol-3-phosphate concentrations were: (●) 2.5 mM, (□) 5 mM, (■) 7.5 mM, (○) 10 mM, (△) 15 mM, and (▲) 25 mM. The enzyme activity was measured in frozen–thawed brown adipose tissue mitochondria (0.0513 mg protein/ml) as described under Materials and Methods.

more pronounced with antimycin A than with myxothiazol. The antimycin A-insensitive reduction rate is about 10% of the overall rate.

The sigmoidal shape of inhibition curves with antimycin A is also obtained when the substrate concentration is changed between 2.5 and 25 mM (Fig. 2b).

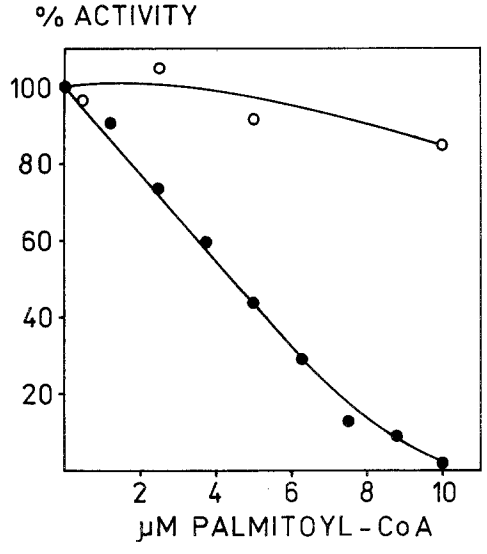

Fig. 3. Inhibition of ubiquinol-2 cytochrome *c* reductase (○) and glycerol-3-phosphate cytochrome *c* oxidoreductase (●) by palmitoyl-CoA. The enzyme activity was measured in frozen–thawed brown adipose tissue mitochondria as described under Materials and Methods.

Inhibition of Ubiquinol Cytochrome *c* Reductase and Glycerol-3-phosphate Cytochrome *c* Oxidoreductase Activity by Palmitoyl-Co A

Bukowiecki and Lindberg (1974) observed that the activity of glycerol-3-phosphate dehydrogenase of brown adipose tissue mitochondria was considerably depressed by palmitoyl-Co A. The authors suggested that palmitoyl CoA acts specifically at the level of the dehydrogenase complex. Our studies were therefore further extended by experiments in which palmitoyl CoA was used as specific inhibitor of glycerol-3-phosphate cytochrome *c* oxidoreductase activity. It is evident from our results presented in Fig. 3 that glycerol-3-phosphate cytochrome *c* oxidoreductase is inhibited by palmitoyl-Co A whereas ubiquinol-2 cytochrome *c* reductase is only slightly decreased.

We tested also the effects of thenoyltrifluoroacetone (inhibitor of succinate CoQ oxidoreductase) and salicylhydroxamic acid (inhibitor of glycerol-3-phosphate dehydrogenase activity in trypanosomes) on glycerol-3-phosphate dehydrogenase activity (not shown), but they were not effective. These results are in accordance with the results of Cottingham and Ragan (1980a, b) in the isolated enzyme.

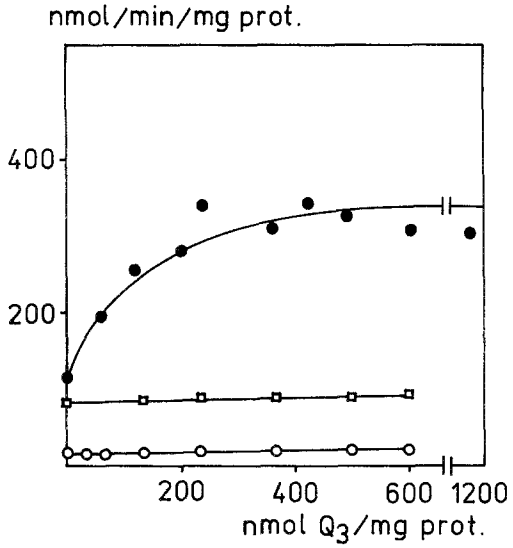

Fig. 4. Effect of added CoQ_3 on glycerol-3-phosphate cytochrome *c* oxidoreductase (●), succinate cytochrome *c* oxidoreductase (□), and antimycin A inhibition of stimulated glycerol-3-phosphate oxidoreductase (○). Antimycin A (25 ng) was added before substrate. The enzyme activities were measured in freshly isolated brown adipose tissue mitochondria (0.1 mg protein/ml) as described under Materials and Methods.

Saturation of Glycerol-3-phosphate Cytochrome *c* Reductase by the Q-Pool

A computer analysis of electron transfer in the CoQ region for oxidation of either NADH and succinate on the basis of the known affinities for CoQ of the partner enzymes reveals that CoQ in the pool is not saturating for maximal integrated activity (Battino *et al.*, 1990a; Lenaz and Fato, 1986; Lenaz *et al.*, 1990a).

The theoretical calculation was confirmed for NADH-cytochrome *c* reductase by direct reconstitution experiments of mitochondrial membranes fused with phospholipids containing an excess of CoQ_{10}. Direct addition of short-chain homologs, without phospholipid fusion, although not possible for NADH oxidation due to inhibition of activity (Lenaz *et al.*, 1975), was attempted in this study for glycerol-3-phosphate dehydrogenase. The activity was titrated with CoQ_3 (Fig. 4). If we assume that V_m for CoQ_3 is the same as with the endogenous ubiquinones, the strong stimulation of basal activity reveals that endogenous quinone is not saturating. On the other hand, the same concentrations of CoQ_3 do not enhance succinate cytochrome *c* reductase, indicating that the CoQ pool is saturating between complex II and III. Simultaneous determinations of glycerol-3-phosphate and succinate oxidation by cytochrome *c* exhibits results indistinguishable from addition of the former substrate alone (not shown).

Evaluation of K_m and V_{max} in the presence of exogenously added CoQ_3 showed that K_m for glycerol-3-phosphate is increased from 10.8 to 22.2 mM and V_{max} is increased from 339 to 851 nmol/mg protein/min.

The large amounts of CoQ_3 needed for saturation of glycerol-3-phosphate cytochrome *c* oxidoreductase activity are probably the result of incomplete solubilization of CoQ_3 in the mitochondrial membrane (Fato *et al.*, 1986); for this reason, no realistic assumption can be obtained directly from these studies on the K_m of the system for ubiquinones. Nevertheless, application of the pool equation for nonsaturating ubiquinone (Lenaz and Fato, 1986) allows calculation of the K_m for endogenous CoQ_9 + CoQ_{10} of glycerol-3-phosphate ubiquinone reductase. According to this equation,

$$V_{obs} = \frac{[(V_{mr} \cdot V_{mo})/(V_{mr} + V_{mo})] \cdot Q_t}{[(V_{mr} \cdot K_{mo} + V_{mo} \cdot K_{mr})]/(V_{mr} + V_{mo})] + Q_t}$$

where V_{obs} is the observed rate of glycerol-3-phosphate cytochrome *c* oxidoreductase at ubiquinone concentration Q_t in the membrane, V_{mr} and V_{mo} are the maximal rates of glycerol-3-phosphate ubiquinone reductase and of ubiquinol cytochrome *c* oxidoreductase, respectively, and K_{mr} and K_{mo} are the respective K_m for ubiquinone. The K_m of glycerol-3-phosphate ubiquinone reductase for CoQ_9 (K_{mr}) can be calculated if all other variables are known. For this purpose, V_{obs} (glycerol-3-phosphate cytochrome *c* oxidoreductase) is directly obtained from this study, and V_{mo} is taken as the maximal rate of ubiquinol-2 cytochrome *c* reductase; K_{mo} was taken as 1.2 mM in the lipids (Zhu *et al.*, 1982). Since glycerol-3-phosphate dehydrogenase assay does not yield reliable values of V_m for endogenous quinone reduction, the value of V_{mr} was obtained from the pool equation

$$V_m^{obs} = V_{mr} \cdot V_{mo}/V_{mr} + V_{mo}$$

where V_m^{obs} is the maximal stimulated rate with CoQ_3. All the rates were expressed as electron equivalents per second and the concentrations were expressed as M in the lipids, assuming that 1 mg mitochondrial protein corresponds to 0.5 mg of phospholipids, and that 1 mg of phospholipids is contained in 1 μl of lipid bilayer. Inserting the above value into the equation, a K_{mr} of

3.14 mM for CoQ_9 in the phospholipid is obtained for glycerol-3-phosphate ubiquinone reductase.

CoQ Distribution in Hamster Brown Adipose Tissue

The HPLC analysis of hamster brown adipose tissue mitochondria revealed a low content of CoQ and the presence of two Coenzyme Q homologs, i.e., CoQ_9 and CoQ_{10}, as reported for the other tissues of various rodents (Battino *et al.*, 1990b, 1991b).

From the total CoQ, 0.752 nmol/mg mitochondrial protein, 72% was found as CoQ_9 and 28% as CoQ_{10}.

Contrary to other tissues, we found in brown adipose tissue a very high content of extramitochondrial CoQ. We found 60% of the total CoQ content in mitochondria and 40% of the total CoQ content in postmitochondrial supernatant. The high CoQ content in the postmitochondrial fraction is in accord with the data of Joel and Ball (1962).

DISCUSSION AND CONCLUSION

Glycerol-3-phosphate dehydrogenase is the most simple branch of the respiratory chain but one of the least known enzymes connected with the electron-transfer system of the mitochondrial inner membrane. In the extensive paper of Cottingham and Ragan (1980b), it was shown that the partially purified enzyme is capable of reconstitution with CoQ_{10} and mitochondrial ubiquinol cytochrome *c* oxidoreductase to give antimycin A-sensitive glycerol-3-phosphate cytochrome *c* oxidoreductase activity. The authors did not find any evidence that other polypeptides are involved in the electron-transfer system between glycerol-3-phosphate dehydrogenase and the rest of the respiratory chain. The absence of Q binding protein supported the idea that hydrogen atoms are transferred directly to the Q-pool inside the mitochondrial membrane.

Our results give further evidence for this hypothesis and eliminate the possibility of the direct connection of the glycerol-3-phosphate dehydrogenase with complex III through specific Q molecules. It is quite evident that inhibitory curves of antimycin A and myxothiazol (Fig. 1A, B) have a linear shape in the case of ubiquinol cytochrome *c* reductase because the inhibitor acts directly on the enzyme molecule. The hyperbolic inhibition of ubiquinol cyt. *c* reductase by antimycin in the brown adipose tissue mitochondria contrasts with the titrations of Zhu *et al.* (1982) of succinate oxidase inhibition versus saturation in bovine heart submitochondrial particles. Apparently in the brown adipose tissue mitochondria, contrary to heart mitochondria, antimycin A can also be used to test pool behavior. Because the glycerol-3-phosphate dehydrogenase cannot fully saturate the respiratory chain and the enzyme itself is not affected by antimycin A, we should obtain in the case of glycerol-3-phosphate cytochrome *c* oxidoreductase a sigmoidal curve with the lag proportional to the excess of bc_1 complex over glycerol-3-phosphate dehydrogenase (Kröger and Klingenberg, 1973b). Experimental data presented in Fig. 2B fully support this hypothesis using various substrate concentrations. We obtained in all curves the same concentration range for the lag in which the enzyme activity is not inhibited by antimycin A: in all cases the maximal inhibition was obtained at the same concentration of inhibitor. The inhibitor titer for maximal inhibition of glycerol-3-phosphate cytochrome *c* reductase was much higher than that required for inhibition of ubiquinol cytochrome *c* reductase. The simple explanation of this discrepancy is in the extremely high affinity of the inhibitors for their binding sites in the bc_1 complex, so that the amount bound is strongly affected by the number of complexes in the assay medium (i.e., the protein concentration, which was considerably greater for glycerol-3-phosphate cytochrome *c* reductase than for ubiquinol cytochrome *c* reductase; cf. Materials and Methods).

As expected, a sigmoidal inhibition curve was not obtained when palmitoyl-CoA was used; the reason is that the inhibitor acts directly on the glycerol-3-phosphate dehydrogenase molecule which is rate limiting already when uninhibited, prior to the entry of reducing equivalents into the Q-pool.

We may thus conclude that glycerol-3-phosphate dehydrogenase can be considered as an example of the simple branch of the respiratory chain transporting reducing equivalents to the Q-pool inside the mitochondrial membrane and through Q to Complex III.

This study also shows that the endogenous Q-pool of the mitochondrial membrane of brown adipose tissue is not saturating for obtaining maximal electron transfer for glycerol-3-phosphate cytochrome *c* oxidoreductase. Since brown adipose tissue mitochondria have a low content of CoQ, it is possible that the low ubiquinone level in these mitochondria, and conversely its high content in the postmitochondria fraction, are the result of the considerable fat content and of the high lipid solubility of the quinone. The low

CoQ level of these mitochondria may explain why the activity is increased 3-fold by exogenous CoQ_3. Evidently due to absence of Q binding protein, glycerol-3-phosphate dehydrogenase is more sensitive to low endogenous CoQ than NADH or succinate dehydrogenases. Lack of saturation for CoQ of electron transfer seems to be a common property of most respiratory enzymes, as a result of the relatively high K_m of mitochondrial enzymes using oxidized or reduced CoQ. Although application of the pool equation including CoQ concentration in the membrane (Lenaz and Fato, 1986; Ragan and Cottingham, 1985) and using published values for the "K_m" of succinate oxidation for CoQ (Norling *et al.*, 1974) indicates that the quinone is not saturating for maximal succinate cytochrome *c* reductase activity (Lenaz *et al.*, 1990), the results of this study show experimentally that CoQ_3 is unable to further enhance this activity above control level. The reason for such discrepancy is not clear and may concern tissue specificity or alternatively methodological differences. Since the natural levels of long-chain CoQ homologs in the membrane may approach the maximal amounts that can be accumulated in a physiologically active way, it follows that saturation of activity may never be reached.

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