Biochimica et Biophysica Acta 852 (1986) 100–105
Elsevier

BBA 42134

The role of phospholipids in the binding of antimycin to yeast Complex III

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(Received 30 June 1986)

Key words: Respiratory chain; Antimycin binding; Complex III; Coenzyme Q; Phospholipid; (Yeast)

Treatment of yeast Complex III with hexane or repeated fractionation with ammonium sulfate-cholate abolishes the ability of antimycin to bind to the complex. Antimycin binding is partially restored by addition of phospholipids to the depleted complex; this action of phospholipids can be enhanced by including Q_6 in the reconstitution mixture.

Introduction

Recently, there has been an increase in the use of respiratory inhibitors as tools to investigate electron transfer in mitochondrial Complex III in large part due to the discovery of several new and potent inhibitors [1–7]. Even so, the classical inhibitor antimycin A still appears to be the most effective, binding stoichiometrically to Complex III and decreasing the ubiquinol cytochrome *c* reductase activity by mor than 95%.

Despite extensive knowledge of the relationship between the chemical structure and potency of antimycin as a Complex III inhibitor [8–10], a large number of experiments have failed to define the site of interaction of antimycin with any of the subunits of the enzyme complex [7,11–14] and our current understanding of the binding site is quite limited. Thus, even though cytochrome *b* has long been suspected of being a site of action, purified yeast cytochrome *b* completely fails to bind antimycin [15]. Very recent photo-affinity studies using an azido derivative of antimycin [16] and measurement of the binding studies of antimycin to carefully prepared subunits of the enzyme [17] both suggest that band 7, a 12 kDa subunit, may be a component of the binding site.

The difficulty in identifying the peptide(s) that bind antimycin, despite its exceptional affinity for the native complex, has raised the possibility that nonprotein components may be necessary for the integrity of the antimycin binding site. Here we present evidence showing that phospholipids plays a role in the binding of antimycin to Complex III isolated from yeast mitochondria.

Materials and Methods

Yeast Complex III (native Complex III) was prepared according to Siedow et al. [18]; a typical preparation has a coenzyme Q content of 1–1.5 mol/mol c_1. Cytochrome *b* was isolated from yeast Complex III by our published procedure [15]. Complex III depleted of coenzyme Q was prepared by two methods. The first combines lyophilization followed by seven cycles of hexane extraction of Complex III, a procedure similar to that of Szarkowska [19]. The second method involves repeated treatment of Complex III with ammonium sulfate and cholate as detailed by Yu

Abbreviation: Tween-20, polyoxyethylene sorbitan monolaurate.

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et al. [20]. The coenzyme Q-depleted complex obtained by either method typically contains less than 0.05 mol coenzyme Q per mol c_1. Gas chromatography/mass spectrometry analylses were carried out using a Varian Model 3400 gas chromatograph interfaced to an Extrel ELQ-400 mass spectrometer. Thin-layer chromatography was performed using the single plate system described by Gilfillan et al. [21]. Two solvent systems were used: $CHCl_3$/ MeOH/ petroleum ether/ acetic acid/boric acid (120 : 60 : 100 : 30 : 5.4, v/v/v/v/w) for analysis of phospholipids and hexane/petroleum ether/4-acetic acid (80 : 20 : 1, v/v/v) for neutral lipids and sterols.

Ubiquinone-6 (coenzyme Q_6) and antimycin were purchased from Sigma. Coenzyme Q_6 was prepared as a 5 mM solution in absolute ethanol. Antimycin was prepared as 0.5 or 1 mM solution in dimethylsulfoxide; its concentration was determined from absorbance measurements at 320 nm ($\epsilon = 4.8$ mM $\cdot$ 1 cm^{-1}).

Soybean asolectin was obtained in granule form from Associated Concentrates. Sources of pure phospholipids are as follows: Cardiolipin, phosphatidylethanolamine and phosphatidylserine are products of Avanti Polar-Lipid Inc.; phosphatidylcholine is a Sigma product. Before each experiment, dried phospholipids were suspended to a concentration of 10 mg/ml in water by sonication. Cardiolipin, when used for fluorescence quenching experiments (or enzyme reconstitution) was prepared as a clear solution (10 mg/ml) in 5% Tween-20. Ergosterol and squalene are also Sigma products and were prepared as 10 mM solutions in water by sonication.

Enzyme activity was measured by following the reduction of 50 μM ferricytochrome c by 25 μM 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinol (PBQH2) (pH 7.4), $T = 25$ °C [18]. Quantitation of coenzyme Q_6 was performed using a combined methanol-petroleum ether extraction and HPLC-ultraviolet measurement as described in Ref. 22.

Reconstitution of CoQ-depleted Complex III was carried out essentially as described by Yu et al. [20]. When present, coenzyme Q_6 was first added at a ratio of 2 mol/mol c_1 to the coenzyme Q-depleted enzyme (2–4 mg/ml concentration); this was followed by the addition of 0.2–0.3 mg phospholipid/mg Complex III. To obtain maximum reconstitution of activity, samples were stirred at 4 °C for 24 h. The amount of Q_6 reincorporated into the complex was determined by HPLC after centrifugation (100 000 $\times$ g, 2 h) and washing twice with 0.1 M phosphate buffer (pH 7.4) containing 0.5% cholate.

Antimycin-binding was monitored by quenching of fluorescence using an SLM-400 fluorimeter [15]. Lack of antimycin binding to coenzyme Q-depleted Complex III was confirmed by the binding-precipitation protocol [15]; various amounts of antimycin were incubated with fixed amount of CoQ-depleted Complex III for 10 min, the reaction mixtures were then centrifuged (48 000 $\times$ g, 2 h) and free antimycin was quantitated by fluorescence measurements of the supernatants.

Results and Discussions

Native yeast Complex III binds antimycin with a stoichiometry which is 1 : 1 with the content of cytochrome c_1 (15) (Fig. 1: △). Upon removal of coenzyme Q this ability to bind antimycin is completely eliminated and cannot be restored by incubating the coenzyme Q-depleted complex with coenzyme Q_6 alone (Fig. 1: △ and □). This result has been obtained with both methods of coenzyme Q-depletion, viz. extraction with hexane (Fig. 1, bottom panel) and ammonium sulfate-cholate fractionation (Fig. 1, top panel) and has been confirmed using the binding-precipitation method [15]. Examination of the hexane extract by TLC revealed two major components, one of which migrated with authentic Q_6. The identity of the second spot has been established by gas chromatography/mass spectrometry, which revealed the presence of several sterols in the hexane extract. The major components were identified as ergosterol, together with lesser amounts of squalene. These sterols probably account for the ultraviolet absorbing species whch is eluted after Q_6 during HPLC quantitations of this ubiquinone [22]. No phospholipid was found in the hexane extract. Thin-layer chromatography of chloroform-methanol extracts of the native and hexane-treated complexes were very similar and showed comparable amounts of cardiolipin, phosphatidylethanolamine and phosphatidylcholine.

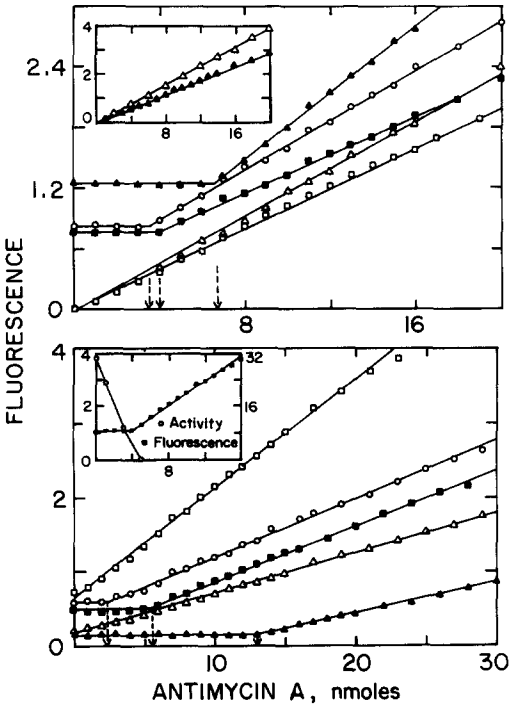

Fig. 1. Top panel: antimycin binding to coenzyme Q-depleted Complex III obtained by ammonium sulfate-cholate extraction. (▲) Native Complex III (7 nmol in 0.1 M potassium phosphate (pH 7.4), containing 0.5 hl cholate). Coenzyme Q-depleted complex (8.3 nmol in the same buffer as native complex). Treatment of each sample: △, none; □, addition of 17 nmol CoQ_6 and 2-h incubation; ○, addition of 0.6 mg cardiolipin and 4-h incubation; ■, reconstitution by 17 nmol Q_6 and 0.6 mg cardiolipin for 5 h. The reactions were performed at 4°C. The enzyme activity relative to the native complex for the four samples are 0.9%, 1.8%, 18%, 53%, respectively. The residual coenzyme Q_6 in the coenzyme Q-depleted sample used in this experiment is less than 1% that of the native sample. Either buffer or buffer containing 17 nmol Q_6 and 0.6 mg cardiolipin was used as the fluorescence blank. The amount of antimycin bound to the native (6.7 nmol), reconstituted (4.2 nmol) and cardiolipin-replenished (3.6 nmol) samples are indicated by the vertical arrows. Inset to top panel: antimycin binding to purified cytochrome *b* (15 nmol) (△) before or after mixing with 15 nmol coenzyme Q_6 plus 0.2 mg phospholipid mixture (▲). Bottom panel: antimycin binding to coenzyme Q-depleted Complex III obtained by hexane extraction. Native Complex III (13 nmol) and four samples of coenzyme Q-depleted Complex III (11.9 nmol each) were used for the quenching experiment. Treatment of the coenzyme Q-depleted samples were almost identical to that described in the legend of Fig. 2, except for slight variations in incubation time. The incubation time for coenzyme Q-depleted, coenzyme Q-depleted plus coenzyme Q_6, coenzyme Q-depleted plus cardiolipin and reconstituted were 0.25, 2 4 and 4 h, respectively; and the enzyme activities of these four samples relative to the native complex were 2.5%, 2.8% 23.9%, and 44%. The amount of antimycin bound to native, reconstituted and cardiolipin-repleted samples are 13.0, 5.7 and 2.8 nmol, respectively. Inset to bottom panel: The relationship between activity loss (○------○) and fluorescence quenching (■- - - - -■) was recorded using a separate sample (7 nmol) of reconstituted Complex III. The fluorescence (left ordinate) is shown in arbitrary units; the enzyme activity (right ordinate) is in units of the standard assay.

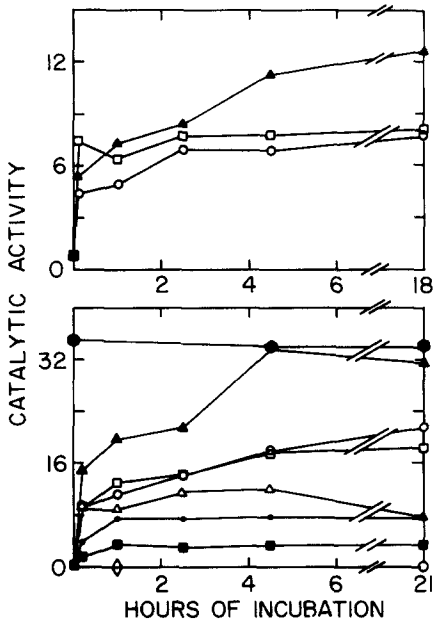

Fig. 2. The efficiency of Q_6 and various phospholipids on the stabilization and reconstitution of coenzyme Q-depleted Complex III. 5 nmol of coenzyme Q-depleted Complex III (in 0.2 ml 50 mM potassium phosphate buffer (pH 7.4) containing 0.5% cholate and 1 mM EDTA) was used for each sample. Aliquots were removed from each reconstituted mixture for activity assay after 10 min and 1, 2.5, 4.5 and overnight incubation. Top panel: The experimental conditions were the same as for the reconstitution experiment except that coenzyme Q_6 was absent. The symbols correspond to those in the bottom panel. Bottom panel: ●, native complex III; ◇, coenzyme Q-depleted enzyme; ○, coenzyme Q-depleted sample plus coenzyme Q_6 (5 nmol); ▲, plus Q_6 + phospholipid mixture; □, plus Q_6 + Cardiolipin; ○, plus Q_6 + asolectin; ■, plus Q_6 + phosphatidylcholine; ●, plus Q_6 + phosphatidylethanolamine; △, plus Q_6 + phosphatidylserine. The phospholipids were present at 0.2 mg/mg protein.

Despite the failure to restore activity, significant amounts of Q_6 can be incorporated. For example, upon incubation of hexane-extracted enzyme with either 1, 2 or 10 Q_6/c_1, 0.7, 1.3 and 5 equivalents of Q_6 could be detected in the protein

complex. Values of 0.7, 1.0 and 3.6 equivalents were obtained when cardiolipin was also included in the incubation mixture. Similar results were obtained with ammonium-sulfate cholate extracted enzyme.

As preliminary data indicated that phospholipids could restore antimycin binding, we tested phospholipids found in the inner membrane of yeast mitochondria [23] (individually and in mixtures) for their ability to reconstitute Complex III as gauged by the restoration of antimycin-sensitive catalytic activity.

Coenzyme Q-depleted samples obtained by ammonium sulfate-cholate fractionation lose almost all enzyme activity (Fig. 2, bottom panel); by contrast, native Complex III maintains full catalytic activity when incubated at 4°C for the time necessary to prepare the coenzyme Q-depleted sample and accomplish reconstitution.

All phospholipids tested restored enzymatic activity to some degree. The mixture of phosphatidylcholine, phosphatidylethanolamine and Cardiolipin * was the most effective both in stabilizing the coenzyme Q-depleted complex (Fig. 2, top panel) and in reconstitution of enzyme activity (Fig. 2, bottom panel). Reconstitution of 90% of the original catalytic activity was obtained using this mixture. Asolectin and cardiolipin alone restore about 60% of the native activity, a significantly higher value than those obtained when either phosphatidylethanolamine, phosphatidylcholine or phosphatidylserine were used alone (Fig. 2, bottom panel). Neither ergosterol nor squalene were effective in restoring enzymatic activity nor did they enhance the reconstitutive ability of the phospholipids.

Because of the efficacy of cardiolipin in reconstituting enzyme activity, and its easy solubilization by 5% Tween-20 **, we chose to use it for fluorescence measurements of antimycin binding, thus minimizing the light scattering problem originating from the turbid samples produced by addition of the other phospholipids.

In the presence of cardiolipin, a significant portion of the ability of Complex III to quench the fluorescence of antimycin was restored (Fig. 1, ○). Furthermore, the restoration of fluorescence quenching was strongly correlated with the extent of recovery of enzyme activity (Fig. 1).

When coenzyme Q-depleted complex prepared by ammonium sulfate-cholate extraction is used for the measurements of antimycin binding, the extent of antimycin fluorescence quenched by enzyme reconstituted with only cardiolipin and that reconstituted by cardiolipin plus Q_6 is not significantly different (Fig. 1, top panel). However, with coenzyme Q-depleted enzyme obtained by hexane extraction, significant differences were observed between the cardiolipin- and cardiolipin-plus-Q_6-treated samples (○ and □ in Fig. 1, bottom), with the extent of fluorescence quenching by the former being about one-half that of the latter. This difference is also found when asolectin is used as the source of phospholipid for reconstitution of ammonium sulfate extracted complex (data not shown). It should be noted that antimycin does not bind to purified cytochrome *b* even in the presence of coenzyme Q_6 plus phospholipids (Fig. 1, inset to top panel). These results show that added phospholipids are necessary for the recovery of antimycin binding and that the ability of phospholipid to restore antimycin binding can be enhanced by the presence of Q_6.

We previously demonstrated a good correlation between the extent of antimycin binding and the loss of enzyme activity in native Complex III [15]. Similar experiments with reconstituted enzyme complex yield the same correlation (Fig. 1, inset to bottom panel). The observation that the end-point for fluorescence quenching is the same as that for inhibition of enzyme activity demonstrates that incomplete recovery of activity upon reconstitution is due to irreversible inactivation of a fraction of the preparation. Were all of the preparation to exhibit a reduced catalytic activity, then a significant fraction of activity would have remained at the equivalence point for fluorescence quenching.

The loss of antimycin-binding capacity of coenzyme Q-depleted complex is reflected in kinetic experiments on the reoxidation of fully reduced enzyme by ferricyanide [24]. In both native Complex III and the coenzyme Q-depleted sample, the loss in absorbance at 561.5 nm leads

* The molar ratio of phosphatidylcholine, phosphatidylethanolamine and cardiolipin was 2 : 1 : 1.

** 5% Tween-20 alone did not produce any reconstitution of activity.

that observed at 553.5 nm during the initial period of reaction. However, whereas antimycin markedly depresses the reoxidation rate of cytochrome *b* in the native complex, it has no effect upon the oxidation of cytochrome *b* in the coenzyme Q-depleted sample. This result is to be expected if antimycin does not bind to the coenzyme Q-depleted sample.

Our data clearly show that phospholipids are required for maximum expression of antimycin binding to yeast Complex III and that the extent of binding depends upon the chemical nature of the lipids used in reconstitution. However, as the original phospholipids are not removed by hexane extraction, the loss of antimycin-binding and the restoration of this binding by added phospholipid can only be explained by asserting that the original disposition of the antimycin binding site has been disorganized by treatment with the organic solvent and that this process is reversed upon supplementing the quinone-depleted complex with the exogenous lipid. There is, however, no evidence that the added lipid is integrated into the antimycin binding site. Indeed, it cannot even be concluded that phospholipids are immediately involved in the binding of antimycin, an indirect interaction between phospholipids and the components of the antimycin binding site, being an equally acceptable explanation of our data. At the same time, the requirement for Q is obscure. Even though substantial amounts of Q_6 can be restored to the depleted complex in the absence of phospholipid, it has not been established whether any of this Q_6 is present at some natural site or is simply nonspecifically bound to the protein complex as a consequence of its nonpolar character. This latter property might also underlie the ability of Q_6 to enhance the reconstitution power of phospholipids, at least in hexane-extracted enzyme. The difference between hexane-extracted and ammonium sulfate-cholate extracted enzyme would then reside in the spectrum of residual phospholipid and, possibly, the conformational state of the polypeptides which is achieved in the two cases.

It must be stressed that many of the properties of the metal centers appear to be unaffected by extraction with hexane. These properties include the optical, EPR and stoichiometric titration [25], the MCD and potneteiometric behavior [26] and the reaction of the reduced complex with oxidants [24]. However, reduction of the cytochromes by Q_1H_2 is abolished in the hexane-treated complex; this is most simply explained by assuming that the endogenous quinone is the initial acceptor of reducing equivalents from the electron donor (Kauten, R.J., Tsai, A.L. and Palmer, G., unpublished results).

Nevertheless, our data do show that, at least in the case of the yeast enzyme, the integrity of the antimycin binding site depends on both protein and non-protein components.

Acknowledgements

Supported by the National Institutes of Health (GM21337) and the Robert A. Welch Foundation (C-636). We are indepted to Dr. Jan Pyrek for the identification of the sterols by gas chromatography/mass spectrometry.

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