ON THE SIGMOIDAL RELATIONSHIP BETWEEN INHIBITION OF RESPIRATION AND ANTIMYCIN TITER

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1. Introduction

Bryła et al. [1–3] have reported that treatment of submitochondrial particles (heart–muscle preparation) with anionic detergents (i.e. cholate, deoxycholate, or dodecylsulfate) converted the relationship between antimycin titer and inhibition of succinate-cytochrome *c* reductase from sigmoidal to linear. Similar results were reported with the antimycin-induced enhancement of reducibility and the red shift of the absorption spectrum of cytochrome *b*. In contrast, the sigmoidal titration curves were retained when the submitochondrial particles were treated with cationic or nonionic detergents [4]. On the basis of these observations, Bryła et al. proposed that antimycin acts as an allosteric inhibitor, binding preferentially to a conformation form (designated as the R state) of the cytochromes *b*-c_1 segment (Complex III) which is monomeric and less active in electron transfer than the oligomeric conformation (designated as the T state). Anionic detergents would convert the antimycin-titration curves from sigmoidal to linear by dissociating Complex III from the oligomeric T conformation to the monomeric R conformation. In this communication, data are presented which support a hypothesis originally suggested by Potter and Reif [5], that the sigmoidal inhibition curve may result from the presence of a rate-limiting reaction which precedes or follows the antimycin-sensitive reaction in the sequence of reactions comprising the overall respiratory process.

2. Methods

Mitochondria were isolated from beef heart either by a modification [6] of the procedure described by Hatefi et al. [7] or a modification of the procedure described by Blair [8]. Submitochondrial particles were prepared by ultrasonic disruption of mitochondria [9]. Purified Complex III was prepared according to the procedure described by Rieske [10]. Trypsin-inactivated Complex III was prepared by incubation (1½ hr at 24° plus 1 hr at 0°) of dithionite-reduced Complex III (4 mg protein) with trypsin (2 mg crystalline). Further proteolysis was stopped by addition of soybean trypsin inhibitor (5 mg) after which the mixture was dialized for 15 hr against 0.25 M sucrose–0.05 M phosphate, pH 7.0 and concentrated by dialysis against dry Sephadex G-100 resin. Reduced coenzyme Q (QH_2)-cytochrome *c* reductase activity was estimated at 30° by a procedure previously described [11]. At 0° the procedure was modified to obtain continuous spectrophotometric traces of reduction of cytochrome *c* by the use of spectrophotometer cells equipped with quartz inserts so as to give a 3 mm light path. In this procedure, reaction-traces were made with a recording spectrophotometer equipped with a time-drive attachment. Succinate-cytochrome *c* reductase activities were estimated at 30° in the same reaction medium and by the same procedure as used for QH_2-cytochrome *c* reductase determinations except that the QH_2-2 was replaced by 10 mM succinate.

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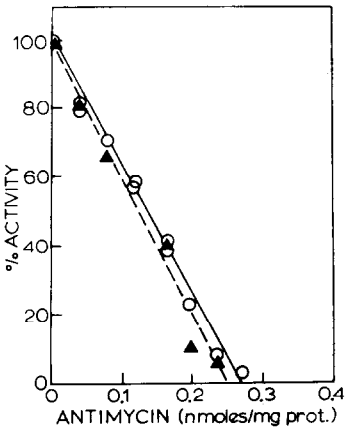

Fig. 1. Relationship between antimycin-titer and inhibition of QH_2-cytochrome *c* reductase activity of submitochondrial (sonic) particles. QH_2-cytochrome *c* reductase activities were determined after preincubation of the particles with antimycin. (o–o–o): Untreated particles. (▲–▲–▲): Particles (1.1 mg protein) solubilized in a medium containing 0.5% deoxycholate, 0.175 M sucrose, and 0.1 M phosphate, pH 7.0 in a total volume of 0.5 ml.

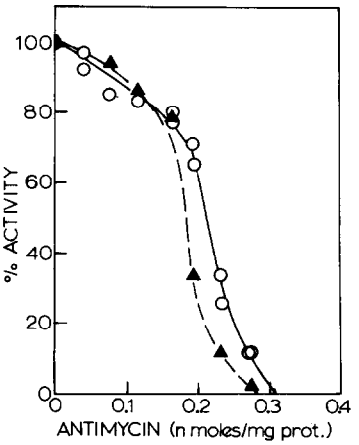

Fig. 2. Relationship between antimycin titer and inhibition of succinate-cytochrome *c* reductase activity of submitochondrial particles. The enzymic activity was determined at 30° in the same medium as used for QH_2-cytochrome *c* reductase determination with 0.01 M succinate replacing QH_2-2. (o–o–o): Untreated particles. (▲–▲–▲): Particles treated with deoxycholate as described in fig. 1.

3. Results and discussion

In the investigations of Bryła et al. respiratory activity of submitochondrial particles was measured in terms of the rate of reduction of externally added cytochrome *c* by succinate. Because this reaction involves a sequence of at least two kinetically independent steps, we have employed in addition measurements of the rate of reduction of added cytochrome *c* by QH_2-2. This assay system should better represent the respiratory activity of the cytochromes *b*-c_1 segment alone.

Fig. 1 shows the obtained QH_2-cytochrome *c* reductase activity of submitochondrial (sonic) particles as a function of the added titer of antimycin. In this experiment a linear relationship was obtained. As expected, solubilization of the particles with deoxycholate did not alter the linearity of the inhibition. In fig. 2 curves are shown of antimycin titer vs. succinate-cytochrome *c* reductase activity in preparations identical except for reducing substrate to those used in the experiments represented by fig. 1. In these cases sigmoidal inhibition curves were obtained. The amount of deoxycholate sufficient to solubilize the submitochondrial particles was not considered sufficient to cause transformation of the antimycin-titration curve from sigmoidal to linear as reported by Bryła et al.

An essential feature of the evidence of Bryła et al. supporting the "allosteric" hypothesis is the conversion of the antimycin-inhibition relationship from sigmoidal to linear in the presence of high concentrations of anionic detergents. Fig. 3 shows the effect of increasing concentrations of cholate on the QH_2-cytochrome *c* reductase and the succinate-cytochrome *c* reductase activities of submitochondrial particles. After an initial increase in activity due to solubilization of the mitochondrial membranes cholate was observed to inhibit QH_2-cytochrome *c* reductase at lower concentrations than those required to inhibit succinate-cytochrome *c* reductase. Both activities approached complete inhibition at ca. 8 mg cholate per mg protein.

It was reported also by Bryła et al. [4] that cationic or nonionic detergents failed to transform the antimycin-inhibition curve from sigmoidal to linear. Fig. 4 shows the effects of Triton X-100 on the activities of QH_2-cytochrome *c* reductase and succinate-cytochrome *c* reductase in submitochondrial particles.

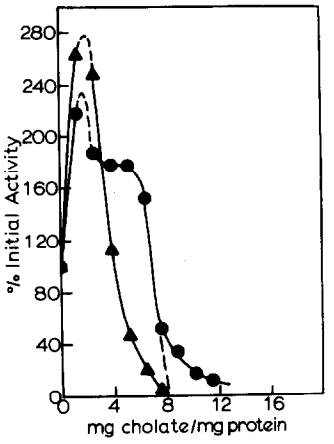

Fig. 3. Effect of cholate on the QH_2-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities of submitochondrial particles. Submitochondrial particles (1.5 mg protein in 0.1 ml of 0.1 M phosphate, pH 7.4) were treated with 0.1 ml of K cholate solution of a concentration required to yield the desired ratio of cholate to protein. Identical aliquots were tested for QH_2-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities at pH 7.0 and 30°. (▲–▲–▲): QH_2-cytochrome *c* reductase. (●–●–●): Succinate-cytochrome *c* reductase.

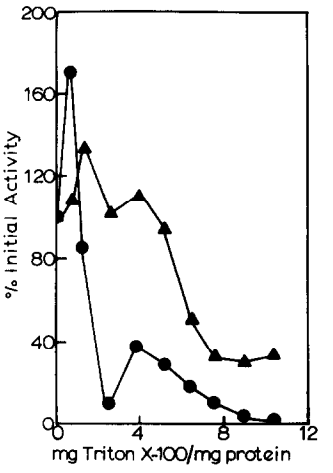

Fig. 4. Effect of Triton X-100 on the QH_2-cytochrome *c* reductase and the succinate-cytochrome *c* reductase activities of submitochondrial particles. Except for Triton X-100 replacing K cholate the preparation of submitochondrial particles and procedure were identical to those described for fig. 3. (▲–▲–▲): QH_2-cytochrome *c* reductase. (●–●–●): Succinate-cytochrome *c* reductase.

In this case, the succinate-cytochrome *c* reductase was preferentially inhibited by the nonionic detergent Triton X-100.

These results are consistent with the hypothesis that the sigmoidal nature of the antimycin titration of respiratory activity is due to the presence in the respiratory chain of a rate-limiting factor which precedes or follows in sequence the antimycin-sensitive component. As expected, according to this hypothesis, the respiratory activity of the QH_2-cytochrome *c* segment in all cases was inhibited linearly by antimycin. According to this hypothesis, anionic detergents could convert the antimycin-inhibition curve of the succinate-cytochrome *c* reductase from sigmoidal to linear by preferentially inhibiting the QH_2-cytochrome *c* segment so as to shift the rate-limiting step from the succinate-Q segment to the QH_2-cytochrome *c* segment of the overall process. Triton X-100 by preferentially inhibiting the succinate-Q segment, which is already rate-limiting, would not be expected to change the antimycin-inhibition pattern.

These results and interpretations are in agreement with the studies of Lee et al. [12] and Klingenberg and Kröger [13, 14], from which they advanced the hypothesis that a mobile pool of Q acts as an electron carrier between rate-limiting flavodehydrogenases and nonrate-limiting cytochrome chains which are inhibited stoichiometrically by antimycin.

The kinetic data in this report offer no explanation for the nonlinear dependence of the substrate reducibility and the red shift of the spectrum of cytochrome *b* on the concentration of added antimycin as reported by Bryła et al. In these cases it is assumed that cytochrome *b* resides within the respiratory segment which interacts with antimycin. Of possible relevance to this situation is the effect of the oxidation state of the antimycin-sensitive component on the binding of antimycin to the respiratory chain. Experimentally, the binding of antimycin to intact mitochondria was measured after reduction of the respiratory components with succinate as compared with the binding of antimycin to mitochondria with the respiratory components essentially oxidized. Oxidized Complex III inactivated by di-

Table 1
Effect of oxidation state in respiration on the binding of antimycin to the respiratory chain of intact mitochondria.

Exp.	Addition in sequence	QH_2-cyt *c* reductase activity (arbitrary units)
1	BHM + (KCN + NaN_3) + Succ. + AA + Complex III	9.2
2	BHM + AA + (KCN + NaN_3) + Succ. + Complex III	1.0
3	BHM + (KCN + NaN_3) + Succ. + AA	0.4
4	BHM + AA + (KCN + NaN_3) + Succ.	0.0
5	Complex III	0.4
6	BHM	14.8

The reaction mixture (0.1 ml) consisted of: A) Beef-heart mitochondria (BHM) (0.24 nmoles cyt c_1*); B) KCN (ca. 5 mM) and NaN_3 (ca. 10 mM); C) K succinate (12.5 mM); and D) Antimycin (AA) (0.3 nmoles). After 5 min 0.1 ml of solution of trypsin-inactivated Complex III (1.0 mg protein, 3.0 nmoles cyt c_1) was added. After 15 min at 24° the mixture was diluted to 1.0 ml and the mitochondria dissolved in a solution containing 0.25 M sucrose, 2 mg deoxycholate, and 0.05 M phosphate, pH 7.4. This mixture was assayed for QH_2-cytochrome *c* reductase activity according to a procedure referred to in "Methods".

* Cytochrome c_1 content is assumed to represent the concentration of antimycin-binding component.

gestion with trypsin was used as a competitive binder of antimycin in the manner described by Thorn [15]. This preparation of Complex III, although devoid of electron-transport activity, was still capable of stoichiometric interaction with antimycin as determined by the ability of antimycin to inhibit fully the cleavage of the complex by guanidinium salts [16]. To insure further that the inactivated Complex III would remain in an oxidized form even in the presence of reduced components of the intact respiratory chain of mitochondria, intact mitochondria were used in the reaction mixture. Antimycin binding to the intact respiratory chain of the mitochondrial preparation was measured by the degree of inhibition of QH_2-cytochrome *c* reductase after solubilization of the mitochondrial membranes with deoxycholate. Table 1 summarizes the results of this experiment. It is noted that mitochondria after treatment with succinate did not compete with inactivated Complex III for the single equivalent (based on cytochrome c_1 content) of antimycin as well as mitochondria treated with antimycin before reduction of the respiratory components with succinate.

Apparently, antimycin is less firmly bound to the reduced form of the antimycin sensitive component than to its oxidized form. A model based on this information may explain the nonlinear response of cytochrome *b* to increasing titers of antimycin. Addition of a substoichiometric titer of antimycin could increase the binding of antimycin added subsequently by inhibiting the reduction of the antimycin-sensitive component by succinate. Here again, it is assumed that free exchange of reducing equivalents between adjacent respiratory assemblies occurs via the mediation of coenzyme Q. The lessened and hyperbolic responses of cytochrome *b* to antimycin in particles reduced with dithionite or menaquinol-0 [1] is consistent with the above model since these reducing agents would reduce the antimycin-sensitive component directly; therefore, this reduction would not be influenced by the antimycin titer.

Another factor which may influence the linearity of the response of cytochrome *b* to a titration with antimycin is the dependence of the midpoint potential of cytochrome *b* on the oxidation state of a component distinct from cytochrome *b* [9]. This component may also be identical to the oxidation-reduction component which appears to control the binding of antimycin to the respiratory chain.

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