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STUDIES ON THE MECHANISM OF INHIBITION OF THE MITOCHONDRIAL ELECTRON TRANSPORT BY ANTIMYCIN

IV. EFFECT OF SURFACE-ACTIVE AGENTS ON THE ANTIMYCIN-INHIBITION CURVE AND AVAILABILITY OF SULFHYDRYL GROUPS OF THE HEART MUSCLE PREPARATION

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SUMMARY

1. Pretreatment of submitochondrial particles with anionic detergents, such as deoxycholate and dodecyl sulfate, results in a change in the curve describing inhibition by antimycin of the succinate–cytochrome *c* reductase from sigmoidal towards linear.

2. On treatment of the preparation with either nonionic (Triton X-100 or Tween 80) or cationic (Cetavlon) detergents, the sigmoidal inhibition curve is retained. However, the preparation preincubated with Tween 80 is one half as sensitive to antimycin as the untreated one despite the fact that the activity of the preparation is not affected by this detergent.

3. In the presence of the anionic detergents, much higher amounts of sulfhydryl groups of the preparation are titratable by 5,5′-dithiobis(2-nitrobenzoic acid) than those of the control preparation. Addition of antimycin is without effect.

4. Preincubation of the preparation with Cetavlon results in only a small increase in the amount of sulfhydryl groups, whereas the nonionic detergents are without effect on the sulfhydryl content of the preparation.

5. The results indicate that the anionic detergents at the concentration transforming the antimycin-inhibition curve from sigmoidal towards linear result in a rapid increase of the sulfhydryl content of the heart-muscle preparation.

INTRODUCTION

Recently, we have reported that pretreatment of submitochondrial particles with cholate[1–3] results in a change in the curves describing: (i) inhibition by antimycin of the succinate–cytochrome *c* reductase activity; (ii) reducibility of cytochrome *b* and (iii) the displacement to the red of the α-band of ferrocytochrome *b*, from sigmoidal towards linear. On the basis of these results we have proposed[1–3] that antimycin is an allosteric inhibitor of the respiratory chain, that the antimycin-sensitive

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

component of the chain is an oligomer or polymer and that transformation of the sigmoidal curves towards linear is due to a dispersion of the oligomeric or polymeric structure by cholate. The effect of other detergents on the antimycin-inhibition curve of the particulate preparation has not been studied. It was also of interest to determine whether conditions that favored such a dispersion might increase the sulfhydryl titer of the particulate preparation either in the presence or absence of antimycin.

The present paper describes the effect of surface-active agents on the antimycin-inhibition curve and on the availability of the heart-muscle preparation to titration by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

MATERIALS AND METHODS

Beef heart-muscle preparation was made by the method of KEILIN AND HARTREE[4] modified by SLATER[5]. Protein was determined by the method of CLELAND AND SLATER[6] using egg albumin as standard.

Cytochrome *c* was isolated from beef heart[7].

Antimycin A, Type III, was obtained from Sigma. The concentration of an ethanolic solution was determined from its absorbance at 320 nm, using an absorption coefficient of $4.8 \cdot 10^6$ $cm^2 \cdot mole^{-1}$ (ref. 8).

Sodium deoxycholate was supplied by Polfa (Poland), sodium dodecyl sulfate was from Fisher Scientific Co., Cetavlon (cetyl trimethyl ammonium bromide) was obtained from Imperial Chemical Industries Ltd., Triton X-100 (polyoxyethylated *tert.*-octylphenol (E_{9-10})) was supplied by Rohm and Hass Co., Tween 80 (polyoxyethylene sorbitan monooleate) was from Koch-Light and Co. Ltd., and guanidine hydrochloride was obtained from Reachim (U.S.S.R.) and Koch-Light and Co. Ltd. DTNB was supplied by Sigma and dithiothreitol (Cleland's reagent) was prepared as described by EVANS *et al.*[9] and found to have the desired properties.

Assay of activity and treatment of the preparation with detergents

Succinate–cytochrome *c* reductase activity was measured as previously described[10]. Inhibition by antimycin was determined after 1 min preincubation of the preparation with antimycin before addition of substrate.

Treatment with detergents was carried out by 2 min preincubation of the preparation (14 mg protein per ml) with the indicated amount of detergent dissolved in 0.1 M phosphate buffer (pH 7.6).

Determination of sulfhydryl groups

Sulfhydryl groups were determined by a modification of the procedure of BUTTERWORTH *et al.*[11], as follows. To a suspension of the protein (about 3 mg) 1 μmole of DTNB was added in 0.1 M sodium phosphate buffer (pH7.6), either with or without detergent. The mixture was incubated at room temperature for 2 min and then 7.5 ml of 5 % $HClO_4$ were added. The precipitated thiophenylated protein was separated by centrifugation. The precipitate was washed several times with 10 ml portions of an ethanolic solution of 1 % $HClO_4$ until the supernatant solution no longer yielded a yellow color on neutralization with NaOH and reduction with dithiothreitol. The protein was then dissolved in 1.5 ml of 6 M guanidine hydrochloride, neutralized with NaOH, and the thiophenol was released by incubation for 1 h with 0.3 ml of 0.01 M

dithiothreitol. To the solution 3.5 ml of 9% $HClO_4$ were added. The precipitated protein was centrifuged and washed 3 times with 1% $HClO_4$ in ethanol, the supernatant solution being retained. The washed precipitated protein was suspended in 1 ml of water, 0.01 ml of 30% H_2O_2 was added, and the suspension was heated in a boiling-water bath for 1 min. The supernatant solution was made alkaline with NaOH, the volume being adjusted to 10 ml. The liberated thiophenylate was determined by measuring the absorbance at 412 nm ($\varepsilon_m = 13600$)[12].

The protein was then determined by the biuret method of GORNALL *et al.*[13].

RESULTS

Effect of surface-active agents on the inhibition curve

Fig. 1 shows the effect of detergents on the inhibition by antimycin of the succinate–cytochrome *c* reductase activity of the heart-muscle preparation. In agreement with our previous observations[1–3] concerning the effect of cholate on the antimycin-inhibition curve, pretreatment of the preparation with other anionic detergents, such as deoxycholate or dodecyl sulfate, resulted in the transformation of the sigmoidal inhibition curve towards linear (Fig. 1A). On the other hand, on treatment of the preparation with either cationic (Cetavlon) or nonionic detergents (Triton X-100 and Tween 80) the antimycin-inhibition curve remained sigmoidal (Fig. 1B). However, the sensitivity to antimycin of the preparation treated with Tween 80 was half that of the control preparation. The activity of the succinate–cytochrome *c* reductase in the absence of antimycin was unaffected by Tween 80 irrespective of the amount of detergent added.

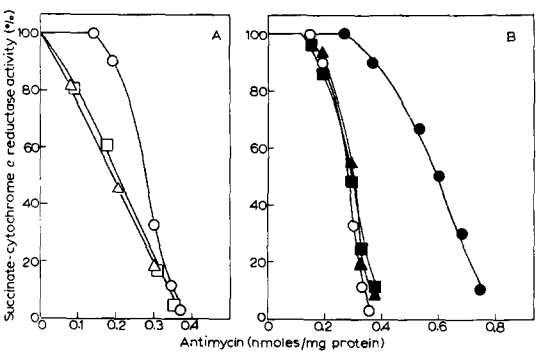

Fig. 1. Effect of anionic (A) or cationic and nonionic (B) detergents on the inhibition by antimycin of the succinate–cytochrome *c* reductase activity of the heart-muscle preparation. ○—○, control preparation; △—△, preparation treated with 13.7 mg deoxycholate per mg protein (66 mg deoxycholate per ml); □—□, preparation treated with 0.7 mg dodecyl sulfate per mg protein (4.8 mg dodecyl sulfate per ml); ■—■, preparation treated with 0.2 mg Cetavlon per mg protein (2 mg Cetavlon per ml); ▲—▲, preparation treated with 1.4 mg Triton X-100 per mg protein (18 mg Triton X-100 per ml); ●—●, preparation treated with 27.2 mg Tween 80 per mg protein (80 mg Tween 80 per ml). Activities in the absence of antimycin were: 0.104, 0.108, 0.097, 0.100, 0.101 and 0.104 unit/min, respectively, where 1 unit is equal to 1 μmole of succinate oxidized per mg protein.

Effect of surface-active agents on the availability of sulfhydryl groups to titration by DTNB

There are reports on the effect of both detergents and antimycin on availability

of sulfhydryl groups of Complex III (ref. 14). In the present study a preparation containing the intact respiratory chain was used. In this preparation, as well as in the reconstituted succinate–cytochrome *c* reductase[15], the curve describing the inhibition by antimycin of electron transport is strongly sigmoidal, in contrast to the linear curve found with Complex III (refs. 16–19).

Table I shows the availability of sulfhydryl groups to titration by DTNB in the heart-muscle preparation treated with surface-active agents. Detergents were applied in concentrations that do not affect the succinate–cytochrome *c* reductase activity. Concentrations of the anionic detergents used in these experiments resulted in a change of the antimycin-inhibition curve from sigmoidal towards linear. As can be seen, the amount of sulfhydryl groups of the preparation titratable by DTNB was

TABLE I

EFFECT OF SURFACE-ACTIVE AGENTS AND GUANIDINE HYDROCHLORIDE ON THE AVAILABILITY OF SULFHYDRYL GROUPS OF THE HEART-MUSCLE PREPARATION TO TITRATION BY DTNB

Determinations of sulfhydryl groups were performed on 4 separate heart-muscle preparations. Results of typical experiments are given. Samples of 2.5 mg protein of the preparation were used.

Treatment	*Detergent/ protein ratio (mg/mg)*	*Sulfhydryl content*	
		nmoles/ mg protein	%*
None		32	100
Deoxycholate	13.0	53	165
Dodecyl sulfate	0.5	56	175
Cetavlon	0.2	38	118
Triton X-100	1.5	34	106
Tween 80	26.6	34	106
Guanidine hydrochloride (2.5 M)		103	322

* The sulfhydryl content of the control preparation was taken to be 100%.

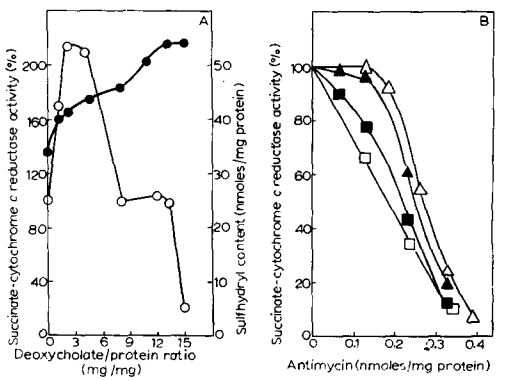

Fig. 2. Effect of increasing concentrations of deoxycholate on the sulfhydryl content and the succinate–cytochrome *c* reductase activity (A) and on the inhibition by antimycin (B) of the heart-muscle preparation. ●—●, amount of sulfhydryl groups available to titration by DTNB; ○—○, succinate–cytochrome *c* reductase activity; △—△, antimycin-inhibition curve of the control preparation; ▲---▲, ■---■ and □---□, antimycin-inhibition curves of the preparations treated with 2, 10 and 13 mg deoxycholate per mg protein (7.7, 59 and 65 mg/ml), respectively. The activity of the control preparation was 0.114 unit/min.

higher (approx. 1.7 times) in the presence of the anionic detergents (deoxycholate and dodecyl sulfate) than in their absence. Pretreatment of the preparation with the cationic detergent Cetavlon resulted in only a small increase of the sulfhydryl groups (about 1.2-fold), while the sulfhydryl content of the preparation was not significantly affected by the nonionic detergents Triton X-100 or Tween 80.

Addition of antimycin did not cause any significant change in the amount of sulfhydryl groups of the preparation treated with either of the investigated detergents.

In the presence of guanidine hydrochloride the amount of sulfhydryl groups of the preparation available to titration by DTNB was three times higher than that of the control preparation. In view of these results it was of interest to follow the unmasking of sulfhydryl groups of the preparation treated with increasing concentrations of deoxycholate and dodecyl sulfate. The increase of the detergent concentration to 2 mg deoxycholate per mg protein (Fig. 2A) or 0.4 mg dodecyl sulfate per mg protein (Fig. 3A) resulted in an increase of both the succinate–cytochrome *c* reductase activity and the amount of sulfhydryl groups available to titration by DTNB. Antimycin-inhibition curves were only little affected by these amounts of detergents (Figs. 2B and 3B). In the presence of the higher levels of detergents (up to 8 mg deoxycholate or 0.6 mg dodecyl sulfate per mg protein) the enzymic activities of the preparations were decreased, whereas the sulfhydryl content was not significantly affected (Figs. 2A and 3A). However, if the amounts of detergents were equal to those that resulted in a transformation of the curve describing inhibition by antimycin of the preparation from sigmoidal to linear (*i.e.* 13 mg deoxycholate per mg protein or 0.7 mg dodecyl sulfate per mg protein, as shown in Figs. 2B and 3B, respectively), an increase in the availability of sulfhydryl groups to titration by DTNB was again observed (Figs. 2A and 3A). The enzymic activity in the absence of antimycin was little affected by these concentrations of detergents. On the other hand, addition of the higher levels of detergents exceeding 13 mg deoxycholate per mg protein (Fig. 2A) or 0.7 mg dodecyl sulfate (Fig. 3A) caused inactivation of the

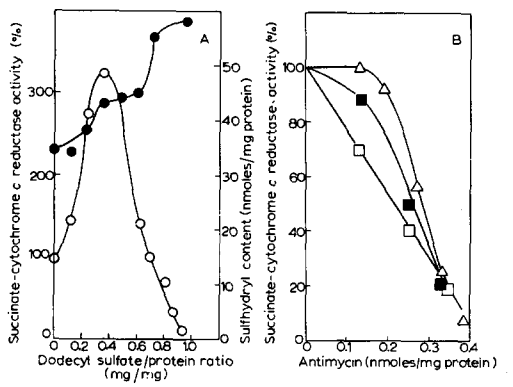

Fig. 3. Effect of increasing concentrations of dodecyl sulfate on the sulfhydryl content and the succinate–cytochrome *c* reductase activity (A) and the inhibition by antimycin (B) of the heart-muscle preparation. ●—●, amount of sulfhydryl groups available to titration by DTNB; ○—○, succinate–cytochrome *c* reductase activity; △—△, antimycin-inhibition curve of the control preparation; ■---■ and □---□, antimycin-inhibition curves of the preparations treated with 0.4 and 0.7 mg dodecyl sulfate per mg protein (3.6 and 5.1 mg/ml), respectively. The activity of the control preparation was 0.114 unit/min.

succinate-cytochrome *c* reductase activity and only a small increase of the sulfhydryl content of the preparation.

DISCUSSION

Of the detergents investigated only anionic types (*i.e.* cholate[1–3], deoxycholate and dodecyl sulfate) had the ability to transform the antimycin-inhibition curve of the succinate–cytochrome *c* reductase from sigmoidal towards linear as well as to increase the availability of sulfhydryl groups of the preparation to titration by DTNB. A similar effect of anionic detergent had been observed by GRANDA AND SCANU[20] who showed that neither cationic benzalkonium chloride nor a nonionic Triton had the solubilizing effect of the anionic sodium dodecyl sulfate on the low-density lipoprotein apoproteins of human serum. In agreement with the suggestion of GRANDA AND SCANU[20], it seems likely that either the charge on the anionic detergents is essential or that the hydrophobic portions of the anionic and cationic detergents are not sterically fitted for interaction with the hydrophobic region of the protein. According to GREEN AND FLEISHER[21], the bile salts are unique in that they can rupture the bonds between proteins and phospholipids in a reversible fashion without modifying the catalytic properties of the protein involved in these bonds. This suggestion seems to be supported by the restoration of the sigmoidal shape of the antimycin-inhibition curve due to removal of the cholate by dialysis[1].

Of the nonionic detergents only Tween 80 does not activate the succinate–cytochrome *c* reductase activity. This may result from a rapid reconstitution of the crista membrane after treatment[22]. However, the reconstitution seems not to be complete, since the preparation treated with this detergent is twice less sensitive to antimycin than the control preparation. The reason for this phenomenon is not known.

The existence of two steps in the release of sulfhydryl groups resulting from exposing of the heart-muscle preparation to increasing concentrations of the anionic detergents is of particular interest. The first step, brought about by low levels of detergents, is accompanied by the stimulation of the succinate–cytochrome *c* reductase activity, probably owing to weakening of the hydrophobic bonds between phospholipids and proteins. The second step occurs when the detergents have reached certain critical concentrations and results in a change of the antimycin-titration curve of the preparation from sigmoidal towards linear. The sulfhydryl groups available to titration by DTNB at higher levels of detergents are probably situated in the interior of protein molecules or, in view of the polymeric nature of several enzymes of the respiratory chain, within the aggregates, as suggested by OKUNUKI *et al.*[23,24] for the polymeric cytochrome *a*.

Unmasking of sulfhydryl groups due to the addition of detergents in the concentrations which transform the shape of the antimycin-inhibition curve is observed in the heart-muscle preparation despite the fact that this preparation contains the intact respiratory chain and not only the fragment of the chain binding antimycin.

In contrast to the data of BAUM *et al.*[14] for Complex III, addition of antimycin to the heart-muscle preparation before treatment with detergent did not result in a decrease in the sulfhydryl content of the preparation. This may suggest that the sulfhydryl groups are not involved in the conformational changes of the protein binding the allosteric inhibitor. However, the possibility that, in the presence of anti-

mycin, the decrease or increase of sulfhydryl groups of the preparation is very small and therefore masked by the sulfhydryl groups present out of the b–c_1 fragment of the chain, cannot be excluded.

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Recently, KLINGENBERG AND KROGER[25] have postulated that the pool function of ubiquinone may be able to explain the non-linear dependence of inhibition on the concentration of antimycin A, rather than the allosteric effect of this inhibitor on the respiratory chain as postulated by us[1–3]. In accordance with the pool model of ubiquinone, they explained the fact that in preparations treated with cholate[1] the sigmoidicity is abolished and replaced by a linear decrease of electron transfer activity by a restricted diffusion of ubiquinone in the membrane in the presence of cholate. Thus the membrane might be fragmented by cholate into small areas which permit the diffusion of ubiquinone between only a few molecules of the dehydrogenases and cytochrome chains.

This hypothesis is, however, inconsistent with both the effects of different types of detergents on the antimycin-inhibition curve described in the present paper and with the effects of Triton X-100 and deoxycholate on NADH and succinate-cytochrome c reductase activities[26].

First, the effect of detergents on the antimycin-titration curve appears to be rather specific since it is restricted only to anionic detergents (when used at high concentration) whereas the inhibition of ubiquinone reduction by dehydrogenases in the presence of detergents is nonspecific[27] and is induced by much lower concentrations. Second, only anionic detergents, when applied at the concentration transforming the antimycin-inhibition curve from sigmoidal towards linear, result in a rapid increase of sulfhydryl content of the preparation. The sigmoidicity of the antimycin-titration curve is restored by partial removal of cholate by dialysis[1].

Third, while both the nonionic Triton X-100 and the anionic deoxycholate affect oxidation of NADH and succinate by cytochrome c reductase in a similar fashion[26], only deoxycholate results in a change in the curve describing inhibition by antimycin from sigmoidal towards linear.

Thus it appears that the concept of ubiquinone function can not explain the non-linear dependence of the inhibition on the concentration of antimycin. All available experimental data are in accordance with the hypothesis of allosteric interaction between antimycin and the respiratory chain[1–3].

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