Factors Affecting the Binding of Antimycin A to Complex III of the Mitochondrial Respiratory Chain*

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SUMMARY

The nature of the binding of antimycin A to Complex III of the mitochondrial respiratory chain was investigated according to two procedures. In the first procedure, tritium-labeled antimycin A was extracted from its binding to Complex III either by acetone or by concentrated solutions of taurocholate. Seventy per cent of the complex-bound antimycin A was extracted by either reagent. The acetone-extracted antimycin A was found to be fully inhibitory to the reduced coenzyme Q-cytochrome c reductase activity of Complex III. Moreover, even though most of the antimycin A was extracted from Complex III by taurocholate, its inhibitory effect on the cleavage of the complex was retained. The second procedure utilized enzymically active Complex III as a reagent to detect antimycin A that failed to be bound to, or was released from, an enzymically inactive Complex III. The binding of antimycin A to intact Complex III was found to be essentially irreversible, little or no transfer of antimycin A from antimycin-treated complex to untreated complex being observed. High levels of detergent (3 to 5% taurocholate) in the reaction mixture permitted some transfer of the antimycin A. Conditions that normally caused cleavage of Complex III (i.e. bile salts plus ammonium sulfate, or 1.0 M guanidine), or digestion with trypsin, destroyed the capacity of the complex to bind antimycin A. Reagents that cleaved Complex III despite the treatment of the complex with antimycin A (i.e. 3.0 M guanidine, 0.2 M guanidine plus freezing, or taurocholate plus mersalyl) also caused a release of antimycin A from the complex. Noninhibitory derivatives of antimycin A (i.e. the O-methyl ether of antimycin or deformylantimycin A) offered little or no competition to antimycin A in its binding to Complex III.

A property that characterizes mitochondrial respiration is its sensitivity toward antimycin A (1). In fact, this inhibition by antimycin has been used extensively as a test for the presence of mitochondria in cellular extracts, or of electron transfer assemblies derived from mitochondria. The ubiquity of the antimycin-sensitive factor is illustrated by its occurrence in the respiratory equipment of chloroplasts (2, 3) and that of certain bacteria, and also in a nonrespiratory enzyme, hepatic aldehyde oxidase (4). In spite of the general occurrence of antimycin sensitivity in respiring organelles, extensive data have shown that the interaction of antimycin with the respiratory chain is highly site-specific. The locus of inhibition in mitochondrial respiration has been shown to lie between cytochromes b and c1, both in the intact respiratory assemblies (1) and in the isolated cytochrome b-c1 segment, designated Complex III in this laboratory (5). The site specificity of antimycin is emphasized by the observation that the antibiotic is a stoichiometric inhibitor of the enzymic activity (reduced coenzyme Q-cytochrome c reductase) of Complex III (6), as well as of respiration in intact respiratory assemblies (7). The involvement of the antimycin sensitive site in the structural integrity of Complex III, as well as in its enzymic function, was shown in this laboratory by the observation that stoichiometric levels of antimycin prevented cleavage of the complex in the presence of bile salts plus ammonium sulfate (6). It has been suggested that the antimycin-sensitive site may be functional in the initial energy-conserving process at the second phosphorylation site in mitochondria (8). Consequently, the elucidation of the chemical nature of the antimycin-sensitive site is of primary interest for understanding both the structure and function of Complex III. As a bonus, this information may also provide the long sought description of the first high energy step of the mitochondrial respiratory chain.

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The abbreviations used are: antimycin, antimycin A; guanidine, guanidinium hydrochloride; CoQ, coenzyme Q, ubiquinone; CoQII, reduced coenzyme Q, QO, 2-nonyl-4-hydroxyquinoline N-oxide.
intermediate in the energy conservation process at the second site of phosphorylation.

The initial question, applicable to the chemical nature of the antimycin-sensitive site, concerns the type of binding between this site and antimycin. Although the stoichiometric nature of the reaction between antimycin and its site of inhibition indicates a highly specific and irreversible interaction, several investigators have reported that under certain conditions the inhibitory effects of antimycin on respiration could be abolished or reversed (9-12). However, because these "reversals of inhibition" were observed with enzymic systems involving more than one segment of the respiratory chain, such results are difficult to assess with respect to the binding of antimycin.

The availability of purified Complex III has provided us with an unambiguous system for the evaluation of the binding of antimycin to its site of interaction. Antimycin inhibits the catalytic activity of this complex in a linear and stoichiometric manner. This stoichiometric inhibition by antimycin also applies to the cleavage of Complex III either by bile salts plus ammonium sulfate or by guanidinium salts (13); therefore, the interaction of antimycin with the complex can be assessed even under conditions that normally cause loss of catalytic activity.

The data in this report indicate that antimycin is bound to Complex III in a specific and irreversible manner; however, no covalent linkages are involved in the binding. Also, no alteration in the inhibitory activity of antimycin is suffered after removal from its interaction with Complex III. Cleavage of Complex III in either the absence or the presence of antimycin destroys the capability of the complex to bind antimycin. The functional groups within the structure of antimycin that are essential for its inhibitory activity also appear to be essential for the binding of antimycin to Complex III.

**EXPERIMENTAL PROCEDURE**

**Materials**—Complex III was purified from bovine heart mitochondria by a procedure described previously (14). Heat-inactivated Complex III was prepared by the following procedure. Complex III (20 mg of protein per ml in 0.66 M sucrose-0.005 M Tris-HCl, pH 8.2) was incubated at 38-40° for 1 hour. A small amount of precipitated protein was removed by centrifugation at 1000 × g after this treatment usually only 5 to 10% of the initial activity remained; however, if 0.1 M phosphate, pH 7.5, was used as buffer, a much more prolonged incubation (more than 4 hours) was required to obtain a comparable inactivation. Antimycin was obtained from Kanegafuchi Chemical Industries Company, Ltd., Osaka, Japan. Tritium-labeled antimycin was prepared by the technique of Dorfman (15). Exposure to tritium gas, the crude labeled antimycin was chromatographed on a column of silicic acid with methanol-chloroform, 1:1, as the solvent system. The fluorescence of antimycin as activated by a long wave ultraviolet light was used to monitor the elution of antimycin from the column. Most of the decomposition products (also those most highly substituted with tritium) remained on the column. The antimycin in the methanol-chloroform eluate was recovered after evaporation of the solvent and then was crystallized successively in petroleum ether-ethyl ether (four times) and ethanol (three times) until recrystallization resulted in no further change in specific radioactivity of the antimycin. The final specific radioactive labeling was 1.9 mCi per mmole. Thin layer chromatography of the 3H-antimycin on silica gel in two dimensions (first with acetone other, 1:1, then with dioxane petroleum ether-acetic acid, 45:50:0.5) gave one fluorescent spot that contained 80% of the radioactivity; almost all of the remaining 20% was located in the immediate trailing edges of this spot. The methyl ether of antimycin and deformylantimycin A were obtained through the courtesy of Dr. F. M. Strong. Tryptsin (once crystallized) was obtained from Worthington. Phospholipase A was used as the extract of boiled venon from Crotalus atrox (Ross Allen’s Reptile Institute). Guanidine hydrochloride and urea were recrystallized before use.

**Methods**—Protein was determined by the biuret procedure of Gornall, Bardawill, and David (16). The phospholipid content of protein fractions was estimated from the analysis of total phosphorus by the method of Fleischer et al. (17). Cytocrones b and c1 in preparations of Complex III were estimated spectrophotometrically by a procedure described previously (13).

Samples containing 3H-labeled antimycin were analyzed for tritium by two procedures. Acetone-extractable 3H-antimycin was deposited in a scintillation vial by evaporation of the acetone solvent. The residue was dissolved directly in a scintil-lator mixture consisting of 5.0 g of 2,5-diphenyloxazole (PO) and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene-(dimethylPOPOP) dissolved in 1 liter of toluene. The total 3H-antimycin in samples of Complex III was determined from the tritium recovered as 3HOH from a total combustion of the samples (18). The water from combustion of the samples was dissolved in a scintillator solution containing naphthalene (60 g per liter), 2,5-diphenyloxazole (4.0 g per liter), 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (200 mg per liter), and methanol (100 ml per liter), all dissolved in p-dioxane. Samples were counted in either a Nuclear-Chicago or a Packard Tri-Carb scintillation counter.

CoQH2-cytochrome c reductase activities were determined by the procedure of Rieske (19), adapted from the original procedure described by Green and Burkhard (20). The assay as used in the early phases of this study utilized about 0.5 μg of enzyme protein in the assay mixture and a reaction time of 10 sec, at 38°. A considerable improvement in reliability was obtained by lowering the temperature of the assay to 0° while concomitantly increasing the amount of enzyme used to about 4.0 μg and increasing the reaction time to 1.0 min. These modifications resulted in more than a 2-fold increase in the linear range of cytochrome c reduced by CoQH2. Samples of Complex III were prepared for assay of enzyme activity by dilution to a protein concentration of 0.1 mg per ml in 0.2 to 0.4% sodium taurocholate (or in some cases in 0.05 to 0.1% potassium deoxycholate). Usually 0.04 ml (4 μg of protein) of the diluted enzyme was added to the assay mixture.

**RESULTS**

**Extraction of 3H-Labeled Antimycin from Inhibited Complex III**—The first question to be answered was whether any permanent change in either the antimycin molecule or the complex occurred as a result of the interaction of antimycin with Complex III. First, Complex III was mixed with varying titers of 3H-labeled antimycin; then the antimycin was extracted with acetone and tested for specific inhibitory activity (i.e. moles of Complex III inhibited per mole of antimycin added) toward...
enzymanically active Complex III. Table I lists the results of this
experiment. At all titers of antimycin, whether subto-
litrometric with, or in molar excess to, the cytochrome c1 content
of Complex III, the extracted antimycin was found to be as
inhibitory as was untreated antimycin. Therefore, if antimycin
undergoes any chemical change as a result of its interaction
with Complex III, this change either is readily reversible or
does not affect the inhibitory capacity of antimycin.
Second, to test whether Complex III retained any effects of
antimycin after removal of the inhibitor, the enzyme was
treated with 1 eq of H-antimycin (1.0 mole of antimycin per mole
of cytochrome c) and then was extracted successively with 10% 
sodium taurocholate. After each treatment excess taurocholate,
together with extracted antimycin, was removed from the enzyme
by passage of the extraction mixture through a column of 
Sephadex G-50. After four extractions, Complex III was
recovered from the final eluate by precipitation with ammonium 
sulfate. Aliquots of this protein were assayed for their content
of tritium, cytochrome c1, and the degree of cleavage achieved by
incubation of the samples in the presence of taurocholate plus
ammonium sulfate. The data of Table II indicate that, with respect to recovered cytochrome c1, 70% of the H-antimycin
was removed from the sample of Complex III by this procedure.
Also, data from a parallel experiment indicated that 75 to 85% of
the phospholipid present in the sample of Complex III was
simultaneously extracted. Although much of the antimycin
was removed from Complex III by this extractive procedure,
when Complex III thus treated was subjected to conditions of
cleavage (incubation at 20°C in the presence of taurocholate plus
ammonium sulfate) very little cleavage was achieved despite a
considerable precipitation of Complex III. To assess the
effect of the residual 0.3 eq of antimycin on the cleavage of
control sample of Complex III was treated with 0.3 eq of anti-
mycin and was then subjected to cleavage by the taurocholate 
plus ammonium sulfate mixture. Although in this case cleavage
was retarded considerably, it was still significantly greater than
the cleavage achieved with the extracted samples.

**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytochrome c1</th>
<th>p moles antimycin added</th>
<th>Inhibitory activity recovered (%)</th>
<th>Radioactivity recovered (p moles/mole)</th>
<th>Inhibitory activity recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>20</td>
<td>14.5</td>
<td>73</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>40</td>
<td>29.0</td>
<td>68</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>60</td>
<td>35.0</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>80</td>
<td>54.0</td>
<td>71</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>100</td>
<td>64.0</td>
<td>72</td>
<td>64</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Sample of Complex III</th>
<th>Antimycin added</th>
<th>Antimycin remaining after extraction</th>
<th>Cytochrome c1 ratio in supernatant solution after cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extracted</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.44</td>
</tr>
<tr>
<td>2</td>
<td>1.1(H-antimycin)</td>
<td>0.33</td>
<td>2.1</td>
</tr>
<tr>
<td>3</td>
<td>1.1(unlabeled antimycin)</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>0.33</td>
<td>0.33*</td>
<td>1.47</td>
</tr>
</tbody>
</table>

* No extraction.
investigators have reported the reactivation of antimycin-inhibited respiration by such reagents as serum albumin (9), inactivated succinate oxidase (10), coenzyme Q (11), and a protein prepared from chicken liver (12). Attempts to demonstrate a reactivation of antimycin-inhibited Complex III by treatment with these reagents have failed uniformly.

To test further whether the binding of antimycin to Complex III could be reversed, a technique was used which was analogous to that described by Thorn (10) for heart muscle preparations. This technique involved the use of active Complex III to test enzymatically for the degree of binding of antimycin to Complex III which was inactive enzymically. Complex III may be inactivated by mild heat treatment without affecting the antimycin-sensitive site, as indicated by retention of the ability of antimycin to block the cleavage of the complex promoted by 1.2 mM guanidine (13).

With this type of assay system, we first tested the extent to which 1 eq of antimycin remained bound to inactivated Complex III in the presence of 1 eq of enzymically active complex, and vice versa. Transfer of antimycin from active complex to inactivated complex would result in a mixed activity greater than that of active complex alone after treatment with antimycin. Likewise, transfer of antimycin from inactivated complex to active complex should result in a lower activity in the mixture than with active enzyme alone. Part of the mixtures were incubated in the presence of taurocholate (3.3 or 5.0%) in order to duplicate partially the conditions of extraction of Complex III with taurocholate (cf. Table II). The results of this experiment are listed in Table III. Almost equal distribution of antimycin between active and inactive complex is indicated by a resultant activity almost precisely halfway between the individual activities when 1 eq of antimycin was added to a mixture containing 1 eq each of inactivated and active Complex III. By the use of this intermediate activity (A + I + AA as explained in the legend of Table III) as a criterion of complete equilibration of antimycin in the mixture of inactivated and active Complex III, it was possible to assess the extent of transfer of antimycin from the one species of Complex III to the other, in either the absence or the presence of detergent. Table III shows that little, if any, transfer of antimycin occurred in the taurocholate-free mixture. Little activity was recovered when heat-inactivated Complex III was added to antimycin-inhibited enzyme (compare activities of A + AA + I and A + AA). Moreover, little activity was lost when active enzyme was added to heat-inactivated enzyme which had been treated with antimycin (compare activities of I + AA + A and A + I). In the presence of taurocholate, however, the same procedure gave results that could be interpreted as a transfer of antimycin from heat-inactivated Complex III to the active complex.

### Table III

**Equilibration of antimycin between Complex III and heat-inactivated Complex III**

<table>
<thead>
<tr>
<th>Components and order of addition</th>
<th>Relative enzymic activity at taurocholate concentrations of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>100</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
</tr>
<tr>
<td>A + AA</td>
<td>15</td>
</tr>
<tr>
<td>(A + AA) + I</td>
<td>15</td>
</tr>
<tr>
<td>1 + AA</td>
<td>6</td>
</tr>
<tr>
<td>(A + I)</td>
<td>13</td>
</tr>
<tr>
<td>(A + D) + AA</td>
<td>64</td>
</tr>
<tr>
<td>(I + AA) + A</td>
<td>104</td>
</tr>
</tbody>
</table>

<sup>a</sup> A = 1 eq of enzymically active Complex III; I = 1 eq of heat-inactivated Complex III; AA = 1 eq of antimycin A (AA = amount of cytochrome c<sub>1</sub> contained in 1 reaction equivalent of Complex III).

<sup>b</sup> Values given for the relative enzymic activities (CoQH<sub>2</sub>-cytochrome c reductase) have been normalized with respect to the enzymic activity of 1 reaction equivalent of Complex III.

<sup>c</sup> Results from two separate experiments are listed.

**Fig. 1.** Titration of Complex III with antimycin in the presence of 5% sodium taurocholate. Complex III (20 mg of protein dissolved in 1.0 ml of sucrose-Tris buffer) was treated with 60 µl of a 0.9 mM solution of antimycin in ethanol. Mixtures of this solution (containing 1.0 mole of antimycin per mole of cytochrome c<sub>1</sub>) and active Complex III were blended to yield the ratios plotted on the abscissa. Each of the series of solutions (of increasing titers of antimycin) was diluted with 1 volume of 10% sodium taurocholate. After 30 min at 0°, 0.04-ml volumes were diluted to 4.0 ml in 0.1% potassium deoxycholate and were assayed for CoQH<sub>2</sub>-cytochrome c reductase activity (see "Methods").
In this case a significant inhibition of the active enzyme occurred when antimycin-treated Complex III was mixed with enzymatically active complex (compare activities of I + AA + A and A + I). In fact, the activities approached those of the equilibrium mixtures (compare activities of I + AA + A and A + I + AA). The transfer of antimycin from active to heat-inactivated complex in the presence of taurocholate was less pronounced (compare activities of A + AA + I and A + AA), although a small, but consistent, activation was observed.

Because of the apparent increase in the dissociability of antimycin from Complex III in the presence of taurocholate, the inhibition of enzymic activity of Complex III was determined after incubation with increasing amounts of antimycin in the presence of 5% taurocholate. As shown in Fig. 1, the complex appeared to retain full sensitivity to antimycin even after incubation (at 0°C) in the presence of 5% taurocholate for 30 min. The linearity of the inhibitory response as a function of the titer of antimycin also was assurance that, even in the presence of high concentrations of taurocholate, the relative enzymic activity of active Complex III after its addition to antimycin-treated, inactive complex would reflect the degree of binding of antimycin to inactivated complex.

**Effect of Various Treatments on Binding of Antimycin to Complex III**—By an extension of the technique used in the measurement of the equilibrium dissociation of antimycin from Complex III, we were able to measure the relative binding of antimycin to Complex III after exposure of the complex to a variety of reagents and conditions. Exposure of Complex III to conditions which caused loss of antimycin binding concomitantly caused a complete loss of enzymic activity; this eliminated the necessity of using heat-inactivated complex as the reactant complex. Under these conditions, however, it was usually necessary to dilute to a large extent the treated complex prior to its admixture with the enzymically active complex. This modification of procedure was required to avoid any inactivation of active enzyme by the test reagent other than the antimycin added together with the inactivated enzyme. The dilution procedure also eliminated any tendency of antimycin to equilibrate between undamaged binding sites on the test complex and the detecting complex, respectively, when in the presence of high concentrations of bile salts. Because of variability in the response of Complex III to the various treatments, the assay procedure was modified somewhat with each treatment to be compatible with the activity and stability of the detecting enzyme (active Complex III). The results of these experiments are given in Table IV. Of the treatments listed, only boiling in the presence of 15% taurocholate, exposure to 3 M guanidine, or freezing in the presence of 0.2 M guanidine resulted in a significant release of antimycin from its association with the treated Complex III. Exposure of Complex III to pH 5 (in case of heat-inactivated complex), to 10% taurocholate for 16 hours at 25°C, or to 1 M guanidine resulted in a large loss of capability to bind antimycin; however, these treatments did not cause a significant release of antimycin that had been bound to the complex prior to treatment. Treatment of the complex with 6 M urea for 1 hour at 25°C caused a complete loss of enzymic activity but had little effect either on the capability of the complex to bind antimycin or on the release of the inhibitor. Apparently, after detachment from the complex by the action of 3 M guanidine, the antimycin, for the most part,

### Table IV

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Modification of conditions</th>
<th>Loss of activity</th>
<th>Loss of binding of antimycin</th>
<th>Release of antimycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea, 0.5 M</td>
<td>None</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Urea, 3 M</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urea, 6 M</td>
<td>None</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Urea, 6 M</td>
<td>1 hr at 25°C</td>
<td>80</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Guanidine, 0.25 M</td>
<td>None</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Guanidine, 1.0 M</td>
<td>None</td>
<td>76</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Guanidine, 3.0 M</td>
<td>None</td>
<td>92</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Guanidine, 0.2 M</td>
<td>15 hrs at -20°C</td>
<td>84</td>
<td>84</td>
<td>70</td>
</tr>
<tr>
<td>Arginine-HCl, 0.2 M</td>
<td>None</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Tetramethyl ammo-nium chloride, 1.0 M</td>
<td>None</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Sodium taurocho-late, 10%</td>
<td>16 hrs at 25°C</td>
<td>100</td>
<td>94</td>
<td>23</td>
</tr>
<tr>
<td>Sodium taurocho-late, 15%</td>
<td>0.5 min at 100°C</td>
<td>100</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Citrate, 0.1 M, pH 5.0</td>
<td>No Tris buffer</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Citrate, 0.1 M, pH 5.0</td>
<td>Complex III inactivated by heat</td>
<td>84</td>
<td>84</td>
<td>84</td>
</tr>
</tbody>
</table>

* The enzymic activities of CoQH2-cytochrome c reductase were determined on samples diluted and assayed as described under "Methods." In cases when activity was unaffected, the binding and release of antimycin were also unaffected; therefore such determinations were omitted in some cases.

* Loss of binding of antimycin was determined by removing a sample of the treated enzyme containing 2 mg of Complex III protein, adding 6 μl of 1 mM antimycin in ethanol, diluting with water to a noninhibitory level (i.e. 0.5 M for urea, 0.25 M for gua-nidine, or 3% for taurocholate), adding 0.1 mg of fresh Complex III, and assaying. After addition of antimycin and after addition of fresh Complex III, a 3-min equilibration at 0°C was allowed. Loss of binding is expressed as the percentage of inhibition of the activity of the freshly added Complex III, assayed at a level of 4 μg of active enzyme.

* Release of antimycin was determined on a separate sample of Complex III to which antimycin (1 μ mole per mole of cytochrome c1) was added prior to treatment with the reagent. The same sequence outlined in Footnote b was then used without further addition of antimycin.

* Activity was reduced approximately 20% in presence of ammonium sulfate at 0.2 saturation at 0°C.
was released into the aqueous medium; most of the inhibitory activity of the antimycin added to Complex III was detected in the supernatant fluid after precipitation and removal of the guanidine-treated protein by sedimentation.

Although taurocholate alone, even at high concentrations, was relatively ineffective in causing the release of antimycin into the aqueous medium (after dilution), treatment of Complex III with a mercurial in addition to the taurocholate was effective in promoting the release of antimycin from the complex. Under properly poised conditions, a colorless protein also was detached from Complex III following the release of antimycin. The release of antimycin into the aqueous medium and the subsequent precipitation of the colorless protein during incubation of Complex III in the presence of taurocholate plus mersalyl are shown in Fig. 2. The absence of any precipitation of the colorless protein until almost all of the antimycin was released suggests that this protein may have become accessible to the mersalyl only after the site of binding of antimycin to the complex was destroyed.

The facile cleavage of Complex III by guanidine, coupled with the destruction of antimycin binding to the complex by this agent, suggested that protein structures other than those

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**Fig. 2.** Effect of mersalyl on the release of bound antimycin and “core protein” from Complex III. The reaction mixtures were made up by combining the following components: (a) Complex III (2.6 ml of a solution in 0.66 M sucrose containing 20 mg of protein per ml); (b) 2 M Tris-HCl, pH 8.3 (0.13 ml); (c) water (0.65 ml); (d) antimycin (0.156 ml of 1.0 mM antimycin in ethanol); (e) ammonium sulfate (1.56 ml of a saturated (0°C) solution); (f) sodium taurocholate (2.6 ml of a 50% solution); and (g) sodium mersalyl (0.13 ml of a 0.05 M solution). The mixture was kept at 0°C. At the indicated times, 0.3-ml samples (containing 2 mg of protein) were diluted with 4 volumes of 0.25 M sucrose. The insoluble “core protein” which separated was estimated from the turbidity of the diluted sample (i.e., absorbance at 700 nm). A 5-ml volume of 0.05 M dithiothreitol was then added to the diluted sample to neutralize the inhibitory effects of the mersalyl, followed by 0.1 ml of the solution of active Complex III. After time was allowed for equilibration (3 min at 0°C), 0.32 ml of the assay sample mixture was diluted to 4.0 ml in 0.4 M sodium taurocholate. The CoQH2-cytochrome c reductase activity remaining in the added, untreated Complex III was determined by the method described in the text. The loss of activity was a measure of the degree of release of antimycin from the mersalyl-treated complex; Δ, release of protein from the complex; ○, release of antimycin.

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**Fig. 3.** Effect of tryptic digestion of Complex III on the catalytic activity, the capacity for binding antimycin, and the release of bound antimycin from the complex. The reaction mixture was prepared by combining the following components: Complex III (0.0 mg of protein dissolved in 0.5 ml of 0.66 M sucrose), 0.5 ml of 0.5 mM sodium phosphate (pH 7.5), and 0.5 ml of 0.2% trypsin. For activity measurements of CoQH2-cytochrome c reductase, 0.06-ml samples (0.1 mg of protein) of the digestion mixture were withdrawn at the indicated times and were diluted to 4.0 ml with 0.4% sodium taurocholate; a 0.04-ml sample (containing 4 μg of protein) of this diluted enzyme was used in the modified assay (see Methods). Estimations of the capability of the digested complex to bind antimycin were made with 0.3-ml samples (containing 6 μmole of cytochrome c1) of the digestion mixture, to which were added 6 μl of a 1.0 mg/ml ethanolic solution of antimycin (6 μmole of antimycin). After 3 min at 0°C, 0.1 ml of the solution of Complex III (see above) without trypsin was added to the digested sample. After a 3-min period for equilibration, the mixture was diluted to 4.0 ml in 0.4% sodium taurocholate; a 0.04-ml sample (4 μg of protein of active Complex III) was assayed for CoQH2-cytochrome c reductase activity. This activity was related to antimycin binding according to the description in the text. For determination of the release of antimycin from the complex, the procedure was the same as for the determination of the antimycin-binding capacity except that the antimycin was added to Complex III (1.0 mole per mole of cytochrome c1) prior to the digestion. The percentage release of the antimycin was calculated by means of the following relationship.

% release of antimycin = (100 - % binding of antimycin)

Contributed by primary linkages may be involved in antimycin binding. In addition, the lipophilic nature of antimycin raises the question of an involvement of phospholipid in the binding of antimycin to the complex. To test both possibilities, we resorted to digestions of Complex III by trypsin, which hydrolyzes primary peptide linkages, and by phospholipase A, which hydrolyzes the α-glyceride linkages of phospholipids. The enzymic activity of the complex, the ability of the complex to bind antimycin, and the release of bound antimycin from the complex were measured as functions of the time of digestion of the complex by either trypsin or phospholipase. The results of the experiment with trypsin are illustrated in Fig. 3. Treatment with trypsin resulted in rapid enzymic inactivation, with a slower loss of the ability of the complex to bind antimycin. Moreover, although trypsin destroyed the antimycin-
The activity was estimated according to the procedures described in the text. 0.1% deoxycholate or in 0.2% taurocholate, and the enzymic activity was estimated according to the procedures described in the text.

Table V

<table>
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<tr>
<th>Additiona</th>
<th>Incubation time</th>
<th>Enzymic activity</th>
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<td>100b</td>
</tr>
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<td>30</td>
<td>95c</td>
</tr>
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</table>

a Expressed as moles per mole of cytochrome c1.
b The reaction mixture was diluted in 0.2% taurocholate and was assayed at 38°C.
c The reaction mixture was diluted in 0.1% deoxycholate and was assayed at 38°C.

The ability of antimycin to titrate precisely the antimycin-binding site of Complex III suggests that a tenacious association is formed between antimycin and the isolated complex. The results of the studies reported here have reinforced this concept. Only relatively high concentrations of detergent, or conditions that result in denaturation of the antimycin-binding site, have caused dissociation of antimycin from Complex III. These results are in apparent conflict with the conclusions reached by other investigators, based on investigations of relatively intact respiring particles. Poter and Reif (9) observed a spontaneous recovery of succinate oxidase activity in the liver tissue of rats which had been treated with inhibitory amounts of antimycin. They found that the albumin fraction of the serum reactivated succinate oxidase activity of various tissues that had been inhibited by antimycin. Because of these results, they described antimycin as a pseudoreversible inhibitor of succinate oxidase. Thorn (10) observed that the succinate oxidase activity of heart muscle preparations which had been inactivated completely by antimycin recovered most of the lost activity upon addition of an equal quantity of the enzyme preparation which had been inactivated irreversibly with respect to succinate oxidase by either 2-amino-4-hydroxyquinoline N-oxide or 2,3-dimercaptopropanol. It is noteworthy that in the classic experiments of Potter and Reif and those of Thorn, as well as in subsequent reports of reactivation of antimycin-inhibited respiration (11, 12), the oxidation of succinate was used as a criterion of respiratory activity. Estabrook (7) and Pumphrey (22) demonstrated that most of the antimycin sites in respiring particles could be titrated with antimycin before a significant inhibition of the oxidation of succinate or DPNH occurred. It is possible, therefore, that a complete reactivation of succinate oxidase could be achieved as a result of a marginal reversal of antimycin binding to Complex III within the particle. Another factor, which may explain in part the difference between purified Complex III and relatively intact respiratory chains with respect to their capability to be reactivated, is the possible presence of an energy-coupling system in the more nearly intact respiratory particles. If the antimycin-sensitive site is involved in the primary site of energy coupling, the degree of coupling of the respiring particle may influence the dissociability of antimycin from its site of inhibition. A requirement for the reactivation of antimycin-inhibited mitochondria by an antimycin-binding protein obtained from chicken liver is reported to be that the respiration of the mitochondria be well coupled to energy transduction (23). Perhaps a high energy intermediate involved in the initial coupling process competes favorably with antimycin for binding at the antimycin site.
Despite the irreversible nature of antimycin binding to Complex III, it is apparent from the experiments reported here that no stable covalent linkages are involved in the binding of antimycin to isolated Complex III. Moreover, the observation that antimycin, after extraction from its binding to Complex III, retained its inhibitory capacity indicates that antimycin itself is not altered irreversibly as a result of its interaction with the complex. However, the suggestion of Estabrook (7) that antimycin undergoes an alteration from an inactive to an active form during its interaction with the respiratory chain cannot be ruled out.

A puzzling result of this study is the inability of Complex III to be cleaved by taurocholate plus ammonium sulfate after 70% of the antimycin has been extracted from the inhibited complex. This observation suggests that antimycin caused a permanent change in the complex even though the antimycin was removed and was itself unchanged. This viewpoint is difficult to reconcile with the results of our investigations on the cleavage of Complex III (13). Under selected conditions Complex III, despite prior treatment with antimycin, could be cleaved by guanidine. A possible cause for the inability of Complex III to be cleaved after the extraction of the antimycin may have been the formation of disulfide linkages during the prolonged extraction with the detergent. We have observed that the accessibility of certain sulfhydryl groups in Complex III to specific sulfhydryl reagents was greatly reduced after the complex was treated with antimycin and then with detergents (24). In this connection, the observation that mersalyl can promote the release of antimycin from the complex during treatment with taurocholate plus a mercurial (25). Although antimycin has a somewhat lipophilic structure, which may aid in its adsorption to the complex, the actual mechanism of binding to the antimycin-sensitive site probably does not involve phospholipids. In fact, our observation that antimycin was released into the aqueous phase upon treatment of Complex III with 3 M guanidine suggests that the participation of phospholipid, even in the superficial adsorption of antimycin by the complex, was not significant. Because the apolar portion of the antimycin molecule is necessary for the irreversible inhibition of Complex III, however, some type of hydrophobic region probably is present in association with the antimycin-sensitive site. Another explanation for the lack of effect of phospholipase on the binding of antimycin is that the products of hydrolysis of the lipids may remain effective as solubilizers of antimycin.

The failure of phospholipase digestion to affect either the binding of antimycin to Complex III or the release of the inhibitor from the complex has a significant bearing on the role of phospholipid in the binding of antimycin to the complex. Although antimycin has a somewhat lipophilic structure, which may aid in its adsorption to the complex, the actual mechanism of binding to the antimycin-sensitive site probably does not involve phospholipids. In fact, our observation that antimycin was released into the aqueous phase upon treatment of Complex III with 3 M guanidine suggests that the participation of phospholipid, even in the superficial adsorption of antimycin by the complex, was not significant. Because the apolar portion of the antimycin molecule is necessary for the irreversible inhibition of Complex III, however, some type of hydrophobic region probably is present in association with the antimycin-sensitive site. Another explanation for the lack of effect of phospholipase on the binding of antimycin is that the products of hydrolysis of the lipids may remain effective as solubilizers of antimycin.

The nature of chemical changes in Complex III which lead to cleavage has been discussed in a companion report (13). Because of the apparently intimate relationship between the integrity of the antimycin-sensitive site and the ability of the complex to bind antimycin, the effects of enzymic digestion on antimycin binding may provide additional clues to the chemical changes which occur when the antimycin-sensitive site is disrupted. Although digestion with trypsin destroyed the ability of the complex to bind antimycin, this treatment failed to release antimycin from its binding to the complex. It appears that antimycin, when bound to the complex, protected the antimycin-sensitive site from attack by trypsin much as it protected the antimycin-sensitive site against attack by bile salts plus ammonium sulfate, or by 1 M guanidine. It is presently unknown whether antimycin protected the antimycin-sensitive site by a direct stabilization or shielding, or by an indirect effect involving the maintenance of a stable conformation in the entire complex. In the case of digestion by trypsin, however, the former hypothesis appears to be the more probable, since there was little quantitative difference in the number of peptide linkages hydrolyzed (as estimated by the titer of ninhydrin-reacting groups released by trypsin) whether or not the complex was treated with antimycin. This may indicate that there was essentially no difference in over-all conformation (and the accessibility of peptide linkages) of Complex III between the untreated and the antimycin-treated preparations. This conclusion is supported by our failure to observe any alteration in the sedimentation pattern of Complex III in the double sector cell upon treatment of the complex with antimycin.

The failure of phospholipase digestion to affect either the binding of antimycin to Complex III or the release of the inhibitor from the complex has a significant bearing on the role of phospholipid in the binding of antimycin to the complex. Although antimycin has a somewhat lipophilic structure, which may aid in its adsorption to the complex, the actual mechanism of binding to the antimycin-sensitive site probably does not involve phospholipids. In fact, our observation that antimycin was released into the aqueous phase upon treatment of Complex III with 3 M guanidine suggests that the participation of phospholipid, even in the superficial adsorption of antimycin by the complex, was not significant. Because the apolar portion of the antimycin molecule is necessary for the irreversible inhibition of Complex III, however, some type of hydrophobic region probably is present in association with the antimycin-sensitive site. Another explanation for the lack of effect of phospholipase on the binding of antimycin is that the products of hydrolysis of the lipids may remain effective as solubilizers of antimycin.

The lack of competition of antimycin O-methyl ether and of deformylantimycin toward the binding of antimycin to Complex III suggests that both the free phenolic group and the formylamino group are absolute requirements for the binding of antimycin to Complex III. Because these groups also are required for the inhibitory activity of antimycin, it is apparent that the binding of antimycin to Complex III is associated directly with the inhibition of enzymic activity of the complex. Although the hydrophobic diacate ring of antimycin modifies the inhibitory nature of the compound, it apparently cannot compete successfully with the contributions to the binding made by the phenolic group or the formylamino group. Moreover, because QO may offer some competition to antimycin in its binding to Complex III, and also is inhibitory to enzymic activity, it may provide a feasible approach to a determination of the type of interactions involved in the binding of antimycin or other
inhibitors to Complex III. Structurally dissimilar compounds that compete with antimycin in binding to the complex should be examined for common properties that are essential to their inhibitory and binding capabilities.

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REFERENCES
Factors Affecting the Binding of Antimycin A to Complex III of the Mitochondrial Respiratory Chain
John S. Rieske, S. H. Lipton, Harold Baum and H. I. Silman


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