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Detection of Antimycin-Binding Subunits of Complex III by Photoaffinity-Labeling with an Azido Derivative of Antimycin

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Abstract

Deformamidoazidoantimycin A (DAA), a photoactive derivative of antimycin A containing an azido group substituting for the formamido group attached to the phenyl ring, was synthesized. The ultraviolet spectrum of DAA was almost identical to that of antimycin A, indicating little alteration of the electronic structure of the substituted phenyl ring by the azido substitution. However, the inhibitory effectiveness of DAA toward ubiquinol-cytochrome *c* reductase (Complex III) purified from bovine heart (K_i = ca. 0.5 μM) was considerably less than that of antimycin ($K_i \leqslant 3$ pM), indicating a direct rather than a supporting role of the formamido group in the inhibitory activity of antimycin. Exposure of purified Complex III to [^{3}H]DAA plus ultraviolet light caused a major labeling by tritium of SDS-PAGE band 7 (m = 13 kDa by SDS-PAGE) and lesser but significant labeling of bands 3, 6, 8, and 9. Pretreatment of Complex III with antimycin greatly suppressed the labeling of bands 5, 6, and 7 but caused an apparent increased labeling of bands 8 and 9 by [^{3}H]DAA, respectively. The labeling of band 7 by [^{3}H]DAA also was strongly suppressed by reduction of Complex III by either sodium borohybride or ascorbate. Based on magnitude of labeling by [^{3}H]DAA and the degree of suppression of labeling by antimycin, the protein of band 7 qualified as the principal component for specific binding of antimycin with the protein of band 6 (m = 16 kDa) showing a lesser but significant amount of specific binding.

Key Words: Ubiquinol-cytochrome *c* reductase; Complex III; subunits; polyacrylamide gel electrophoresis; antimycin-binding site; photoaffinity labeling; deformamidoazidoantimycin A.

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Introduction

It is well known that antimycin[4] affects primarily the substrate reducibility and the spectroscopic properties of cytochrome *b* in the respiratory chain. Accordingly, cytochrome *b* has generally been considered as the antimycin-binding subunit of Complex III. However, because of the intricate structure of the complex and the ability of antimycin to protect the complex against dissociation in the presence of chaotropic reagents, indicating conformational stabilization of structure (Rieske *et al.*, 1967), the possibility exists that the perturbation of cytochrome *b* by antimycin is indirect, not involving a specific binding to the cytochrome. This consideration led to an attempt by this laboratory to utilize a photoactive derivative of antimycin as a direct photoaffinity label of the antimycin-binding subunit of Complex III.

Preliminary results utilizing an azido derivative of antimycin indicated that a polypeptide band in the SDS-PAGE gel corresponding to 11.5 kDa specifically incorporated the probe (Das Gupta and Rieske, 1973). However, these results were flawed by insufficient resolution of low-molecular-mass subunits by the SDS-PAGE procedure then in use. The development of improved SDS-PAGE procedures resulting in greater resolution of the low-molecular-mass subunits and an improved procedure for preparation of DAA enabled this laboratory to reexamine this labeling experiment in greater detail. A report of the results of this new study has been presented elsewhere (Ho *et al.*, 1982).

Materials and Methods

Complex III was purified from bovine-heart mitochondria by the procedure described by Rieske (1967). Fractions enriched respectively in core proteins, cytochrome *b*, cytochrome c_1, and the iron–sulfur protein were prepared by modification of the procedures described by Baum *et al.* (1967). Bovine serum albumin, ovalbumin, horse heart cytochrome *c*, and bovine pancreatic insulin were obtained from Sigma Chemical Co. Sperm whale myoglobin was obtained from Pierce Chemical Co. Antimycin A was obtained as a mixed complex of antimycins A_1–A_4 through the courtesy of Dr. Claude Vezina, Ayerst Laboratories, Montreal, Canada. Sodium dodecyl sulfate (SDS) was purchased from BDH through Gallard-Schlesinger Chemical Co. Acrylamide and methylene-bisacrylamide were recrystallized

[4] Abbreviations used: Antimycin, antimycin A; DAA, deformamidoazidoantimycin A; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; decyl QH_2, 2-*n*-decyl-3-methyl-5,6-methoxy-1,4-benzohydroquinone; PAGE, polyacrylamide gel electrophoresis.

from acetone. All other reagents were obtained from commercial sources and were of the highest purity available.

SDS-PAGE Procedure

SDS-PAGE employed in this study was a modification of the procedures of Furthmayr and Timpl (1971) and Weber and Osborn (1969) emphasizing the counterion effect (Rodbard and Chrambach, 1971). A stock acrylamide solution (30% in acrylamide and 0.8% in *N,N'*-methylene-bisacrylamide was prepared and stored in the dark at 0°C. A 7.5% gel was prepared by mixing 25 ml of the stock acrylamide solution, 75 ml 0.2 M $NaHPO_4$, pH 7.0, and 0.5 ml 20% sodium dodecyl sulfate and diluted to a total volume of 100 ml. The acrylamide mixture was polymerized in cylindrical tubes (5. mm i.d. × 7–12 cm) subsequent to addition, with thorough mixing of 0.75 ml of freshly prepared 10% ammonium persulfate solution.

Protein samples to be electrophoresed were precipitated by either 90% acetone or by addition of saturated ammonium sulfate to 0.48 saturation after which the protein was collected by sedimentation. The sedimented pellet was dissolved in a dissociating buffer consisting of 0.1 M sodium phosphate, pH 7.0, 5% (w/v) sodium dodecyl sulfate, and 1.0% (v/v) mercaptoethanol and incubated for no more than 3 hr at ambient temperature.

Electrophoresis was usually run for 8–12 hr at no more than 8 mA per tube with a lower reservoir buffer of 0.1 M sodium phosphate, pH 7.0, and an upper reservoir buffer of 0.1% sodium dodecyl sulfate and 0.15 M TRIS phosphate, pH 7.0. After electrophoresis, the gels were removed from the tubes and fixed with 20% trichloroacetic acid. Residual sodium dodecyl sulfate was removed by diffusion in a diffusion destainer for 8 hr in a solution of methanol : acetic acid : water in volume ratios of 5 : 1 : 5, respectively. Gels were stained with Coomassie Blue G 250 according to a modified procedure of Blakesley and Boezi (1977). Gels were scanned at 600 nm in a Gilford Model 2000 spectrophotometer equipped with a gel scan attachment. Gels were calibrated for mobility-molecular mass with bovine serum albumin, ovalbumin, horse-heart cytochrome *c*, bovine insulin, and sperm whale myoglobin.

Preparation of [^{3}H]deformamidoazidoantimycin (DAA)

Deformylantimycin hydrochloride prepared from the antimycin complex according to the procedure described by van Tamelen *et al.* (1961) was isotopically labeled by exposure of the powdered crystalline compound to

tritium gas followed by successive recrystallizations to a constant specific radioactivity of 12.8 mCi/mmole. A 50-mg portion of the deformylantimycin hydrochloride was dissolved in 1.5 ml ethanol: 0.5 ml 8 N HCl. At 0°C, 100 mg $NaNO_2$ dissolved in 0.5 ml water was added after which the solution was diluted with 10 volumes of cold water. The yellow diazonium salt was extracted by two 5-ml volumes of $CHCl_3$ after which the $CHCl_3$ layer was treated with solid crystals of NaN_3 until the yellow color was discharged and the evolution of nitrogen ceased. The $CHCl_3$ solution was washed three times with 10 ml water after which the $CHCl_3$ was dried with anhydrous $MgSO_4$ and removed under vacuum. The oily residue was dissolved in a small volume of ethanol which was then treated with decolorizing charcoal and filtered. A small amount of water was added to the alcohol solution after which the solution was cooled to 0°C. Colorless crystals of DAA appeared shortly: mp 95–96°C, 71% yield. All of these operations were performed in subdued light or in the dark when practicable.

Other Procedures

Protein was determined by a modification of the biuret procedure of Gornall *et al.* (1948) or by a modified Lowry procedure (Markwell *et al.*, 1978) with bovine serum albumin as a standard. Ubiquinol-cytochrome *c* reductase activities were measured at 5°C or at 20°C by a procedure described by Rieske (1967) modified to allow continuous recording of reduction of cytochrome *c* and the use of a decyl analog of ubiquinol (decylQH_2). Sigma Type VI cytochrome *c* was used without prior dialysis.

Photoaffinity labeling of Complex III with [^{3}H]DAA was effected by a 15-min exposure of the reaction mixture to light from a spectrophotometer deuterium lamp. (Spectral measurements with aqueous solutions of DAA indicated that complete decomposition of the azido group occurred within 6 min during exposure to the ultraviolet light.) The reaction mixture consisting of 1.6 mg (4.8 nmol) Complex III protein and 17 nmol [^{3}H]DAA in 0.1 ml of solution was contained in a silica spectrophotometer cell packed in ice. After irradiation the sample was "cold chased" by addition of an excess of unlabeled DAA. The sample subsequently was extracted three times with ether to remove unbound DAA. The ether-extracted sample was dissolved in the dissociating buffer, pH 7.0, and then separated into subunit bands by PAGE.

Tritium levels in the PAGE gels were counted individually as 1.0-mm slices digested with 1.0 ml 15% H_2O_2 for 1 hr at 90°C prior to addition of 10 ml Aquasol-2 (New England Nuclear) as the liquid-scintillation fluid.

A quantitative estimate of the bound tritium in each PAGE band was

made by a measurement of the area under the smoothed peak derived from the radioactivity (counts) vs. gel slice number plot. The baseline was taken as the minimum counts recorded between bands 5 and 6 where there was no overlapping of protein bands. This baseline was assumed to represent irreversible nonspecific adsorption of tritium label in the gel matrix. Similarly, an estimate of the protein content of each PAGE band was made by an estimate of the area under the peak as recorded by the densitometric scan of the gel which had been stained by Coomassie Blue. Deconvolution of closely spaced, overlapping bands was accomplished manually by iteration.

Results

Chemical Properties of DAA

The tentative structure of DAA and the structure of antimycin are given in Fig. 1. The infrared spectrum of DAA was almost identical to that of deformylaminoantimycin except for a prominent, slightly asymmetric band at 2100 cm^{-1} which is characteristic for the asymmetric stretching frequency of the azido group (Gurst, 1971). The ultraviolet absorbance spectrum of an acidified alcoholic solution of DAA was almost identical to that of antimycin, displaying an intense absorption at 225 nm, a shoulder at 250 nm, and an isolated, broad absorption centered at 317 nm. Antimycin under

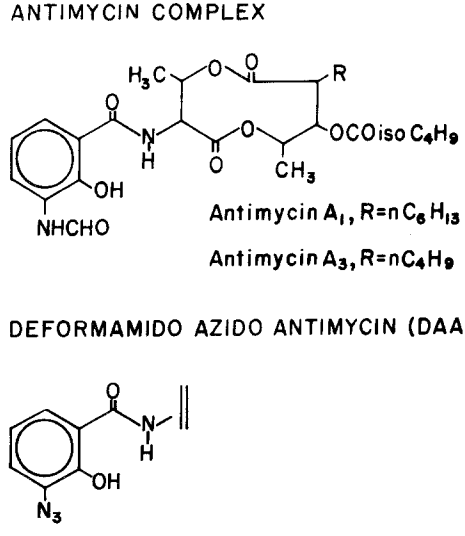

Fig. 1. Structures of antimycin and deformamidoazidoantimycin (DAA).

similar solvent conditions displays absorption bands at 227 and 317 nm and a shoulder at 250 nm. It is of interest that the ethanolic solution of DAA exhibited an extinction coefficient at 317 nm of 6,000 $M^{-1} cm^{-1}$, essentially identical to the value reported for antimycin A_1 (Schilling *et al.*, 1970).

Exposure of DAA (< 0.1 mM) in aqueous solution to ultraviolet light of a spectrophotometer deuterium lamp resulted in rapid decomposition of DAA as indicated by a loss of absorption bands at both 225 and 317 nm.

Inhibitory Properties of DAA

The structure of DAA consists of antimycin with the photoactive azido group replacing the formamido group (Fig. 1). Although the formamido group is required for the inhibitory activity of antimycin (Van Tamelen *et al.*, 1961), inhibitory properties are retained when the formamido group is replaced by an electron-withdrawing group such as the nitro group (Neft and Farley, 1971). On this basis, the replacement of the formamido group with an azido group held promise of yielding an inhibitory compound. Furthermore, the azido group has been observed to be similar to acylamino groups in its interaction with the benzene ring (Smith *et al.*, 1962).

As shown in Table I, DAA inhibited the electron transport activity of purified Complex III although at considerably higher concentrations than observed with similar inhibitions by antimycin. The calculated inhibition constant, K_i, for the inhibition of cytochrome *c* reductase activity of Complex III was approximately 0.5 μM at both 5 and 20°C in contrast to an estimated dissociation constant, K, of < 0.1 pM for the binding and antimycin to isolated Complex III (Berden and Slater, 1972) and an inhibition constant, K_i, of $\leqslant 3$ pM for decylQH_2-cytochrome *c* reductase activity of the complex (Ramesh, V., and Rieske, J. S., unpublished). In other respects, DAA behaved similar to antimycin, being able to support the oxidant-induced reduction of cytochrome *b* as noted in the earlier communication (Das Gupta and Rieske, 1973).

Table I. Inhibition of decylQH_2-cytochrome *c* Reductase Activity of Purified Complex III by DAA[a]

Temperature (°C)	Complex III (μM)	DAA (μM)	Percent inhibition	K_i (μM)
5	0.16	1.87	77	0.58
5	0.16	3.74	90	0.51
5	0.16	7.48	93	0.49
20	0.04	1.87	79	0.49
20	0.04	3.74	89	0.46
20	0.04	7.48	93	0.54

[a]Assays were performed as described in Materials and Methods.

Photoaffinity Labeling of Complex III by [^{3}H]DAA

Although DAA displayed much less affinity for Complex III than antimycin, it still displayed sufficient binding to the complex to make identification of the subunits involved by photoaffinity-labeling procedures feasible. Complex III was incubated with a 3.5-fold molar excess of [^{3}H]DAA and then exposed for 15 min to focused light from a spectrophotometer deuterium lamp. Becuse of the relatively low specific radioactivity of [^{3}H]DAA and the reduced affinity of DAA for Complex III, it probably would have been desirable to have used a greater ratio of [^{3}H]DAA to Complex III. However, to avoid an unacceptable amount of nonspecific labeling of the complex, the relatively low ratio of [^{3}H]DAA to Complex III was maintained. The labeling profile of the SDS-PAGE bands[5] is shown in Fig. 2A. Although all of the subunits showed tritium labeling, the principal radioactivity appeared in band 7 except for the radioactivity at the solvent front attributable to unbound [^{3}H]DAA and its decomposition products or possibly to DAA bound to phospholipid. Considerably less, but significant, labeling of bands 3, 6, and 9 occurred. Because of the proximity of band 8 to band 7 it was difficult to estimate the relative degree of labeling of band 8 by this experiment alone. However, as seen in the antimycin-treated complex (see next paragraph and Fig. 2B), band 8 also received significant labeling by [^{3}H]DAA. Interestingly, the largest protein subunits of the complex, core proteins I and II, cytochromes *b* and c_1, and the iron–sulfur protein, represented by bands 1–5, received the lowest degree of labeling. Very little labeling of the core proteins (bands 1 and 2) occurred, even though these proteins make up almost 50% of the protein bulk of the complex.

Although the relatively intense labeling of band 7 alone would suggest a specific binding of [^{3}H]DAA to the protein making up this band, a further test for specific labeling was conducted by repeating the labeling procedure with Complex III pretreated with antimycin. Antimycin, because of its high affinity for the antimycin-binding site, should serve as an effective shield against the specific binding of DAA. The [^{3}H]DAA-labeling profile of the PAGE gel is shown in Fig. 2B. A large decrease in labeling of band 7 in comparison with the other bands is shown, with band 8 retaining the most prominent labeling. Significant decreases in labeling of bands 4, 5, 6, and 9 also appeared to be caused by prior treatment of the complex with antimycin. As shown in Fig. 2C, when antimycin was added after treatment of the

[5] In this study PAGE gel bands are numbered in order of increasing mobility by Arabic numerals. Protein subunits of Complex III are numbered in order of decreasing molecular weight by Roman numerals. Bands 1–7 are generally conceded to represent subunits of Complex III correspondingly numbered I–VII. It is yet uncertain that bands 8 and 9 consist of subunit proteins of Complex III. Subunits I–V have been assigned to core proteins I and II, cytochrome *b*, cytochrome c_1, and the iron–sulfur protein, respectively.

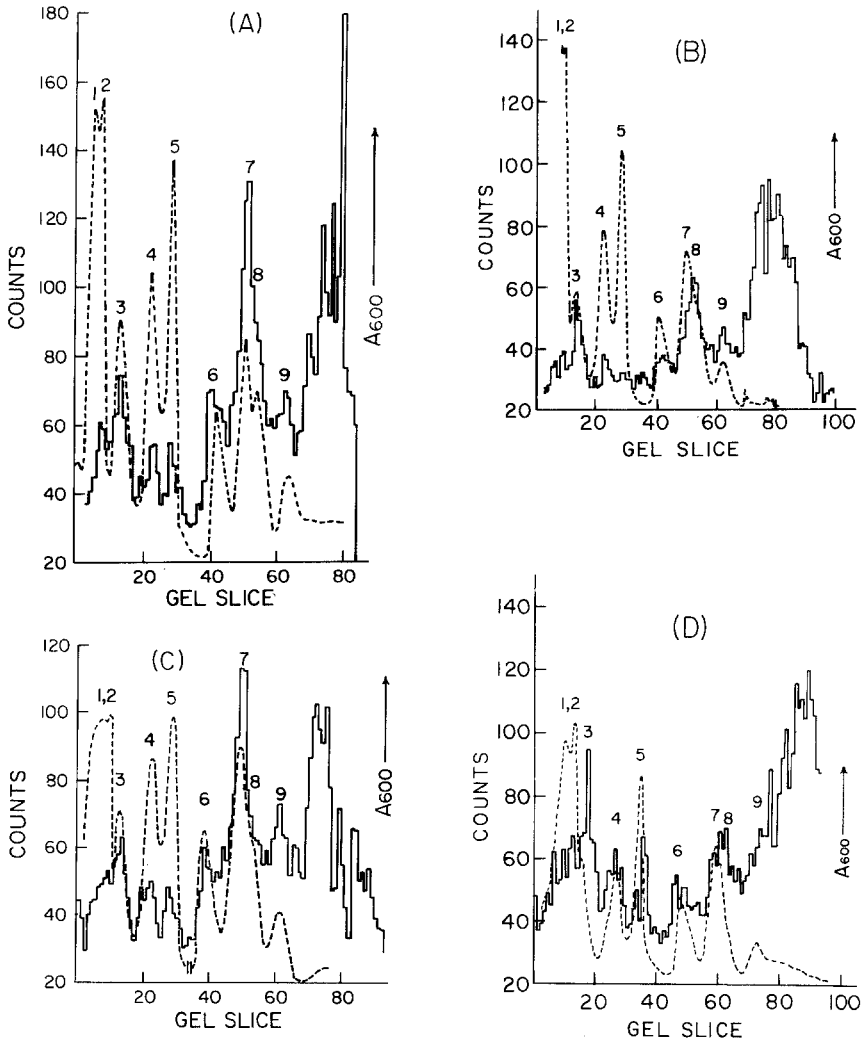

Fig. 2. Photoaffinity labeling of polypeptides of Complex III by [^{3}H]DAA. Samples of Complex III were exposed to [^{3}H]DAA and subjected to SDS-PAGE and [^{3}H] analysis according to procedures described in Materials and Methods. Radioactivity measurements were not corrected for background radioactivity which averaged 20 cpm in slices of gel not containing sample. The dashed-line traces represent densitometric scans of the stained gels prior to slicing and counting. PAGE polypeptide bands are numbered in order of increasing mobility. Bands 1–7 represent subunits I–VII of Complex III (see footnote 5). (A) PAGE labeling profile of untreated Complex III; (B) PAGE labeling profile of Complex III treated with a stoichiometric amount of antimycin prior to photolabeling with [^{3}H]DAA; (C) PAGE labeling profile of Complex III treated with a stoichiometric amount of antimycin subsequent to photolabeling of the complex with [^{3}H]DAA; (D) PAGE labeling profile of Complex III treated with $NaBH_4$ prior to photolabeling with [^{3}H]DAA. Gel slices containing polypeptide bands were visually identified before separation of the slices for counting.

complex with [^{3}H]DAA the labeling profile was similar to that obtained by treatment with [^{3}H]DAA alone. This indicates that DAA was covalently linked to the protein subunits and therefore not displaceable by the much tighter-binding antimycin.

Table II summarizes numerically the results shown in Figs. 2A–2D. In perusing these data, caution must be exercised because of the uncertainties inherent in the SDS-PAGE procedures, especially in the use of densitometric scans of cylindrical gels which suffer from a certain lack of reproducibility due to band distortions in both shape and intensity of staining. In addition, different proteins appear to vary in staining capability thus causing uncertainty in the comparison of the protein contents of the different gel bands. However, because of the intense staining of band 7 relative to its assumed protein content based on subunit stoichiometry and molecular mass as compared with the other bands, the value of [^{3}H]/(protein) for band 7 as shown in Table II would be in error on the low side. Also, because of the low obtainable radioactivity of the [^{3}H]DAA and other experimental requirements, it was necessary to obtain radioactivity scans by the radioisotope counting of individual gel slices with its inherent errors rather than by autoradiography of slab gels. However, despite these uncertainties the numerical data are generally consistent in showing that band 7 represents the predominant antimycin-binding subunit with a subunit represented by band 6 having a significant secondary binding capability. These conclusions are borne out by comparison of the specific-binding coefficients of the proteins of SDS-PAGE bands shown in line 7 of Table II, which are combined measures of specific labeling by [^{3}H]DAA and the labeling suppression by antimycin. Also, it is to be noted that band 3, which is assigned to the apoprotein of cytochrome *b*, is relatively highly labeled by [^{3}H]DAA, but this labeling appears to be little affected by prior treatment of Complex III with antimycin (compare values listed in lines 2 and 4 of Table II). Several bands received relatively low labeling by [^{3}H]DAA, yet this labeling was lowered further by prior treatment of Complex III with antimycin. These were bands 1 and 2, 4, and 5 assigned to the core protein, cytochrome c_1, and the iron–sulfur protein, respectively (compare the values listed in lines 1 and 8 of Table II). In contrast, the labeling of band 8 appeared to be significantly increased by prior treatment of the complex with antimycin. These results suggest that antimycin may alter the accessibility of Complex III subunits to DAA directly by occupying the same site that binds DAA, or indirectly by altering the quaternary conformation of the complex or the tertiary conformations of the affected subunits.

A little-understood phenomenon observed in this study was the effect of reducing agents on the labeling of subunits of Complex III by [^{3}H]DAA. Reduction of the complex by either Na borohydride or ascorbate significantly

Table II. Relative Photoaffinity Labeling of the PAGE Bands of Complex III by [^{3}H]DAA[a]

Description	PAGE band							
	1 + 2	3	4	5	6	7	8	9
1. III + DAA, [^{3}H][b]	133	250	114	105	210	450	176	204
2. III + DAA, [^{3}H]/(Prot.)[c]	0.84	3.2	1.4	1.3	5.2	6.7	4.6	5.8
3. III + ANT + DAA, [^{3}H][b]	62	124	43	24	57	86	186	100
4. III + ANT + DAA, [^{3}H]/(Prot.)[c,d]	0.50	3.0	0.74	0.38	2.0	1.7	7.8	6.7
5. III + DAA + ANT, [^{3}H]/(Prot.)[c,d]	–	3.2	1.3	1.5	4.4	5.7	4.1	13
6. III + $NaBH_4$ + [^{3}H]DAA, [^{3}H][b,d]	220	250	160	130	130	95	190	–
7. III + $NaBH_4$ + [^{3}H]DAA, [^{3}H]/(Prot.)[c]	1.8	8.3	4.6	3.0	5.0	2.4	17	–
8. $\langle$[^{3}H]/(Prot.)$\rangle_{ANT+DAA}$/$\langle$[^{3}H]/(Prot.)$\rangle_{DAA}$	0.60	0.94	0.53	0.29	0.39	0.25	1.7	1.2
9. $\langle$[^{3}H]/(Prot.)$\rangle_{NaBH_4+DAA}$/$\langle$[^{3}H]/(Prot.)$\rangle_{DAA}$	2.1	2.6	3.3	2.3	0.96	0.36	3.7	–
10. Specific binding index[e]	1.4	3.4	2.6	4.5	13	27	2.7	4.8

[a] General procedures are given in Materials and Methods and specific experimental details are given in the captions to Figs. 2A–2D.
[b] Values are expressed as integrated counts-min^{-1}.
[c] Protein concentrations are integrated absorbances of the stained bands expressed in arbitrary units.
[d] Complex III was treated with reagents in the order listed.
[e] Expressed as the values in line 2 divided by the values in line 8.

lowered the antimycin-sensitive labeling of band 7 yet appeared to have little effect on, or actually to increase with respect to gel staining, the labeling of most of the other polypeptides of the complex. Figure 2D and Table II, lines 6, 7, and 9, show the results obtained with borohydride-reduced Complex III. Similar results were obtained after reduction of Complex III with ascorbate (data not shown).

Discussion

The synthesis of DAA as a photoaffinity probe of the binding site of antimycin was based partially on the premise that substitution of the formamido group of antimycin (see Fig. 1) with an azido group would cause little change in the pK of the phenolic hydroxyl group as based on the report of Smith *et al.* (1962). Both the formamido group and the phenolic hydroxyl are required for the inhibitory activity of antimycin (Van Tamelen *et al.*, 1961). However, the retention of inhibitory activity of certain analogs of antimycin after substitution of the formamido group with a nitro group (Neft and Farley, 1971) suggested that the formamido group functions as an electron-withdrawing group thus activating the binding capability of the adjacent phenolic hydroxyl group.

Our finding that the ultraviolet spectrum of DAA is almost identical to that of antimycin confirms the observation of Smith *et al.* (1962) that the azido group exerts effects similar to acylamino groups on the electronic properties of the phenyl ring. However, the low inhibitory effectiveness of DAA ($K_i = 0.5\,\mu M$) compared with that of antimycin measured under similar conditions ($K_i \leqslant 3\,pM$), suggests that the formamido group is involved directly as a bonding group to Complex III rather than indirectly only as a modulator of the phenolic hydroxyl group via the phenyl ring. In this case the amino acid residue to which DAA is attached in the photolyzed sample may be identical to the residue that binds with the formamido group of antimycin.

Despite the relatively low inhibitory activity of DAA toward the electron-transfer activity of Complex III, the photoaffinity labeling of Complex III by [^{3}H]DAA indicates that only polypeptides corresponding in mobility to PAGE band 7 (subunit VII) and to a lesser extent band 6 (subunit VI) qualify as specific binding sites for DAA. The labeling profiles observed in this study would infer that these polypeptides are primary binding sites for antimycin although not necessarily the only binding sites in Complex III. The earlier experiment involving [^{3}H]DAA as a photoaffinity label of the antimycin-binding subunit found the label principally associated with SDS-PAGE band 8 (Das Gupta and Rieske, 1973) rather than bands 6 and 7 as

observed in this study. This discrepancy may have been due to the poorer resolution of bands 7–9 and overlapping by the unbound or phospholipid-bound [^{3}H]DAA and its decomposition products near the solvent front in the SDS-PAGE gels utilized in the earlier study.

The marked suppression by antimycin of the labeling of subunits having relatively low affinity for DAA, especially the iron–sulfur protein (subunit V), which has been shown to be independently separable from the antimycin-binding site (Rieske *et al.*, 1964, 1967), raises a question concerning the validity of the labeling suppression by antimycin alone as a criterion of specific binding of DAA. This problem of suppression of nonspecific binding of DAA by antimycin, possibly through conformational perturbations, probably can only be eliminated by use of a photoactive derivative of antimycin having an affinity for the specific antimycin-binding site comparable to that of antimycin. However, in the case of PAGE band 7, its relatively high labeling by [^{3}H]DAA together with a 75% or greater suppression of this labeling by antimycin is compelling evidence that the protein making up this band (subunit VII) contains the primary binding site for antimycin and DAA.

As discussed in previous reports (Ho, 1979; Rieske, 1980; Rieske *et al.*, 1984), antimycin appears to function as a multivalent inhibitor, being able to perturb, almost independently, several measurable functions of the complex. It was proposed that antimycin is simultaneously bound to, or in contact with, several polypeptide subunits of the complex. If this is the case, then it is possible that photoactive groups placed at different positions on the antimycin molecule would label different polypeptide subunits.

The loss of specific labeling of PAGE band 7 by [^{3}H]DAA in reduced Complex III is little understood. This phenomenon superficially indicates that DAA and, by analogy, antimycin are not specifically associated with a protein present in band 7 when the complex is reduced. However, Berden and Slater (1972) reported that the binding of antimycin to oxidized and to fully reduced complex in submitochondrial particles was essentially the same, although a significant decrease in binding was observed with the succinate-reduced particles. This apparent discrepancy can be explained by multivalent binding of antimycin if the decrease of subunit bonding at the formamido group of antimycin is compensated by an increased bonding with other groups on the antimycin molecule upon reduction of the complex. However, it is more likely that this effect of reduction on the photoaffinity binding of DAA is related in some way to the similar effects of reduction and antimycin treatment, respectively, on the stabilization of the complex against dissociation by bile salts plus ammonium sulfate or by guanidine.

In earlier studies (Baum *et al.*, 1967; Das Gupta and Rieske, 1973) as well as in this study (data not shown), the protein of band 7 was observed to

separate with the soluble cytochrome c_1 fraction obtained upon cleavage of Complex III by incubation of the complex with taurocholate plus ammonium sulfate or with guanidine.[6] This protein (m = 13 kDa) appears to be distinct from the two low-molecular-mass proteins found to be associated with purified cytochrome c_1 (Wakabayashi *et al.*, 1982; Schägger and Von Jagow, 1983). These proteins displayed molecular masses of 11 and 5 kDa, respectively, based on SDS-PAGE mobility. Also, it must be pointed out that band 7 has been attributed by some investigators to a protein associated with cytochrome *b* during fractionation of the complex (Marres and Slater, 1977). However, it has been noted that the use of 8 M urea in the SDS-PAGE buffer as reported by Marres and Slater reversed the order of migration of proteins associated with cytochromes *b* and c_1, respectively (Capaldi *et al.*, 1977). The apparent specific labeling of band 6 by [^{3}H]DAA as observed in this study may be a reflection of this uncertainty of assignment of bands 6 and 7. In our studies, the protein of band 6 has generally been associated with the cytochrome *b* fraction. A specific labeling of both subunits VI and VII of the complex suggests that these subunits may be closely linked between cytochromes *b* and c_1. This arrangement may also explain the stabilizing effect of antimycin with respect to the cleavage of the complex if antimycin binds to both subunits VI and VII.

Also, it may be of interest that the protein of band 7 has been associated with DCCD inactivation of Complex III presumably by being crosslinked via DCCD to the iron–sulfur protein (subunit V) (Nalecz *et al.*, 1983). Although a similar mechanism for the inhibitory effects of antimycin may be entertained, the early finding that the iron–sulfur protein can be dissociated readily from antimycin-treated Complex III (Rieske *et al.*, 1964) would invalidate a mechanism closely analogous to the crosslinking effect of DCCD.

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[6] In these earlier studies the PAGE band now designated as band 7 was designated as band 6 since band 3 was not counted due to its poor staining and resolution.

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