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Mucidin and Strobilurin A Are Identical and Inhibit Electron Transfer in the Cytochrome bc_1 Complex of the Mitochondrial Respiratory Chain at the Same Site as Myxothiazol†

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Received July 15, 1985

ABSTRACT: Mucidin and strobilurin A, antifungal antibiotics isolated from the basidiomycetes *Oudemansiella mucida* and *Strobiluris tenacellus*, respectively, inhibit electron-transfer reactions in the cytochrome bc_1 complex of the mitochondrial respiratory chain. The two compounds have identical effects on oxidation–reduction reactions of the cytochromes *b* and c_1 in isolated succinate-cytochrome *c* reductase. They inhibit reduction of cytochrome c_1 by succinate but do not inhibit reduction of cytochrome *b*. When added in combination with antimycin, either inhibitor blocks reduction of both cytochromes *b* and c_1. Mucidin and strobilurin A differ from antimycin in that they inhibit, rather than promote, oxidant-induced reduction of cytochrome *b*. They also differ from antimycin in that they do not block reduction of cytochrome *b* by succinate when cytochrome c_1 is previously reduced by ascorbate and they do not inhibit oxidation of cytochrome *b* by fumarate. These effects of mucidin and strobilurin A are, however, qualitatively identical with those of myxothiazol, an antibiotic that inhibits respiration by binding to cytochrome *b* [Von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T., & Trumpower, B. L. (1984) *J. Biol. Chem. 259*, 6319–6326]. Mucidin and strobilurin A have identical UV and mass spectra, and they elute together on high-pressure liquid chromatography. We thus conclude that these antibiotics, although isolated from different bacteria, are structurally identical. Our results indicate that strobilurin A and mucidin inhibit electron transport at the same site as myxothiazol and not at the antimycin site, as previously reported [Subik, J., Behren, M., & Musilek, V. (1974) *Biochem. Biophys. Res. Commun. 57*, 17–22].

Antibiotics that inhibit respiration provide an important approach for elucidating the pathway of electron transfer and mechanism of energy transduction in the cytochrome bc_1 complex of the mitochondrial respiratory chain. Thus, the effects of antimycin (Bowyer & Trumpower, 1981; De Vries et al., 1983) and myxothiazol (Meinhardt & Crofts, 1982; De Vries et al., 1983; Von Jagow et al., 1984) on oxidation–reduction reactions of the redox components of the bc_1 complex provide evidence that there are two pathways of cytochrome *b* reduction. This finding supports a protonmotive Q cycle pathway of electron transfer in the bc_1 complex (Mitchell, 1975, 1976; Bowyer & Trumpower, 1981; Meinhardt & Crofts, 1982; De Vries et al., 1983).

All antibiotic inhibitors of the bc_1 complex that have been characterized to date can be classified into one of two groups,

† This investigation was supported by NIH Grant GM 20379. B.L.T. is grateful to the Humboldt Stiftung for support provided by a Humboldt Preis.
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0006-2960/86/0425-0775$01.50/0 © 1986 American Chemical Society

according to which of the two pathways of *b* reduction they block. Antimycin blocks the pathway of *b* reduction through "center i", in the terminology of the Q cycle (Mitchell, 1976), by binding at a site proximal to the heme of *b*-562 (Berden & Opperdoes, 1972; Dutton et al., 1972; Roberts et al., 1980; Bowyer & Trumpower, 1981) and destabilizing ubisemiquinone (Ohnishi & Trumpower, 1979). Funiculosin, reputedly an "antimycin-like" inhibitor (Nelson et al., 1977; Rieske, 1980; Ksenzenko et al., 1983), presumably also blocks reduction of cytochrome *b* through the center i pathway.

Myxothiazol blocks the pathway of cytochrome *b* reduction through "center o" by binding proximal to the heme of *b*-566 (Becker et al., 1981) and apparently blocking oxidation of ubiquinol at a site on the iron–sulfur protein of the bc_1 complex (Von Jagow et al., 1984). This pathway of *b* reduction is linked to reduction of iron–sulfur protein and cytochrome c_1, consequently, myxothiazol also blocks reduction of cytochrome c_1 (Meinhardt & Crofts, 1982; De Vries et al., 1983; Von Jagow et al., 1984).

Neither antimycin nor myxothiazol will inhibit *b* reduction when added separately, since an alternative pathway of *b* reduction remains operative. However, when added together, the two inhibitors block reduction of both cytochromes *b*-562 and *b*-566 in addition to cytochrome c_1 (Meinhardt & Crofts, 1982; De Vries et al., 1983; Von Jagow et al., 1984). This phenomenon provides a useful diagnostic test to establish that inhibitors act on separate pathways of *b* reduction.

The importance of such antibiotics is increased by the recent application of genetic methods to further delineate their sites of action. Numerous mutants of yeast and mouse cells resistant to antimycin and myxothiazol have been isolated, and these mutations have been mapped to the mitochondrial cytochrome *b* gene (Roberts et al., 1980; Thierbach & Michaelis, 1982; Howell et al., 1983). As the genetic approaches are extended to locate the mutant loci within the sequence of the cytochrome *b* gene (Nobrega & Tzagaloff, 1980; Anderson et al., 1982) and these results are interpreted together with biochemical information on these inhibitors, it should be possible to locate the center i and center o domains on cytochrome *b* and to relate these to the location of the hemes of *b*-562 and *b*-566. However, the eventual success of these approaches requires that similarities and differences among the various antibiotics are recognized and that their effects on electron-transfer reactions in the bc_1 complex are correctly described.

Mucidin is an antifungal antibiotic isolated from mycelial cultures of the basidiomycetes *Oudemansiella mucida* (Musilek et al., 1969). It inhibits respiration and is thus cytotoxic to a wide variety of yeasts and fungi (Musilek et al., 1969; Subik et al., 1974b). Because of these properties, it has apparently been used clinically for topical treatment of dermatomycoses (Vondracek et al., 1970; Sedmera et al., 1981).

Mucidin inhibits oxidation of succinate and pyruvate by rat liver mitochondria and does not inhibit ATPase activity (Subik et al., 1974a,b). Addition of mucidin to respiring mitochondria causes increased reduction of the *b* cytochromes and oxidation of the *c* cytochromes. These effects are similar to those caused by antimycin. It was thus concluded that mucidin acts between the *b* and *c* cytochromes at the same site as antimycin and HQNO (Subik et al., 1974a).

When first isolated, mucidin was reported to be a crystalline, dextrorotatory solid (Musilek, 1970). The structure of mucidin was not known. However, on the basis of the reported properties of the two inhibitors, it was concluded that mucidin is different from strobilurin A (Anke et al., 1979), which is an oil at room temperature and optically inactive (Anke et al., 1977; Schramm et al., 1978).

Strobilurin A is an antifungal antibiotic isolated from *Strobiluris tenacellus* (Anke et al., 1977). It is one of a family of antibiotics, including strobilurins B and C and oudemansins A and B, produced by the genera *Strobilurus*, *Mycena*, *Oudemansiella*, *Hydropus*, and *Cyphellapsis* (Schramm et al., 1978; Anke et al., 1983), which contain a β-methoxyacrylate system as a common structural feature (Becker et al., 1981; Schramm et al., 1982). The structure of strobilurin A has been deduced by NMR and mass spectroscopy (Schramm et al., 1978), and the stereochemistry of the 9,10 double bond in the side chain has been established by chemical synthesis (Anke et al., 1984).

Strobilurin A inhibits ubiquinol-cytochrome *c* reductase activity of isolated bc_1 complex and causes a shift to longer wavelength of the α-band in the optical spectrum of cytochrome *b*-566 (Becker et al., 1981). These effects are qualitatively similar to those of myxothiazol. Thus, it has been assumed that strobilurin A acts at the same site as myxothiazol (Becker et al., 1981).

In preliminary experiments to test the sites of action of mucidin and strobilurin A, we noted that they appeared to inhibit at the same site in the bc_1 complex, contrary to what might be expected from previously published results (see above). A paper then appeared in which it was stated that mucidin and strobilurin A are the same compound (Sedmera et al., 1981). However, as discussed below, there remained several inconsistencies in the published properties of mucidin. It also remained to be established whether these antibiotics act at the same site as antimycin (Subik et al., 1974a) or myxothiazol (Becker et al., 1981). We thus compared selected physical-chemical properties of mucidin and strobilurin A and examined their effects on oxidation–reduction reactions of the cytochromes *b* and c_1 in isolated succinate-cytochrome *c* reductase complex.

Experimental Procedures

Materials. Antimycin and ascorbic acid were obtained from Sigma. Mucidin was obtained from Dr. N. Howell, who has used the inhibitor to select resistant mutants (Howell et al., 1983) and who received the compound from Dr. V. Musilek (Prague). Strobilurin A was provided by Dr. T. Anke (Kaiserslautern). Mucidin and strobilurin A were oils at room temperature. Both were dissolved in ethanol to a concentration of 1 mM, on the basis of the extinction coefficient of strobilurin A (Anke et al., 1977). Succinate-cytochrome *c* reductase was isolated from beef heart mitochondria (Von Jagow et al., 1984) and stored at −10 °C in buffered 50% glycerol.

Mass Spectroscopy. Mass spectra were obtained with a Finnigan 4023 spectrometer. Chemical ionization was carried out at 70 eV and a source temperature of 250 °C. The emission current was 0.30 A, and the electron multiplier was set at 1400 V. Mass was calibrated with perfluoro-*n*-butylamine. The spectrometer was tuned to give ion intensities for bis(pentafluorophenyl)phenylphosphine recommended by the Environmental Protection Agency (Eichelberger et al., 1975). Anthracene-d_{10} and 2,2′-binaphthylchrysene-d_{12} were included as internal standards. Reagent gas flow, methane or isobutane, was adjusted to give an ionizer pressure of 0.1 or 0.05 Torr (uncorrected), respectively.

High-resolution mass spectra were obtained at the Massachusetts Institute of Technology on a CEC 21-110B mass spectrometer using a heated inlet system and an IBM 1800 computer system. Ionization was carried out at 70 eV and a source temperature of 200 °C.

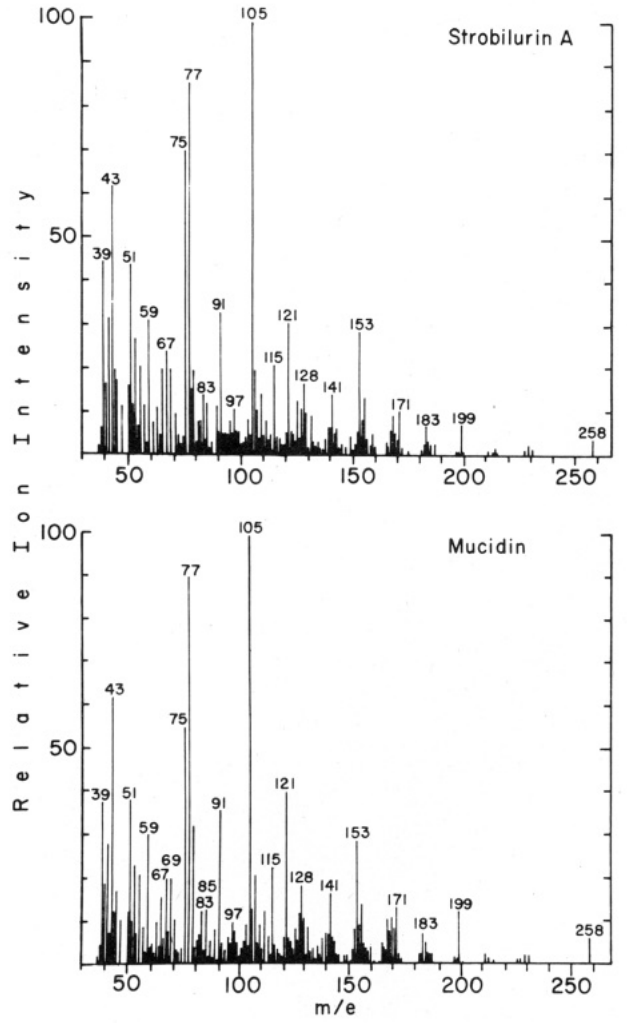

FIGURE 1: Mass spectra of mucidin and strobilurin A. Spectra were obtained as described under Experimental Procedures.

High-Pressure Liquid Chromatography. Analytical HPLC was performed on a Beckman Model 332 unit with a Hitachi 155-40 variable-wavelength detector and a Model C-RIA Shimadzu Integrator recorder (North & Mitchell, 1981). Approximately 7 nmol of mucidin or strobilurin A was applied to a 4.5 mm × 25 cm Altex Ultrasil ODS (10-μL) column from 1 μL of ethanol. The column was eluted with a linear gradient of 50–100% acetonitrile in methanol.

Optical Spectroscopy. Ultraviolet and visible absorption spectra of mucidin and strobiluirin A were recorded on a Cary 118C spectrometer. Reduction and oxidation of the cytochromes were measured on an Aminco DW 2a spectrophotometer interfaced to a digital storage oscilloscope (Von Jagow et al., 1984). As in previous experiments (Bowyer & Trumpower, 1981), the oxidation–reduction status of the cytochromes was checked before and after each reaction. Concentrations of reductase complex and details of the reaction mixtures are described in the figure legends.

RESULTS

Mass Spectra. Strobilurin A and mucidin have essentially identical fragmentation patterns in the mass spectrometer, as shown in Figure 1. Both compounds give rise to a molecular ion at m/e 258, consistent with a molecular formula $C_{16}H_{18}O_3$, and a base peak at m/e = 105. The spectra are identical in the occurrence and relative intensities of all ions larger than m/e = 115 and, likewise, of all ions of greater than 20% abundance. The only difference between the two spectra is the relative abundance of several low molecular weight ions,

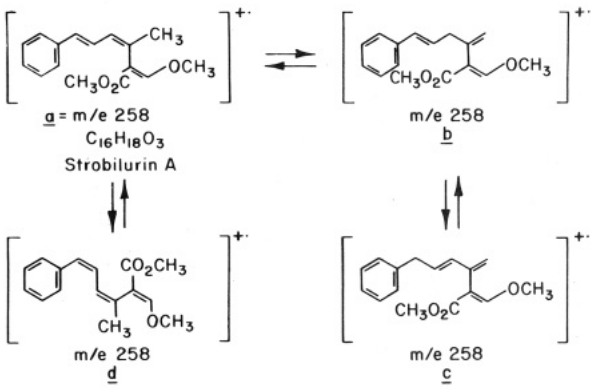

FIGURE 2: Structures of strobilurin A and three parent ion isomers that arise by double bond isomerization and migration in the mass spectrometer.

for example at m/e = 107, 85, and 69. These are present in minor amounts and are attributable to a small amount of low molecular weight impurity in the mucidin, as discussed below.

The structure of strobilurin A was originally deduced by spectroscopic methods (Schramm et al., 1978), and the stereochemistry at the 9,10 double bond in the side chain was established by chemical synthesis (Anke et al., 1984). This structure accounts for the ions observed in the mass spectrometer, the structures of some of which have been assigned by analogy to previously deduced fragmentation patterns (Schramm et al., 1978) and others whose structures are proposed herein.

The mass spectrum of strobilurin A is complicated by the fact that both double bond isomerization and migration can occur (Lauwers et al., 1973; McLafferty, 1980) in the mass spectrometer, leading to parent ions a–d (Figure 2), each of which can fragment or rearrange differently. These fragmentations are discussed, and the structures of the resulting ions are shown, in the supplementary material (see paragraph at end of paper regarding supplementary material).

High-Pressure Liquid Chromatography. When strobilurin A and mucidin were analyzed by high-pressure liquid chromatography, their elution times (4.95 and 4.89 min, respectively) were within experimental error of each other. When mixed, the two compounds eluted as a single symmetrical peak (4.88 min). The elution profile of mucidin showed a trace amount of impurity that eluted ahead (0.63 min) of mucidin. This impurity amounted to 7–8% of the UV-detectable material eluted from the column.

Optical Spectra. Strobilurin A and mucidin also have essentially identical UV absorption spectra, with an absorption maximum at 295 nm, a prominent shoulder at 300 nm, and additional peaks at 238 and 231 nm. The spectra are in good agreement with that reported for strobilurin A (Schramm et al., 1978). The only difference between the spectra of the two compounds was a higher base line of nondescript absorbance in the spectrum of mucidin, attributable to the impurity referred to above.

Effects of Strobilurin A and Mucidin on Reduction of Cytochromes b and c_1 by Succinate. The trace in Figure 3a shows reduction of cytochrome *b*, which results when succinate is added to isolated succinate-cytochrome *c* reductase. Under these conditions, reduction of cytochrome *b* is not inhibited by mucidin (Figure 3b) or strobilurin A (Figure 3c).

In contrast, reduction of cytochrome c_1 is inhibited by mucidin (Figure 3e) and by strobilurin A (Figure 3f), and their effects are quantitatively comparable. These antibiotics are thus similar to myxothiazol, which also blocks reduction of c_1 but not of *b* (Meinhardt & Crofts, 1982; De Vries et al.,

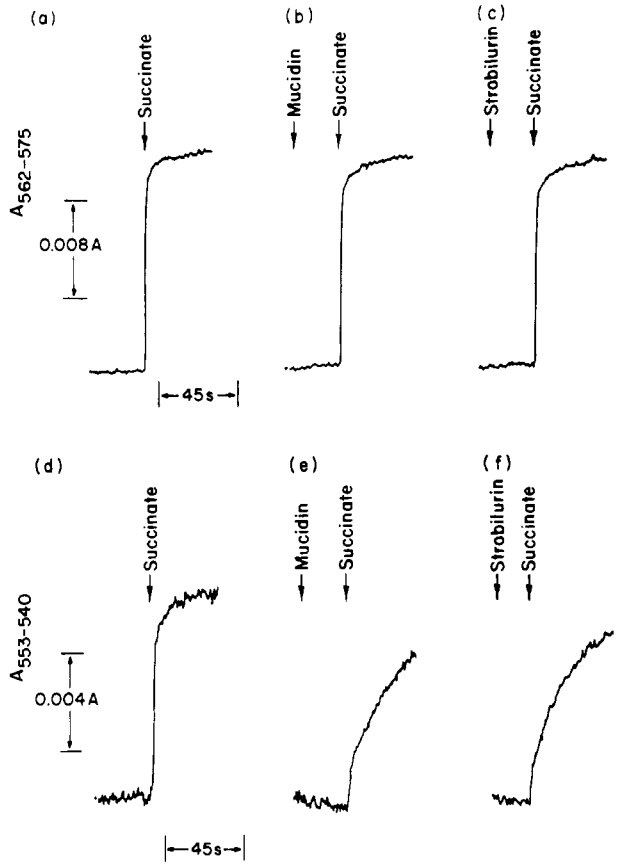

FIGURE 3: Effects of mucidin and strobilurin A on reduction of cytochromes b and c_1 by succinate. Traces a–c show reduction of cytochrome b, and traces d–f show reduction of cytochrome c_1. Reductase complex was suspended at 0.6 μM cytochrome c_1 in 100 mM sodium phosphate and 0.5 mM EDTA, pH 7.5, containing 0.5% sodium cholate. The oxidation–reduction status of the cytochromes was checked before and after each reaction by scanning the spectrum. Where indicated, 5 mM succinate, 40 μM mucidin, and 40 μM strobilurin A were added.

1983; Von Jagow et al., 1984). This inhibition of c_1 reduction is quantitatively different than that by myxothiazol, in that 2 μM myxothiazol completely blocks this reaction (see Figure 1; Von Jagow et al., 1984), while a slow reduction of c_1 proceeds even at 40 μM mucidin or strobilurin A (Figure 3). It should be noted, however, that this slow rate represents a more extensive inhibition than is apparent, since c_1 reduction in the absence of inhibitors (Figure 3d) is complete in 100–200 ms (Erecinska & Wilson 1972), and thus, the true uninhibited rate is masked by the 1-s mixing time in the spectrophotometer.

Mucidin and strobilurin A are also like myxothiazol in that they inhibit reduction of cytochrome b when added in combination with antimycin (Figure 4). In this instance, also the inhibition by mucidin (Figure 4b) and strobilurin A (Figure 4c) is quantitatively similar, and both are less potent than myxothiazol, which completely blocks b reduction in the presence of antimycin (Von Jagow & Engel, 1981; Von Jagow et al., 1984).

The two pathways of b reduction differ in that the myxothiazol-sensitive pathway is linked to reduction of cytochrome c_1 (Meinhardt & Crofts, 1982; De Vries et al., 1983; Von Jagow et al., 1984). In the absence of myxothiazol, this pathway can also be blocked by prereduction of c_1, and under these conditions the alternative pathway of b reduction can be blocked by antimycin (Trumpower & Katki, 1975). The traces in Figure 5 show that mucidin and strobilurin A do not block the antimycin-sensitive pathway after prereduction of cytochrome c_1 but do block the myxothiazol-sensitive pathway,

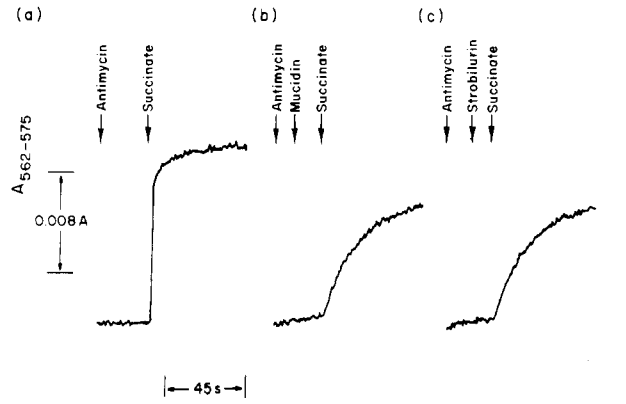

FIGURE 4: Effects of mucidin and strobilurin A on reduction of cytochrome b by succinate in the presence of antimycin. Reductase complex was suspended as in Figure 3. Where indicated, 5 mM succinate, 2 μM antimycin, 40 μM mucidin, and 40 μM strobilurin A were added.

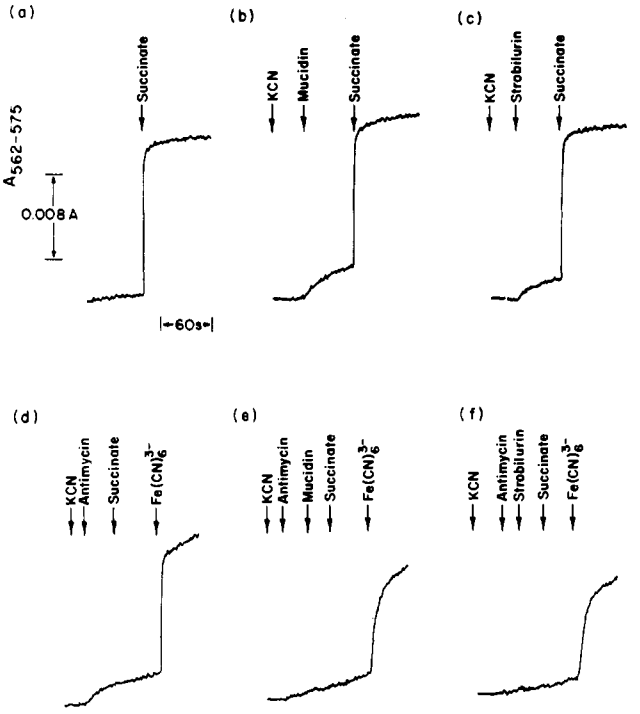

FIGURE 5: Effects of mucidin and strobilurin A on reduction of cytochrome b by succinate after prereduction of cytochrome c_1. Reductase complex was suspended as in Figure 3. Where indicated, 1 mM KCN, 3 mM succinate, 10 μM ferricyanide, 2 μM antimycin, 40 μM mucidin, and 40 μM strobilurin A were added.

which is linked to the redox status of cytochrome c_1.

The preparations of reductase complex used for these experiments contained small amounts of endogenous reducing equivalents, possibly ubiquinol. If KCN was added to block slow reoxidation of c_1, presumably by trace amounts of cytochrome oxidase, it was thus possible to prereduce the c_1 without adding ascorbate. The trace in Figure 5a is a control, showing reduction of cytochrome b by succinate when c_1 is initially oxidized. The traces in Figures 5b,c show that mucidin or strobilurin A does not block reduction of cytochrome b by succinate if c_1 is previously reduced. The trace in Figure 5d is a second control, showing that antimycin does block reduction of b by succinate if c_1 is prereduced.

All three of the inhibitors cause a slow autoreduction of a small amount of the cytochrome b. Myxothiazol has this same effect [Figure 4c in Von Jagow et al. (1984)]. The cause of this autoreduction is not known but may be related to a con-

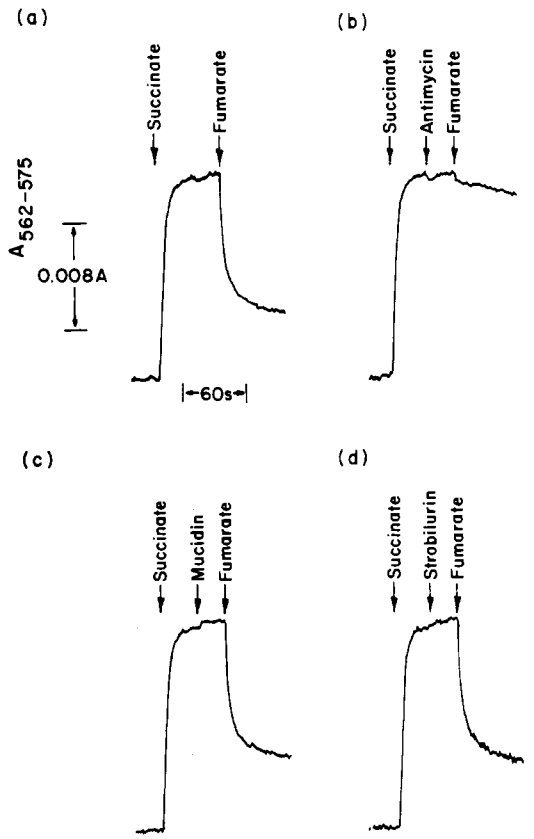

FIGURE 6: Effects of mucidin and strobilurin A on oxidation of cytochrome *b* by fumarate. The cytochromes were reduced by 20 μM succinate and reoxidized by 20 mM fumarate. Inhibitors were added at the same concentrations as in Figure 3.

formational change within the bc_1 complex (Rieske, 1976).

When reduction of cytochrome *b* by succinate is blocked by antimycin after prereduction of c_1, subsequent oxidation of c_1 opens the antimycin-insensitive pathway and thus ellicits "oxidant-induced reduction" of cytochrome *b*. This oxidant-induced reduction is blocked by myxothiazol (Von Jagow et al., 1984). The traces in Figure 5e,f show that mucidin and strobilurin A inhibit oxidant-induced reduction of cytochrome *b*, although again they are less effective than myxothiazol (Von Jagow et al., 1984).

Effects of Strobilurin A and Mucidin on Oxidation of Cytochrome b by Fumarate. When cytochrome *b* is reduced by succinate, it can be reoxidized by fumarate (Figure 6a). The range of potentials spanned by the succinate/fumarate couple is such that approximately 60% of the succinate-reducible *b* can be reoxidized by fumarate. Although there are two pathways for reduction of cytochrome *b*, there is only one pathway for its reoxidation by fumarate, and this is inhibited by antimycin (Von Jagow et al., 1984). The lack of a myxothiazol-sensitive pathway of *b* oxidation is due to the fact that the succinate-reducible *b* is primarily *b*-562 (Von Jagow et al., 1984), the potential of which is not sufficiently low to drive reoxidation of the high-potential c_1 and iron–sulfur protein, which is mechanistically coupled to reoxidation of cytochrome *b* through the myxothiazol-sensitive route.

In contrast to the effect of antimycin (Figure 6b), neither mucidin (Figure 6c) nor strobilurin A (Figure 6d) inhibits oxidation of cytochrome *b* by fumarate. Thus, in this regard also, these two antibiotics are like myxothiazol.

Discussion

The primary purpose of our experiments has been to establish the site(s) of action of mucidin and strobilurin A in the bc_1 complex of the mitochondrial respiratory chain. Following initial reports that these antibiotics are different compounds (Musilek, 1970; Anke et al., 1979), it was recently stated in a paper that they are identical (Sedmera et al., 1981). This tentative identity was not documented, and there are several discrepancies in the reported mass spectrum of mucidin. Although it was stated that mucidin gives rise to an m/e = 77 ion, representing the phenyl group (Nerud et al., 1982), this ion is not present in the published spectrum (Vondracek et al., 1983). In addition, the mass spectra of mucidin (Vondracek et al., 1983) and strobilurin A (Schramm et al., 1978) obtained in different laboratories are markedly different. For these reasons, we also compared selected properties of these two antibiotics and have provided a detailed analysis of their mass spectra.

Our results show that, when obtained under comparable conditions, the mass spectra of mucidin and strobilurin A are essentially identical. By analogy to previously deduced fragmentation patterns, all of the major ions have been identified. These are consistent with the fragmentation pattern expected from strobilurin A, the structure of which has now been established by chemical synthesis (Anke et al., 1984).

The only difference between the mass spectra of the two antibiotics is the presence of several low molecular weight ions in the spectrum of mucidin. These are attributable to a small amount of impurity, which also contributes nondescript absorbance to the UV spectrum. This impurity was separable from mucidin by high-pressure liquid chromatography, but due to the limited amounts of sample available, it was not possible to use this as a preparative procedure.

Mucidin and strobilurin A have identical inhibitory effects on electron-transfer reactions in the bc_1 complex. When added at concentrations estimated to be identical on the basis of their UV absorbance, the two antibiotics inhibit with equal efficiency. These observations further confirm their identity, and the latter also indicates that the low molecular weight impurity in the mucidin does not contribute to its inhibitory properties.

It seems clear that mucidin is identical with strobilurin A. We suggest that hereafter the name strobilurin A be used for this antibiotic, since the structure of the antibiotic by this name was established by chemical synthesis (Anke et al., 1984), and the two structurally related antibiotics that have been isolated and characterized are named strobilurins B and C (Anke et al., 1983).

Strobilurin A appears to inhibit the same pathway of cytochrome *b* reduction as myxothiazol. It inhibits reduction of cytochrome c_1, and when added in combination with antimycin, it inhibits reduction of both cytochromes *b* and c_1. Strobilurin A is also like myxothiazol (Von Jagow et al., 1984) but different from antimycin in that it inhibits oxidant-induced reduction of cytochrome *b* and does not inhibit oxidation of cytochrome *b* by fumarate. These results are consistent with the finding that strobilurin A and myxothiazol similarly inhibit production of superoxide anion in the bc_1 complex, a reaction that is enhanced by antimycin and funiculosin (Ksenzenko et al., 1983).

It is thus likely that strobilurin A binds to the same site as myxothiazol. These two antibiotics have a β-methoxyacrylate system in common (Becker et al., 1981; Schramm et al., 1982), and both alter the optical spectrum of cytochrome *b*-566 (Becker et al., 1981). Genetic studies also indicate that strobilurin A and myxothiazol bind at a common site and that this site is distinct from the antimycin binding site. Mouse cells resistant to myxothiazol showed some cross-resistance to strobilurin A ("mucidin") but not to antimycin, funiculosin,

or acetylcolletotrichin (Howell et al., 1983). In the same studies, several mutants selected with antimycin were resistant to funiculosin and acetylcolletotrichin but not to myxothiazol or strobilurin A.

There are also numerous yeast mutants that are resistant to antimycin or myxothiazol. However, none of these are cross-resistant to the alternative inhibitor (Briquet & Goffeau, 1981; Thierbach & Michaelis, 1982). Likewise, yeast mutants resistant to strobilurin A (mucidin) are not resistant to antimycin (Subik, 1975; Subik et al., 1977).

Acknowledgments

We thank Monica Tragler for discussions helpful in interpreting the mass spectra and Dr. Tim Anke for providing his manuscript prior to publication. We also thank Drs. Catherine E. Costello and Thomas Dorsey, Masschusetts Institute of Technology, for the high-resolution mass spectra, supported by NIH Grant FR 00317 from the Division of Research Facilities and Resources.

Supplementary Material Available

A description of the fragmentation of strobilurin A in the mass spectrometer and seven figures showing structures of the fragment ions (5 pages). Ordering information is given on any current masthead page.

Registry No. Succinate-cytochrome *c* reductase, 9028-10-8; mucidin, 52110-55-1; myxothiazol, 76706-55-3; antimycin, 11118-72-2.

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