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COMPLEX I AND COMPLEX III OF MITOCHONDRIA HAVE COMMON INHIBITORS ACTING AS UBIQUINONE ANTAGONISTS

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Summary. Mitochondrial complex I and complex III have common inhibitors with ubiquinone-like structure. The tridecyl analog of stigmatellin, which inhibits mitochondrial complex III at nanomolar concentrations, also inhibits the NADH:ubiquinone reductase activity of complex I at micromolar concentrations. The inhibitor titer depends upon the concentration of the mitochondrial particles and extrapolates to 0.2 μM at zero particle concentration. The stigmatellin analog is more powerful than its parent compound and is noncompetitive with exogenous ubiquinones, rotenone and piericidin.

Myxothiazol, which is another potent inhibitor of complex III, is also found to inhibit the activity of complex I with a titer comparable to that of the tridecyl analog of stigmatellin. Additionally, piericidin, which is the most powerful inhibitor of complex I, inhibits the ubiquinol:cytochrome *c* reductase activity of complex III at micromolar concentrations in mitochondrial particles and at submicromolar concentrations in the isolated enzyme complex.

Many different types of membrane enzymes catalyze the reduction of ubiquinone (Q) or the oxidation of ubiquinol (QH_2). Complex I, NADH:Q oxidoreductase type 1 (1-4), is the principal ubiquinone reductase in animal mitochondria, where ubiquinol is exclusively oxidized by complex III, QH_2:cytochrome *c* oxidoreductase or bc_1 complex (5,6). There are a large variety of compounds, often having quinone-like structure, that inhibit the bc_1 complex (5-8). Some of these compounds also inhibit the Q reductase reaction at the Q_B site in the photosynthetic reaction center of purple bacteria and plants (9-12). The most powerful of these common inhibitors is probably stigmatellin (6,10-13).

The presence of common inhibitors is suggestive of similar protein structures (10,11,14-16) and this has been exploited for modeling the quinone and inhibitors' binding sites in cytochrome *b* of the bc_1 complex

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with the atomic structure of the bacterial reaction center (9) as a template (15,16). The same rationale is extensively applied for modeling the herbicide binding sites in plant photosystems (10,11). A similar approach has been used for identifying the Q-binding regions in the bacterial glucose:Q oxidoreductase and the ND1 subunit of complex I (17). In this line, it would be desirable to discover other quinone-like compounds that are powerful inhibitors of multiple Q-reacting enzymes including the bacterial reaction center (14).

Here we report that stigmatellin and myxothiazol, originally characterized as potent inhibitors of complex III (6,13,18), also inhibit the NADH:Q reductase activity of complex I in animal mitochondria. Additionally piericidin, which is the most powerful inhibitor of complex I (1-4,19,20), has also been found to inhibit the QH_2:cytochrome *c* reductase activity of complex III.

The present results suggest that the hydrophobic subunits of complex I and III may have similar structural features which are responsible for the binding of common Q-like inhibitors.

METHODS

Beef and sheep heart mitochondria and submitochondrial particles were prepared as described (21,22). Isolated bc_1 complex from beef heart was prepared as previously reported (23).

NADH:Q reductase and NADH:ferricyanide reductase activities of frozen-thawed mitochondrial particles were assayed essentially as described by Yagi (24). Some assays of NADH:Q reductase activity were performed also as described in (25). The mitochondrial preparations were diluted to about 2 μM cytochrome *b*, determined as in (23), incubated for several minutes with 100-300 μM NADH, and treated with 2-3 mol antimycin and 4-5 mol myxothiazol per mol cytochrome *b* to block completely the reactions between cytochrome *b* and exogenous ubiquinones (26). Ubiquinol-2:cytochrome *c* reductase activity was measured as previously described (23,26). Protein concentration was measured with the biuret method.

Inhibitors were purchased commercially when available (e.g. stigmatellin from Fluka), whereas ubiquinones were generously given by Eisai Company, Tokyo, Japan. Funiculosin was a kind gift from Sandoz, Basel, Switzerland, and the tridecyl analog of stigmatellin (cf. (13)) was provided by ICI Agrochemicals, Bracknell, UK. 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) and mucidin were provided by Prof. B.L. Trumpower, Dartmouth College, NH, USA, and Dr. J. Subik, University of Bratislava, Slovakia, respectively. Piericidin was kindly donated by Dr. T. Friedrich, University of Düsseldorf, Germany. Inhibitors' concentration was determined as described (8,20).

RESULTS

We have evaluated in detail the effects of the inhibitors of the bc_1 complex on the rate of NADH oxidation by exogenous quinones. The

concentrations of the inhibitors were initially kept slightly overstoichiometric with respect to the content of cytochrome *b* in the mitochondrial particles to block completely the redox interaction of the *b* cytochromes with exogenous quinones (26). Antimycin or other center **i** inhibitors of the bc_1 complex (6) (e.g. hydroxy-quinoline-N-oxide, HQNO) affect only marginally the NADH: Q reductase - antimycin is indeed employed routinely in this assay of complex I (20,24). Myxothiazol, stigmatellin and other center **o** inhibitors of the bc_1 complex (6,8) also have little effect on the NADH:Q reductase activity, although they generally reduce this activity when added together with antimycin, cf. (27). This effect of center **o** inhibitors at nM concentrations is small with ubiquinone-1 (10-20% of the control rate), but large with duroquinone (over 60% of the control rate, results not shown).

Hence, the NADH:Q reductase assay suffers from interferences of the reactions occurring at either center **i** or center **o** in the bc_1 complex, because of the interaction of exogenous quinones with reduced cytochrome *b* at either center (26). However, by increasing the concentration of some center **o** inhibitors well above the levels that completely block the bc_1 complex and any quinone-cytochrome *b* interaction, the rate of NADH:ubiquinone-1 reductase activity is further decreased and almost completely inhibited at around 3 μM (Fig 1 and Table I). Antimycin and most other inhibitors of complex III do not significantly alter the NADH:Q reductase activity at the same micromolar concentrations (Table I).

Interestingly, the tridecyl analog of stigmatellin (13) is 5-fold more powerful than its parent compound in inhibiting the NADH:ubiquinone-1 reductase activity of complex I in beef mitochondrial particles, whereas mucidin, which has the same β-methoxy-acrylate group as myxothiazol (8), is much less powerful than myxothiazol (Table I). Therefore, the chemical nature of the hydrophobic tail of these compounds plays a crucial role in determining the inhibitory properties on the NADH:Q reductase.

Fig. 2A shows the titration with the tridecyl analog of stigmatellin of both the NADH:ubiquinone-1 and the ubiquinol-2:cytochrome *c* reductase in beef submitochondrial particles. The I_{50} for the former activity is 2.5 μM and for the latter activity is 0.035 μM. In both assays, the I_{50} depends upon the concentration of the mitochondrial particles. A value of 0.2 μM is extrapolated for the NADH:Q reductase at zero particle concentrations. The inhibition by the analog of stigmatellin is non-competitive with respect to exogenous ubiquinones and additive to the effect of either rotenone or piericidin.

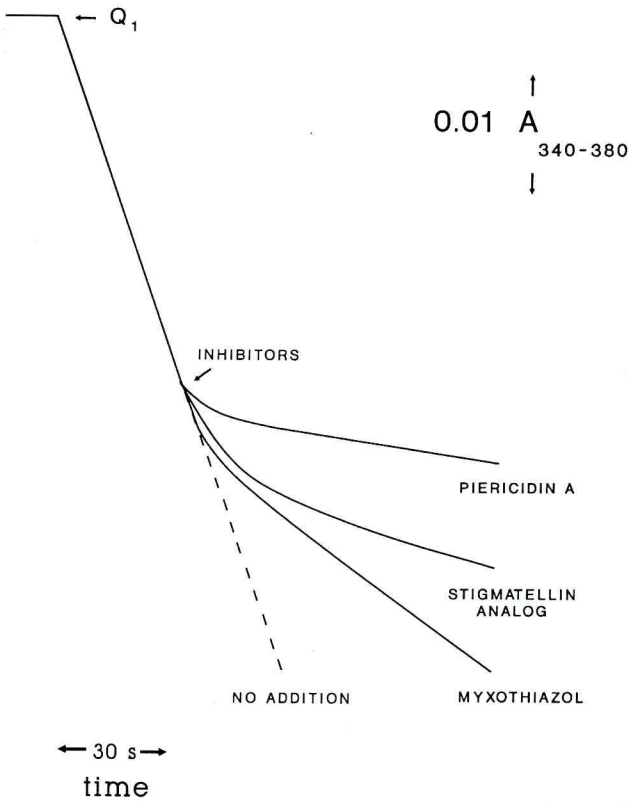

Fig. 1. Effect of quinone-like compounds on the NADH:Q reductase. Experimental conditions were those described in (25) with 30 μM ubiquinone-1 and 0.035 mg/ml of beef heart submitochondrial particles. The particles were pretreated with antimycin and 4 mol of myxothiazol per mol of cytochrome *b*. The final concentration of myxothiazol and the analog of stigmatellin after addition into the assay cuvette was 1.5 μM, whereas that of piericidin was 0.6 μM. The non-inhibited rate was 0.24 μmol/min per mg of protein.

Nevertheless, the rotenone- or piericidin-insensitive NADH:Q reductase is not affected by the stigmatellin analog and myxothiazol. Moreover, these inhibitors do not alter the rate of NADH:ferricyanide reductase in mitochondrial particles.

Having discovered that powerful inhibitors of the bc_1 complex also inhibit the activity of complex I, we investigated whether a powerful inhibitor of complex I would inhibit the activity of complex III. Piericidin is indeed found to inhibit the ubiquinol:cytochrome *c* reductase at μM concentrations in mitochondrial particles (Fig. 2A). The I_{50} of piericidin is about three-orders of magnitude higher for the activity of complex III than for the activity of complex I (Fig. 2B, cf. (19)). However, the titer of piericidin on the activity of isolated bc_1 complex is much lower than in mitochondrial particles, since a I_{50} of

Table I

Effect of bc_1 inhibitors on NADH:Q reductase of beef submitochondrial particles

Inhibitor	concentration (μM)	% inhibition
HQNO	3.1	0
funiculosin	2.9	0
antimycin	3.0	9.4
diuron	3.0	0
UHDBT	2.8	8.1
mucidin	3.3	15.5
stigmatellin	3.2	39.0
stigmatellin tridecyl analog	3.1	86.6
myxothiazol	3.0	84.0

Activity with 54 μM ubiquinone-1, 150 μM NADH and 9 nM in cytochrome *b* of the particles. Inhibitors were added directly in the assay without incubation. The particles were pretreated with 5.4 μM antimycin, 9 μM myxothiazol and 300 μM NADH at 2 μM cytochrome *b*. The rate in the presence of 1 μM piericidin was subtracted. Note that except for diuron, all the other compounds inhibit completely the activity of complex III at the same concentration.

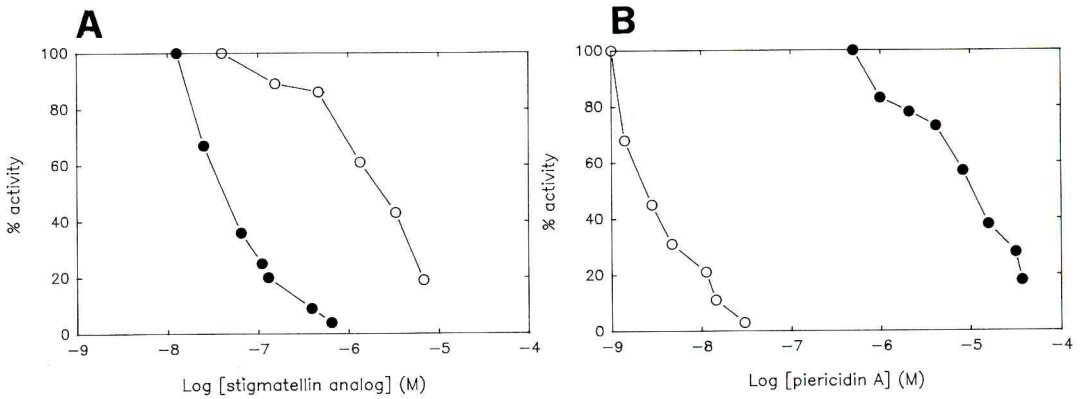

Fig. 2. Inhibition of the activity of complex I and of complex III with the tridecyl analog of stigmatellin (A) and piericidin (B). Experimental conditions are those described by Yagi (24) with 130 μM NADH and 48 μM ubiquinone-1 for the activity of complex I (o——o), and with 9.5 μM ubiquinol-2 and 22 μM cytochrome *c* for the activity of complex III (●——●) (23). Beef heart submitochondrial particles (0.063 mg/ml) were treated with 3 mol antimycin and 4 mol myxothiazol per mol of cytochrome *b* and activated by NADH (20) in the assay of complex I. The inhibitors were incubated for at least 5 minutes with submitochondrial particles in the titration of the two activities. The non-inhibited rate was 0.2 μmol/min per mg of protein for the NADH:Q reductase and 3.3 μmol/min per mg of protein for the ubiquinol-2:cytochrome *c* reductase. The non physiological rates of piericidin insensitive Q reduction (about 9% of the overall rate) and antimycin plus myxothiazol-insensitive cytochrome *c* reduction (less than 2% of the overall rate) were subtracted.

0.3 μM was measured under the same conditions as those in Fig. 2 (results not shown).

DISCUSSION

Stigmatellin, particularly its tridecyl analog, and myxothiazol inhibit both complex I and complex III. This may have interesting implications for the identification of the binding sites of ubiquinone in complex I, especially because stigmatellin acts as a ubiquinone antagonist also in photosynthetic reaction centers (10-12). Stigmatellin related compounds, such as 4-alkyl acridones (11), inhibit both complex I and photosynthetic reaction centers with I_{50} values comparable to that of the tridecyl analog of stigmatellin (28). Such compounds, however, are poor inhibitors of complex III (28).

That myxothiazol inhibits the NADH:Q reductase activity is not completely new, but it was previously interpreted to reflect an interaction between complex I and center **o** of complex III (27). This interpretation should be reconsidered in the light of the present results. Moreover, in the original work reporting the inhibitory effect of myxothiazol on NADH respiration of beef mitochondrial particles it was found that the I_{50} was identical to that of antimycin (18), whereas it has been clarified subsequently that myxothiazol is a less powerful inhibitor than antimycin on complex III alone (8,26). A similar, but quantitatively larger, difference in inhibitory potency of the tridecyl analog of stigmatellin is seen between its effect on NADH respiration and on the activity of complex III, cf. (13) and (29). Hence, it may be concluded that both myxothiazol and the stigmatellin analog inhibit complex I when assayed in the integrated NADH oxidase activity as in (13,18).

Studies are in progress for characterizing the action of stigmatellin and myxothiazol on purified complex I. A preliminary characterization of the effects of piericidin on the isolated complex III indicates that it may act like a center **o** inhibitor, since it reduces the rate of cytochrome *b* reduction by succinate in the presence of antimycin, in accordance with early reports (19).

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