37. Bennoun, P., Delasme, M. and Kück, U. (1991) Genet. **127**, 335–343
38. Geier, B. M., Schägger, H., Brandst, U. and Von Jagow, G. (1992) Eur. J. Biochem. **208**, 375–380
39. Brandt, U. and Von Jagow, G. (1991) Eur. J. Biochem. **195**, 163–170
40. Engel, W. D., Michalski, C. and Von Jagow, G. (1983) Eur. J. Biochem. **132**, 395–402
41. Trumpower, B. L. and Edwards, C. A. (1979) J. Biol. Chem. **254**, 8697–8706
42. Brandt, U., Haase, U., Schägger, H. and Von Jagow, G. (1991) J. Biol. Chem. **266**, 19958–19964

Received 27 July 1993

Natural variation in the potency and binding sites of mitochondrial quinone-like inhibitors

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Introduction

In mitochondria, ubiquinone (Q) functions as a lipophilic substrate of various transmembrane redox complexes, among which the most important are NADH:Q reductase (Complex I) and ubiquinol:cytochrome-*c* reductase (Complex III or bc_1 complex) [1–3]. In both complexes the hydrophobic subunits that are synthesized in mitochondria form the Q-reacting sites: cytochrome *b* for complex III and some NADH dehydrogenase (ND) subunits of complex I [1–5]. The transmembrane proteins interacting with Q also form the binding site for a variety of lipophilic compounds that inhibit the function of the complexes by interfering with the normal reactivity of Q. Often such compounds have a quinone-like structure [1, 2, 4–7].

Complex I and III are of crucial importance for the energy of the cell and this is why their mitochondrially encoded subunits interacting with Q are so conserved [2–4]. Despite this conservation, large differences in the affinity for quinone-like inhibitors are present in different species owing to variations in the protein sequences [4, 8]. The inhibitors are foreign compounds that bind specifically to some parts of the Q-reacting proteins so as to obstruct either the entrance of the substrate, or the exit of the product, in the Q-reacting pocket. The action of the inhibitors, therefore, is not necessarily competitive with the binding of Q or ubiquinol [4, 7, 8].

Abbreviations used: Q, ubiquinone; ND, NADH dehydrogenase; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole; MOA, methoxyacrylate; DBMIB, dibromothymoquinone; TDS, tridecyl analogue of stigmatellin.

Some organisms have exploited the natural variation in the Q-binding proteins for obtaining environmental advantages, e.g. by secreting substances that selectively inhibit the mitochondrial function of other organisms. A good example is given by the tropical plants of the *Derris* genus, which secrete rotenone and thereby kill damaging insects. While rotenone is a powerful inhibitor of insect Complex I [8], it is a very weak inhibitor of plant complex I [9]. The design of agrochemicals targeted to mitochondrial Complex I or Complex III could also exploit the natural variation in the Q-binding proteins for achieving selectivity and low toxicity towards plants and mammals.

Our work in Bologna has been focused on the natural variations in the potency of inhibitors of complex III for deducing structure–function correlations in cytochrome *b* [4, 10–13]. The mutual relationship and relative potency of quinone-like inhibitors has also been studied following the observation that some of these compounds inhibit both Complex III and Complex I [14]. The information obtained from our studies defines a more detailed picture of the interaction of these inhibitors with the mitochondrial complexes.

Experimental procedures

The potency of the various inhibitors was measured by titrating the activities of NADH:Q reductase [14] and ubiquinol:cytochrome-*c* reductase [4, 10, 12] both of mitochondrial particles from different species [4] and of Complex I and III isolated from beef heart. The mutual exclusivity of the inhibitors was determined from the additivity of their inhibition of steady-state activity by using the theoretical analysis of Chou and Talalay [15]. The relative

potency of inhibitors was always normalized to the content of the bc_1 complex as described [4, 12].

Results and discussion

Inhibitors of Complex III

Of all the compounds inhibiting mitochondrial Complex III, antimycin is still the most specific and its titre is rarely overstoichiometric with respect to the content of the complex [4, 6]. Natural resistance to antimycin is restricted to some cyliate protozoans, especially *Tetrahymena* [12], and to parasitic worms [16]. Antimycin interaction at the Q_i centre of the cytochrome *b* structure overlaps the interaction of 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) and partially overlaps that of funiculosin (Figure 1) [4]. The latter inhibitor also affects the Q_o centre and its interaction overlaps that of hydroxylquinones such as 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) [4, 13, 17] (Figure 1).

The potency of funiculosin is much more variable than that of other Q_i centre inhibitors. For instance, horse and rabbit (both animals of economic interest) are strongly insensitive to funiculosin compared with most mammals, both *in vitro* [4] and *in vivo* [18]. Thus, funiculosin might be a reference compound for developing agents against fungal or parasitic infections of these animals.

Hydroxyquinolines like HQNO are relatively weak inhibitors of Complex III, compared with both antimycin and funiculosin in animal and yeast mitochondria [4], but strong inhibitors in plant mitochondria [4, 11]. This hypersensitivity in plants should be considered whenever compounds related to quinolines are investigated as potential agrochemicals.

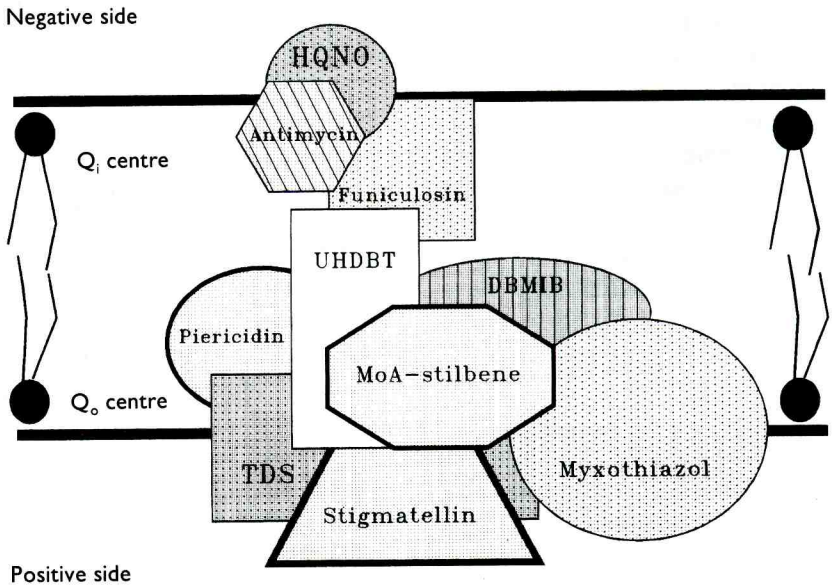
The changes in potency of funiculosin and HQNO in various species can be correlated to a few amino acid substitutions in the cytochrome *b* protein that lie near or at the negative side of the membrane [4, 13]. The most straightforward of such correlations is the increase in the volume of the side-chain at position 194 and the decrease in funiculosin affinity, e.g. mutation of Ala to Val at position 194 (A194V) in horse [4].

Strong natural resistance of Complex III towards Q_o centre inhibitors has been found in sea urchins and protozoans [10, 12] and is clearly correlated to one or several amino acid substitutions in the highly conserved region of cytochrome *b* that connects transmembrane helix C with transmembrane helix D. Indeed, these substitutions

Figure 1

Interaction sites of Complex III inhibitors

When inhibitors are mutually exclusive, as deduced by the steady-state assay according to Chou and Talalay [15], their geometrical symbols are shown to overlap each other. When inhibitors are mutually non-exclusive, their symbols are shown to be separate from each other. Note that MOA-stilbene and TDS (the tridecyl analog of stigmatellin [25, 27]) are mutually exclusive with all the other Q_o centre inhibitors. The interaction site of mucidin is almost superimposable to that of MOA-stilbene, but closer to that of myxothiazol because it is not competitive with piericidin.



match those seen in resistant mutants [4, 10, 12, 19–20].

Mitochondria from different plants and vegetal tissues have variable titres of myxothiazol, strobilurins and stigmatellin which often exceed those seen in either animal or yeast mitochondria by one order of magnitude [4, 12]. However, this decreased potency is only apparent because the titres of the same inhibitors in Complex III isolated from plant mitochondria are similar to those in Complex III from animal mitochondria (M. Degli Esposti, unpublished work; see also [11, 21]). The apparent weakness of Q_o-centre inhibitors in plants cannot be correlated to natural variations in their cytochrome *b* sequences, which are also identical to each other for the majority of the extramembranous loops forming the Q_o centre [4, 12]. We are now investigating how the different lipid and/or protein composition of plant mitochondria affects the action of Q_o-centre inhibitors. Such studies could be useful for evaluating phytotoxic effects of strobilurin compounds used as fungicides in different cultivated plants.

Modelling of cytochrome *b* has been attempted in different ways to help understand the structure of this protein and its functional sites. The availability of sequence information from over 900 species has been of great value in these deductions [4]. A consensus of opinion on the probable packing of the eight transmembrane helices has now been reached (see [4, 5]), but the folding and secondary structure of the extramembrane loops is still quite uncertain [4] – a refined crystal structure is needed. Unfortunately, Q_o-centre inhibitors usually bind to the extramembrane loops at the positive side of the membrane, as indicated by the mapping of the mutations conferring resistance to these compounds [4, 19, 20]. Therefore, the structural details of the binding sites of the inhibitor in cytochrome *b* are largely unknown.

To obtain additional information on the binding sites of the inhibitors, we have utilized the analysis of mutual exclusivity [15] in Complex III purified from beef heart [17]. This approach also gave information on the dynamic interaction of the inhibitors, since it is based on the additivity of their inhibition of the steady-state function of the enzyme [15]. Besides the classical Q_o-centre inhibitors mucidin (strobilurin), myxothiazol, UHDBT and stigmatellin [6], we have also studied dibromothymoquinone (DBMIB) [22], Methoxyacrylate (MOA)-stilbene [23] and piericidin [14]. Piericidin is probably the 'oldest' Q_o-centre inhibitor with a quinone-like structure as its double-kill effect with antimycin on the reduction of cytochrome *b* was first reported in 1968 [24]. This work has been subsequently neglected, mainly because piericidin is also the most powerful inhibitor of Complex I [1, 7, 24].

Some properties of piericidin action on isolated Complex III (K_i *ca.* 0.1 μM) are different from those of the majority of Q_o-centre inhibitors, since piericidin affects the rate of substrate reduction of cytochrome *b* more than that of cytochrome c_1 (M. Degli Eposti et al., unpublished work). It is thus possible that the interaction site of piericidin is partially different from that of other quinone-like compounds at the Q_o centre. This is confirmed by the pattern of mutual exclusivity with several inhibitors obtained by steady-state analysis (Figure 1). Piericidin is mutually exclusive (i.e. competing for the same interaction site) with MOA-stilbene, UHDBT and the tridecyl analogue of stigmatellin [25] (termed TDS), but it is non-exclusive with stigmatellin, DBMIB and myxothiazol (Figure 1). Stigmatellin is also non-exclusive with myxothiazol and DBMIB, which in turn is mutually exclusive with all other Q_o-centre inhibitors including myxothiazol (Figure 1).

Several results obtained by the exclusivity analysis on Complex III activity are consistent with independent results obtained from binding and spectroscopic studies [23, 25–27]. This indicates that the binding site of stigmatellin is not identical to that of myxothiazol. Evidence along this line also comes from the lack of cross-resistance to stigmatellin in some myxothiazol-resistant mutants [20, 27] and in naturally resistant organisms [4, 12]. The functional data of mutual exclusivity define details of the interaction sites of chemically different inhibitors of complex III and also distinguish the action of analogues having the same pharmacophore, such as stigmatellin and TDS. This approach, therefore, may be valuable for developing or implementing agrochemicals targeted to Complex III.

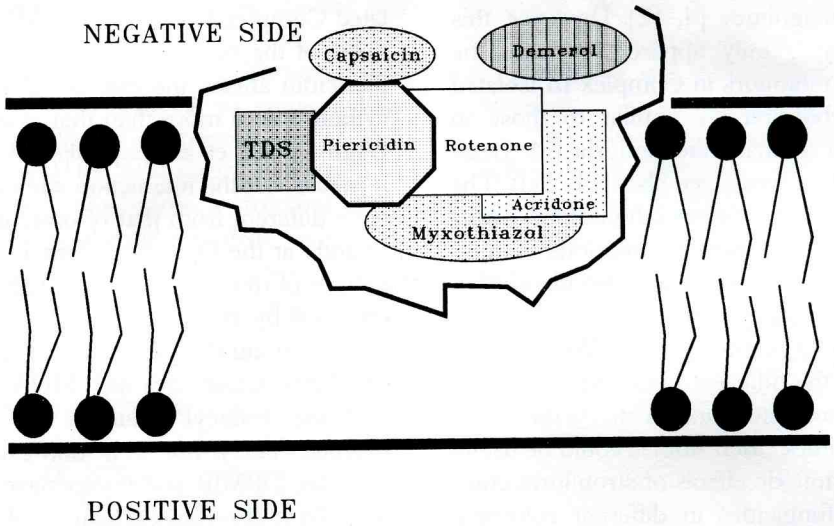
Inhibitors of Complex I

As already mentioned, Complex I has some quinone-like inhibitors in common with Complex III. Besides piericidin [14, 24], myxothiazol and TDS are Q_o-centre inhibitors of Complex III and also rotenone-like inhibitors of Complex I [14]. In isolated beef Complex I, TDS and myxothiazol have a K_i of approximately 0.1 μM and behave non-competitively with respect to exogenous quinones. However, their site of action is not identical, since TDS is more powerful in the isolated complex than in mitochondrial particles (similarly to pieri-

Figure 2

Interaction sites of complex I inhibitors

The mutual exclusivity or non-exclusivity of the inhibitors deduced from the steady-state assay according to Chou and Talalay [15] are represented as in Figure 1. Note that the site of Demerol (meperidine) is separate from that of all other inhibitors.



cidin), whereas myxothiazol is less powerful in the isolated complex than in mitochondrial particles (similarly to rotenone).

To evaluate the mutual interaction of these new inhibitors of Complex I with representative inhibitors acting at the Q junction of this complex, we have undertaken the same study of additivity on steady-state activity [15] as that described previously for Complex III inhibitors. The results clearly indicate the presence of multiple interaction, and presumably binding, sites for various Complex I inhibitors, as illustrated in Figure 2.

In particular, TDS, capsaicin and Demerol are mutually nonexclusive or nonoverlapping with the interaction site of all other inhibitors tested, including rotenone and piericidin. In the isolated complex supplemented with phospholipids, however, TDS and capsaicin tend to be partially competitive with piericidin. For this reason, their interaction site is shown to be adjacent to that of piericidin in Figure 2. That Demerol, the analgesic meperidine [8], interacts at a site different from that of other inhibitors is consistent with the fact that this compound is capable of inhibiting reactions, such as menadione reduction, that are rotenone-insensitive and probably located before the Q junction in the complex [8, 28]. Other compounds such as substituted thienylvinylindoles [29] seem to have an action similar to Demerol.

In conclusion, our results of mutual exclusivity of inhibitors (Figure 2) confirm emerging indications that the rotenone site of Complex I is more complicated than is usually considered – 'complex I by name, complex I by nature.' Several overlapping and non-overlapping interaction sites exist for the quinone-like inhibitors in mitochondrial NADH:Q reductase, presumably reflecting an intricate multisubunit structure [3, 7]. This may explain why such a large set of chemically different compounds is capable of inhibiting this reaction of Complex I. To obtain more structure–function information on the Q-reaction site of this important mitochondrial enzyme, we are now exploring natural variations in the affinity of several inhibitors in conjunction with structural deductions from the aligned sequences of ND subunits [4, 30]. Hopefully, these studies will yield information that is also useful for developing agrochemicals, such as insecticides targeted to Complex I.

Research was partially sponsored by MURST and Instituto Superiore Sanita', Rome, Italy. Piericidin was a generous gift from Dr T. Friedrich, University of Dusseldorf, Germany, and MOA-stilbene from Dr P. Rich, Glynn Research, Bodmin, U.K. TDS was provided by Dr A. M. Colson, University of Louvain-la-Nueve, Belgium (see [27]), acridone from D. W. Oettmeier, University of Bochum, Germany and mucidin from Dr J. Subik, University of Bratislava, Slovakia. Funiculosin was donated by Dr P. Bollinger, Sandoz Pharma, Basel, Switzerland.

1. Hatefi, Y. (1985) Annu. Rev. Biochem. **54**, 1015–1069
2. Weiss, H., Friedrich, T., Hofaus, G. and Preis, D. (1991) Eur. J. Biochem. **197**, 563–576
3. Walker, J. E. (1992) Q. Rev. Biophys. **25**, 253–324
4. Degli Esposti, M., DeVries, S., Crimi, M., Ghelli, A., Patarnello, T. and Meyer, A. (1993) Biochim. Biophys. Acta **1143**, 243–271
5. Crofts, A., Hacker, B., Barquera, B., Yun, C. H. and Gennis, R. B. (1992) Biochim. Biophys. Acta **1101**, 162–165
6. Von Jagow, G. and Link, T. (1986) Methods Enzymol. **126**, 253–271
7 Singer, T. P. and Ramsay, R. R. (1992) in Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), pp. 145–162, Elsevier, Amsterdam
8. Filser, M. and Werner, S. (1988) Biochem. Pharmacol. **37**, 2551–2558
9. Ikuma, H. and Bonner, W. D. (1967) Plant Physiol. **42**, 1535–1541
10. Degli Esposti, M., Ghelli, A., Butler, G., Roberti, M., Mustich, A. and Cantatore, P. (1990) FEBS Lett. **263**, 245–257
11. Degli Esposti, M., Flamini, E. and Zannoni, D. (1985) Plant Physiol. **77**, 758–764
12. Ghelli, A., Crimi, M., Orsini, S., Gradoni, L., Zannotti, M., Lenaz, G. and Degli Esposti, M. (1992) Comp. Biochem. Physiol. **103B**, 329–338
13. Degli Esposti, M., Ghelli, A., Crimi, M., Baracca, A., Solaini, G., Tron, T. and Meyer, A. (1992) Arch. Biochem. Biophys. **295**, 198–204
14. Degli Esposti, M., Ghelli, A., Crimi, M., Estornell, E., Fato, R. and Lenaz, G. (1993) Biochem. Biophys. Res. Commun. **190**, 1090–1096
15. Chou, T. C. and Talalay, P. (1981) Eur. J. Biochem. **115**, 207–216
16. Takamiya, S., Furushima, R. and Oya, H. (1984) Mol. Biochem. Parasitol. **13**, 121–134
17. Rieske, J. S., Ramesh, V. and Tripathy, B. C. (1984) in Biomedical and Clinical Aspects of Coenzyme Q (Folkers, K. and Yamamura, Y., eds.), vol. IV, pp. 99–108, Elsevier, Amsterdam
18. Ando, K., Matsuura, I., Newata, Y., Endo, H., Sasaki, H., Okytomi, T., Saehi, T. and Tamura, C. (1978) J. Antibiot. A **31**, 533–538
19. Howell, N. and Gilbert, K. (1988) J. Mol. Biol. **203**, 607–618
20. Di Rago, J. P., Coppée, J. P. and Colson, A. M. (1989) J. Biol. Chem. **264**, 14543–14548
21. Berry, E. A., Huang, L. and De Rose, V. (1991) J. Biol. Chem. **266**, 9064–9077
22. Degli Esposti, M., Rotilio, G. and Lenaz, G. (1984) Biochim. Biophys. Acta **767**, 10–20
23. Brandt, U., Schägger, H. and Von Jagow, G. (1988) Eur. J. Biochem. **173**, 499–506
24. Jeng, M., Hall, C., Crane, F. L., Takahashi, N., Tamura, S. and Folkers, K. (1968) Biochemistry **7**, 131–1322
25. Thierbach, G., Kunze, B., Reichenbach, H. and Hofle, G. (1984) Biochim. Biophys. Acta **765**, 227–235
26. Mansfield, R. W. and Wiggins, T. E. (1990) Biochim. Biophys Acta **1015**, 109–115
27. Tron, T., Crimi, M., Colson, A. M. and Degli Esposti, M. (1991) Eur. J. Biochem. **199**, 753–760
28. Hatefi, Y., Stempel, K. E. and Hanstein, W. G. (1986) J. Biol. Chem. **244**, 2358–2365
29. Andreani, A., Rambaldi, M., Locatelli, A., Andreani, F., Poglayen, G. and Degli Esposti, M. (1992) Eur. J. Med. Chem. **27**, 729–734
30. Abagnato, C. (1992) Doctorate thesis, University of Bologna

Received 27 July 1993