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The reaction sites of rotenone and ubiquinone with mitochondrial NADH dehydrogenase

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Abstract

This article summarizes recent studies in the authors' and other laboratories of selective inhibitors acting at the 'rotenone' site and at the Q binding site in the NADH-Q oxidoreductase segment of the respiratory chain. A wide array of inhibitors act at the rotenone site to block electron flux from the enzyme to the Q pool. Using evidence from studies with rotenone, piericidin A, and analogs of the neurotoxic *N*-methyl-4-phenylpyridinium, we have proposed two binding sites for these inhibitors, both of which must be occupied for complete inhibition of NADH oxidation.

Key words: Complex I; MPP^+; NADH dehydrogenase; NADH-Q oxidoreductase; Q_{10}; Piericidin A; Rotenone; Ubiquinone

1. Introduction

NADH-Q oxidoreductase (NADH dehydrogenase) is by far the most complex enzyme of the mammalian respiratory chain, consisting of a large number of differing subunits [1] and 5 operational entities. These are the substrate site, the FMN binding domain, the 4 or 5 Fe-S clusters, the 'rotenone site', and the ubiquinone binding site. This report deals with recent studies on the latter two regions.

2. The rotenone site

2.1. Historical

Barbiturates were the first group of inhibitors discovered to act in the NADH dehydrogenase segment of the respiratory chain [2]. Later [3] the much more potent inhibitor, rotenone, was shown to block respiration in the same manner. Although other sites were suggested [3–5], it is now generally accepted that both rotenone and barbiturates act between the highest potential Fe-S cluster and Q [6–8]. Piericidin A inhibits NADH oxidation in the same general location as rotenoids and barbiturates [9,10] but is considerably more potent. The displacement of [^{14}C]rotenone by piericidin A from the binding site confirmed that all these inhibitors bind either at the same site or close to it [11]. The most recent group of compounds which have been shown to block respiration at the rotenone site are MPP^+ (*N*-methyl-4-phenylpyridinium), the neurotoxic bioactivation product of MPTP, and its analogs [12,13]. The most potent of these inhibitors of NADH oxidation in inner membrane preparations are the long-chain 4'-alkyl substituted analogs, with IC_{50} values in the 3 to 6 μM range [14] and some even in the nanomolar range. If present in sufficient excess, hydrophobic MPP^+ analogs can prevent the binding of piericidin and rotenone and, concurrently, prevent the inhibition by these agents [14,15].

2.2. Evidence for dual binding sites

Piericidin A and rotenone, being highly hydrophobic, bind avidly to lipophilic membranes but washing membrane preparations with bovine serum albumin dissociates them from unspecific but not from specific

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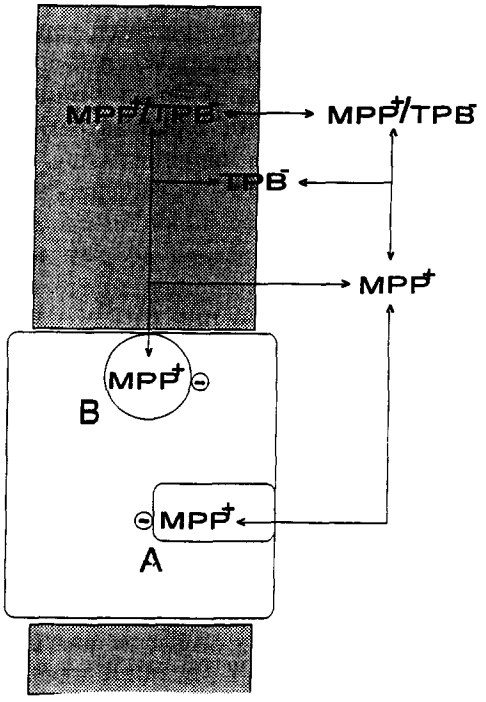
binding sites [16]. The first indication that the inhibition by piericidin and rotenone involves two binding sites came from the observation [16] that the titration curve relating specifically bound piericidin or rotenone to inhibition was sigmoidal, suggestive of dual, cooperative binding sites. Scatchard plots for the binding per mol of FMN (an approximation of the concentration of NADH dehydrogenase in inner membrane preparations) gave a value of about 2, which declined to near 1 on a treatment of the membranes with mercurials or bile salts and organic solvents, as in the isolation of the Complex I preparation [17], with coincident changes in the titration curves from sigmoidal to hyperbolic [18]. Calculations of the stoichiometry of the binding sites, based on FMN content, have been questioned because of inherent technical problems and a 1:1 ratio proposed, instead. While there is some justice to the criticism, an equimolar ratio fails to explain the sigmoidal inhibition curves, or their conversion to hyperbolic ones on partial inhibition or conformational alteration. More recently, van Belzen et al. [19] tried to circumvent the problem by relating piericidin titration to center 2, determined by EPR, but they failed to take into account that piericidin is bound to both the specific site(s) and unspecific ones. Thus, the question remains open.

In recent studies a new approach to the question of the number of binding sites leaves little doubt that, indeed, two binding sites are involved in inhibition at the 'rotenone site'. The inhibition of NADH oxidation in inner membrane preparations (ETP) by hydrophilic MPP^+ derivatives is biphasic: partial (35–45%) inhibition appears within 5 min, followed by a slow, gradual increase in the inhibition over a period of several hours [20]. If, on the other hand, a catalytic amount of tetraphenylboron (TPB^-) is added, complete inhibition is reached almost immediately. With more hydrophobic MPP^+ analogs extensive or complete inhibition appears rapidly without TPB^-. These results were interpreted in terms of the model shown in Scheme 1.

The rotenone site is visualized here as consisting of two binding regions, an external one exposed to the aqueous medium and a less accessible, internal one in the membrane (Scheme 1). Occupation of both sites is required for complete inhibition. Hydrophilic inhibitors readily enter the external site and yield partial (around 40%) inhibition but can only slowly penetrate the internal, hydrophobic binding pocket, unless carried into the membrane by ion-pairing with TPB^-. In contrast, hydrophobic MPP^+ analogs readily diffuse into the membrane, so that nearly complete inhibition is reached rapidly without the intervention of TPB^-. Another interesting feature is that hydrophilic analogs (except for MPP^+ itself) give hyperbolic inhibition curves when reacting only with the external site but usually yield sigmoidal titration curves when TPB^- is used to accelerate the development of the inhibition [20].



Scheme 1. Hypothetical scheme for the two MPP^+ binding sites in NADH-Q oxidoreductase. The stippled area represents the mitochondrial inner membrane with the matrix face on the right. The white box represents the NADH-Q oxidoreductase complex with one inhibitor site (A) accessible to hydrophilic compounds and the other (B) buried in the hydrophobic milieu. From Ref. [20].

A test of the validity of Scheme 1 is shown in Fig. 1. In this experiment the lipophilic 4'-decyl-MPP^+ was used to inhibit NADH oxidase activity. Complete inhibition was reached at ~8 μM analog within 5 min, with an IC_{50} value of about 2 μM (open triangles). When a slight molar excess (10 μM) TPB^- was added

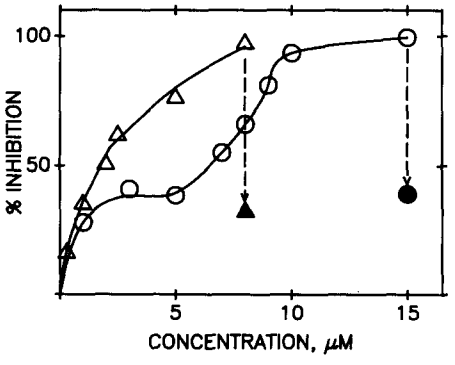


Fig. 1. Inhibition of the NADH oxidase activity of ETP by 4'-decyl-MPP^+ and its reversal. ETP was incubated for 5 min. at 30°C with various concentrations of the inhibitor with or without 10 μM TPB^-. Oxygen uptake was measured polarographically. Open triangles, no TPB^- during the preincubation; open circles, 10 μM TPB^- present. The dashed lines indicate the change in the inhibition observed when 10 μM TPB^- is added to the inhibited sample. From Ref. [21].

to the inhibited enzyme, the inhibition immediately declined to 35% (shaded triangle). When 10 μM TPB^- was initially present, a very different titration curve appeared (open circles), with a plateau between 2–5 μM inhibitor at about 35% inhibition. Only when the concentration of 4'-decyl-MPP^+ added equalled that of the ion-pairing agent did the inhibition rise to 100%. Subsequent addition of a second 10 μM aliquot of TPB^- again lowered the inhibition to 35–40%, the plateau level reached in the first part of the experiment (solid circle). Thus, TPB^- can both accelerate the inhibition and reverse it. This experiment clearly suggests that this analog can readily saturate both sites to give full inhibition but TPB^-, by strong ion-pairing with the inhibitor, competes with a binding group at the internal, hydrophobic site, dissociating the inhibitor from it.

Other studies suggest that two ionizing groups may play a role in the dual binding site: a positively charged one ($pK_a = 6.8$), possibly an imidazole group, and a thiol with a pK_a of ~ 8.4 [20]. The latter appears to be located in the hydrophobic binding site. It is probably the hydrophobic site that is lost on treatment of ETP with mercurials or detergents, resulting in partial loss of rotenone/piericidin sensitivity [16,18].

2.3. Studies on the rotenone site with ion-sensitive electrodes

The evolving picture of the dual binding sites for rotenone, piericidin, and MPP^+ congeners described above was based on enzyme activity assays but can now be supplemented by physical studies based on the work of Davey et al. [22] and Aiuchi et al. [23] with an MPP^+-selective electrode. Using a thermostatted chamber fitted with both O_2 and MPP^+ analog-sensitive electrodes, we measured the uptake and release of hydrophobic MPP^+ derivatives in the micromolar range with great stability. Fig. 2 illustrates that the uptake of 4'-heptyl-MPP^+ by the inner membrane is virtually instantaneous, whereas the development of the inhibition of NADH oxidation takes 3 min, presumably the time required for the inhibitor to penetrate to the inhibitory site within the membrane.

2.4. Topography and attempts at localization

The structural diversity of the compounds which block electron transport between NADH-Q oxidoreductase and Q (barbiturates, rotenoids, piericidins, the structurally related myxalamids [24], MPP^+ analogs, 4-hydroxypyridines [25], and capsaicin) makes it difficult to visualize how they could all react at the same site(s). Nevertheless, Chung et al. [25] proposed some general features of a common binding region for most of these compounds. Conceivably, binding in the vicinity of each other but not to the same group amino acids, may bring about identical inhibitory effects. If one of the rotenone sites is a hydrophobic pocket, as several authors have proposed, binding at various points on that cleft might competitively interfere with the binding of other inhibitors.

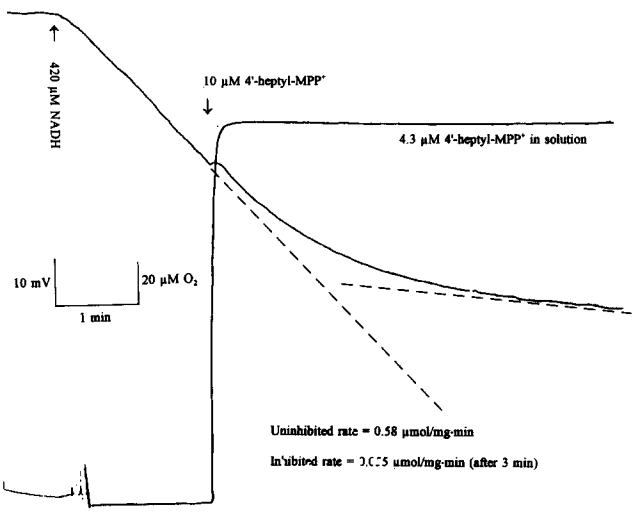


Fig. 2. Uptake of 4'-heptyl-MPP^+ by ETP and development of inhibition of NADH oxidase.

The early concept that piericidin A inhibits Q reduction because it competes with or displaces Q owing to common structural features, has not stood the test of time. The proposal [26] that piericidins, as well as other inhibitors acting at the same region of Complex I, act by bringing about a conformation change in the enzyme unfavorable for electron flux to Q, seems more plausible. The subunit location of the rotenone site within the enzyme also remains to be conclusively established. As discussed elsewhere [1], the evidence that the 33 kDa subunit is involved, based on photoaffinity binding of azido-labelled rotenoids [27,28], is not unambiguous.

3. The quinone binding site(s)

3.1. Q binding sites in NADH dehydrogenase

Several peptides have been suggested to be involved in Q binding. A ubiquinone binding peptide of 15 kDa was isolated from an iron-protein fragment of Complex I [29]. In *Neurospora crassa*, a 9.5 kDa peptide has been identified as the Q-binding site in the complete enzyme by labelling with the photoreactive ubiquinone analogue [30]. Since rotenone does not interfere with the binding of the photolabelled Q analogue to the 9.5 kDa *N. crassa* subunit [30] and MPP^+ inhibits Q reduction in the beef enzyme with a mixed kinetic pattern [31], there is no hard evidence that the two

sites (Q and inhibitor binding) are on the same subunit. Indeed, the subunit labeled by photosensitive rotenone analogs (33 kDa) is much larger [27,28].

Mobility behavior of a spin-labelled Q derivative bond to phospholipid depleted or reconstituted Q-cytochrome *c* reductase suggested that the alkyl tail extended into the hydrophobic periphery of the complex and had some degree of mobility [32]. Similar general conclusions were reached in an NMR study [33]. Only the head part of the Q molecule would need to be protein bound to stabilize the ubisemiquinone radical signals which have been detected in Complex I [34]. The characteristics of the EPR signals indicated that two forms of the radical were present. These observations were consistent with a Q cycle mechanism for the proton-translocating Complex I [35].

3.2. Reactions with exogenous Q and inhibitor analogues

Although it has been shown that exogenous Q_1 can interact directly at the redox sites in pentane-extracted Complex I [36], the presence of endogenous Q in normal preparations means that most measurements with exogenous Q acceptors involve not the redox binding site itself, but another site where the exogenous Q can accept electrons from the endogenous pool. Thus, although informative, structure-function studies of inhibitors based on assays using external Q analogs with preparations containing endogenous Q_{10} do not necessarily reflect the properties of the endogenous Q site.

Piericidin and piericidin-like molecules such as the 4-hydroxypyridines and 4-hydroxyquinolines have been postulated to act at the Q binding site because of their structural resemblance to Q (see Fig. 1) [25]. However, rotenone, barbiturates, and MPP^+ analogs have quite disparate structures, yet have been shown definitively to act at the same site as piericidin [10,14]. Competitive inhibition of NADH-oxidase by piericidin was observed in pentane-extracted ETP with respect to Q_{10} [37] but the amount of piericidin bound was not affected by the amount of Q_{10} in the membranes [37], so that binding is not mutually exclusive. The binding of pethidine analogs which interact with the ND1 gene product (33 kDa) as do rotenone analogs is prevented not only by rotenone but also by Q and by NADH [38]. The NADH substrate site is remote from the Q and rotenone sites, so conformational effects may be more likely than mutual interference. Capsaicin analogs have been proposed to act at the Q site but the evidence is weak. In common with rotenone and piericidin, they inhibit only NADH dehydrogenases which have coupling site 1, suggesting common requirements for binding not found in the simpler systems [39–41].

In our attempts to explore the Q binding site for exogenous Q we have recently assessed the effect of 2-alkyl-4,6-dinitrophenols. These are structurally similar to Q (see Fig. A) and inhibit Complex III and the b-c_1 complex of PS-II. The inhibition of NADH dehydrogenase in ETP by these dinitrophenol analogs was competitive with Q, unlike the mixed inhibition observed for MPP^+ [31]. The study of a series of these inhibitors indicated, first, that the inhibition of Complexes I and III require similar structures. Second, it identified features required for inhibition (Table 1). The inhibitory potency for NADH dehydrogenase was increased with α-branching and by lengthening the carbon chain at the 2-position (equivalent to the isoprenoid side chain of Q). The best of the series tested had 4 carbons in the main chain (#8 in Table 1). The importance of α-branching has been suggested to be due to conformation effects which make the main chain extend perpendicular to the benzene ring plane [42].

Table 1
Structure of 2-substituted 4,6-dinitrophenols and their inhibition of NADH oxidase activity in beef heart submitochondrial particles

Compound	Substituent (2-position)	π [a]	I_H (μM)	Maximum inhib. (%)
1	H	0	62	84
2	CH_3	0.56	170	82
3	CH_2CH_3	1.02	164	48
4	$CH(CH_3)_2$	1.53	7	62
6	$CH(CH_3)CH_2CH_3$	1.96	3	55
6A [b]	$CH(CH_3)CH_2CH_3$	1.96	23	90
7	$CH(CH_3)_3$	1.98	67	63
8	$CH(CH_3)CH_2CH_2CH_3$	2.54	0.10	73
9	$CH(CH_2CH_3)_2$	2.54	0.19	64
10	$CH_2CH(CH_3)_2$	1.96	85	64
15	$CH_2CH_2CH(CH_3)CH_2CH_3$	3.08	0.83	88
16	$CH(CH_3)(CH_2)_3CH_3$	3.08	0.43	80
23	$CH_2(CH_2)_4CH_3$	3.21	38	73

Data from Ref. [31].
[a] The π value is the hydrophobicity index estimated from the partition coefficient in octanol/water.
[b] Compound 6A has a Cl group instead of an OH group.

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