ROLE OF MULTIPLE BINDING SITES IN THE INHIBITION OF NADH OXIDASE BY PIERICIDIN AND ROTENONE

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Summary: Scatchard plots indicate that ^{14}C-piericidin A and -rotenone bind at 2 specific sites per mole of NADH dehydrogenase in ETP, but the titer found for complex I or mersalyl-treated ETP more closely approximates 1. The curves for inhibition of NADH oxidase by piericidin and rotenone are sigmoidal; this results from an unequal contribution of the 2 specific sites to the inhibition. An unspecific binding site also contributes to the inhibition in a manner reversed by washing the particles with bovine serum albumin (BSA). In contrast, inhibition of NADH-CoQ reductase activity is due entirely to binding at specific site(s) because BSA does not restore activity.

The precise locus of inhibition by piericidin A, rotenone, and barbiturates is not settled although most studies indicate an action on the 0_2 side rather than the substrate side of the NADH dehydrogenase flavoprotein (1-5). The suggestion (4) that the inhibition site is between the flavoprotein and a hypothetical non-heme iron protein, which was based on optical spectra the interpretation of which is ambiguous (6), is inconsistent with EPR data (6-9) which localize the inhibition between the EPR-active non-heme iron of the dehydrogenase and CoQ.

Rotenone and piericidin, once considered to be highly selective in blocking a specific site to inhibit NADH oxidase, are now known to inhibit several enzymes; for example, piericidin A, the more selective reagent (10), inhibits succinoxidase (11) but a higher concentration (10^5 times) is required than that needed for a corresponding degree of inhibition of NADH oxidase (10). The binding of ^{14}C-labeled rotenone and piericidin to mitochondria, ETP, and ETP_H is linear over a wide concentration range, including those far in excess of the amount required for maximal effects on NADH oxidation (10,12). While most of the bound

piericidin and rotenone are released on washing the labeled particles with BSA, there is relatively little reversal of rotenone inhibition and almost none with piericidin. These results are interpretable in terms of binding at some sites from which BSA readily releases these inhibitors (unspecific sites) and others from which BSA does not release them significantly (specific sites) (10). The titration curves for binding at the specific site(s) and for NADH oxidase inhibition are almost identical indicating that the specific site(s) is the critical locus for the inhibition of NADH oxidase. The content of the specific site(s) relative to that of NADH dehydrogenase, calculated from such curves, is 1.5 to 1.6 times in ETP and nearly unity in simpler particles, such as complex I (10).

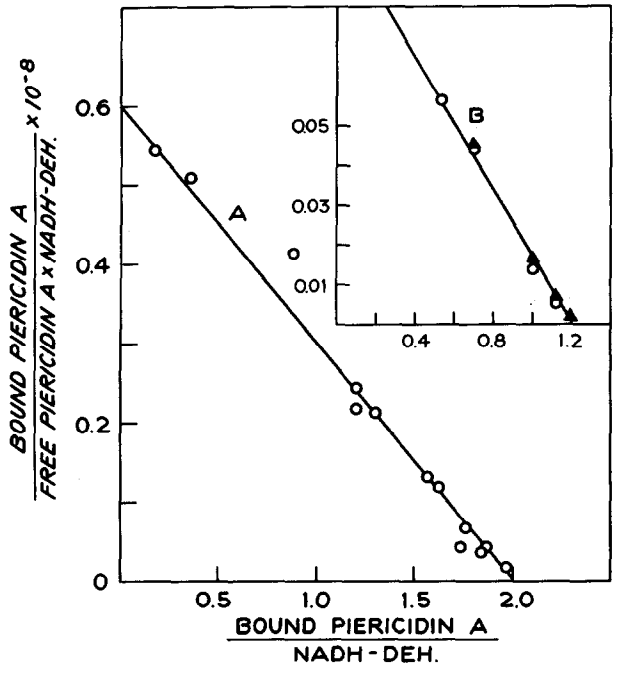

Fig. 1. Scatchard plots for the specific binding of ^{14}C-piericidin A. The main graph (A) refers to ETP and the inset (B) to complex I (▲) and to the NADH-cytochrome reductase complex (complexes I + III) (●), prepared as per reference 14. Titrations were in sucrose-P_i buffer; there were 2 washings with 2% BSA in sucrose followed by 1 with 0.25 M sucrose. The NADH dehydrogenase content was calculated as in previous work (10). With ETP, the protein concentration was 1 or 2 mg/ml during titration and the concentration of inhibitor was varied from 13 mμM to 1.3 μM. Samples were incubated for 1 hr at 0° before the BSA wash. For labeling complex I and complexes I + III, the particles at 2.8 mg protein/ml were incubated for 10 min at 25°, using 2 mg NADH/ml, and washed with BSA-sucrose solution, as above.

A more complete consideration of the binding data is needed to evaluate the significance of the correlation between the piericidin-rotenone titer and the NADH dehydrogenase content. The Scatchard plots (13) shown in Fig. 1 of the specific binding site(s) for ^{14}C-piericidin A in ETP give an extrapolated value (main plot) of 2 sites per mole of NADH dehydrogenase in ETP while in NADH-cytochrome reductase and complex I preparations (14) the value (inset) approximates one site per mole. The experimental values found in this study agree well with those of Horgan et al. (10), who also found that the specific site titers are the same for rotenone and piericidin in different particles; so, the number of specific binding sites for rotenone is assumed to be 2 in ETP and 1 in complex I.

When using ETP preparations, sigmoidal curves are obtained for inhibition of NADH oxidase by piericidin, with or without BSA washes (Figs. 2 and 3), and by rotenone. The inhibition curves, but not the binding curves, are sigmoidal; this indicates that the 2 specific binding sites in ETP contribute unequally to the inhibition of NADH oxidase and the difference is not the result of a cooperative effect of the 2 sites as far as specific binding is concerned.

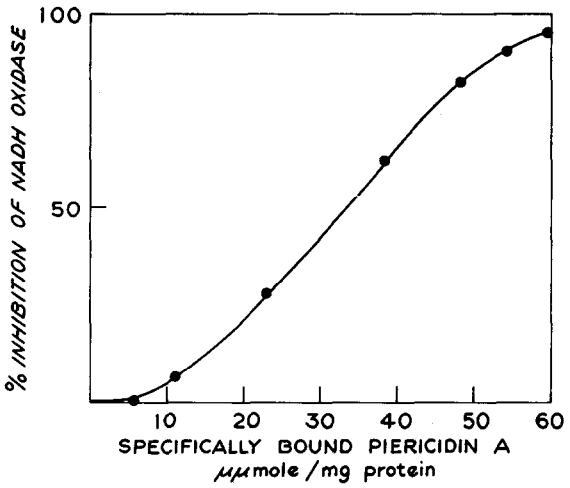

Fig. 2. Inhibition of NADH oxidase activity of ETP by specifically bound piericidin A. Samples of ETP (20 mg protein, 1.5 μmoles/min/mg protein NADH oxidase activity at 23°) were titrated with ^{14}C-piericidin A and washed as in Fig. 1. Remaining oxidase activity was determined polarographically and the radioactivity was determined by scintillation counting.

(The concentrations used are lower than in prior inhibition studies in this or other laboratories and this fact explains why the sigmoidal response which is evident only at low inhibitor concentrations escaped detection until now.) CoQ reductase is similar to NADH oxidase in sensitivity to inhibition by low concentrations of piericidin but at high concentrations the oxidase activity is more profoundly inhibited (Fig. 3); with rotenone, the differential response is pronounced through the entire concentration range (Fig. 4).

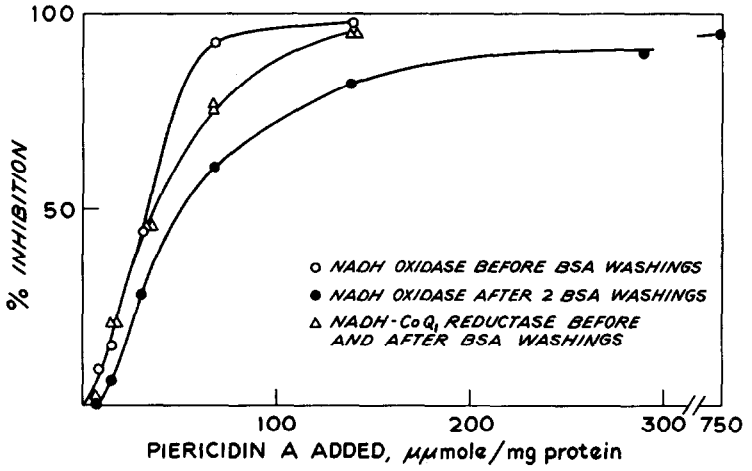

Fig. 3. Comparison of inhibition of NADH oxidase and NADH-CoQ_1 reductase activities of ETP by piericidin A and of the effect of washing with BSA. ETP samples (as in Fig. 2) were titrated with ^{14}C-piericidin A as in Fig. 1, and were assayed for oxidase and CoQ_1 reductase activities before and after 2 washings with BSA in sucrose, and with sucrose. CoQ_1 reduction was assayed spectrophotometrically at 340 mμ, 30°, in 0.1 M tris-sulfate, pH 8.0, with 0.1 mM CoQ_1, 0.2 mM NADH, and 0.3 mM KCN present; specific activity of CoQ reductase was 0.7 μmole/min/mg.

The reversibility of inhibition varies with the inhibitor and preparation. Horgan et al. (12) noted that, contrary to expectation (15), NADH oxidase completely inhibited with rotenone is reactivated gradually and quite extensively on successive washing of the particles with BSA. While BSA washing causes no reactivation at piericidin concentrations 3-4 times greater than that needed to completely inhibit NADH oxidase (10), at lower piericidin concentrations there is marked reversal of NADH oxidase inhibition but not of NADH-CoQ_1 reductase

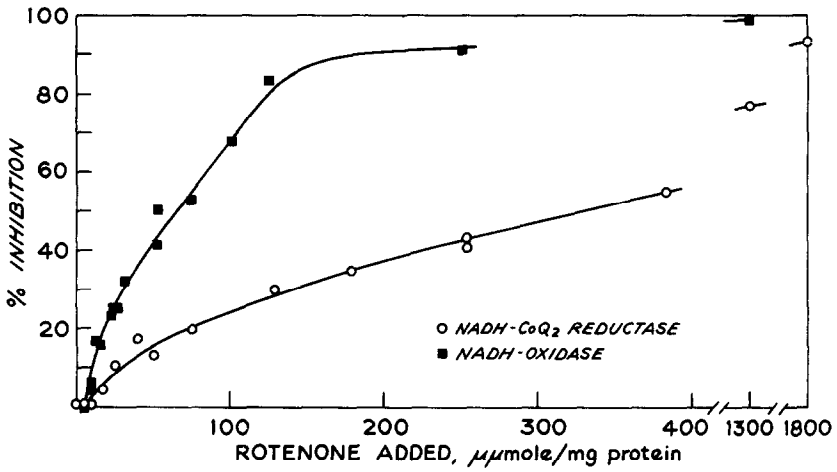

Fig. 4. Titration of NADH oxidase and CoQ_2 reductase activities of ETP with rotenone. ETP samples were titrated, as in previous work (12), in sucrose-P_i-BSA buffer with rotenone in the presence or absence of 1 mg NADH/ml. The reaction time before centrifugation was 60 min at 0° without NADH or 10 min at 25° with NADH; resuspension was in sucrose-P_i buffer (12) before assay; BSA washing was omitted. The conditions of the CoQ_2 assay are the same as for CoQ_1 in Fig. 3.

inhibition on washing with BSA (Fig. 3). Any reactivation by BSA occurs during the first wash; subsequent BSA washings neither remove labeled piericidin nor decrease the degree of inhibition. (The reversibility of piericidin inhibition was not evident in earlier studies because of the high levels of inhibitor and inhibition involved.)

The mechanism of the inhibition of mitochondrial NADH oxidation appears to be more complex than previously envisaged and to involve multiple binding sites. Two specific binding sites that contribute unequally to the inhibition may explain the sigmoidicity of the inhibition curves for piericidin A and rotenone in ETP preparations; the specific site saturated first seems to cause relatively little loss of activity. The affinity of these 2 specific sites for piericidin is similar because the Scatchard plot is linear. In addition to piericidin bound at the specific sites, some unspecifically bound piericidin also appears to contribute to the inhibition as evidenced by the finding that

BSA washing causes significant reactivation but only at low concentrations of piericidin. Hence, the particular unspecific binding site which contributes to the inhibition also appears to be saturated early in titrations (Fig. 3).

The energy-linked reduction of NAD by succinate, in ETP_H preparations, and the NADH oxidase activity give sigmoidal inhibition curves of similar sensitivity with piericidin A and BSA washing causes reactivation in each case. However, these systems differ in sensitivity from the reaction of NADH with CoQ_1 and CoQ_2, and BSA causes no reversal of the CoQ reductase inhibition which, therefore, involves only specific binding. The unspecific binding of piericidin and rotenone which contributes to the oxidase but not to the CoQ reductase inhibition may be at a point higher up in the respiratory chain, on the 0_2 side of the CoQ. This hypothesis is consistent with the block previously found in the cytochrome b-c_1 region (7), but fails to account for the fact that the succinoxidase system is not inhibited under these conditions. Therefore, it is more likely that all binding sites which contribute to NADH oxidase inhibi-

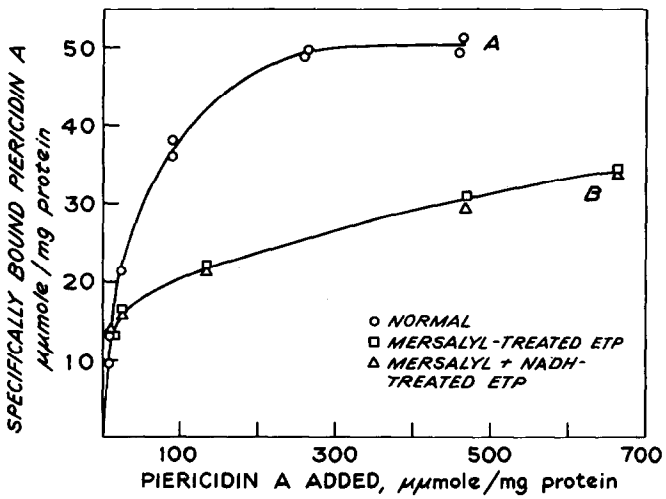

Fig. 5. Effect of mersalyl on specific binding capacity of ETP for piericidin A. Labeling with ^{14}C-piericidin A and washings were as in Fig. 1. Treatment prior to piericidin addition was: ETP in 80 mM P_i-50 μM EDTA, pH 7.4: o—o, control (15 min at 0°): □—□, 10 min at 0° with 30 μM mersalyl: Δ—Δ, 0.2 mM NADH added 3 times at 0°, at 3 min intervals to "precondition" the particles (9), followed by addition of 30 μM mersalyl and standing for 10 min. All samples were centrifuged and resuspended in sucrose-P_i for titration with piericidin A.

tion occur prior to the junction of NADH and succinate oxidases. Perhaps some component of NADH oxidase such as a lipid is essential in the unspecific binding of piericidin and rotenone and the resulting oxidase inhibition but is not critical in the electron flux to external CoQ.

Modification of the dehydrogenase in ETP preparations by mersalyl treatment, with or without preconditioning with NADH (9,16), profoundly alters the binding curve (Fig. 5) in a manner which may involve only 1 of the 2 specific sites because the titer in Scatchard plots falls from 2 to about 1.2 (3). However, once the piericidin is specifically bound it is not released as a result of the conformational changes induced by mersalyl. It appears that NADH dehydrogenase in its native conformation may be involved in the specific binding but that other membrane components are also contributing factors.

Acknowledgment

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