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New evidence for the dimeric nature of NADH:Q oxidoreductase in bovine-heart submitochondrial particles

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The initial velocity of NADH oxidation by bovine-heart submitochondrial particles was measured at pH 8.0 after pretreatment of these particles with different amounts of the inhibitor piericidine A together with 0.035 mM NADH. The amount of piericidine A required to fully inhibit the NADH oxidation activity extrapolated to exactly 1.0 per Fe-S cluster 2 of NADH:Q oxidoreductase. When no reducing equivalents from NADH were present during the pretreatment, this ratio was 1.2. The difference is explained by assuming that NADH:Q oxidoreductase binds piericidine A more effectively in the reduced state than in the oxidized state. It was also found that after Q_{10}-extraction and reincorporation of submitochondrial particles, the amount of piericidine A required to fully inhibit the NADH oxidation activity of the particles increased with the amount of Q_{10} present during reincorporation. This is explained by assuming that binding of piericidine A, to the inhibitory site of NADH:Q oxidoreductase requires Q_{10}. When 0.035 mM NADPH instead of NADH was present during the pretreatment of submitochondrial particles with piericidine A, the amount of inhibitor per cluster 2 required to fully inhibit the initial NADH-oxidation activity extrapolated to 0.5. This result strongly suggests that NADH:Q oxidoreductase is a functional dimer.

Introduction

NADH:Q oxidoreductase (EC 1.6.99.3) from bovine-heart mitochondria, also called Complex I, catalyzes the first step in the respiratory chain, the oxidation of NADH and the transfer of the two reducing equivalents to Q_{10}, linked to the outward translocation of protons with an $H^+/2e^-$ stoichiometry of four to five [1]. The enzyme is extraordinarily large and complex. The purified enzyme has a molecular mass of about 700 kDa per FMN [2] and consists of 26 polypeptides [3–5]. In addition to the flavine it contains at least four EPR detectable Fe-S clusters as carriers of reducing equivalents [6]. The hydrodynamic properties of the isolated enzyme point to a dimeric structure [7]; in spite of this, there is no structural evidence that NADH:Q oxidoreductase is dimeric in the intact inner membrane, but there are a number of indications that the enzyme might be a functional dimer. Most indicative is the fact that in isolated NADH:Q oxidoreductase, as well as in submitochondrial particles, the Fe-S cluster 1 ($g_{x,y,z}$ = 1.92, 19.4, 2.02) is present at a spin concentration precisely one-half of that of cluster 2 ($g_{x,y,z}$ = 1.92, 1.92, 2.05) or FMN [8,9]. Other evidence comes from the pathway of electron transfer in NADH:Q oxidoreductase during NADPH oxidation at pH 6.2. Cluster 1 and cluster 3 ($g_{x,y,z}$ = 1.88, 1.94, 2.10) are only partially reduced by NADPH, while clusters 2 and 4 ($g_{x,y,z}$ = 1.86, 1.93, 2.04) are fully reduced [10]. A detailed kinetic study of this reaction has led to the proposal that Complex I consists of two monomers, one of which lacks cluster 1 [11–14]. Oxidation of NADH proceeds through either monomer with kinetics that are not resolvable with the available techniques. NADPH, however, reduces clusters 1 and 3 only slowly and to a limited extent, indicating preferential oxidation by the cluster 1-deficient monomer. Additional evidence for a functional dimer came from the identification of two interacting NADH binding sites per molecule [15].

Up to now, inhibitor studies have given no evidence that NADH:Q oxidoreductase would be a functional dimer. Rotenone and piericidine A, both potent inhibitors of the mitochondrial oxidation of NADH, are able to inhibit NADH:Q oxidoreductase in submitochondrial particles at stoichiometric concentrations [16].

Abbreviations: BSA, bovine serum albumin; Mes, 4-morpholineethanesulphonic acid; SMP, submitochondrial particles.

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Both inhibitors compete for the same specific site; piericidine A seems to bind more effectively [17]. Maximal inhibition of oxidized submitochondrial particles by piericidine A occurred at a titer of 1- to 1.5-times the estimated concentration of NADH:Q oxidoreductase [17], which does not support a dimeric functioning of NADH:Q oxidoreductase. The binding of rotenone and piericidine A is not covalent [18] and it was with a photoaffinity analogue of rotenone, that the binding site was located to be on a mitochondrially encoded polypeptide of a molecular mass of 33 kDa [19,20]. The site of inhibition of rotenone or piericidine A in the pathway of electron transfer from NADH to Q_{10} is probably close to the site of reduction of Q_{10}, since the Fe-S clusters of the enzyme are fully reduced in the presence of these inhibitors [21–23]. Even the reduction of Fe-S cluster 2 (midpoint potential −20 mV [24]) by NADH in submitochondrial particles is not retarded by pretreatment with rotenone. Thus, the rotenone-inhibition site is suggested to be on the oxygen side of cluster 2. In the presence of rotenone or piericidine A and a limited amount of NADH cluster, 2 remains reduced even after exhaustion of NADH and the complete oxidation of all other Fe-S clusters [25]. This means that under these conditions, cluster 2 cannot be oxidized by forward electron transfer provided by an electron leak through the rotenone inhibition site. These observations suggest that the rotenone-interaction site is close to cluster 2. The binding of the inhibitors to the enzyme appears to require the presence of phospholipid [26]. Also, a competition between Q_{10} and piericidine A for binding to NADH:Q oxidoreductase in submitochondrial particles has been proposed [27] (Q_{10} reduction is also dependent on phospholipids [28]). These data indicate a close relationship between the specific binding site of piericidine A and the site of Q_{10} reduction, in addition to the close relationship with cluster 2.

In the present report we provide further experimental evidence for the existence of such a close relationship. In addition, the proposal of a dimeric structure of NADH:Q oxidoreductase is reinforced by new data on the titration of the NADH-oxidation activity in bovine-heart submitochondrial particles by the inhibitor piericidine A.

Materials and Methods

NADPH, NADH, NAD^+ and $NADP^+$ were purchased in the purest form available from Boehringer (Mannheim, F.R.G.). Butadione was obtained from E. Merck (Darmstadt, F.R.G.), BSA from Koch-Light Laboratories (Colnbrook, U.K.) and Q_{10} from Sigma (U.S.A.). Piericidine A isolated from *Streptomyces mobaraensis* [29], was a gift from Dr. S. de Vries. All other chemicals were of analytical grade.

Bovine-heart submitochondrial particles were prepared essentially as described in Ref. 30 and treated with butadione as has been described [14]. Assays for the oxidation activities with NADH and NADPH were carried out polarographically at 30 °C, using a Clark electrode for measuring oxygen consumption in the media: 0.25 M sucrose/100 mM Tris-HCl/1 mM butadione (pH 8.0) or 0.25 M sucrose/100 mM Mes-KOH/1 mM butadione (pH 6.2). The cell volume was 1.60 ml and about 0.5 mg of protein of submitochondrial particles was added together with 5 mM NADH or NADPH. Q_{10} was extracted from submitochondrial particles after extensive washing with 150 mM KCl solution and lyophilization by six washes of *n*-pentane as has been described [31]. Reincorporation of varying amounts of Q_{10} was performed as has been described [31]. Protein concentrations were determined by the biuret reaction [32].

Stopped-flow experiments were performed at 30 °C with a Union Giken stopped-flow apparatus with a four-jet tangential mixer. The initial velocity of NADH oxidation was monitored at 340 nm in the 5 s range. The first vessel contained 0.5 mg of protein of submitochondrial particles per ml to which in some cases 0.035 mM NADH, NADPH, NAD^+ or $NADP^+$ was added together with varying amounts of piericidine A. The contents were incubated 5 min before starting the measurements. Piericidine A and 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S13) were added as concentrated ethanol solutions in such a way that no more than 0.1% (v/v) ethanol was added to the assay mixture. The second vessel contained 0.065 mM of NADH. The buffers used were the same as in the polarographical measurements.

The amount of Fe-S cluster 2 in submitochondrial particles was determined by EPR spectroscopy [9].

Results

Titration of the NADH-oxidation activity of Q_{10}-reincorporated submitochondrial particles by piericidine A

The results of experiments in which the NADH-oxidase activities of lyophilized submitochondrial particles or pentane-extracted/Q_{10}-replenished submitochondrial particles were titrated with the inhibitor piericidine A are shown in Fig. 1. Before lyophilization, the submitochondrial particles had a NADH-oxidation activity (pH 8.0) of 1250 nmol NADH per min per mg of protein. This activity diminished to 695 nmol per min per mg of protein after lyophilization and to less than 5 nmol per min per mg protein after Q_{10} extraction. Reincorporation of Q_{10} in the presence of respectively 1.5, 3, 6, 12, 24 and 36 nmol of Q_{10} per mg of protein resulted in activities of 167, 313, 347, 368, 372 and 374 nmol per min per mg protein. This was in accordance with the results found by Gutman et al. [27]. The results

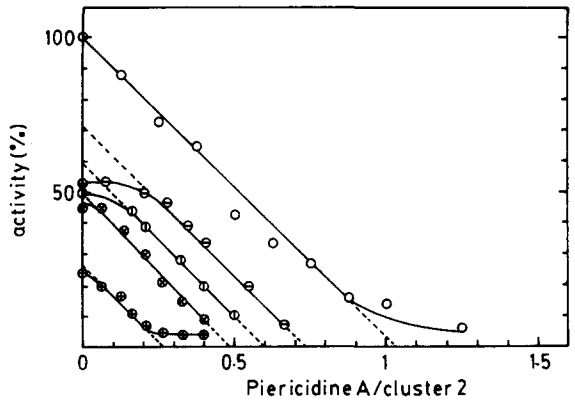

Fig. 1. The inhibition of NADH oxidation activity by piericidine A (pH 8.0) in submitochondrial particles containing different amounts of Q_{10}. Submitochondrial particles (0.25 mg of protein) were suspended in a cuvet containing 3 ml 0.25 M sucrose/100 mM Tris-HCl (pH 8.0). NADH (0.1 mM) was added and after its oxidation, the oxidation of another 0.1 mM NADH was monitored at 340 nm at a temperature of 22° C. ○——○, lyophilized submitochondrial particles; ⊕——⊕, Q_{10}-extracted submitochondrial particles that were replenished with 1.5 nmol of Q_{10} per mg of protein; ⊗——⊗, idem, but replenished with 3 nmol of Q_{10} per mg of protein; ⊙——⊙, idem, but replenished with 6 nmol of Q_{10} per mg of protein; ⊖——⊖, idem, but replenished with 12 nmol of Q_{10} per mg of protein. Full activity (100%) of the lyophilized submitochondrial particles was 695 nmol NADH oxidized per min per mg of protein.

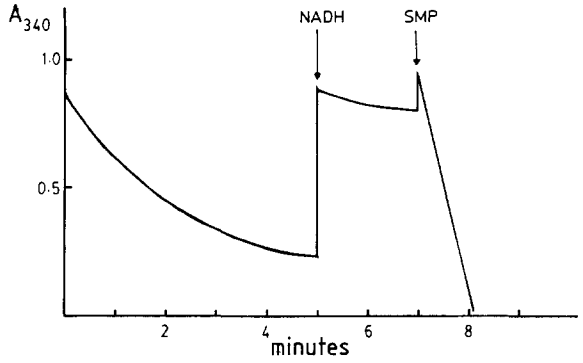

Fig. 2. The NADH consumption by a mixture of Q_{10}-extracted submitochondrial particles with specifically bound piericidine A and normal submitochondrial particles. Q_{10}-extracted submitochondrial particles were incubated at a protein concentration of 38 mg per ml with 8 μM piericidine A for 5 min at 4° C in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4), collected by centrifugation (30 min at 100000 × g) and washed twice with the same buffer containing 2% (w/v) BSA to remove nonspecifically bound piericidine A. 0.25 mg of protein of these particles and 0.25 mg of protein of untreated particles were mixed in a 4 ml cuvet containing 3 ml 0.25 M sucrose/100 mM Tris-HCl (pH 8.0). The consumption of 0.1 mM NADH was monitored at 340 nm at a temperature of 22° C. When the rate of NADH consumption was almost zero extra NADH (0.1 mM) was added, followed by 0.25 mg of protein of untreated particles (SMP).

of the titrations of the NADH oxidation activities by piericidine A of particles replenished with 12, 24 or 36 nmol of Q_{10} per mg of protein were identical. The amount of cluster 2 in this batch of submitochondrial particles was not determined by EPR spectroscopy, but estimated to be 0.12 nmol per mg protein in accordance with the concentration of cluster 2 routinely determined by EPR in submitochondrial particles. The NADH oxidation activity of submitochondrial particles after lyophilization was taken to be 100% in Fig. 1 and these particles gave a linear relationship between the amount of piericidine A added and the remaining NADH oxidation activity, except for the last 5–10% of the activity that required an excess of piericidine A to be fully inhibited (data not shown). Extrapolating this linear part to full inhibition resulted in a ratio of piericidine A per cluster 2 of about 1. It should be mentioned that the method to determine the amount of cluster 2 has an inaccuracy of about 10% [8]. As seen in Fig. 1, the linear parts of the titration curves with the Q_{10} replenished particles were all parallel to that of the lyophilized particles, but extrapolated to lower ratios of piericidine A per cluster 2. Fig. 2 shows an experiment in which Q_{10}-extracted submitochondrial particles, to which piericidine A was bound at the specific site, were mixed with an equal amount of untreated submitochondrial particles after which the oxidation of NADH was monitored at 340 nm. After some time this resulted in a 98% decline of the NADH oxidation activity of the untreated particles. Addition of an extra amount of NADH and the subsequent addition of untreated submitochondrial particles demonstrated that neither NADH nor oxygen depletion were the cause of this decline in activity.

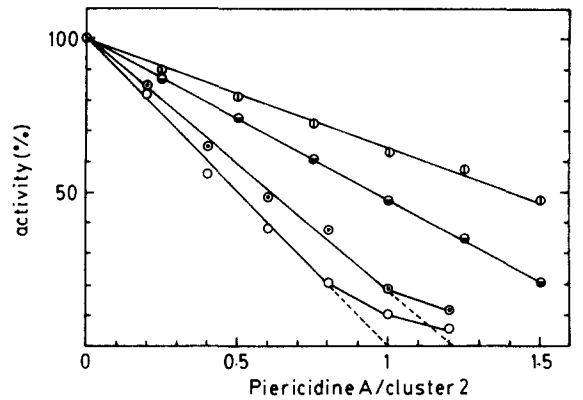

Fig. 3. The inhibition by piericidine A of the NADH oxidation activity of submitochondrial particles (pH 8.0) pretreated in different ways. After the pretreatment the initial velocities were measured as described in Materials and Methods. The NADH oxidation activities are shown relative to that of the untreated submitochondrial particles (1235 nmol per min per mg of protein). ○——○, pretreatment with varying amounts of piericidine A plus 0.035 mM of NADH for 5 min; ⊙——⊙, pretreated with piericidine A only (5 min); ◒——◒, pretreatment by subsequent addition of BSA (1 mg per ml), piericidine A and 0.035 mM of NADH (5 min); ⊙——⊙, pretreatment by the subsequent addition of BSA (1 mg per ml) and piericidine A (5 min).

The effect of reduction of submitochondrial particles on the inhibition by piericidine A

Fig. 3 shows that submitochondrial particles preincubated with piericidine A together with a small amount of NADH were inhibited more efficiently than particles incubated with piericidine A in the absence of reducing equivalents. The inhibition of NADH oxidation activity of submitochondrial particles pretreated with piericidine A plus 0.035 mM NADH extrapolated to a value of 1 molecule of piericidine A per Fe-S cluster 2, while this ratio was about 1.2 for particles pretreated with piericidine A only. This experiment confirms the fact that piericidine A binds in stoichiometric amounts to the specific site of inhibition of NADH:Q oxidoreductase in submitochondrial particles. It is also known, however, that nonspecific binding to submitochondrial particles can occur [16–18,33]. We therefore suspected that the difference between the titration curves of particles pretreated with the inhibitor in the presence or absence of NADH was mainly caused by a difference in distribution between specific sites (resulting in inhibition) and nonspecific sites (noninhibitory), binding at the specific site being more efficient in the reduced enzyme. To examine this possibility, we included 1 mg per ml BSA during the pretreatment with the inhibitor in order to increase the amount of nonspecific binding sites and indeed, more piericidine A was needed in order to fully inhibit the NADH oxidation activity. Likewise, in agreement with this idea, preincubation together with NADH again gave better inhibition. Preincubations with piericidine A performed in the presence of 0.035 mM NAD^+ and $NADP^+$ instead of NADH had no effect (data not shown), but incubation with NADPH gave unexpected results.

The effect of NADPH on the inhibition of submitochondrial particles by piericidine A

In Fig. 4 the results are shown of two oxygen-uptake experiments (pH 8.0), in which submitochondrial particles pretreated with a ratio of 0.5 mol of piericidine A per mol of cluster 2 in the presence of either NADH or NADPH were used. In the absence of piericidine A, preincubation with NADPH did not influence the oxygen-uptake activity, nor did it introduce a lag phase. In the presence of piericidine A, preincubation with NADPH introduced a lag phase. The initial velocity observed during this lag phase proved to be dependent upon the amount of piericidine A added, as shown in Fig. 5. Full inhibition of the initial velocity did now occur at a ratio of piericidine A to cluster 2 equal to 0.5. However, maximal steady-state NADH oxidation activity, that was reached 3 to 4 min after addition of 5 mM NADH (Fig. 4, trace B) required 1.05 mol of piericidine A per mol of cluster 2 (not shown). No lag phase appeared when both NADH and NADPH were present during preincubation and the amount of piericidine A

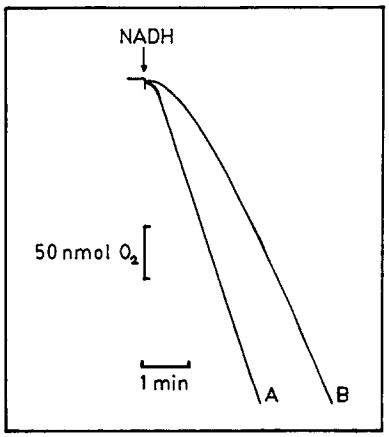

Fig. 4. The oxygen consumption of submitochondrial particles preincubated with 0.5 mol of piericidine A per mol of Fe-S cluster 2 of NADH:Q oxidoreductase in the presence of NADH or NADPH. (A) preincubated together with 0.035 mM NADH. (B) preincubated together with 0.035 mM NADPH. The reaction was started by addition of 5 mM NADH (pH 8.0).

per cluster 2, needed for full inhibition, extrapolated again to a ratio of 1. When the pretreatment was devised a little differently, in that first 0.035 mM NADH was added together with 0.035 mM NADPH, time was given for complete oxidation of this amount of NADH, whereafter piericidine A was added; the amount of inhibitor required for complete inhibition of the initial NADH oxidation activity was 0.5 mol per mol of cluster 2 (not shown). The addition of 30 μM of the uncoupler 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S13) to the particles had no effect in any of these experiments. When these experiments were repeated at pH 6.2, no lag phase could be observed after incubation of submitochondrial particles with piericidine A together with NADPH and the titrations with

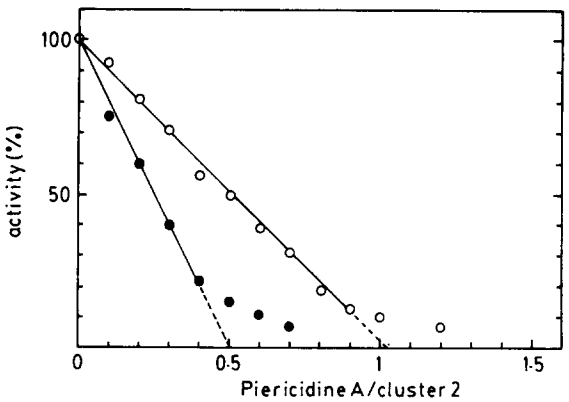

Fig. 5. Initial NADH oxidation activities of submitochondrial particles pretreated with piericidine A in the presence of NADH or NADPH (pH 8.0). The NADH oxidation activities are shown relative to that of the untreated submitochondrial particles (1065 nmol per min per mg of protein). ○———○, piericidine A preincubated together with 0.035 mM NADH; ●———●, piericidine A preincubated together with 0.035 mM NADPH.

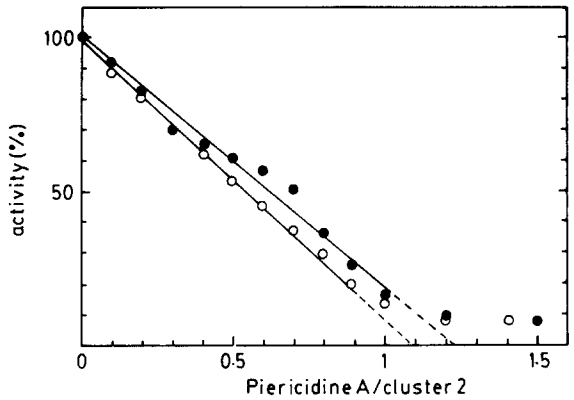

Fig. 6. Titration with piericidine A of the initial NADH oxidation activities of submitochondrial particles (pH 6.2). The NADH oxidation activities are shown relative to that of the untreated submitochondrial particles (860 nmol per min per mg of protein). ○———○, pretreated with piericidine A together with 0.035 mM NADH; ●———●, pretreated with piericidine A together with 0.035 mM NADPH.

piericidine A-treated submitochondrial particles in the presence of either NADH or NADPH, extrapolated to ratios of 1.1 and 1.2, respectively (Fig. 6). In Fig. 7 the inhibition of the initial velocity by pretreatment with piericidine A in the presence of NADH or NADPH (pH 8.0) was again measured, but this time the pretreatment was extended by the addition of 1 mg of BSA per ml in order to see whether the specifically-bound piericidine A could be released from the particles. Fig. 7 shows that this was indeed possible, but not under all circumstances. When NADH was present during the binding of 1 mol of piericidine A per cluster 2, the initial velocity of the residual NADH oxidation activity only increased from 12% to 15% within 1 h (Fig. 7, trace A). However, when NADPH was present during the binding of 0.5 mol piericidine A per mol of cluster 2, the addition of BSA caused a clear release of the nearly complete inhibition of the initial velocity by the inhibitor (Fig. 7, trace C). This effect was not observed when the submitochondrial particles were incubated with the same amount of piericidine A together with NADH instead of NADPH (Fig. 7, trace D). When no reducing equivalents were present during the pretreatment with piericidine A the inhibition appeared to be less, and remained almost the same during prelonged incubation with BSA (Fig. 7, trace B). Increasing the amount of BSA in these experiments increased the initial NADH oxidation activities when preincubation was carried out in the presence of NADPH, and also in the absence of reducing equivalents (data not shown), indicating that the amount of piericidine A bound to the specific site on the submitochondrial particles in the absence of reducing equivalents could be perturbed by BSA. No effect of elevated BSA concentration on the NADH oxidation was observed when the binding of piericidine A was performed in the presence of NADH.

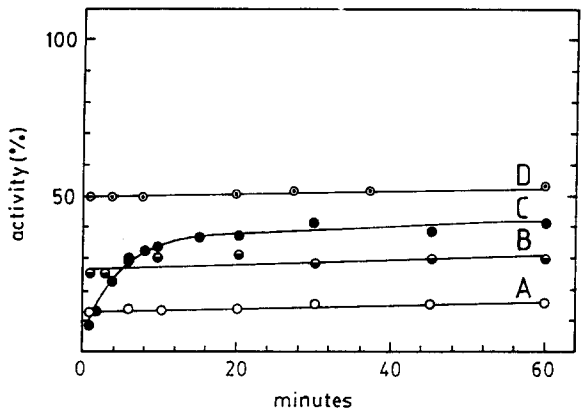

Fig. 7. Effect of the presence of BSA on initial NADH oxidation activities of submitochondrial particles (pH 8.0) pretreated with piericidine A in the presence or absence of NADH or NADPH. (A) ○———○, pretreated with 1 mol of piericidine A per mol of cluster 2 and 0.035 mM NADH before addition of BSA. (B) ◒———◒, pretreated with 1 mol of piericidine A per mol of cluster 2 before addition of BSA. (C) ●———●, pretreated with 0.5 mol of piericidine A per mol of cluster 2 and 0.035 mM NADPH before addition of BSA. (D) ⊙———⊙, pretreated with 0.5 mol of piericidine A per mol of cluster 2 and 0.035 mM NADH before addition of BSA. Samples drawn at the indicated times were assayed for the initial NADH oxidation activity. The NADH oxidation activities are shown relative to that of the untreated submitochondrial particles (1130 nmol per min per mg of protein).

Discussion

The results in Fig. 1 suggest that piericidine A is preferentially binding to those NADH:Q oxidoreductase molecules that are present in Q_{10}-containing (and thus active) respiratory chain assemblies, i.e., Q_{10} seemed to be required for tight binding. The presence in the submitochondrial particles of amounts of Q_{10} less than normal, resulted in lower amounts of piericidine A required to fully inhibit the NADH oxidation activity. The fact that the titration curves of the Q_{10}-extracted particles replenished with more than 6 nmol of Q_{10} per mg protein were not linear at low piericidine A concentrations, was probably due to the fact that the Q_{10}-extraction procedure induced a rate limiting step for the NADH oxidation in these Q_{10}-replenished particles at a site other than the NADH:Q oxidoreductase segment. Fig. 1 also indicates that only 75% of the Complex I molecules are repaired by Q_{10}-replenishment. The fact that parallel lines were found, indicates that the activity is directly proportional to the intact (i.e., Q_{10}-containing) piericidine-specific binding site, so the turnover number per intact piericidine A site is not affected by Q_{10}-extraction from lyophilized particles. The results of Fig. 2 indicate that Q_{10}-depleted submitochondrial particles were still able to bind piericidine A in stoichiometric amounts, although less tight than untreated particles. When mixed with an equivalent amount of un-

treated submitochondrial particles and in the presence of excess NADH, piericidine A seemed to transfer to the untreated submitochondrial particles, causing the NADH oxidation activity of the mixture to become strongly inhibited. Piericidine A binding to the specific inhibition site is apparently more efficient in the presence of Q_{10}. This strengthens the conclusion from the experiments in Fig. 1, namely that binding of piericidine A to NADH:Q oxidoreductase involves Q_{10}. This seems to be in contradiction with the results of Gutman and colleagues [27]. They observed that in Q_{10}-replenished particles, the inhibition by piericidine A was competitive with respect to Q_{10}. However, they used an aqueous reincorporation method and needed extremely high amounts of Q_{10} in their assays which, according to them, probably precipitated or formed micelles. It can be envisaged that micelles of Q_{10} will bind piericidine A, removing it from its specific site and in that way mimicking competition. Our observations indicate that piericidine A does not simply act by dislocation of Q_{10} from an active site on the enzyme. On the contrary, our results indicate that Q_{10} strengthens binding of the inhibitor. It has been reported that binding of piericidine A at the specific site requires lipids [16,17,26]. It was also observed that Q_{10} reduction by NADH:Q oxidoreductase was dependent on lipids [28,34], which would serve as a solvent for Q_{10} and probably increased the conformational stability of the enzyme. Piericidine A binding prevented phospholipase A from solubilizing the flavoprotein moiety of the enzyme and also from losing the specific inhibition site [26], which is in line with the idea that essential phospholipids are required for the binding of piericidine A. Together, these facts suggest that the binding of piericidine A to NADH:Q oxidoreductase involves protein, Q_{10} and lipids, which must all be in close proximity to the specific binding site.

The fact that under certain conditions, only one molecule piericidine A per 2 Fe-S clusters 2 is required to fully inhibit the initial velocity of NADH oxidation by NADH:Q oxidoreductase in submitochondrial particles (Figs. 4 and 5), is perhaps the best demonstration of our proposal that NADH:Q oxidoreductase is dimeric in its functioning in the natural membrane system of submitochondrial particles. The fact that this phenomenon could only be observed when particles were pretreated with piericidine A in the presence of NADPH at pH 8.0, but not at pH 6.2, can easily be understood from our previous experiments [11–14]. It was observed that when NADPH was added to submitochondrial particles half of the Fe-S clusters 2, 3 and 4 were reduced at pH 8.0, while no NADPH oxidation activity could be observed under these circumstances. But when NADPH was added at pH 6.2, where there is a clear NADPH oxidation activity, clusters 2 and 4 could be fully reduced. NADH is able to reduce all Fe-S clusters fully at both pH values. Since Fig. 3 indicates that reduced NADH:Q oxidoreductase binds piericidine A more efficiently at its specific site than oxidized NADH:Q oxidoreductase, and taking into account that the specific binding site for piericidine A is in very close proximity of cluster 2 [21–23], we make the assumption that this increased affinity for binding of piericidine A on reduction of NADH:Q oxidoreductase is associated with the reduction of cluster 2. Therefore, reduction of only half of the clusters 2, increases the binding affinity of only half of the specific binding sites of NADH:Q oxidoreductase. As Fig. 5 demonstrates, the blocking of only one of the two specific binding sites is enough to fully inhibit enzyme activity. This specifies the basic catalytic unit of the enzyme as containing two clusters 2. Of course, when excess NADH is subsequently added to measure the oxidation activity, both clusters 2 become reduced and now compete for binding of piericidine A. In time, this leads to a redistribution of the inhibitor between these two sites and experimentally, it was noticed that the inhibition was released to the extent observed after pretreatment with piericidine A in the presence of NADH. Since at pH 6.2, NADPH is able to reduce all clusters 2, just like NADH, both binding sites will have a high-binding affinity at this pH and the amount of piericidine A required to fully inhibit the enzyme activity will be stoichiometric to cluster 2 again (Fig. 6).

There is, however, a point of contradiction when one accepts the working hypothesis for the functioning of Complex I proposed previously [14]: during incubation with piericidine A in the presence of NADPH (pH 8.0), the inhibitor would preferentially bind to the protomer containing the Fe-S clusters that was suggested not be involved in the oxidation of NADH. Consequently, no effect on the NADH oxidation activity would be expected, which is clearly not the case. Therefore, the original hypothesis has to be modified and improved by proposing that apparently both clusters 2 must cooperate in reducing Q_{10}, each donating one of the two required electrons. Inclusion of this assumption can explain all results on the pre-steady-state reduction of NADH:Q oxidoreductase obtained thusfar [11–14]. It explains that at pH 8.0, NADPH is not oxidized by NADH:Q oxidoreductase, although it is able to reduce half of the Fe-S clusters 2 and 4 very rapidly. It also makes clear that blockage of only this half of the enzyme by piericidine A results in the complete inhibition of the initial NADH oxidation activity. In Fig. 8 the improved working hypothesis for the functioning of NADH:Q oxidoreductase in submitochondrial particles is shown. It contains two Fe-S clusters 2, 3 and 4 per functional unit, two flavins and two piericidine A binding sites, but only one cluster 1. Q_{10} is only reduced when two electrons, one donated from each cluster 2, can simultaneously reach the molecule. No electrons are

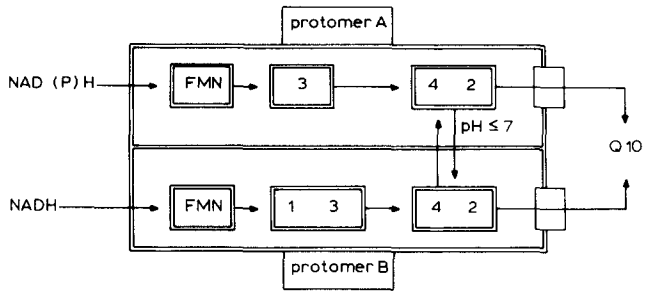

Fig. 8. Schematic representation of the oxidation and reduction reactions carried out by NADH:Q oxidoreductase under various conditions. The arrows indicate the proposed routes of electron transfer within the enzyme. The empty boxes at the Q_{10}-side of the enzyme symbolize the piericidine A binding sites.

transferred that would result in semiquinones with a lifetime longer than about 5 ms. The latter assumption is given in by the fact that semiquinones that could be associated with the action of NADH:Q oxidoreductase were never observed during NADH or NADPH oxidation in submitochondrial particles in any of our experiments [11–14].

The scheme adopted in Fig. 8 implies, however, that binding of one molecule of piericidine A on any of the two clusters 2 would always result in the complete inhibition of NADH oxidation activity. Under some conditions (Fig. 5), namely pretreatment of particles with piericidine A in the presence of NADPH at pH 8.0, this is indeed the case, but when pretreatment with the inhibitor was carried out in the presence of NADH (or NADPH at pH 6.2) two mol of piericidine A per two mol of cluster 2 was required. This apparent contradiction can be resolved by assuming a cooperativity for the binding of piericidine A at the two specific binding sites when both clusters 2 are reduced. If this would not be the case, then piericidine A titrations of the NADH oxidation activity of submitochondrial particles preincubated with NADH would not give a straight line but a curved one. The action of the inhibitor (i) on the enzyme can be formulated by the following equilibria:

$$\text{e-e} + \text{i} = \text{e-e-i} \qquad K_1 = [\text{e-e-i}]/[\text{e-e}]\cdot[\text{i}]$$
$$\text{e-e} + \text{i} = \text{i-e-e} \qquad K_2 = [\text{i-e-e}]/[\text{e-e}]\cdot[\text{i}]$$
$$\text{e-e-i} + \text{i} = \text{i-e-e-i} \qquad K_3 = [\text{i-e-e-i}]/[\text{e-e-i}]\cdot[\text{i}]$$
$$\text{i-e-e} + \text{i} = \text{i-e-e-i} \qquad K_4 = [\text{i-e-e-i}]/[\text{i-e-e}]\cdot[\text{i}]$$

Only free enzyme (e-e) is active and from the equation it can be deduced that a straight relation between inhibition and the added amount of inhibitor will only be found if K_3 and K_4 are sufficiently larger than K_1 and K_2. Simulations in which it was assumed that $K_1 = K_2$ and $K_3 = K_4$, showed that K_3 had to be at least one order of magnitude larger than K_1 for the inhibition titration to become experimentally indistinguishable from a straight line. In Fig. 7 it was shown that the binding of piericidine A to NADH:Q oxidoreductase in the presence of 0.035 mM NADH was quite strong, since addition of BSA did not relieve the inhibition of NADH oxidation activity of submitochondrial particles by piericidine A once the inhibitor was bound. In the absence of NADH and in the presence of BSA, there is a competition for the binding of piericidine A between BSA and the particles as is shown in Fig. 3. When the experiment of Fig. 3 was repeated with a stoichiometric amount of piericidine A in the presence of BSA, and a longer incubation time was used, in which every 5 min a small amount of NADH (0.035 mM) was added in order to reduce NADH:Q oxidoreductase, the inhibition progressed in time and eventually became virtually complete (data not shown). It thus appeared that the sequence of additions was important. When piericidine A was presented to the particles with the subsequent addition of 0.035 mM NADH, binding was fast and virtually irreversible: addition of BSA did not relieve the inhibition (Fig. 7, trace A). When however, piericidine A was presented to a mixture of submitochondrial particles and BSA, more than half of the piericidine A ended up bound to BSA (Fig. 3). On subsequent addition of aliquots (0.035 mM) of NADH the binding to the particles increased due to reduction of cluster 2 and the progress in the inhibition clearly indicated the (slow) transfer of piericidine A to the particles. In Fig. 7, trace C, it is also shown that the binding of piericidine A to particles reduced by NADPH was partially released by addition of BSA. This means that binding to only one of the two available specific sites results in a less efficient binding. The fact that a larger amount of BSA in this experiment led to higher initial velocities (data not shown), which is not the case when NADH instead of NADPH was used during pretreatment, also indicated this. This means that binding to only one of the two available specific sites results in a less efficient binding, which reinforces the proposal of cooperativity between the two specific binding sites when both clusters 2 are reduced. Fig. 7, traces A and B indicate that in oxidized particles piericidine A is bound to specific and nonspecific sites, confirming that piericidine A binds less efficiently to its specific site in oxidized enzyme than in reduced enzyme (Fig. 7, trace A), as was already deduced from Fig. 3.

As was mentioned above, semiquinones that could be associated to NADH:Q oxidoreductase were not observed by us in submitochondrial particles. Semiquinones have been observed in isolated NADH:Q oxidoreductase [36]. This observation led to the proposal of a Q_{10}-binding protein, and since the EPR signal of the semiquinone radical proved to be heterogenous, and four Q_{10} molecules per FMN were found to bind to the isolated NADH:Q oxidoreductase, it was suggested that two kinds of Q_{10}-binding proteins existed which were both able to bind two Q_{10} molecules. In a later

report this Q_{10}-binding protein was isolated from NADH:Q oxidoreductase and proved to bind only one molecule of Q_{10} [37]. This discrepancy in the EPR behavior of Q_{10} in SMP and the isolated enzyme is not understood. The existence of a Q_{10}-binding protein in NADH:Q oxidoreductase makes the postulation of a Q-cycle like in the bc_1 complex attractive [38], though such a Q-cycle would involve semiquinones. We want to emphasize that our working hypothesis (Fig. 8) does not exclude a Q-cycle as proposed by Suzuki and King [36]. A consequence of our findings is that any hypothesis on the electron-transfer pathway and proton translocation of NADH:Q oxidoreductase, like the ones put forward in an extensive study of Krishnamoorthy and Hinkle [40], should take its dimeric behavior into account.

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