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Three Classes of Inhibitors Share a Common Binding Domain in Mitochondrial Complex I (NADH:Ubiquinone Oxidoreductase)*

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We have developed two independent methods to measure equilibrium binding of inhibitors to membrane-bound and partially purified NADH:ubiquinone oxidoreductase (complex I) to characterize the binding sites for the great variety of hydrophobic compounds acting on this large and complicated enzyme. Taking advantage of a partial quench of fluorescence upon binding of the fenazaquin-type inhibitor 2-decyl-4-quinazolinyl amine to complex I in bovine submitochondrial particles, we determined a K_d of 17 ± 3 nM and one binding site per complex I. Equilibrium binding studies with [^{3}H]dihydrorotenone and the aminopyrimidine [^{3}H]AE F119209 (4(*cis*-4-[^{3}H]isopropyl cyclohexylamino)-5-chloro-6-ethyl pyrimidine) using partially purified complex I from *Musca domestica* exhibited little unspecific binding and allowed reliable determination of dissociation constants.

Competition experiments consistently demonstrated that all tested hydrophobic inhibitors of complex I share a common binding domain with partially overlapping sites. Although the rotenone site overlaps with both the piericidin A and the capsaicin site, the latter two sites do not overlap. This is in contrast to the interpretation of enzyme kinetics that have previously been used to define three classes of complex I inhibitors. The existence of only one large inhibitor binding pocket in the hydrophobic part of complex I is discussed in the light of possible mechanisms of proton translocation.

The proton-pumping NADH:ubiquinone oxidoreductase (EC 1.6.99.3, complex I) is the first membrane-bound electron transport complex of the mitochondrial respiratory chain. Electron transfer from NADH to ubiquinone is coupled to the translocation of two protons per electron across the inner mitochondrial membrane (1, 2). Thereby, complex I accounts for up to 40% of the proton-translocating capacity of the respiratory chain.

Complex I is present in the mitochondria of most eukaryotic organisms and many bacteria. In mammals, it consists of 43 different subunits with a molecular mass of ~1,000 kDa (3). The homologous procaryotic complex I has a minimal number of 14 different subunits with a total molecular mass of ~500 kDa (4, 5).

Electron microscopic analysis of the *Neurospora crassa* (6), *Escherichia coli* (7), and bovine complex I (8) indicates an L-shaped structure with two domains arranged perpendicular to each other that are called peripheral and membrane arm (9). In mitochondrial complex I, seven nuclear-encoded proteins with strong homology to their bacterial counterparts form the peripheral part (10, 11). These subunits carry the NADH binding site and the redox groups, namely noncovalently bound FMN and the iron-sulfur centers N-1 to N-5 (12). The membrane arm contains the remaining seven subunits homologous to the bacterial complex, which are encoded by the mitochondrial genome in eucaryotes.

Despite recent progress in structural knowledge, little is known about the electron pathway, the proton translocation mechanism, and the binding sites and mode of action of the large number of specific inhibitors of complex I. However, it seems inevitable to conclude from the available evidence that the proton translocating machinery resides largely in the membrane part, although all known prosthetic groups have been assigned to the peripheral part of the enzyme (13). This has revived earlier ideas (14) that a mechanism similar to the proton motive ubiquinone cycle operating in the cytochrome bc_1 complex (15) confers proton translocation in complex I (13, 16). These hypothetical mechanisms inherently predict that the hydrophobic part of complex I carries two or three independently operating reaction sites for ubiquinone.

Many structurally diverse hydrophobic compounds have been described to inhibit complex I and are considered to interfere with ubiquinone reduction (12, 17, 18). Kinetic studies suggest that these inhibitors can be grouped into two (19) or even three (20) classes, represented by piericidin A (class I/A-type), rotenone (class II/B-type), and capsaicin (C-type), respectively. It remains unclear, however, whether these classes in fact reflect three distinct inhibitor and quinone binding sites. Two different semiquinone species have been reported by EPR spectroscopy during the steady state reaction of complex I (21), but there is still some controversy whether these reflect two ubiquinones or two forms of the same ubiquinone (22). The problem with the large number of studies employing Michaelis-Menten type kinetics (19, 23–27) is that the physical properties of the substrate, the inhibitors, and the membrane-bound enzyme as well as the complexity of the underlying catalytic mechanism make interpretation of these data difficult and ambiguous. Especially, because of their amphiphilic properties, ubiquinone and the inhibitors tend to accumulate in the small hydrophobic membrane phase so that the actual target site concentrations are very difficult to determine. This would be essential to calculate meaningful kinetic parameters.

Therefore, we have developed two independent approaches to investigate equilibrium inhibitor binding to complex I. This allowed us to test directly if representative complex I inhibitors interact with each other at their cognate binding sites and how these binding sites relate to each other.

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EXPERIMENTAL PROCEDURES

Inhibitors—All inhibitors were used as ethanolic stock solutions. The synthetic capsaicin analogue CC 44[1] (28) and 5′-β-epirotenone (29) were kind gifts from H. Miyoshi, Kyoto; DQA (SAN 549 (30)), fenazaquin, pyrimidifen, and fenpyroximate 4(*cis*-4-*tert*-butylcyclohexylamino)5-chloro-6-ethylpyrimidine (AEF117223) were obtained from AgrEvo, Frankfurt; Kresoxim-Methyl Brio® was a kind gift from BASF, Ludwigshafen, Germany; piericidin A was a kind gift from A. Dupuis, Grenoble; rolliniastatin-1 and rolliniastatin-2 were kind gifts from M. Degli Esposti, Clayton, Australia. [isopropyl-^{3}H]Dihydrorotenone, 1.89 TBq mmol^{-1}, was synthesized by Amersham Pharmacia Biotech. 4(*cis*-4-[^{3}H]Isopropyl cyclohexylamino)5-chloro-6-ethyl pyrimidine ([^{3}H]AE F119209), 2.06 TBq mmol^{-1}, was synthesized by Roussel Uclaf, Romainville, France. All other chemicals were purchased from Sigma or Carl Roth GmbH & Co. (Karlsruhe) in analytical quality.

Preparation of Bovine Submitochondrial Particles—Mitochondria were isolated as described by Smith (31). Bovine submitochondrial particles (SMP) were prepared essentially as described by Thierbach and Reichenbach (32). Mitochondria were diluted in 250 mM sucrose, 10 mM potassium phosphate, 10 mM Tris/HCl, 2 mM EGTA, 2 mM $MgCl_2$, pH 7.4, to a protein concentration of ~10 mg/ml. Batches of about 25 ml were treated 10 times for 15 s with a Branson sonifier 250 (Branson, Danbury, CT) at maximum output energy in an ice bath. The sonicated suspension was centrifuged at 10,000 × *g* for 10 min, and the supernatant was centrifuged at 100,000 × *g* for 45 min at 4 °C. The pellet was resuspended in 1 mM EDTA, 1 mM $MgCl_2$, 75 mM sodium phosphate, pH 7.4, and could be stored for months in liquid nitrogen. The content of cytochrome *c* oxidase was determined by the reduced minus oxidized spectrum at 605–630 nm ($\epsilon_{605-630\ nm}$ = 24.0 mM^{-1} × cm^{-1}).

Preparation of Housefly SMP—20 g of housefly (*Musca domestica*) thoraces were homogenized at 4 °C in 150 ml of 154 mM KCl, 1 mM EDTA, adjusted to pH 7.4, following the procedure of Nedergaard and Cannon (33). After filtration through two layers of cheesecloth, the homogenate was centrifuged for 10 min at 500 × *g*. The pellet was discarded, and the supernatant was centrifuged for 10 min at 3000 × *g*. The pellet was resuspended in 20 mM Tris/HCl, pH 8.0, 100 mM KCl, 1.0 mM EDTA, and the protein concentration was adjusted to 10 mg/ml.

Solubilization and Partial Purification of Housefly Complex I—Housefly SMP suspensions were solubilized by the addition of 4% (w/v) dodecylmaltoside and 150 mM KCl for 30 min at 4 °C and centrifuged at 100,000 ×g for 60 min at 4 °C. The supernatant was partly delipidated on a Sephacryl S200 column (26 × 600 mm) in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.1% (w/v) CHAPS at 4 °C. Fractions of the void volume showing *n*-decylubiquinone-dependent NADH oxidation were pooled and further purified on a Q-Sepharose column (26 × 100 mm) equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0.1–1.0 M NaCl in 20 mM Tris/HCl, pH 8.0, 0.1% (w/v) CHAPS. Fractions exhibiting rotenone-sensitive NADH:ubiquinone oxidoreductase activity were pooled and stored in aliquots at −80 °C.

Determination of Catalytic Activity—We used *n*-nonylubiquinone (NBQ) as a substrate for the determination of NADH:ubiquinone activity of SMP, which has been reported as one of the best ubiquinone-10 analogues for this purpose (25, 34). NBQ was prepared essentially following the protocol of Wan *et al.* (35). Steady state activity was recorded in a Shimadzu UV-300 spectrophotometer as NADH oxidation at 340–400 nm ($\epsilon_{340-400\ nm}$ = 6.10 mM^{-1} × cm^{-1}) using a thermostatted cuvette (30 °C) with a final volume of 1 ml. 100 μM NADH and 50 μg of SMP were added to buffer containing 50 mM Tris/HCl, pH 7.4, 5 μM Kresoxim-Methyl Brio® and 2 mM KCN. The catalytic reaction was started by the addition of 60 μM NBQ. Inhibitors were added to the cuvette before the addition of NBQ. Michaelis-Menten parameters were determined by varying the concentration of NADH or NBQ.

Fluorescence Measurements—Fluorescence spectra were recorded on a SPEX Fluorolog 212 fluorometer attached to an AT-type personal computer. The fluorescence quench titrations (FQT) were performed and analyzed as described earlier (36) by directly fitting the data to a formula derived directly from the standard binding equation.[2] DQA ($\epsilon_{291\ nm}$ = 8.14 mM^{-1} × cm^{-1} in ethanol) was automatically added to a stirred cuvette in 1-μl steps from a 15 μM stock solution in ethanol using a Hamilton Microlab M dispensor equipped with a 50-μl syringe. For FQT measurements, bovine SMP were diluted in N_2-saturated buffer (2 mM KCN, 1 mM EDTA, 1 mM $MgCl_2$, 2.5 μM Kresoxim-Methyl Brio®, 75 mM sodium phosphate, pH 7.4) to 2.5 mg of protein/ml, corresponding to ~1.5 μM cytochrome *c* oxidase. Based on a complex I to cytochrome *c* oxidase ratio of 1:10 in bovine SMP (37, 38), the complex I concentration was estimated at 0.15 μM or 0.06 nmol/mg of protein.

Indirect determination of the dissociation constant K_d for the binding of 5′-β-epirotenone by fluorescence quench titration was performed based on the standard binding equation (36). The data were analyzed using the Psiplot software package version 4.61 (Poly Software International).

Radioligand Binding Assays—Specific binding of two radiolabeled inhibitors [^{3}H]dihydrorotenone (39–41) and [^{3}H]AE F119209 to the solubilized and partially purified NADH:ubiquinone oxidoreductase from housefly flight muscle mitochondria (18) was measured as follows.

For saturation binding experiments, 3 μg of protein in 20 mM Tris/HCl, pH 8.0, 0.25% (w/v) CHAPS radioligand concentrations ranging from 0.4 to 100 nM were incubated at 22 °C in a sample volume of 100 μl. In competition experiments, the radioligand concentration was fixed at 6.5 nM, and variable concentrations of competing ligands were added. Unspecific binding was determined using 10 μM unlabeled rotenone or AE F119209, respectively. Methanol at a final concentration of 5% (v/v) in the assay mix was used to mediate the dissolution of radioligands and other inhibitors. After 20 min, 300 μl of 10 mg/ml dextran-coated charcoal (Sigma) in 20 mM Tris/HCl, pH 7.2, were mixed in thoroughly. The charcoal was sedimented by centrifugation at 13,000 × *g* for 3 min. Protein-bound radioligand was measured in the supernatant by liquid scintillation counting. Data were analyzed by standard algorithms with either the EBDA (Biosoft, UK) or the SigmaPlot (Jandel Scientific) software package.

The dissociation constant of complex I inhibitors that competed with equilibrium binding of radiolabeled AE F119209 and dihydrorotenone was determined by measuring the amount of bound radioligand in the presence of increasing concentrations of unlabeled inhibitor. From the resulting logistic plots, apparent B_{50} values were determined as the concentration of competing inhibitor required to displace 50% of the radioligand. The apparent dissociation constants K_d were calculated according to the Cheng-Prusoff equation (42, 43): $K_d = B_{50}/1 + [L]/K_d^{[L]}$, where $K_d^{[L]}$ is the dissociation constant of the radioligand, and [L] is the concentration of free radioligand.

RESULTS

Kinetic Constants and I_{50} Values from Steady State Kinetics—To test for the activity of complex I in our SMP preparation, we determined the Michaelis-Menten parameters for NADH and NBQ. The K_m values were 3.9 ± 0.5 μM for NADH and 2.3 ± 0.2 μM for NBQ. V_{max} was 1.16 ± 0.03 μmol of NADH × min^{-1} × mg^{-1} of protein. These values are comparable with those reported by others (3, 25, 44, 45). No increase in steady state activity was observed by applying the "activation" procedure described in Burbaev *et al.* (46).

I_{50} values were determined as the final concentration of inhibitor required to reduce the NADH oxidation rate to 50% of

[1] The abbreviations used are: CC 44, 4-(*p-tert*-butylphenoxy)benzoic acid-3,4-dimethoxybenzylamide; AE F117233, 4(*cis*-4-*tert*-butylcyclohexylamino)5-chloro-6-ethyl pyrimidine; AE F119209, 4-(*cis*-4-[^{3}H]isopropyl cyclohexylamino)5-chloro-6-ethyl pyrimidine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DQA, 2-decyl-4-quinazolinyl amine; FQT, fluorescence quench titration; NBQ, *n*-nonylubiquinone; SMP, submitochondrial particles.

[2] The formula used to analyze FQT is as follows. The observed fluorescence F_{obs} during FQT is given by

$$F_{obs} = (f_{bound} - f_{free}) \times \{Q - \sqrt{(Q^2 - n_s \times [E_{tot}] \times [I_{tot}])}\} + f_{free} \times [I_{tot}] \quad \text{(Eq. 1)}$$

with

$$Q = \frac{1}{2} \times ([I_{tot}] + K_d + n_s \times [E_{tot}]) \quad \text{(Eq. 2)}$$

and

$$[I_{tot}] = [I_{bound}] + [I_{free}] \quad \text{(Eq. 3)}$$

where f_{bound} and f_{free} are the specific fluorescence of the bound and free inhibitor, $[I_{tot}]$, $[I_{bound}]$, and $[I_{free}]$ are the concentrations of total, bound, and free inhibitor, $[E_{tot}]$ is the total concentration of enzyme, and n_s is the number of binding sites.

TABLE I

I_{50} values and competition binding analysis of complex I inhibitors

The inhibitors are grouped according to the classification by Friedrich *et al.* (19) and Degli Esposti and Ghelli (20). I_{50} values were determined as described under "Experimental Procedures." Displacement was tested by adding 0.3 μM (2-fold molar excess) of inhibitor before FQT. Under these conditions, DQA binding was prevented by all inhibitors tested except by 5′-β-epirotenone and CC 44. In the case of 5′-β-epirotenone, an increase in apparent K_d for DQA with increasing concentrations of competing inhibitor was observed and used to indirectly calculate the K_d for this inhibitor. In the case of CC 44, concentrations up to 10 μM did not affect the DQA titrations, but higher concentrations resulted in unspecific distortions of the titration (see text for further details). Competition experiments with [^{3}H]dihydrorotenone or [^{3}H]AE F119209 were performed as described. ND, not determined.

Inhibitor	I_{50}	Displacement of DQA	K_d	
			Competition with AE F119209	Competition with dihydrorotenone
	nM		*nM*	
Class I/A-type				
DQA	6		ND	ND
AE F117233	ND	ND	2.9	6.7
Fenazaquin	6	Yes	ND	ND
Fenpyroximate	10	Yes	21	24
Piericidin A	5	Yes	5.7	10
Pyrimidifen	2	Yes	7.2	6.8
Rolliniastatin-1	2	Yes	4.8	3.6
Rolliniastatin-2	3	Yes	5.7	2.8
Class II/B-type				
Rotenone	20	Yes	12	22
5′-β-Epirotenone	11,000	Yes	ND	ND
C-type				
CC 44	80	No	No competition	130

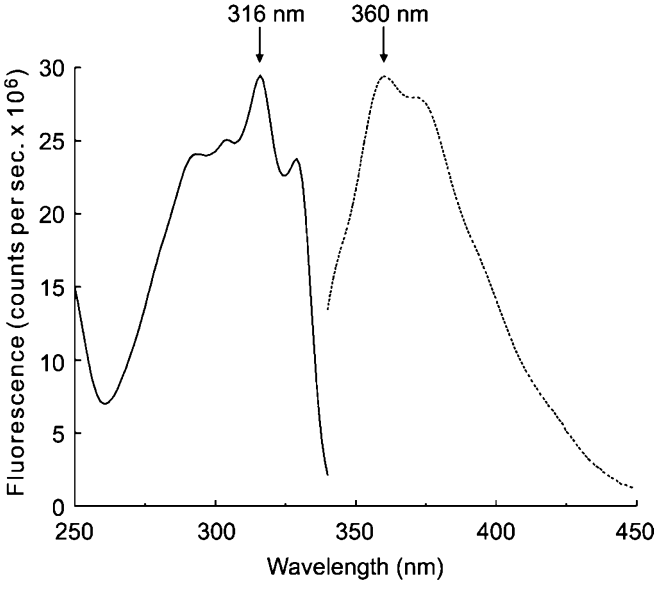

FIG. 1. **Fluorescence spectra of DQA.** The spectra of 10 μM DQA were recorded in 1 mM EDTA, 1 mM $MgCl_2$, 0.1% dodecylmaltoside, 75 mM sodium phosphate, pH 7.4. The background spectra resulting from the buffer were subtracted. ——, excitation spectrum (emission at 360 nm); - - - - - -, emission spectrum (excitation at 316 nm).

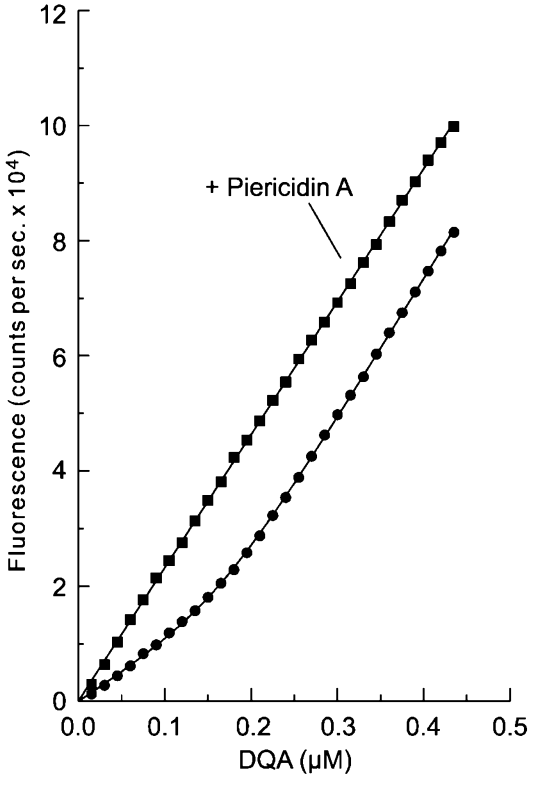

FIG. 2. **Mutual displacement of DQA by different complex I inhibitors.** The experimental conditions were as described under "Experimental Procedures" and in the legend of Table I. ●, bovine SMP, the *line* represents the least squares fit, calculated as described in Brandt and von Jagow (36). ■, bovine SMP, preincubated with 0.3 μM piericidin A.

the uninhibited rate. The data listed in Table I are in good agreement with published values (17, 28, 47). An I_{50} value of 6 nM was determined for DQA, the fenazaquin-type inhibitor used in the FQT binding assay. DQA is an inhibitor specific for complex I, as it had no effect on the activity of succinate dehydrogenase or cytochrome bc_1 complex (data not shown).

Analysis of DQA Binding to Bovine Complex I by Fluorescence Quench Titration—Fig. 1 shows the fluorescence spectra of DQA in aqueous solution. The excitation maximum at 316 nm, and the emission maximum at 360 nm were used to follow DQA binding to complex I. The typical titration given in Fig. 2 shows that the fluorescence of DQA was partially quenched when bound to the enzyme. According to the numerical fit of the data, fluorescence was quenched by 62 ± 3% upon binding, and the K_d was 17 ± 3 nM (n = 15). The concentration of binding sites was found to be 0.15 ± 0.02 μM, which fits perfectly with one binding site per complex I and a ratio between cytochrome *c* oxidase and complex I of 1:10 (37, 38). Neither activation of bovine SMP at 30 °C for 90 min as described in Burbaev *et al.* (46) nor addition of 1% bovine serum albumin or 10 μM Kresoxim-Methyl Brio® or 3% ethanol or 1 mM *N*-ethylmaleimide had any effect on the K_d or the number of binding sites for DQA (data not shown).

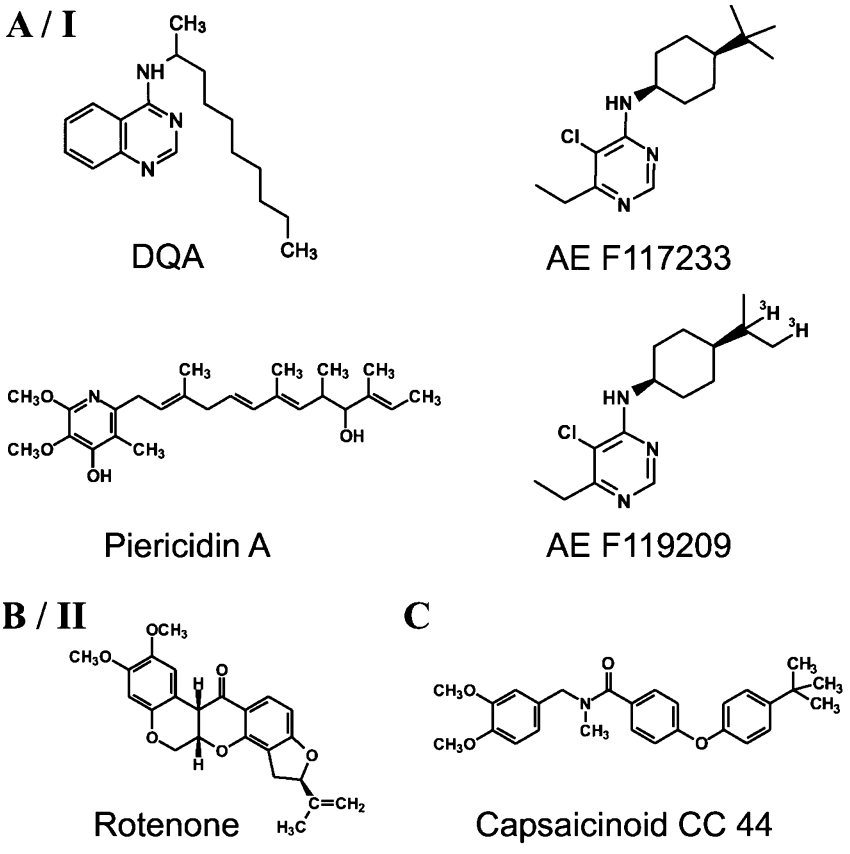

FIG. 3. **Structures of selected inhibitors.**

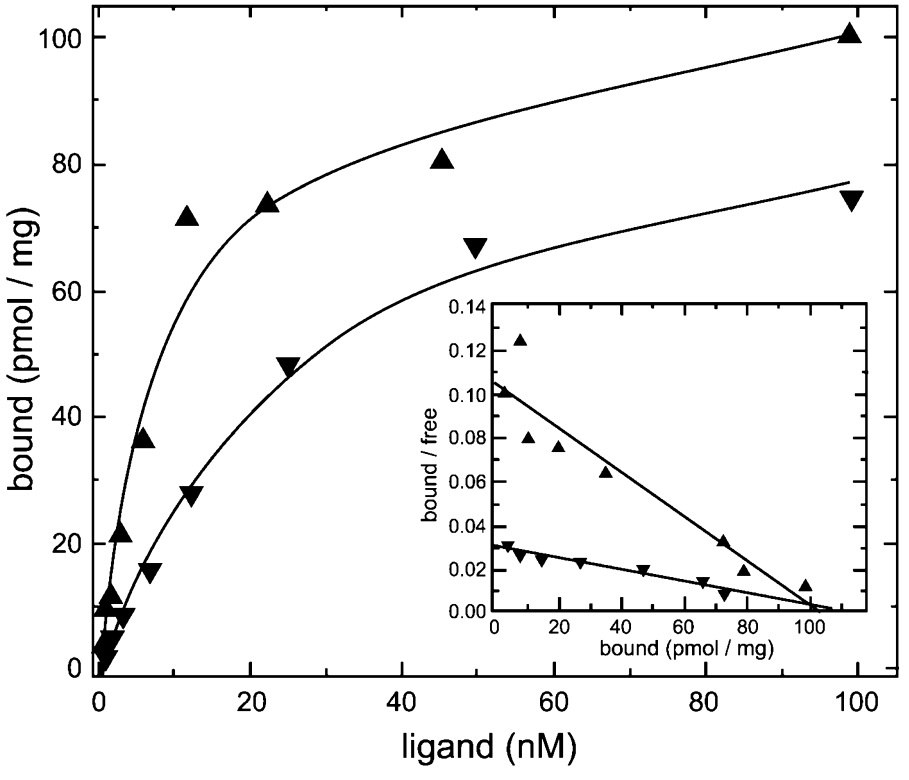

FIG. 4. **Radioligand binding analysis of dihydrorotenone and AE F119209 to *Musca* complex I.** Binding data obtained for both radiolabeled complex I inhibitors to solubilized enzyme are plotted directly and in Scatchard representation (*inset*). ▲, AE F119209. K_d = 9 nM; $bound_{max}$ = 100 pmol/mg. ▼, dihydrorotenone. K_d = 29 nM; $bound_{max}$ = 110 pmol/mg. See text for details.

When SMP were preincubated with 0.3 μM piericidin A (Fig. 2) rotenone, rolliniastatin-1, or rolliniastatin-2, the fluorescence of added DQA was not quenched, indicating displacement of DQA from its binding site. The results for all tested complex I inhibitors are summarized in Table I. Even the rather weak binding rotenone analogue 5′-β-epirotenone (I_{50} = 11 μM) shifted the apparent K_d for DQA when added at concentrations between 1 and 100 μM. From these data a K_d of 6 ± 2 μM (n = 7) for 5′-β-epirotenone was calculated.

Only the capsaicin derivative CC 44 (28) did not affect the FQT titration up to a concentration of 10 μM, indicating that it failed to specifically displace DQA. At higher concentrations of

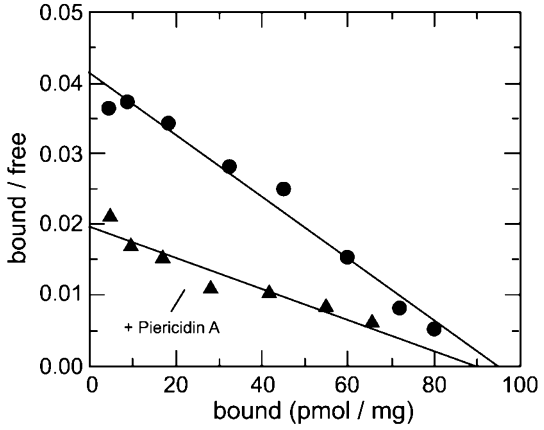

FIG. 5. **Scatchard analysis of dihydrorotenone binding in the presence of piericidin A.** Saturation binding was determined as described in the presence (▲) or absence (●) of 10 nM piericidin A.

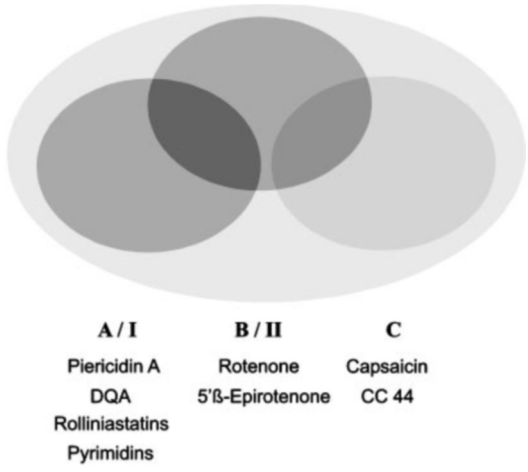

FIG. 6. **Schematic representation of the inhibitor binding domain of complex I.** The binding sites for the three classes of hydrophobic complex I inhibitors as deduced from equilibrium binding studies are depicted to illustrate their relative arrangement in a common binding pocket.

CC 44, the titrations were distorted and could not be fitted to the equation. However, this could not be because of specific displacement, as from an I_{50} of 80 nM for CC 44 (*cf.* Table I) one can predict that 10 μM CC 44 should have had a dramatic effect on the apparent K_d for DQA. This is also illustrated by the fact that at 10 μM even the two orders of magnitude weaker-binding 5′-β-epirotenone had a significant effect.

Binding of AEF119209 and Dihydrorotenone to Partially Purified Musca Complex I—Binding of the tritiated aminopyrimidine AE F119209 (Fig. 3) to the partially purified housefly complex I was found to be specific (90–95% specific binding) and saturable with an apparent dissociation constant of 9 nM as determined by Scatchard transformation of the data (Fig. 4). The maximum number of binding sites B_{max} was 0.1 nmol/mg. Dihydrorotenone also exhibited saturable binding with an apparent K_d of 30 nM and a B_{max} of around 0.1 nmol/mg. The Scatchard plots (Fig. 4, *inset*) and Hill plots (not shown) indicated a homogeneous population of a single binding site for either ligand. Careful analysis of several independent experiments also gave no indication for two binding sites for dihydrorotenone or the aminopyrimidine, as it was not possible to fit the data to two components in any meaningful way.

To study whether dihydrorotenone and the class I inhibitors bound competitively, the saturation binding of labeled dihydrorotenone was measured in the presence or absence of 10 nM of the class I inhibitor piericidin A. Scatchard analysis of the equilibrium binding data (Fig. 5) indicated that the apparent B_{max} of the radioligand was not changed when piericidin A was present, *i.e.* the binding was competitive with respect to the radioligand. The same result was obtained when the aminopyridine inhibitor AE F117233 was used as a competitor for dihydrorotenone (data not shown).

Representatives of each class of complex I inhibitors were tested for their capacity to compete with a fixed concentration of the radioligands AE F119209 or dihydrorotenone under equilibrium binding conditions. The K_d values calculated from these competition experiments were in good agreement with the relative I_{50} values determined by titration of the steady state rate (Table I). With one important exception, both radioligands competed with all tested inhibitors and gave very similar K_d values. The capsaicinoid CC 44 competed with dihydrorotenone but did not affect binding of AE F119209 even at a concentration of 10 μM.

DISCUSSION

The large number of structurally different compounds that have been described to specifically inhibit ubiquinone reduction by proton-translocating NADH:ubiquinone oxidoreductase (17) are potentially very useful to probe the mechanism of this most complicated enzyme of the respiratory chain. These compounds are also of considerable interest as lead structures for the development of insecticides and acaricides (18). However, even the number of independent binding sites is still controversial. The major problem has been that most inhibitor studies with complex I were based on the interpretation of data from steady state kinetics. This approach can only generate indirect evidence that is difficult to validate because of the complexity of the enzyme and experimental complications inherent to the steady state kinetics of complex I (44). Several reports on direct binding studies using radioligands (27, 48) and competition experiments with a limited selection of inhibitors (41) have been published. However, these studies were performed with membrane-bound complex I and suffered from a high degree of nonspecific binding, *e.g.* several washes with bovine serum albumin were necessary to distinguish between specific and nonspecific binding (48), and saturation of the binding sites was not achieved, preventing unambiguous interpretation.

Here we report results from two independent approaches to study equilibrium binding of hydrophobic inhibitors to membrane-bound and partially purified complex I. Both methods were not affected by nonspecific binding effects and gave consistent and reliable results. We found no influence on our FQT measurements by a number of treatments including activation of complex I (46) and addition of bovine serum albumin or the thiol reagent *N*-ethylmaleimide, which were claimed to affect inhibitor binding (48, 50).

To check whether the classification into two (19) or three (20) inhibitor classes represented by piericidin A (class I/A-type), rotenone (class II/B-type), and capsaicin (C-type) in fact reflects two or even three independent binding sites, we have performed direct competition experiments with a representative selection of inhibitors. The data obtained with both methods consistently indicated that all tested hydrophobic inhibitors of complex I share a common binding domain with partially overlapping sites (*cf.* Table I). As illustrated in Fig. 6, the rotenone site (class II/B-type) overlaps with both the piericidin A site (class I/A-type) and the capsaicin site (C-type), but binding of the latter two types of inhibitors does not interfere with each other.

Overlapping binding sites for class I and class II inhibitors have also been suggested from recent results by Darrouzet and Dupuis (51), who have reported a point mutation in complex I

from *Rhodobacter capsulatus* that confers resistance to piericidin A and exhibits cross-resistance to rotenone. The idea of a fairly large ubiquinone binding domain also fits well with recently published data showing that this pocket is sufficiently spacious to accommodate rather bulky exogenous ubiquinones (52).

The observation that some, but not all complex I inhibitors also inhibit bacterial glucose:ubiquinone oxidoreductase (19) can be interpreted in terms of structural similarity of its ubiquinone reactive site to part of the complex I binding pocket. However, some of the conclusions based on enzyme kinetics claiming independent inhibitor binding sites have to be considered as taking the interpretation of this indirect approach too far (45).

We have also found no indications from our equilibrium binding data that there is more than one binding site for piericidin A or rotenone-type inhibitors per complex I as has been concluded indirectly from kinetic studies. (17, 26, 53). The number of binding sites we could identify matched exactly the amount of inhibitor needed to completely block the activity of complex I (38), and in all cases of competitive binding, inhibitors were always displaced completely. Thus, if there where two inhibitor binding sites per complex I, they would have to be indistinguishable in terms of ligand affinity. In the absence of compelling evidence in favor of such unusual binding site heterogeneity, we consider this option as highly unlikely.

It should be noted that in the light of our results, the grouping of complex I inhibitors into three distinct classes, which we have still used to be consistent with the literature, seems somewhat arbitrary, *e.g.*, although kinetic data seem to indicate that the binding sites for piericidin A, DQA, and the aminopyridines are somehow related and largely overlapping, the structural differences between these three compounds suggest that the sites are not identical.

It should be noted that the emerging picture of a fairly large ubiquinone binding pocket with several binding sites for structurally diverse inhibitors in the membrane part of complex I (Fig. 6) is very similar to the now well documented situation (by x-ray crystal structures) in the Q_B site of the bacterial reaction center (54) and in center P of the cytochrome bc_1 complex (55–57).

Taken together, we cannot entirely rule out reversible binding of more than one ubiquinone to complex I at this point. But considering the huge array of structurally diverse high affinity inhibitors known to inhibit ubiquinone reduction completely (17) that we have shown to interact with each other at their cognate binding sites, there is no indication for this. The observation of two distinct semiquinone species by EPR during steady state of complex I can still result from two ubiquinone molecules, one of which is the substrate exchanging with the membrane, whereas the other is tightly bound to the complex acting as a prosthetic group. This situation would be reminiscent to Q_B and Q_A in the bacterial reaction center (58).

If there is in fact only one substrate binding site, this seems difficult to reconcile with the mechanistic models of the reverse ubiquinone-cycle-type that have been put forward recently (13, 16). Such ligand conduction reaction schemes require at least two such sites, one for ubiquinol oxidation and one for ubiquinone reduction. However, the redox-gated ligand conduction mechanism (13) can be modified to a localized mechanism by replacing two substrate sites with a single tightly bound ubiquinone. The modified mechanism is based on the same general mechanistic principles, still employs the redox-dependent protonation and deprotonation of ubiquinone, and features one tightly bound and one substrate ubiquinone (49).

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