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 fenazine-type inhibitor 2-decyl-4-

quinazolinyl amine to complex I in bovine mitochondrial particles, we determined a $K_p$ of $17 \pm 3$ nm and one binding site per complex I. Equilibrium binding studies with $[3H]$dihydrotrotenone and the aminopyrimidine $[3H]$AE F119290 (4-cis-$[3H]$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 capsacain 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 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 $b_{1}c_{1}$ 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 capsacain (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 capsacian analogue CC 44 (28) and 5'-β-epitropane (29) were kind gifts from H. Miyoshi, Kyoto; DQA (SAN 549 (30)), fenazaquin, pH 7.4, to a protein concentration of $3 \times 10^{-5}$ M for NBQ. The pellet was resuspended in 20 mM Tris/HCl, pH 8.0, 100 mM KCl, 1.0 mM potassium phosphate, 10 mM Tris/HCl, 2 mM EGTA, 2 mM MgCl$_2$.

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/HC1, 2 mM EDTA, 2 mM MgCl$_2$, pH 7.4, to a protein concentration of $10\mu g/ml$. 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 and centrifuged at 100,000 g for 45 min at 4°C. The pellet was resuspended 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/HC1, 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 dihydrorotone was determined by measuring the amount of bound radioligand in the assay mix as a function of the concentration of free radioligand. The data were analyzed according to the Cheng-Prusoff equation (42, 43): $K_d = B_{max}/1 + [L]/[K_d]$. 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 constants for NADH and NBQ. $K_m$ 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).

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**RESULTS**

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

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

$$F_{obs} = (F_{saturated} - F_{free}) \times Q - \sqrt{Q^2 - n \times [E_{bound} \times [I_{bound}]] + F_{free} \times [I_{total}]} \tag{Eq. 1}$$

with

$$Q = \frac{1}{2} \times \left[ [I_{free}] + K_d \times [E_{bound}] \right] \tag{Eq. 2}$$

and

$$[I_{total}] = [I_{bound}] + [I_{free}] \tag{Eq. 3}$$

where $F_{saturated}$ and $F_{free}$ are the specific fluorescence of the bound and free inhibitor, $[I_{bound}]$, $[I_{bound}]$, and $[I_{free}]$ are the concentrations of total, bound, and free inhibitor, $[I_{total}]$ is the total concentration of enzyme, and $n_i$ is the number of binding sites.

The abbreviations used are: CC 44, 4-p-tet-butylphenoxbenzoxo acid-3,4-dimethoxybenzaldehyde; AE F117233, 4-cis-4-tet-butylcyclonexylaminio-5-chloro-6-ethylpyrimidine; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DQA, 2-decyl-4-quinazoliny imine; FQT, fluorescence quench titration; NBQ, n-nonylubiquinone; SMP, submitochondrial particles.
the uninhibited rate. The data listed in Table I are in good agreement with published values (17, 28, 47). An I50 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 bc1 complex (data not shown).

### Table I

| Inhibitor                  | I50  | Displacement of DQA | Competition with AE F119209 | Competition with dihydrorotenone |
|----------------------------|------|----------------------|-----------------------------|---------------------------------|
| **Class I/A-type**         |      |                      |                             |                                 |
| DQA                       | 6    | No                   | ND                          | ND                              |
| AE F117233                | ND   | No                   | 2.9                         | 6.7                             |
| Fenazaquin                | 6    | Yes                  | 21                          | 24                              |
| Fenpyroximate             | 10   | Yes                  | 5.7                         | 10                              |
| Piericidin A              | 5    | Yes                  | 7.2                         | 6.8                             |
| Pyrimidifen               | 2    | Yes                  | 4.8                         | 3.8                             |
| Rolliniastatin-1          | 2    | Yes                  | 5.7                         | 2.8                             |
| Rolliniastatin-2          | 10   | Yes                  | 12                          | 22                              |
| **Class I/B-type**         |      |                      |                             |                                 |
| Rotenone                  | 20   | Yes                  | ND                          | ND                              |
| 5'-β-Epirotenone          | 11,000| Yes                 | 12                          | 22                              |
| **C-type**                |      |                      |                             |                                 |
| CC 44                     | 80   | No                   | No competition              | 130                             |

The inhibitors are grouped according to the classification by Friedrich et al. (19) and Degli Esposti and Ghelli (20). I50 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-epirotene and CC 44. In the case of 5'-β-epirotene, an increase in apparent Kd for DQA with increasing concentrations of competing inhibitor was observed and used to indirectly calculate the Kd 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 [3H]dihydrorotenone or [3H]AE F119209 were performed as described. ND, not determined.

**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 Kd 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 Kd or the number of binding sites for DQA (data not shown).
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 (I₅₀ = 51.5 μM) shifted the apparent Kₛ for DQA when added at concentrations between 1 and 100 μM. From these data a Kₛ 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
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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 \mu M 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 \mu M even the two orders of magnitude weaker-binding 5'-\beta-epirotenone had a significant effect.

Binding of AE F119209 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 dihydrotetenone or the aminopyrimidine, as it was not possible to fit the data to two components in any meaningful way.

To study whether dihydrotetenone and the class I inhibitors bound competitively, the saturation binding of labeled dihydrotetenone 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 dihydrotetenone (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 dihydrotetenone 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 dihydrotetenone but did not affect binding of AE F119209 even at a concentration of 10 \mu M.

**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 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 (45) and in center P of the cytochrome bc1 complex (55–57).

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 Qo site of the bacterial reaction center (54) and in center P of the cytochrome bc1 complex (55–57).

Tabeul 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 Qo and Qh 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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