Inhibitor Probes of the Quinone Binding Sites of Mammalian Complex II and Escherichia coli Fumarate Reductase*

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The structural and catalytic properties of beef heart succinate dehydrogenase (succinate-ubiquinone oxidoreductase, complex II) and Escherichia coli fumarate reductase are remarkably similar. One exception is that whereas electron exchange between the mammalian enzyme and its quinone pool is inhibited by thenoyltrifluoroacetonate and carboxanilides, the enzyme from E. coli is not sensitive to these inhibitors. The lack of good inhibitors has seriously hampered the elucidation of the mechanism of quinone oxidation/reduction in the E. coli enzyme. We have previously reported (Tan, A. K., Ramsay, R. R., Singer, T. P., and Miyoshi, H. (1993) J. Biol. Chem. 268, 19328–19333) that 2-alkyl-4,6-dinitrophenols inhibit mammalian complexes I, II, and III, but with different potencies and kinetic characteristics. Based on these studies we have selected a series of 2-alkyl-4,6-dinitrophenols which proved to be very effective non-competitive inhibitors of mammalian complex II, particularly when acting in the direction of quinone reduction, the physiological event. These compounds turned out to be even more potent inhibitors of E. coli fumarate reductase, particularly when acting in the direction of quinol oxidation, again, the physiological event. Kinetic analysis revealed that both enzymes 2 inhibitor binding sites seem to be involved in the oxidation of succinate by quinone, but one seems to be functioning when fumarate is reduced by external quinol. Since the E. coli enzyme can be modified by site-directed mutagenesis, these studies were extended to four mutants of fumarate reductase, impaired by single amino acid substitutions at either of the putative quinone binding sites (QA or QB) of the enzyme. The results were analyzed in terms of the model of these dual sites of quinone binding in fumarate reductase, as well as the nature of the substituent in the 2-position of the dinitrophenol inhibitors.

Beef heart succinate dehydrogenase (succinate-ubiquinone oxidoreductase, complex II) and Escherichia coli fumarate reductase (menaquinol-fumarate oxidoreductase) exhibit remarkably similar catalytic and structural properties. Each complex consists of four subunits, including a flavoprotein subunit containing an Ba-histidyl-FAD moiety, and an iron-sulfur subunit with three distinct Fe-S clusters that transport electrons between the FAD and bound quinones, and two transmembrane polypeptides which also contain the quinone binding sites (1). The anchor subunits show very little sequence homology among species, which probably accounts for the fact that thenoyltrifluoroacetonate and carboxanilides, two types of highly potent inhibitors of electron transfer to quinone in the mammalian succinate dehydrogenase complex (2–4), do not inhibit E. coli fumarate reductase (1). The lack of good inhibitors for the E. coli fumarate reductase has hampered attempts to discern a common mechanism of quinone interaction and the identification of binding sites utilizing site-directed mutagenesis.

Saifor et al. (5) reported that a series of 2-alkyl-4,6-dinitrophenols inhibit both photosystem II and the mammalian bc1 complex by interaction at the quinone sites. Further examination showed that the potency of these inhibitors with mammalian complexes I (NADH-ubiquinone oxidoreductase) and II (ubiquinol-cytochrome c oxidoreductase) was comparable, but that complex II (succinate dehydrogenase) was much less sensitive (6). These observations suggested that tailored 2-substituted 4,6-dinitrophenols may provide the means to probe selectively the structural and functional differences displayed at the quinone binding sites on respiratory chain dehydrogenases.

This work reports studies where derivatives of 4,6-dinitrophenol have now been identified that bring about complete inhibition of mammalian succinate dehydrogenase. Importantly, these analogues proved to be even more effective inhibitors of E. coli fumarate reductase. This report also compares the structure-potency relationships of several 2-alkyl-4,6-dinitrophenols as inhibitors of quinone reduction and quinol oxidation by beef heart complex II and by wild-type and mutant forms of E. coli fumarate reductase impaired by single amino acid substitutions at the putative QA and QB binding sites (7).

EXPERIMENTAL PROCEDURES

Materials—Beef heart electron transport particles (ETP, an inverted submitochondrial particle preparation) and complex II were isolated as in previous work (6). Membrane preparations from wild-type and mutant strains of E. coli were isolated as described elsewhere (7, 8). Construction and nomenclature of the mutant strains were described previously by Westenberg et al. (7).

Assays—Succinate oxidation was determined at 38°C with 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone utilizing 2,6-dichlorophenolindophenol as a terminal electron acceptor (E<sub>m</sub> <sub>c</sub> = 19.1), as in previous work (6). Samples of enzyme were preactivated in assay buffer (50 mM Tris, 0.1 mM EDTA, pH 7.6) containing 20 mM succinate and 1 mM KCN (38°C for 7 min). Fumarate reduction was measured under strictly anaerobic conditions with 2,3-dimethyl-1,4-naphthoquinol as electron acceptor (E<sub>m</sub> <sub>c</sub> = 19.1).

The abbreviations used are: QA, quinone binding site A, and QB, quinone binding site B.
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Inhibition of Fumarate Reductase Mutants—Westenberg et al. (7) concluded from their studies of point mutations in the anchor polypeptides (C and D subunits) of E. coli fumarate reductase that two quinone binding sites (QA and QB) were present and functioned similarly to those in photoreaction centers (10,11). In this mechanism, the quinone acting as primary electron acceptor at QA is nonexchangeable and functions between the oxidized and semiquinone states in passing single electrons to QB. Once fully reduced and protonated, the quinone exchanges with an oxidized quinone of the coenzyme Q pool. Quinol oxidation is assumed to proceed by a reversal of these steps (7). We selected four different mutants for the present study, two with an amino acid substitution in the putative QA site and two with a residue change in QB. All four mutations had been shown to partially inhibit catalysis with quinones. That one of the amino acids substituted might also be part of the inhibitor binding site was tested by screening each mutation for its effect on inhibitor potency (see Table IV).

Table IV presents the apparent $K_i$ values obtained for the 4,6-dinitrophenol derivatives acting on quinone reduction by each of the four mutants. Interestingly, and in contrast to what was observed with the wild-type enzyme, the inhibition of mutant enzymes was incomplete (approximately 80%). The data were analyzed in terms of one inhibition site and a residual, noninhibitable activity ($r$) using the equation,

$$V_{\text{max,obs}} = V_{\text{max}}/(1 + rV_{\text{max}}/K_i)$$

(Eq. 2)

Excellent fit of the data to this equation strongly indicates the operation of a single inhibition site giving the respective $K_i$ values listed in Table IV. Note that the value for $r$ with all inhibitors possessing an alkyl side chain was 0.2. It is not presently known whether any significance may be attached to the fact that this value of $r$ approximates that for $\beta$ (0.3), the fraction of electron flux inhibited at the weaker inhibitor site ($K_{\beta}$) in the wild-type enzyme (Table III). Note also that this noninhibitable pathway of electron flux arising in the mutants is detectable only in the direction of quinone reduction, not quinol oxidation (see below).

The inhibition of quinol oxidation by the mutants was non-competitive in all cases as illustrated for the inhibition of fumarate reductase of mutant E29D by compound 19 in Fig. 4. The interpretation of these data in terms of the type of amino acid replacement and the putative functioning of sites QA and QB will be presented under “Discussion.”

**DISCUSSION**

Continuing our previous studies to develop selective and effective probes of the quinone chemistry in the respiratory chain, we investigated four 2-alkyl-4,6-dinitrophenol derivatives as inhibitors of quinone reduction and oxidation in beef heart succinate dehydrogenase and E. coli fumarate reductase. The 2-alkyl substituents vary in shape, size and hydrophobicity and confer a slightly higher $pK_a$ (4.51) than that of the unsubstituted 4,6-dinitrophenol (4.09). Compounds 17 and 20 appeared to be potent inhibitors of the mammalian and E. coli enzymes. The common structural property of the two compounds is the existence of branching structure at $x$-position of the 2-substituent from the benzene ring. Molecular orbital calculations indicated that $x$-branching structure makes the alkyl chain almost perpendicular to the plane of the benzene ring, resulting in a configuration similar to isoprenoid side
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Fumarate reductase for which very few alternatives are available.

The inhibitor concentrations were: ● 1; ○, 0; △, 1 μM; ▲, 2.5 μM; ■, 5 μM. Inset, Dixon plot.

In the absence of detailed structural information, these observations are rationalized in terms of the mechanism of coenzyme Q reduction occurring in photoreaction centers (10, 11). As described above, the model visualizes two binding sites for quinones: theQA site containing a quinone that is the primary acceptor of electrons from the Fe-S clusters and passes electrons singly to a secondary quinone at theQB site. Once fully reduced, the secondary quinone exchanges with the coenzyme Q pool. To account for the biphasic inhibition curves observed for quinone reduction, it is proposed either that the two inhibitor sites (Ki,q and Ki,q) are located in the span between the Fe-S cluster(s) donating electrons and theQB site OR that blocking electron flow is by the main pathway only and is completely blocked by the normal pathway betweenQA andQB produces leakage of electrons toQB via an alternate route, which can only be prevented by high concentrations of inhibitor (low affinity site). Alternatively, the appearance of this second site may depend on the redox status of the enzyme. Lack of radiolabeled inhibitor has prevented verification of this. In contrast, inhibition does not open up alternate routes during quinol oxidation, electron flow is by the main path only and is completely blocked by a single inhibitory site.

Since the inhibition is noncompetitive with respect to the externally added quinone acceptor, a possible explanation is that the inhibitor sterically blocks electron exchange betweenQA andQB. This environment is likely to be hydrophobic which would explain why the inhibition by different 2-alkyl-4,6-dinitrophenol derivatives depends on the nature of the alkyl substituent. Because all the pKa values (4.51) are the same, it seems unlikely that the different potencies of the inhibitors can be solely ascribed to a difference in the degree of ionization unless thepKa values changes differently for each inhibitor when bound to the enzyme.

Table IV

Inhibition by 2-substituted 4,6-dinitrophenol of fumarate reductase mutants assayed in the direction of quinone reduction

| Enzyme source | Turnover no. | Q site altered | 1 | 5 | 15 | 17 | 19 | 20 |
|---------------|-------------|----------------|---|---|----|----|----|----|
| Wild type     | 100b        | 100            | 87 | 43 | 1.4 | 15 | 1.5 |
| E290D         | 75c         | □              | —  | —  | 1.7 | 0.1 | 0.65 | 0.074 |
| H82Q          | 67c         | □              | —  | —  | 87  | 8.5 | 29  | 5.9 |
| F57V          | 45c         | □              | 1700| 35 | 2.35| 6  | 0.8 |
| Q59W          | 62c         | □              | 2400| 26 | 1.8 | 10 | 1  |

a The Ki values were calculated from the equation [V = Vmax/(1 + Ki) + β × Vmax]. The mean β value was ±0.2. The values for wild type areKi.

b The turnover number was 7900.
c From Westenberg et al. (7).
d Maximum inhibition was 30%.
e Maximum inhibition was 18%.
It is seen in Tables II and III that inhibitor 1, the parent dinitrophenol without a 2-alkyl substituent, has a $K_i$ for mammalian succinate dehydrogenase 5 times lower in the oxidative direction than in the reverse direction (Table II). This might reflect a similar behavior to thenoyltrifluoroacetone and the carboxanilides, the classical inhibitors of beef heart complex II, which have 2 to 3-fold higher affinity for the oxidized than for the reduced form of the enzyme (12). In E. coli fumarate reductase the reverse may be true: the $K_i$ is nearly 15 times lower in the reductive than in the oxidative direction (Table III). The same patterns of inhibition are exhibited by derivatives of dinitrophenol with 2-alkyl substituents, although the potency varies widely. With both mammalian and the E. coli enzymes, the $\alpha$-branching in the side chain of compound 17 leads to increased inhibition, as compared with $\gamma$-branching (compound 15). As mentioned above, $\alpha$-branching forces the 2-substituent to extend almost perpendicular to the ring plane, thus mimicking the favored conformation for the side chain of ubiquinone (5). The presence of a bulky group (compound 19) next to the $\alpha$-branching greatly diminishes this difference, but not if the bulky group is significantly distant from the point of branching (compound 20). In summary, inhibitory potency is enhanced by increasing the hydrophobicity of the derivative but the shape of the substituent must fit the confines of the binding site.

Interestingly, in contrast to completely inhibiting the quinone reductase activity of the wild-type E. coli fumarate reductase, the inhibitors blocked only 80% of the activity in the mutant forms of the enzyme. In addition, the biphasic nature of the inhibition seen with unmodified enzyme no longer is present. It seems that the fraction of noninhibitable activity approximates the fractional activity blocked by the low affinity inhibitor site in the wild-type enzyme. This might possibly indicate that it is this site that is lost or rendered ineffective in the mutants. The mutations did not impair the ability of the inhibitors to block quinol oxidase activity. The inhibition of quinol oxidation remained complete, indicative of one inhibitor site.

It is evident from the $K_i$ values presented in Table III that among the derivatives compound 17 is consistently the best inhibitor of quinol oxidation by the wild-type enzyme, as well as by those mutant forms of E. coli fumarate reductase that were tested (data not shown). This inhibitor, which is effective at submicromolar concentrations, has the structure most closely resembling that of ubiquinone.

In the reverse direction, based on the $K_i$ values obtained from the complementary series of experiments using quinone reduction as the test assay, which are presented in Table IV, it is clear that substitution of Glu-29 with Asp increased the potency of all of the 2-alkyl-substituted dinitrophenol derivatives some 14–20-fold. In this substitution, glutamate is replaced by a smaller amino acid but the carboxylate group and, hence, ability to exchange protons, is maintained. Since other studies have indicated a role for Glu-29 in proton exchange reactions at the Q$_B$ site, it is quite possible that these 2-alkyl 4,6-dinitrophenols inhibit these reactions, either by preventing directly proton donation/abstraction or by interrupting the “bucket brigade” of amino acids carrying protons to and from Q$_B$.

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