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Molecular Characterization of Binding of Substrates and Inhibitors to DT-Diaphorase: Combined Approach Involving Site-Directed Mutagenesis, Inhibitor-Binding Analysis, and Computer Modeling[1]

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ABSTRACT

The molecular basis of the interaction of DT-diaphorase with a cytotoxic nitrobenzamide CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] and five inhibitors was investigated with wild-type DT-diaphorase (human and rat) and five mutants [three rat mutants (rY128D, rG150V, rH194D) and two human mutants (hY155F, hH161Q)]. hY155F and hH161Q were generated to evaluate a hypothesis that Tyr155 and His161 participate in the obligatory two-electron transfer reaction of the enzyme. The catalytic properties of hY155F and hH161Q were compared with a naturally occurring mutant, hP187S. Pro187 to Ser mutation disturbs the structure of the central parallel β-sheet, resulting in a reduction of the binding affinity of the flavin-adenine dinucleotide prosthetic group. With NADH as the electron donor and menadione as the electron acceptor, the k_{cat} values for the wild-type human DT-diaphorase, hY155F, hH161Q, and hP187S were measured as 66 $\pm$ 1, 23 $\pm$ 0, 5 $\pm$ 0 and 8 $\pm$ 2 $\times$ 10^3 min^{-1}, respectively. Because hY155F still has significant catalytic activity, the hydroxyl group on Tyr155 may not be as important as proposed. Interestingly, hY155F was found to be 3.3 times more active than the human wild-type DT-diaphorase in the reduction of CB1954. Computer modeling based on our results suggests that CB1954 is situated in the active site, with the aziridinyl group pointing toward Tyr155 and the amide group placed near a hydrophobic pocket next to Tyr128. Dicoumarol, Cibacron blue, chrysin, 7,8-dihydroxyflavone, and phenindone are competitive inhibitors of the enzyme with respect to nicotinamide coenzymes. The binding orientations of dicoumarol, flavones, and phenindone in the active site of DT-diaphorase were predicted by results from our inhibitor-binding studies and computer modeling based on published X-ray structures. Our studies generated results that explain why dicoumarol is a potent inhibitor and binds differently from flavones and phenindone in the active site of DT-diaphorase.

DT-diaphorase (EC 1.6.99.2), also called NAD(P)H: (quinone-acceptor) oxidoreductase, is a flavoprotein that catalyzes two-electron reduction of quinones and quinonoid compounds to hydroquinones, with either NADH or NADPH as the electron donor (Ernster, 1987). This enzyme is a dimer enzyme, with one flavin-adenine dinucleotide (FAD) prosthetic group per active site. The two identical subunits are in a head-to-tail arrangement. Thus, each active site is made up of parts of both subunits. DT-diaphorase can function physiologically as one of several vitamin K reductases in the vitamin K cycling involved in the hepatic posttranslational modification of vitamin K hydroquinone-dependent blood coagulation factors (Wallin et al., 1978). Oral anticoagulants such as dicoumarol and warfarin (Hollander and Ernster, 1975; Lind et al., 1979) have been found to be potent competitive inhibitors with respect to nicotinamide coenzymes of the enzyme. Flavones isolated from the Chinese herb *Scutellariae radix* (Huang Qin), which has anticoagulating properties, were first shown by Liu et al. (1990) to be potent inhibitors of DT-diaphorase. This enzyme has been shown to be capable of the reduction in vitamin K_3 (menadione) but not vitamin K_1 (Preusch and Smalley, 1990). The reduction in vitamin K_1 by microsomal preparations could not be inhib-

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[1] This article is dedicated to the memory of Dr. Lars Ernster, who passed away recently. DT-diaphorase was originally isolated in Dr. Ernster's laboratory in 1958.

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ABBREVIATIONS: FAD, flavin-adenine dinucleotide; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; PCR, polymerase chain reaction; NRH, dihydronicotinamide riboside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

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ited by 10 μM dicoumarol. Whereas Cibacron blue (Prochaska, 1988), phenindone (Hollander and Ernster, 1975), and flavones (Chen et al., 1993) were all shown to be competitive inhibitors with respect to nicotinamide coenzymes, they were found to inhibit DT-diaphorase in a synergistic fashion when used together with dicoumarol. These results suggest that these inhibitors bind differently in the active site of DT-diaphorase, although they are all competitive inhibitors with respect to nicotinamide coenzymes. However, the molecular basis of the interaction of these inhibitors/anticoagulants to DT-diaphorase is not yet established.

DT-diaphorase is also known to reductively activate cytotoxic antitumor quinones such as mitomycins, anthracyclines, and aziridinyl-benzoquinones (Siegel et al., 1990a,b; Walton et al., 1991), as well as nitrobenzamides such as CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] (Boland et al., 1991). Enzymatic reduction of these antitumor compounds gives rise to reactive intermediates that can then undergo nucleophilic additions with DNA and other macromolecules, suggesting a possible mechanism for their cytotoxicity (Lin et al., 1972). Although these compounds are substrates of DT-diaphorase, their binding characteristics in the active site are not yet known.

Several DT-diaphorase mutants have been generated in our laboratories to study the structure-function relationship of the enzyme (Chen et al., 1992, 1997; Ma et al., 1992a,b; Cui et al., 1995). In addition, the X-ray structure of rat DT-diaphorase has been reported by Li et al. (1995). In this article, we report on the reactivities of prodrugs such as CB1954 with the wild-type DT-diaphorase and five mutants. In addition, the inhibition profiles of five inhibitors (i.e., dicoumarol, Cibacron blue, chrysin, 7,8-dihydroxyflavone, and phenindone) on the wild-type and mutant forms of the enzyme were determined. By analyzing these results and reviewing the X-ray structure of the enzyme, information regarding the molecular basis of the interaction of CB1954 and five inhibitors with DT-diaphorase was obtained. The results are important to further develop prodrugs for cancer treatment and oral anticoagulants to treat heart diseases.

Experimental Procedures

Materials. Dihydronicotinamide riboside (NRH) was prepared by alkaline phosphatase treatment of dihydronicotinamide mononucleotide with a reported method (Friedlos et al., 1992). Alkaline phosphatase was used to remove the phosphate group from dihydronicotinamide mononucleotide, and NRH was purified by reversed-phase HPLC. CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] was synthesized at the Institute of Cancer Research and kindly supplied by P. Burke (Charing Cross Hospital, London, UK).

The five inhibitors, i.e., dicoumarol, Cibacron blue, chrysin, 7,8-dihydroxyflavone, and phenindone, were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). The structures of menadione, CB1954, and five DT-diaphorase inhibitors are shown in Fig. 1. The human and rat forms of the wild-type DT-diaphorase and three rat mutants (rY128D, rG150V, and rH194D) were generated by recombinant DNA methodology and were characterized previously (Ma et al., 1992; Chen et al., 1997).

Mutant Preparations. A polymerase chain reaction (PCR)-based mutagenesis method described by Nelson and Long (1989) was used to generate the three human mutants (hP187S, hY155F, and hH161Q). The desired PCR product was resolved on 1% agarose gel and then extracted with the QIAquick Gel Extraction Kit (Qiagen, Inc., Chatsworth, CA). The gel-purified PCR product was cloned into PCRII vector from the TA cloning kit (Invitrogen Co., San Diego, CA). Mutant clones were then selected by dideoxy sequencing. The resulting mutant constructs were religated into the *Escherichia coli* expression vector pKK233–2 (Pharmacia, Piscataway, NJ), through the *Nco*I and *Hin*dIII restriction sites. For the hP187S mutant, the cloned cDNA was ligated into the PKK-(His)6 vector, which was prepared as described previously (Wu et al., 1998).

Purification of Mutant Proteins. The transformed cells were cultured following a previously described procedure by Chen et al. (1992). The cells were sonicated and centrifuged with the reported procedure. The supernatant from the 90-min centrifugation at 105,000*g* was applied to a 50-ml Affi-Gel Blue (Bio-Rad, Richmond, CA) column, and the column was washed according to the published method, except for the final elution step. The mutant was eluted from the column with a buffer containing 50 mM Tris buffer (pH 7.5), 0.25 M sucrose, 0.5 M KCl, 10 mM NADH, and 5 μM FAD. The active fractions were pooled and concentrated by centrifugation with a centriplus concentrator unit (Amicon, Beverly, MA) with the molecular-weight cutoff at 30,000.

For the His-tagged hP187S mutant protein, the partially purified preparation by the Affi-Gel Blue affinity chromatography was further purified by use of an Ni^{2+}NTAagarose (Qiagen) column. The concentrated enzyme solution was mixed with 1 ml of Ni^{2+}NTAAgarose, which was equilibrated with 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, and 5 μM FAD. The mixture

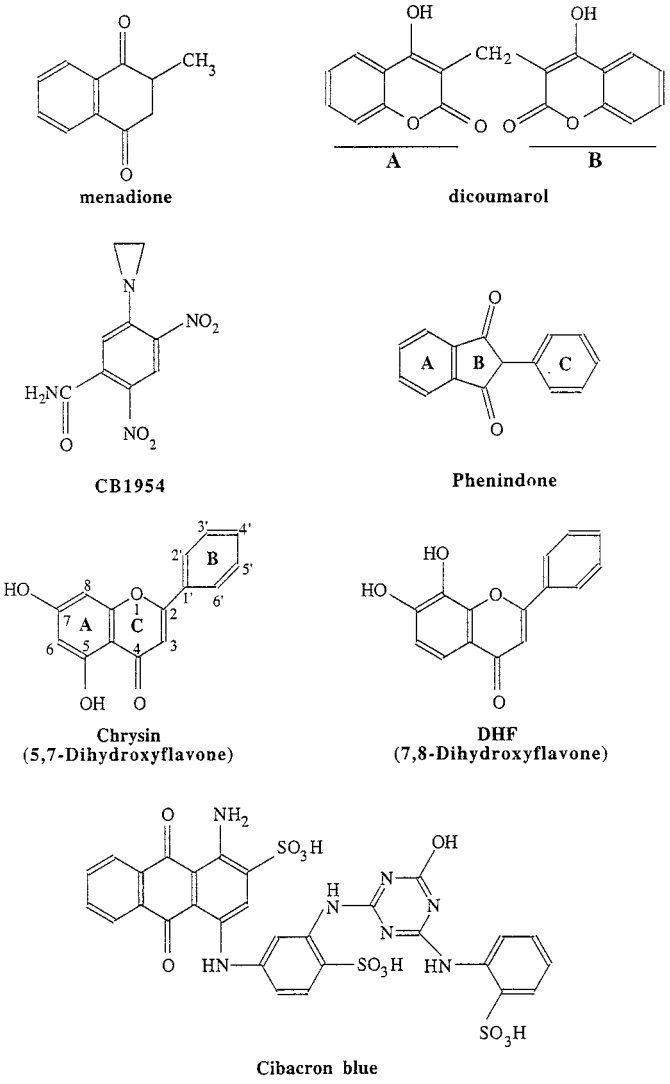

Fig. 1. Structure of menadione, CB1954, and five inhibitors.

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was incubated at 4°C for 40 min. The P187S adsorbed agarose was first washed with 50 mM phosphate buffer (pH 8.0), 300 mM NaCl, followed by 50 mM phosphate buffer (pH 7.0), 300 mM NaCl. The enzyme was finally eluted with an imidazole gradient (0–200 mM) in 50 mM phosphate buffer (pH 7.0), 300 mM NaCl, and 5 μM FAD. The active fractions were pooled and concentrated by ultrafiltration. The final storage buffer for P187S was 50 mM phosphate buffer (pH 7.0)/300 mM NaCl/5 μM FAD.

The purified mutant preparations were analyzed by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970).

Enzyme Assay. NADH (NRH)-menadione reductase activity was determined spectrophotometrically by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at 610 nm [$\epsilon_{(610\ nm)} = 11.3 \times 10^3\ M^{-1} \cdot cm^{-1}$] at 25°C. The assay mixture (1 ml) contained 50 mM potassium phosphate, pH 7.5, 500 μM NADH (or NRH), 10 μM menadione, and 0.3 mg/ml MTT. In the assay, menadione was used as the electron acceptor, and MTT was included to continuously reoxidize the menadiol formed. In addition, the 2,6-dichlorophenolindophenol reductase activity of the preparation was determined following the procedure by Benson et al. (1980). The reduction in CB1954 was analyzed by HPLC. The enzyme preparations were incubated with NADH or NRH (500 μM) and CB1954 at different concentrations (0.1–2.0 mM), in sodium phosphate buffer (10 mM; pH 7) at 37°C. At various times, aliquots (10 μl) were injected onto a Partisil 10 SCX (250 × 4.7 mm) HPLC column and eluted isocratically (1.5 ml/min) with 130 mM unbuffered sodium phosphate. The eluent was continuously monitored for absorption at 340 and 260 nm, and the spectra of the eluting components were recorded with a diode array detector (Waters 996). This separation system could resolve all of the expected reduction products (Boland et al., 1991), and reduction of CB1954 was monitored by either the decrease in its peak area or an increase in the area of the peak corresponding to the reduction product, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. All of the assays were initiated with the addition of the enzyme and were performed in duplicate.

Inhibition Studies. The enzyme was assayed in the presence of various inhibitors at different concentrations. Inhibitors except dicoumarol were dissolved in ethanol, and the maximal volume of ethanol was maintained at 10 μl/ml assay mixture. The activity of the enzyme was not affected by ethanol in amounts up to 10 μl/ml. Dicoumarol was dissolved in 15 mM NaOH. These experiments were performed in triplicate. The K_i values for inhibitors have been derived from Dixon plots (1/v versus [I]). These inhibitors have been shown to be competitive inhibitors of DT-diaphorase with respect to NADH, and the K_m value of NADH for each enzyme preparation was determined and used in this calculation. During the enzyme assay, the concentration of NADH was 200 μM.

Molecular Modeling. Crystallographic coordinates for rat DT-diaphorase with bound FAD, duroquinone, and Cibacron blue (file 1QRD; Li et al., 1995) were obtained from the Protein Data Bank (Berstein et al., 1977). For modeling purposes, the physiological dimer form was used (MacroMolecular file 1qrd__1.mmol). The coordinates were used without further refinement.

The modeling of the binding of prodrugs and inhibitors in the active site was performed with the program InsightII (Molecular Simulations, Inc., San Diego, CA). Crystallographic coordinates for CB1954 (Iball et al., 1975) were obtained from the Cambridge Structural Database (File DNEIBA; Allen et al., 1983) and used without further refinement. All other prodrugs or inhibitors were built via fragment libraries supplied with the modeling software. The initial structures, most of which are semirigid, were first energy minimized to an root-mean-square force of less than 0.001 with the consistent valence force field (Dauber-Osguthorpe et al., 1988). The resulting structures were then subjected to 10 ps of molecular dynamics at 300 K, followed by conjugate gradient minimization to an RMS force of less than 0.001 to complete the refinement cycle. This cycle was repeated 10 times, and the lowest energy conformer was selected from the resulting ensemble of structures.

The energy-minimized substrate and inhibitor molecules were then individually placed with the active site of the enzyme model such that overlap with the bound substrate (or inhibitor) was optimal. The active site was then solvated with an 8-Å layer of pre-equilibrated water. Each compound was again energy minimized but this time in the context of the active site (defined as residues within 10 [Angst] of the substrate or inhibitor). This optimized the position of each inhibitor within the pocket, which was assumed to be rigid. Finally, the minimizations were repeated without constraints so that active-site residues and solvent could move to better accommodate the bound prodrugs or inhibitors (induced fit theory of molecular interactions; Jorgensen, 1991). No predictive tests of the docking models were carried out. The models simply attempt to rationalize the binding data.

Results

Catalytic Properties of DT-Diaphorase Mutants. We have reported previously that the catalytic properties of human and rat forms of DT-diaphorase are different (Chen et al., 1997). The NADH-menadione reductase activity and the NADH-CB1954 reductase activity of the human enzyme were found to be 46 and 14% of those of the rat enzyme, respectively. Previous mutagenesis studies revealed that residue 104 (Tyr in the rat enzyme and Gln in the human enzyme) is an important residue responsible for the different CB1954 reductase activities between the rat and human enzymes. The presence of Gln at position 104 instead of Tyr in the human DT-diaphorase allows the flavin prosthetic group to move deeper into the protein (Chen et al., 1997; Fig. 2B). Such a change may modify the rate of electron transfer between FAD and a substrate such as CB1954.

X-ray structure analysis of rat DT-diaphorase has suggested that the carbonyl group of the nicotinamide from NADH makes two hydrogen bonds: one with the hydroxyl group of Tyr126 and the other with the hydroxyl group of Tyr128 (Li et al., 1995). Gly150 and His161 are thought to interact with oxygens $O_4'N$ and $O_2'N$ of the ribose adjacent to nicotinamide, respectively. His194 may form hydrogen bonds between its Nϵ atom and two oxygens of the pyrophosphate. Cui et al. (1995) performed mutagenesis studies at Tyr128, Gly150, and His194. The catalytic properties of the three rat mutants used in this study, i.e., rY128D, rG150V, and rH194D, have been published (Cui et al., 1995). With 2,6-dichlorophenolindophenol as the electron acceptor, the activities of rY128D and rG150V were determined to be 16 and 23% of the wild-type rat enzyme, and the K_m values of these mutants for NADH were found to be 2.7 and 3.3 times that of the wild-type enzyme (Ma et al., 1992b). Furthermore, rY128D was found to be significantly less sensitive to dicoumarol than the wild-type enzyme (Ma et al., 1992b). The K_m value of rH194D for NADPH was found to be much larger than that of the wild-type enzyme (Cui et al., 1995). These mutagenesis experiments confirm that Tyr128, Gly150, and His194 are important residues in the nicotinamide coenzyme binding site of DT-diaphorase.

We have prepared the human mutants hH161Q and hY155F. As mentioned above, X-ray structural analysis suggested a direct interaction of His161 with nicotinamide coenzymes (Li et al., 1995). In addition, Tyr155 and His161 have also been thought to be involved in the charge relay mechanism to facilitate the obligatory two-electron transfer reaction (Li et al., 1995). The imidazole of His161 is close to the

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nicotinamide ring, so a positive charge is thought to move over a very short distance from the imidazole ring of His161 to the nicotinamide. This process is reversed when the hydride is transferred to the quinone. Therefore, it is not surprising that the k_{cat} value of hH161Q is only 8% that of the wild-type human DT-diaphorase (Table 1). The hydroxyl group of Tyr155 is believed to form a hydrogen bond with O2F of the flavine ring (Li et al., 1995) and to participate in the two-electron transfer reaction by interacting with His161. The importance of the hydroxyl group of Tyr155 is questioned, because hY155F still has 35% of the wild-type activity (Table 1). As discussed below, the CB1954 reductase activity of hY155F is three times that of the wild-type enzyme. In Table 1, the catalytic properties of these human mutants are compared with those of a naturally occurring mutant, hP187S. The K_m values of these three human mu-

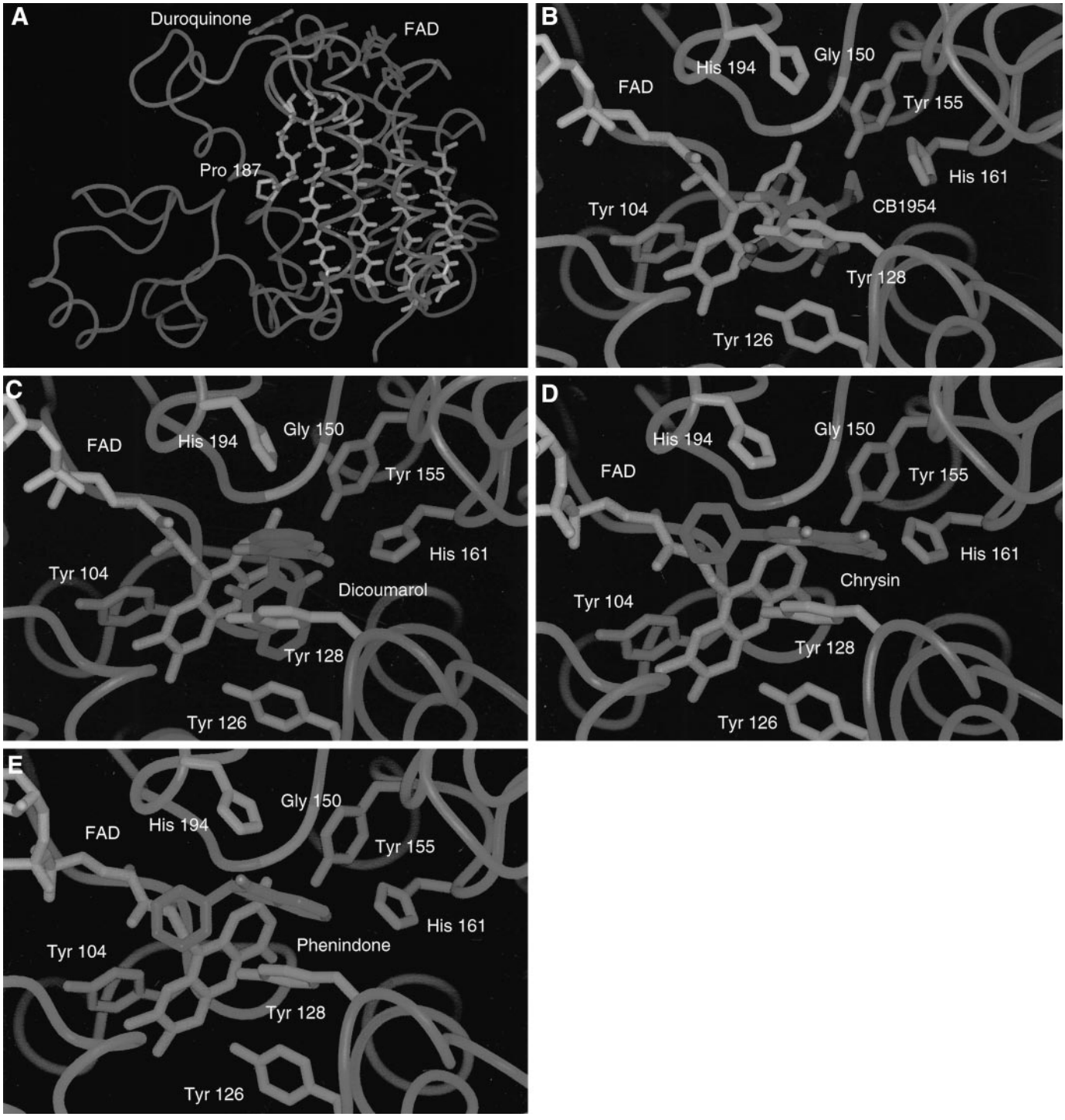

Fig. 2. A, crystal structure of rat DT-diaphorase. Pro187 is shown in white. A Pro187 to Ser mutation would disturb the structure of the central parallel β-sheet (yellow), resulting in a reduction in the binding affinity of FAD (green). B, predicted binding orientation of CB1954 (shown in colors according to the atom type). The sites of mutation are shown in purple. C, predicted binding orientation of dicoumarol. D, predicted binding orientation of chrysin. E, predicted binding orientation of phenindone.

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tants for NADH and menadione are similar to those of the wild-type human enzyme, except for hP187S, the K_m for menadione of which is slightly higher (Table 1). hP187S was also found to be significantly less active than the wild-type human enzyme. Previous studies found that a low activity for hP187S results from a weaker affinity for FAD than the wild-type enzyme (Wu et al., 1998). Pro187 is not positioned in the active site, but the mutation disturbs the structure of the central parallel β-sheet, resulting in a reduction in the binding affinity of the FAD prosthetic group (Fig. 2A). Similar K_m values for both NADH and menadione and k_{cat} values lower than the wild-type human enzyme again support the hypothesis that the His161 to Gln mutation mainly affects the catalytic process rather than the binding affinities of NADH and menadione.

It is possible that the Tyr155 to Phe mutation or the His161 to Gln mutation interrupts the obligatory two-electron transfer reaction but that these mutants can catalyze the reduction of menadione through a one-electron transfer mechanism. Superoxide can be generated through redox cycling of semiquinone. To examine such a possibility, we measured superoxide production during the reduction of menadione by the wild-type human DT-diaphorase hY155F and hH161Q. Significantly more superoxide would be detected for the mutants compared with the wild-type enzyme if the mutants catalyzed the reduction of menadione through a one-electron reduction mechanism. However, the analysis revealed that the wild-type form and two mutants generated low but similar levels of superoxide during the catalysis (results not shown). The superoxide measurements further indicate that the hydroxyl group of Tyr155 is not essential for enzyme catalysis.

The CB1954 reductase activities of rY128D, rG150V, rH194D, and hY155F have also been measured and compared with those of the two forms of the wild-type enzyme (see Table 2). Whereas rY128D and rG150V have similar menadione reductase activity (as discussed above), rY128D has a significantly higher CB1954 reductase activity than rG150V. In addition, hY155F was found to be 3.3 times more active in the reduction of CB1954 than the human wild-type enzyme (Table 2). However, as shown in Table 1, the k_{cat} value of hY155F to reduce menadione is 40% that of the human wild-type enzyme. These results suggest different binding orientations for menadione and CB1954 in the active site of DT-diaphorase. In addition, whereas both NADH and NRH were found to be effective electron donors for rY128D, rH194D, hY155F, and the rat wild-type DT-diaphorase to reduce CB1954 (Table 3), NRH was significantly more effective than NADH for rG150V to reduce the drug. We did not determine the kinetic parameters of hH161Q and hP187S in the CB1954 reduction, because these mutants are significantly less active, and a large portion of the enzyme preparations would be needed for the analysis. Nevertheless, an attempt was made to measure the CB1954 reductase activity of hP187S, and it was estimated that this mutant reduced CB1954 at a rate approximately 10% that of the wild-type human enzyme (data not shown).

Inhibition Studies. The inhibitor-binding constants (K_i) of five inhibitors (Cibacron blue, chrysin, 7,8-dihydroxyflavone, phenindone, and dicoumarol) for the wild-type (human and rat) enzyme and five mutants [three rat mutants (rY128D, rG150V, rH194D) and two human mutants (hY155F, hH161Q)] were determined and are shown in Table 4. Each of the five mutations modified the binding of five inhibitors to a different extent.

Discussion

Binding Nature of CB1954. Considering the fact that CB1954 is a substrate of DT-diaphorase, and its size is only slightly bigger than duroquinone, the compound is thought to be situated where duroquinone is found in the X-ray structure, with the ring parallel to the flavin ring. This allows for an efficient electron transfer during the reductive activation. Based on four docking simulations and results from mutant kinetic analysis, we have predicted the preferred orientation of CB1954 within the active site. We propose that CB1954 situates in the active site, with the aziridinyl group pointing toward Tyr-155 and the amide group placed near a hydrophobic pocket next to Tyr128 (Fig. 2B). Because the K_m values of rY128D, rG150V, and rH194D for CB1954 are similar to that of the wild-type rat DT-diaphorase and the

TABLE 1

Kinetic constants of two wild-type (human and rat) and three human DT-diaphorase mutants

For the determination of K_m values for NADH, the electron acceptor was menadione (12.5 μM). For determination of K_m values for menadione, the electron donor was NADH (200 μM). The kinetic analyses were performed in triplicate.

	hDT	rDT	hY155F	hH161Q	hP187S
			μM		
$K_{m,NADH}$	220 ± 10	110 ± 10	160 ± 10	210 ± 20	230 ± 60
$K_{m,menadione}$	2.7 ± 0.3	2.5 ± 0.1	2.7 ± 0.2	2.1 ± 0.2	4 ± 0.2
			$10^3 \times min^{-1}$		
k_{cat}	66 ± 1	144 ± 18	23 ± 0	5 ± 0	8 ± 2

TABLE 2

Kinetic values of wild-type and mutant forms of DT-diaphorase in reduction of CB1954

For determination of K_m values for CB1954, the electron donor was NADH (500 μM). The analyses were performed in triplicate.

	rDT	rY128D	rG150V	rH194D	hDT	hY155F
			μM			
K_m, CB1954	845 ± 30	860 ± 40	995 ± 55	865 ± 30	1370 ± 70	880 ± 50
			$nmol \cdot min^{-1} \cdot mg^{-1}$			
V_{max}	140 ± 7	51 ± 4	8 ± 1	101 ± 6	20 ± 2	66 ± 6

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V_{max} values of these mutants are lower than that of the wild-type rat enzyme, we feel that these mutations reduce the rate of the electron transfer reaction rather than modify the binding affinity of CB1954. On the other hand, the K_m value of hY155F for CB1954 is found to be significantly lower than that of the wild-type human enzyme. In addition, the V_{max} value of hY155F is 3.3 times higher than that the wild-type human enzyme. These results suggest that the binding affinity and the reduction efficiency of CB1954 are improved, with a Phe at position 155. We therefore predict that the aziridinyl group of CB1954 is in this area. The amide group is thought to be near a hydrophobic pocket next to Tyr128, similar to the amide group of the nicotinamide coenzyme in which the carbonyl makes hydrogen bonds to Tyr126 and Tyr128 (Li et al., 1995). Experiments performed in our laboratories have revealed that the amide group of CB1954 can be replaced with a hydrophobic side chain, and such derivatives are better substrates of the human DT-diaphorase (R.K., K.W. and S.C., unpublished results).

Gly150 is thought to interact with oxygens $O_4'N$ of the ribose adjacent to nicotinamide (Li et al., 1995). Results shown in Table 3 indicate that the Gly150 to Val mutation affects the binding of NADH to a greater extent than the binding of NRH, suggesting that the orientations of the ribose moiety of these two nicotinamide coenzymes in the active site of DT-diaphorase are not exactly the same. It is also possible that the Gly150 to Val mutation reduces the size of the active site pocket, preventing the effective binding of NADH, which is a significantly larger molecule than NRH. On the other hand, the relative activities of NADH and NRH for rY128D are similar, which suggests that the binding orientations of the nicotinamide group of the coenzymes within the active site of this mutant are probably not much different. The two subunits of the enzyme are in a head-to-tail arrangement, and each active site is made up of parts from both subunits. As shown in structural models, Tyr128 is from a different subunit than Gly150, Tyr155, His161, and His194.

TABLE 3

Relative activities of reduction of CB1954 via NADH or NRH as electron donor

Assay was performed in the presence of 500 μM NADH or NRH and 2 mM CB1954.

Electron donor	rDT	rY128D	rG150V	rH194D	hY155F
			$nmol \cdot min^{-1} \cdot mg^{-1}$		
NADH	49.1	31.1	3.2	32.8	14.7
NRH	51.5	25.6	17.8	41.6	14.3
Relative rate (NADH/NRH)	1.0	1.2	0.2	0.8	1.0

Binding Nature of Inhibitors. The K_i values of Cibacron blue, chrysin, 7,8-dihydroxyflavone, phenindone, and dicoumarol to rY128D, rG150V, and hH161Q were significantly greater than those for the wild-type human enzymes, indicating that Tyr128, Gly150, and His161 are situated in the binding pockets of these inhibitors. On the other hand, Tyr155 and His194 are probably not in direct contact with the inhibitors, because the binding of inhibitors are not affected by Tyr155 to Phe and His194 to Asp mutations.

The X-ray structure of Cibacron blue bound to rat DT-diaphorase has been published (Li et al., 1995) and was used to help interpret the results of our inhibition studies. The binding of Cibacron blue is significantly reduced by the Tyr128 to Asp mutation and the Gly150 to Val mutation. The K_i values of Cibacron blue for rY128D and rG150V are 110 and 23 times that for the wild-type rat DT-diaphorase, respectively. The X-ray structural analysis revealed that the inhibitor's trizaine ring is sandwiched between these two residues.

As indicated in Fig. 1, dicoumarol is structurally similar to a dimer of the substrate menadione. To facilitate the discussion of the results, the two halves of the molecule dicoumarol are named rings A and B. The K_i values of dicoumarol for rG150V and rY128F are 437 and 70 times that for the wild-type rat enzyme, respectively, and the K_i value for hH161Q is 140 times that for the wild-type human enzyme. Results obtained from inhibition studies with dicoumarol indicate that the strong binding of dicoumarol (K_i = 2 and 0.5 nM for the rat and human NQO1, respectively) can be explained by a π-π interaction of one ring (ring A, as indicated in Fig. 1) with the isoalloxazine ring of FAD and a π-π interaction of ring B with the phenol ring of Tyr128, thus explaining why the binding affinity of dicoumarol is greatly reduced for rY128D (K_i = 140 nM for rY128D; computer models, see Fig. 2C). Like the triazine ring of Cibacron blue, we predict that ring B of dicoumarol is sandwiched between Gly150 and Tyr128, thus explaining the great increase in the K_i value (i.e., 970 nM) of dicoumarol for rG150V. Because the K_i value for hH161Q is also large (i.e., 70 nM) compared with the wild-type human enzyme, His161 is also thought to be part of the dicoumarol binding site. Specifically, our model suggests that the hydroxyl group of ring A packs against this residue.

Rings A and C of chrysin are thought to be near Gly150 and His161, explaining why the K_i values of chrysin for rG150V and hH161Q are 60 and 82 times those of both rat and human wild-type DT-diaphorase (see Fig. 2D). The results of our inhibition studies also lead us to conclude that chrysin (i.e., 5,7-dihydroxyflavone; K_i = 100 nM for both rat and

TABLE 4

K_i values for two wild-type (human and rat) and five DT-diaphorase mutants

The inhibitor dose-response analyses were performed in triplicate. The K_i values for inhibitors have been derived from Dixon plots (1/v versus [I]). These inhibitors have been shown to be competitive inhibitors of DT-diaphorase with respect to NADH, and the K_m value of NADH for each enzyme preparation was determined and used in this calculation.

	K_i						
	hDT	rDT	rY128D	rG150V	hY155F	hH161Q	rH194D
				nM			
Cibacron blue	500	80	9,000	1,850	970	600	230
Chrysin	100	100	400	6,000	120	8,200	90
7,8-OH flavone	30	60	2,100	16,700	20	4,000	100
Phenindone	500	80	4,600	11,200	210	1,200	10
Dicoumarol	0.5	2.0	140	970	0.6	70	2.0

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human DT-diaphorase) and 7,8-dihydroxyflavone (K_i = 60 and 30 nM for rat and human enzyme, respectively) bind to the active site, with the 7-hydroxyl group (in chrysin) placed near His161, thus explaining the large increase in the K_i value for hH161Q (K_i = 8200 and 4000 nM for chrysin and 7,8-dihydroxyflaovone, respectively). Because the K_i value of 7,8-dihydroxyflavone for rG150V (16,700 nM) is much larger than that of chrysin (6000 nM), the 8-hydroxyl group is thought to pack against Gly150. Therefore, we propose that flavone binds to the active site of DT-diaphorase with the C-8 ring carbon near Gly150 and the 4-keto group pointing away from Gly150 toward the solvent.

Interestingly, the binding of phenindone to the human enzyme, like Cibacron blue, is significantly poorer than the rat enzyme (Table 4). It is felt that phenindone binds to the active site in an orientation similar to that of 7,8-dihydroxyflavone, with the fused-ring system involved in a π-π interaction with Tyr128 and a packing interaction with Gly150 (Fig. 2E). This binding orientation explains why the K_i values of phenindone for rY128D and rG150V are significantly higher than that of the wild-type rat DT-diaphorase (see Table 4). Because there is no side chain on ring A, the His161 to Gln mutation modifies the binding of phenindone moderately (K_i = 500 and 1200 nM for the wild-type human enzyme and hH161Q, respectively). Interestingly, phenindone binds to rH194D significantly better than the wild-type rat enzyme. Computer-modeling analysis suggests that this residue sits above the bound inhibitor, near one of the keto groups.

In summary, by evaluating results obtained from a combined approach involving site-directed mutagenesis of DT-diaphorase, enzyme kinetic analysis, inhibitor-binding studies, and computer modeling based on the published X-ray structure of the rat form of the enzyme, we have predicted the binding orientations of the prodrug CB1954 and four inhibitors, dicoumarol, chrysin, 7,8-dihydroxyflavone, and phenindone, in the active site of DT-diaphorase. Our findings may facilitate the design of better oral anticoagulants for treatment of recurring heart attack and novel prodrugs for treatment of cancer.

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Erratum

In Chen S, Wu K, Zhang D, Sherman M, Knox R, and Yang CS (1999) Molecular characterization of binding of substrates and inhibitors to DT-diaphorase: Combined approach involving site-directed mutagenesis, inhibitor-binding analysis, and computer modeling. *Mol Pharmacol* **56:**272–278, Figure 2 on page 275 should have been published in color. We regret any inconvenience caused by this error. The color version of the figure is given below with its legend.

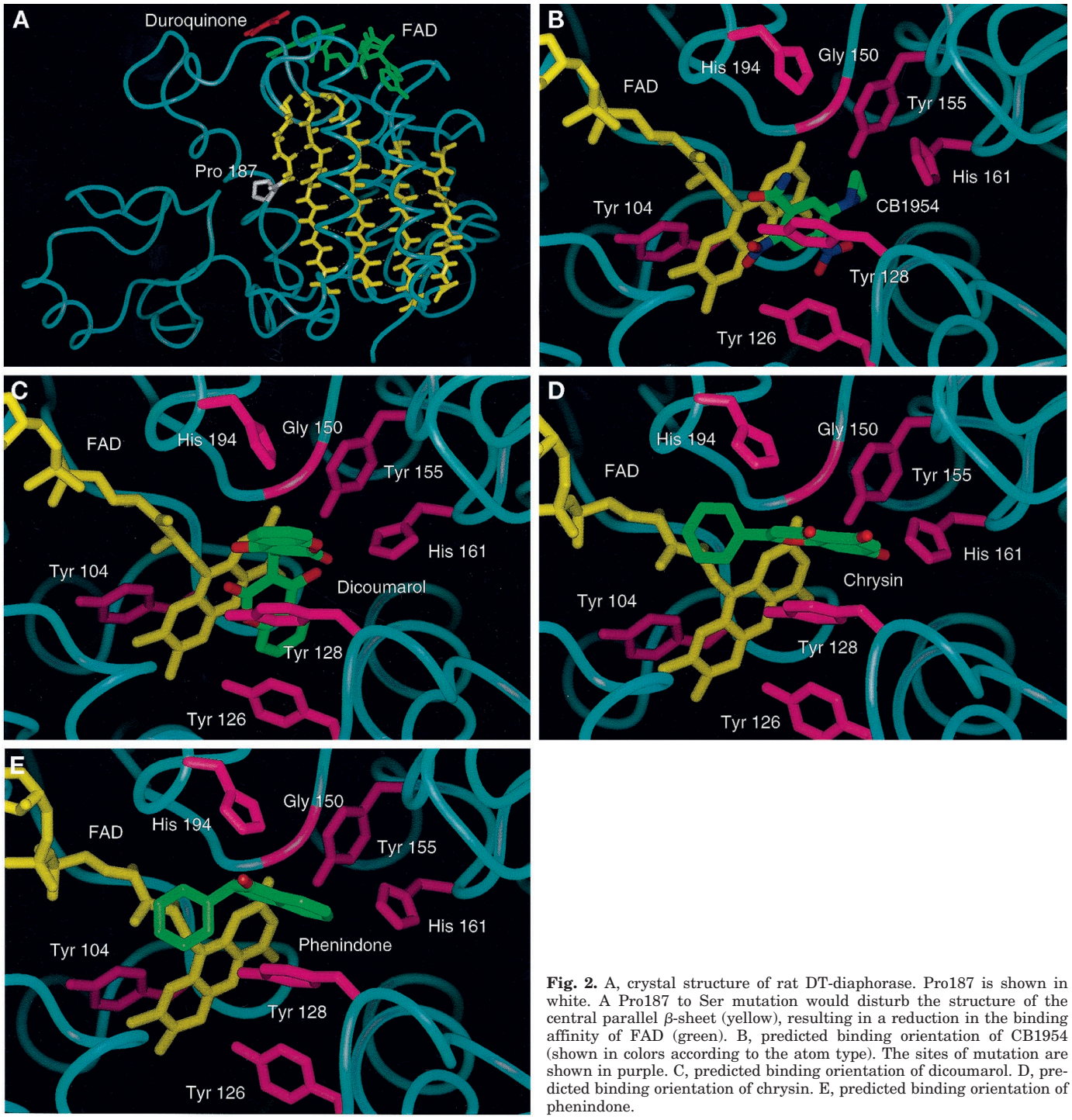

Fig. 2. A, crystal structure of rat DT-diaphorase. Pro187 is shown in white. A Pro187 to Ser mutation would disturb the structure of the central parallel β-sheet (yellow), resulting in a reduction in the binding affinity of FAD (green). B, predicted binding orientation of CB1954 (shown in colors according to the atom type). The sites of mutation are shown in purple. C, predicted binding orientation of dicoumarol. D, predicted binding orientation of chrysin. E, predicted binding orientation of phenindone.

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