Molecular Basis of the Catalytic Differences among DT-diaphorase of Human, Rat, and Mouse*

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DT-diaphorase (EC 1.6.99.2), also referred to as NAD(P)H:quinone-acceptor oxidoreductase, is involved in the reductive activation process of several cytotoxic antitumor quinones and nitrobenzenes. It has been observed in our and other laboratories that the rat enzyme is significantly more effective in activating these drugs than the human and mouse enzymes. These results indicate that the available cytotoxic drugs are better substrates for the rat enzyme and are not the most ideal prodrugs for activation by DT-diaphorase in human tumors. In this study, using site-directed mutagenesis to replace residues in the rat enzyme with the human sequences and residues in the human enzyme with the rat sequences, we have found that residue 104 (Tyr in the rat enzyme and Gln in the human and mouse enzymes) is an important residue responsible for the catalytic differences between the rat and the human (and mouse) enzymes. With an exchange of a single amino acid, the rat mutant Y104Q behaved like the wild-type human enzyme, and the human mutant Q104Y behaved like the wild-type rat enzyme in their ability to reductively activate the cytotoxic drug CB 1954 (5-aziridin-1-yl)-2,4-dinitrobenzamide). The study also confirms the conclusion of the x-ray structural analysis of rat enzyme that residue 130 (Thr in the rat enzyme and Ala in the human and mouse enzymes) is positioned near the binding region of the nicotinamide portion of NAD(P)/H. This structural information is very important for designing suitable drugs and approaches for human cancer chemotherapy mediated by DT-diaphorase.

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rat carboxyl-terminal segments of DT-diaphorase, has been shown to have catalytic properties resembling the rat enzyme, and RM-P, a chimeric enzyme that has rat amino-terminal and mouse carboxyl-terminal segments of DT-diaphorase, has been shown to have catalytic properties resembling the mouse enzyme (13). Based on these results, we propose that the carboxyl-terminal portion of the enzyme plays an important role in the reduction of cytotoxic drugs.

Therefore, it was thought that the catalytic differences among the DT-diaphorase of the three species is due to the amino acid residues that are within the carboxyl-terminal region (after the conserved Pst I site) and identical between the human and mouse enzyme, but different from that of the rat enzyme (see Fig. 2). In this study, a series of rat and human DT-diaphorase mutants with changes in the carboxyl-terminal region were generated and analyzed. The study has revealed that residue 104 (Tyr in rat DT-diaphorase and Gln in human and mouse DT-diaphorase) is an important residue responsible for the catalytic differences between the rat, the human (and mouse) enzymes.

MATERIALS AND METHODS

Site-directed Mutagenesis—A PCR1-based mutagenesis method described by Nelson and Long (14) was used to generate DT-diaphorase mutant cDNAs. Desired PCR product was resolved over a 1% agarose gel and then extracted using 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 dyeoxy sequencing. The resulting mutant constructs were ligated into the E. coli expression vector, pKK233-2 vector (Pharmacia Biotech Inc.), through the NeoI and HindIII restriction sites. The expressed mutants were purified by Affi-Gel Blue affinity chromatography following the previous published procedures (15). The purity of mutant preparations were examined by SDS-polyacrylamide gel electrophoresis (16).

Other—NADH-dehydrogenase reductase activity was determined spectrophotometrically following the published procedure (17). In the assay, menadione was used as the electron acceptor and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium was included to continuously reoxidize the menadione formed. The reduction of CB 1954 by DT-diaphorase was analyzed by HPLC. DT-diaphorase was incubated with NADH (500 μM) and CB 1954 at different concentrations (0.1 to 2 mM), in sodium phosphate buffer (10 mM, pH 7) at 37 °C. At various times, aliquots (10 μl) were injected onto a Partisil SCX (250 × 4.7 mm) HPLC column and eluted isocratically (1.5 ml/min) with 50 mM sodium phosphate containing 1% methanol. The elute was continuously monitored for absorption at 340 and 260 nm, and the spectra of the eluting components were recorded using a diode array detector (ABI 1000S). This separation system could resolve all the expected reduction products (6) and reduction of CB 1954 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.

RESULTS AND DISCUSSION

In this study, five rat mutants, in which the mutated residues are after the conserved Pst I site, were generated. These mutants were converted to the human (mouse) sequence, i.e., Y104Q(r), T130A(r), V204I(r), S218T(r), and L238M(r). In order to prevent confusion, (r) and (h) are included in the name of each mutant to indicate whether it is a rat or human DT-diaphorase mutant, respectively. The mutants were purified to homogeneity, and the purity of the preparations was examined by SDS-polyacrylamide gel electrophoresis (results not shown). Among the five mutants, Y104Q(r) was found to reduce menadione and CB 1954 at a rate similar to that of the human (and mouse) DT-diaphorase, i.e., similar Vmax values (Table I). The Km value of T130A for NADH resembles that of the human (and mouse) enzyme. Therefore, in order to further evaluate the role of residue 104, we prepared the human mutant Q104Y(h). While the mutant Q104Y(h) had a lower menadione reductase activity than the wild-type human DT-diaphorase, its CB 1954 reductase activity was 7-fold that of the wild-type human enzyme, identical to that of the wild-type rat enzyme. Furthermore, a human double mutant Q104Y/ A130T(h) was prepared and found to have a Km value for NADH similar to the wild-type rat enzyme. Therefore, our mutagenesis experiments have provided definitive results indicating that residue 104 of DT-diaphorase plays a critical role in reductive activation of drug CB 1954, and residue 130 is involved in NADH binding.

The x-ray structure of rat DT-diaphorase reveals that the phenolic ring of Tyr-104, together with the main chain carbonyl of Trp-103, provides the main interactions with the “bottom face” of the isooxaloxazine ring, (the face opposite to the one that interacts with substrate and nicotinamide) of FAD (18). In addition, a water molecule hydrogen-bonded to the OH of Tyr-104 is hydrogen-bonded with the O3 of the isooxaloxazine ring and with a phosphate oxygen of FAD. Replacing Tyr-104 by a Gln manifests itself predominantly through effects in the positioning of the isooxaloxazine ring. The change in size of the side chain allows the flavin to move deeper into the protein (by approximately 0.7 Å). Such a change may modify the rate of electron transfer between FAD and the substrate menadione. It is not yet known how the drug CB 1954 modifies the active site of the enzyme. However, the results from this study indicate that the positioning of the isooxaloxazine ring will have a major effect on the reduction of the prodrug CB 1954.

1 The abbreviations used are: PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.

2 M. A. Blanchet, M. Faig, S. Chen, P. Talalay, and L. M. Amzel, unpublished results.
slightly altered positions of the side chains of Tyr-126 and Tyr-128. The observed effects of the mutations agree, in general, with the structural observations: change of Tyr-104 affects the \( V_{\text{max}} \) (by affecting the relation between the flavin and the nicotinamide and the substrate), and change of Thr-130 affects NADH binding.

In addition to the mutations at positions 104 and 130 which led to major changes in the catalytic properties, V204I(r) was found to have a \( K_{\text{m}} \) value for menadione similar to that of the wild-type mouse enzyme (Table I). The x-ray structural analysis of the rat enzyme has revealed that the adenine ring of FAD interacts strongly with Arg-201 (18). It is thought that the mutation may modify the interaction of menadione with the enzyme by a slight change in the FAD binding environment. The human double mutant Q104Y(L238M) was found to have a \( K_{\text{m}} \) value for menadione similar to the wild-type human enzyme. Residue 238 is situated in the carboxyl-terminal domain of the enzyme (18, 19). Both the rat mutant L238M(r) and the human mutant Q104Y(M238L(h)) were found to have large \( K_{\text{m}} \) values for NADH (Table I). In addition, the \( V_{\text{max}} \) value of L238M(r) for CB 1954 was slightly lower than that of the wild-type rat enzyme. Perhaps the most important implication of the results from studies involving rat-mouse chimeric enzymes has generated a possibility is currently being evaluated in our laboratory.

| Residue(s)  | \( K_{\text{m}} \) (NADH) | \( V_{\text{max}} \) (NADH) | \( K_{\text{m}} \) (CB1954) | \( V_{\text{max}} \) (CB1954) |
|------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Rat        | 110 ± 10                  | 2.5 ± 0.1                | 840 ± 40                 | 140 ± 8                  |
| Human      | 220 ± 10                  | 2.7 ± 0.3                | 1370 ± 70                | 20 ± 2                   |
| Mouse      | 210 ± 20                  | 4.3 ± 0.4                | 1280 ± 80                | 20 ± 2                   |
| Y104Q(r)   | 120 ± 10                  | 2.3 ± 0.2                | 950 ± 60                 | 28 ± 2                   |
| Q104Y(h)   | 110 ± 20                  | 2.8 ± 0.4                | 830 ± 40                 | 147 ± 7                  |
| T130A(r)   | 200 ± 20                  | 2.9 ± 0.4                | 890 ± 80                 | 138 ± 6                  |
| Q104Y/A130T(h) | 120 ± 10                  | 1.8 ± 0.3                | 1540 ± 110               | 129 ± 8                  |
| V204I(r)   | 110 ± 20                  | 4.2 ± 0.1                | 890 ± 100                | 149 ± 7                  |
| Q104Y/L204V(h) | 210 ± 20                  | 2.6 ± 0.4                | 860 ± 90                 | 142 ± 11                 |
| S218T(r)   | 140 ± 10                  | 2.5 ± 0.5                | 820 ± 70                 | 138 ± 6                  |
| Q104Y/T218S(h) | 140 ± 30                  | 3.0 ± 0.3                | 980 ± 90                 | 134 ± 14                 |
| L238M(r)   | 320 ± 20                  | 2.9 ± 0.8                | 840 ± 70                 | 89 ± 7                   |
| Q104Y/M238L(h) | 250 ± 10                  | 1.5 ± 0.3                | 1110 ± 80                | 131 ± 7                  |

\[K_{\text{m}} \text{ (NADH)} \text{ (mol/min/mg)} \]

\[K_{\text{m}} \text{ (CB1954)} \text{ (mol/min/mg)} \]

\[V_{\text{max}} \text{ (NADH)} \text{ (mol/min/mg)} \]

\[V_{\text{max}} \text{ (CB1954)} \text{ (mol/min/mg)} \]

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