Crystal Structure of Human DT-diaphorase: A Model for Interaction with the Cytotoxic Prodrug 5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)

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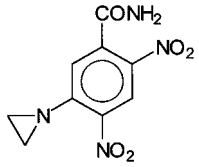
The crystal structure of human DT-diaphorase (NAD(P)H oxidoreductase (quinone); EC 1.6.99.2) has been determined to 2.3 Å resolution. There are only minor differences in shape and volume between the active sites of the rat and human enzymes and in the hydrophobic environment in the vicinity of the substrate. The isoalloxazine ring of the bound FAD is more buried in the human structure. Molecular modeling was used to examine optimal positions for the antitumor prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) in both the human and rat enzyme active sites. This suggests that the position of CB1954 in the active site of the human enzyme is very similar to that in the rat, although there are detailed differences in the predicted patterns of hydrogen bonding between side chains and the drug. Some of the differences are a consequence of the shift in position for the FAD molecule and may contribute to the observed differences in rate of the two-electron reduction of CB1954.

Introduction

DT-diaphorase (NAD(P)H oxidoreductase (quinone); EC 1.6.99.2) is an important detoxifying enzyme that is constitutively expressed in a variety of tissues, with particularly high levels in liver, kidney, and the gastrointestinal tract.[1,2] It catalyzes an obligatory two-electron reduction of a range of quinones and quinonoid compounds. High levels of DT-diaphorase activity are induced when cells are challenged by a variety of potentially carcinogenic and mutagenic compounds,[3] and there is substantial evidence for increased levels of activity in non-small-cell lung and colon carcinomas.[4] Several cytotoxic antitumor quinones, including the mitomycins, anthracyclines, and aziridinylbenzoquinones, are bioactivated by DT-diaphorase[5] to generate reactive intermediates which can undergo nucleophilic additions with DNA, resulting in single-strand breaks. It can also reduce nitrobenzene-containing compounds such as the antitumor prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) (Chart 1) to form a cytotoxic interstrand cross-linking agent by reduction of its 4-nitro group.[6] Cells expressing high levels of DT-diaphorase are more sensitive to drug treatment.[7,8] DT-diaphorase is a tightly associated physiological dimer of identical subunits, each comprising 273 amino acids.[9,10] Each monomer contains a noncovalently bound FAD prosthetic group that is essential for catalytic activity. There are 37 amino acid differences between the rat and human enzymes (Figure 1). The two-domain structure for the monomer proposed on the basis of proteolytic digestion studies[11] was confirmed by the 2.1 Å resolution X-ray structure of the rat enzyme.[12] Crystal structures of two different complexes of the rat liver enzyme were determined in this study: (a) containing Cibacron Blue, a potent competitive inhibitor with respect to NAD(P)H, and the quinonoid compound duroquinone and (b) with $NADP^+$.

Chart 1. Structure of CB1954



Although DT-diaphorase is recognized as of considerable therapeutic value in the reductive activation of prodrugs, significant differences exist in the abilities of the enzyme in different species to activate these antitumor agents. The rat enzyme is more effective than the human in reducing MMC, EO9, streptonigrin, and also CB1954.[6,13−16] All these compounds are therefore better substrates for the rat enzyme than for the human. The molecular basis for these catalytic differences has been recently investigated by site-directed mutagenesis.[17] Together with data from the crystal structure of the rat enzyme complexed with the triazine dye Cibacron blue,[12] this has led to the suggestion that a tyrosine residue (Tyr104), which is substituted by a glutamine in the human enzyme, plays a critical role in the differences in rates of catalysis. Tyr130A is also predicted to be involved and is positioned close to the nicotinamide moiety of NAD(P)H. A full molecular understanding of these differences and their implications is essential for the design of new and improved substrates that can be more effectively reduced by the human enzyme.

Results and Discussion

Overall Structure. Four independent monomers (one tetramer), each containing a noncovalently bound

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        1                                                  50
human   MVGRRALIVL AHSERTSFNY AMKEAAAAAL KKKGWEVVES DLYAMNFNPI
rat      AVRRALIVL AHAERTSFNY AMKEAAVEAL KKKGWEVVES DLYAMNFNPL
        51                                                 100
human   ISRKDITGKL KDPANFQYPA ESVLAYKEGH LSPDIVAEQK KLEAADLVIF
rat     ISRNDITGEP KDSENFQYPV ESSLAYKEGR LSPDIVAEQK KLEAADLVIF
        101                                                150
human   QFPLQWFGVP AILKGWFERV FIGEFAYTYA AMYDKGPFRS KKAVLSITTG
rat     QFPLYWFGVP AILKGWFERV LVAGFAYTYA TMYDKGPFQN KKTLLSITTG
        151                                                200
human   GSGSMYSLQG IHGDMNVILW PIQSGILHFC GFQVLEPQLT YSIGHTPADA
rat     GSGSMYSLQG VHGDMNVILW PIQSGILRFC GFQVLEPQLV YSIGHTPPDA
        201                                                250
human   RIQILEGWKK RLENIWDETP LYFAPSSLFD LNFQAGFLMK KEVQDEEKNK
rat     RVQVLEGWKK RLETVWEESP LYFAPSSLFD LNFQAGFLLK KEVQEEQKKN
        251                   274
human   KFGLSVGHHL GKSIPTDNQI KARK
rat     KFGLSVGHHL GKSIPADNQI KARK
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Figure 1. Aligned amino acid sequences of human and rat DT-diaphorase. The differences between the two are highlighted in red.

FAD cofactor molecule, form the asymmetric unit of human DT-diaphorase (Figure 2a). The monomers are arranged as two noncrystallographic physiological dimers, with the active site being at each dimer interface. All four chains have essentially identical structures. Backbone conformations are all equivalent, within experimental error with a rms deviation between them of 0.3 Å. The rms deviation, including side-chain atoms, is 0.4 Å. There is also no significant difference between backbone conformations for the rat and human diaphorases, with an rms deviation of 0.55 Å, using averaged conformations for both.

FAD Binding Site. There are no significant differences in the backbone conformations of the active-site residues between the four subunits of the human structure. Almost all side-chain conformations are also conserved. Residues Trp105 and Phe106, which are both conserved, and Gln104 form a pocket around the isoalloxazine moiety. Together with the main chain carbonyl of Leu103, these residues are involved in the stabilization of the FAD prosthetic group via interactions with the flavin rings. The overall geometries of the active site are very similar in both the rat and human structures. The rms deviation between the averaged human and rat backbones for residues 100−165 is 0.4 Å (Figure 2b). In view of the conservation of active-site structures for these two species, we conclude that the binding of the Cibacron Blue and duroquinone molecules in the rat structure has had no significant perturbing effect on the active site. A water molecule was found to be bound close to the active site of the human enzyme, hydrogen-bonded to Trp105 and Glu117. This is distant from the position of the bound duroquinone in the rat structure and thus would have little effect on any drug binding.

The FAD molecule adopts identical conformations in both rat and human structures, although there is a shift in its position in the latter, resulting in the FAD molecule being buried slightly more deeply in the active site. In the rat enzyme residue 104 is a tyrosine, which has been postulated by site-directed mutagenesis experiments to be the key residue responsible for the catalytic differences between the rat and human enzymes. A hydrogen bond is formed by a water molecule situated between Tyr104 and a phosphate oxygen of FAD in the rat structure. The absence of this interaction in the human structure, where Tyr104 is replaced by glutamine, directly results in the shift of FAD position. In the human enzyme the flavin is shifted by 0.7 Å with respect to the rat (calculated as a mean of the rms deviations from the rat structure measured in all four monomers). An approximate 0.7 Å shift in position had earlier been suggested;[17] it was then unclear whether this difference is chiefly responsible for the differences in substrate reaction rates between the two enzymes. The phenol ring of Tyr128 adopts a distinct conformation in the two enzymes, possibly as a consequence of the hydrogen bonding to the duroquinone molecule found in the rat structure (see also below). There does not appear to be any other underlying significance to this change in side-chain conformation, or to that at Tyr126. The change at amino acid 130 from threonine (rat) to alanine (human) has not resulted in any backbone changes, contrary to earlier suggestions[17] that this region might exhibit small but significant conformational changes; the rms deviation for the Cα atoms of residues 127−130 is 0.1 Å, comparing rat to human.

The active site in both human and rat enzymes is formed by the interface between two protein chains. There are some differences in the shape and overall volume of the human compared to the rat active site, due to changes in some of the hydrophobic residues forming its boundary. Most noteworthy are Val69 and Leu120 in the rat enzyme, which are changed to Ala69 and Phe120 in the human. These substitutions will doubtless influence the size of substrate able to bind in the active site.

Molecular Modeling of CB1954. Use of the AFFINITY program within the INSIGHTII molecular modeling package resulted in the location of energetically favorable positions and orientation for the CB1954 drug molecule in each active site, for human and rat DT-diaphorase respectively (Figures 3). In each case, only one orientation for the drug is possible, as a consequence of steric clashes in alternative orientations.

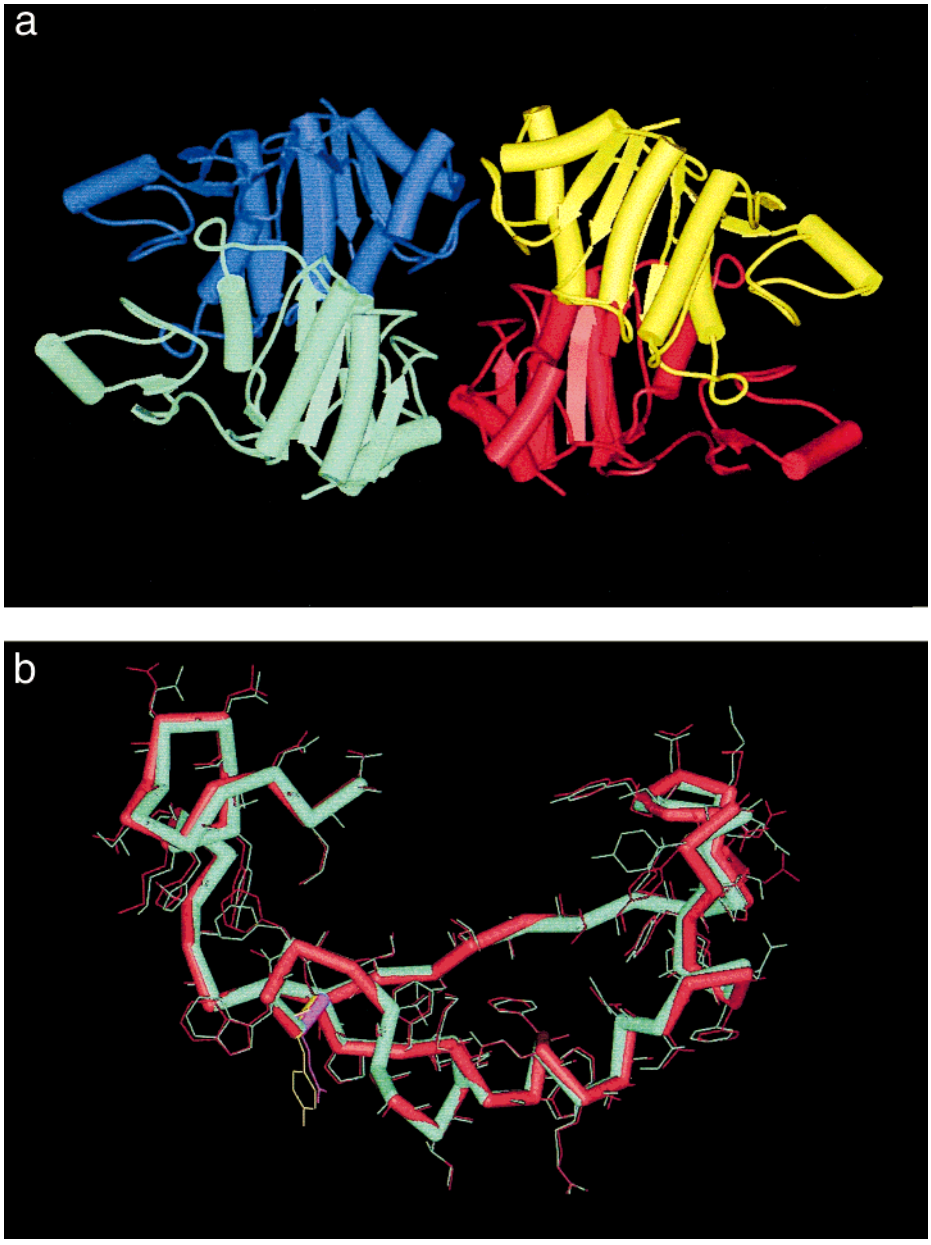

Figure 2. (a) The four subunits in the crystal structure of human DT-diaphorase. (b) Superposition of the Cα atoms for residues 100−165, for rat and human DT-diaphorase. The rat structure is in cyan and the human in red. The side chain of residue Tyr104 of the rat enzyme is shown in yellow, and Gln104 of the human enzyme is shown in magenta.

The calculated binding energies for both enzyme−drug complexes are closely similar. The van der Waals component energies for the human and rat complexes are −54.5 and −54.4 kcal mol^{-1}, respectively, and the electrostatic components are −19.0 and −19.4 kcal mol^{-1}, giving binding energies of −73.5 and −73.8 kcal mol^{-1}. This result is paralleled by the similarity in low-energy positions for the drug in the two active sites, although there is a small displacement in position due to the change in position of the FAD molecule. The CB1954 molecule is close to the position of the duroquinone molecule observed in the rat crystal structure. The orientations of the CB1954 molecule, as seen in the positions of the aziridine and 4-nitro groups, are predicted by the modeling to be closely similar in the two active sites. We suggest that significant factors in governing the drug position are the close van der Waals contact which the aziridine ring has with the aromatic ring of Trp105 and the stacking interactions with the planar isoalloxazine ring of the flavin.

The CB1954 molecule in both active sites is indicated by the modeling to participate in hydrogen bonding between side chains and drug substituent atoms (Figure 3). There are some differences in the details of the arrangements. In the model for the rat enzyme−drug complex (Figure 3a) both oxygen atoms, O3 and O4, of the 4-nitro group hydrogen-bond to Tyr126 and Tyr128, from subunit B. There is also a close electrostatic interaction with His161. In the human complex (Figure 3b), Tyr155 is predicted to be 0.4 Å closer to the amide oxygen atom of CB1954. Only Tyr128, in the human complex, is sufficiently close to the 4-nitro group to hydrogen-bond to atom O1 since the side chain of Tyr128 is swung round the CA−CB bond in the human enzyme compared to the rat. This enables Tyr128 to be within hydrogen-bonding distance of the ring nitrogen

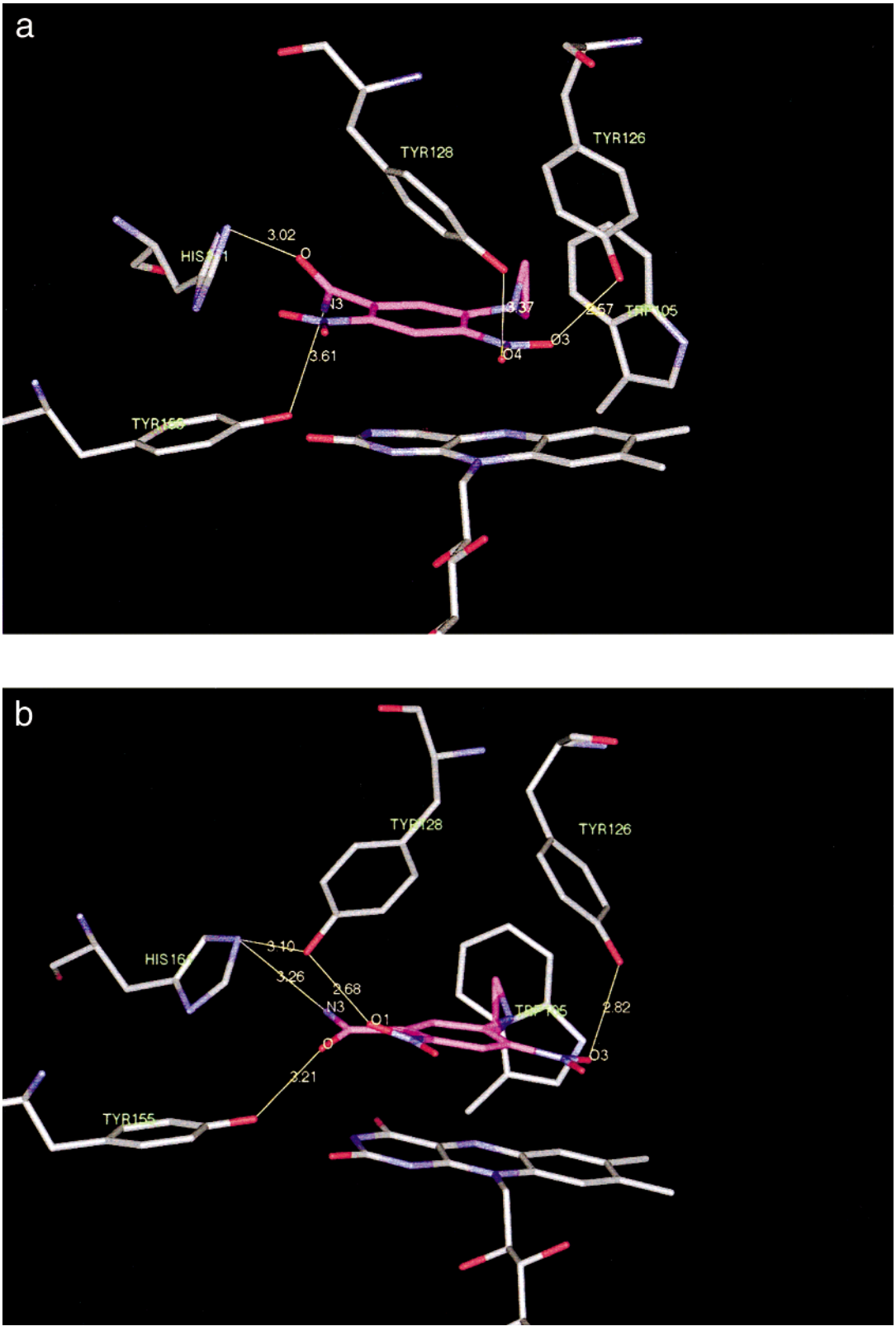

Figure 3. (a) Results of the molecular modeling study, showing the CB1954 and FAD molecules bound in the active site of (a) rat and (b) human DT-diaphorase. Only those residues with direct contacts to the drug molecule are shown. Potential hydrogen bonds are shown as thin yellow lines. The carbon atoms of the CB1954 molecule are highlighted in purple. Two views are shown for each active site, looking along and onto the plane of the isoalloxazine moiety of the FAD molecule.

atom of His161, which is now slightly further (3.3 Å) away from the amide group of CB1954 compared to its position in the rat active site.

Although the CB1954 molecule is predicted to be in overall very similar environments in the human and rat active sites, the differences outlined above may contribute to the observed differences in the drug's rate of two-electron reduction. The postulated mechanism of two-electron transfer[12] involves the transfer of a proton from His161. We note that the net effect of the small

movement in flavin position on going from rat to human is to slightly change the separation between His161 and CB1954, with the drug being closer to His161 in the rat site. We suggest that this small difference may be a factor in facilitating the two-electron transfer, which in the human site may be dissipated through the proximity of Tyr128 to His161. The significance of the hydrogen bonding from the two tyrosine residues 126 and 128 to the 2-nitro position of CB1954 in the rat site is unclear, although it is this nitro group which undergoes nitro reduction by DT-diaphorase to a hydroxylamine.

Methods

Expression and Purification. A sequence containing the cDNA for human DT-diaphorase (a gift from Dr. Anil Jaiswal) was subcloned into plasmid pET11d (Novagen Inc.) and expressed in BL21 (DE3)pLysS cells by induction with IPTG (1 mM) at 37 °C for 10 h. The pelleted cells were resuspended in 50−100 mL of lysis buffer A containing 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2% v/v aprotinin, and the bacterial pellet was disrupted by sonication following freeze/thawing. After centrifugation at 12000*g*, the cell lysate was treated with 1% poly(ethylenimine) (Sigma) to remove nucleic acids before affinity chromatography on Cibacron Blue using a 5-mL Hi trap column equilibrated in buffer A. Contaminating proteins were eluted by developing a linear gradient over 10 column volumes from 0% to 100% buffer B containing 50 mM Tris-HCl, pH 7.4, 1 M NaCl. DT-diaphorase was eluted with reduced NADH (16 mM) in 1 M Tris-HCl, pH 7.4, containing 0.5 mM KCl and 0.25 M sucrose. Homogeneity was assessed by spectroscopic analysis and SDS−PAGE gels. The specific activity was determined using the menadione assay.[18] The molar extinction coefficients at 272 nm (6.65×10^4) and 451 nm (1.06×10^4) were used to determine the concentration.[19] The purified protein was concentrated by ultrafiltration and filtered (0.22 μm) prior to crystallization.

Crystallization. Crystals were grown in sitting drops using the vapor diffusion technique. Equal volumes (4 μL) of protein (9.7 mg/mL) in 100 mM sodium phosphate buffer, pH 7.5, were mixed with reservoir solutions containing 25% w/v poly-(ethylene glycol) 4000. The nonionic detergent cyclohexyl-propyl-β-D-maltoside CYMAL-3 (1 μL of 345 mM, CMC 34.5 mM) was added to the droplet. The crystals grew over several weeks as yellow rectangular plates at 18 °C and were predisposed to twinning. Twinning was believed to be responsible for the crystals cracking during substrate-soaking experiments with CB1954 under nonreducing conditions. These experiments were subsequently abandoned. The final refined cell dimensions are $a = 55.88$ Å, $b = 57.49$ Å, $c = 98.77$ Å, $\alpha = 77.1°$, $\beta = 76.2°$, and $\gamma = 86.9°$. The space group is $P1$ with two dimers (one tetramer) in the asymmetric unit, assuming an estimated solvent content of 0.47 and using the Matthews parameter V_m.[20]

Crystallography. Intensity data were collected on an R-AXIS-II image-plate system (Molecular Structures Corp.) with Yale/MSC mirrors mounted on a Rigaku RU200 X-ray generator operating at 100 mA and 50 kV. A complete dataset was collected to 2.28 Å at room temperature on a single crystal that had been preirradiated in a synchrotron beam; 110 frames of data (oscillation range 1.2°) were processed and reduced with DENZO and SCALEPACK.[21] A total of 193 332 reflections were measured, which reduced to 46 165 unique reflections (81.5% completeness), with a R_{merge} of 0.132. This value is possibly due to crystal decay due to preexposure.

The structure was solved by molecular replacement using the program AMoRe[22] with the structure of the oxidized rat enzyme complexed with Cibacron Blue as the starting model (Protein Data Bank entry code 1QRD). Rigid-body refinement was carried out using the program XPLOR version 3.1, using data to 3.0 Å.[23] Simulated annealing was then carried out on the monomer using strict noncrystallographic symmetry (NCS). The tetramer was internally generated. Strict NCS was then replaced by restrained NCS. All four monomers of the molecular replacement model, each containing a stoichiometrically bound FAD cofactor, were rebuilt manually using the program O, version 5.10.[24] Further cycles of refinement were carried out on the complete tetramer after map fitting. A bulk solvent refinement was carried out to improve the map and correct for solvent scattering. Data from 2.3−40 Å were included, resulting in an R of 26% and R_{free} of 28.6%. Solvent molecules were included in the final stages of refinement. The final residuals were $R = 23.2\%$; $R_{free} = 28.0\%$ with 66 solvent molecules being included. The coordinate data has been deposited in the Protein Data Bank as entry code 1QBG.

Molecular Modeling. The structure of CB1954 was built using the INSIGHTII package, and partial charges were calculated with the AM1 semiempirical quantum chemistry module within INSIGHTII. Its structure was closely comparable with that determined by X-ray crystallography.[25] The optimal fit of CB1954 in the active sites of both human and rat DT-diaphorases was determined with the program AFFINITY.[26] Force-field parameters for both drug and protein residues were taken from the CVFF force field[27] within the DISCOVER module of INSIGHTII. The AFFINITY procedure enabled molecular orientation and position for CB1954 to be explored while monitoring electrostatic and van der Waals drug and protein interactions, while enabling drug and side-chain flexibility to be taken into account. Possible starting orientations were chosen by means of a Monte Carlo algorithm. Individual residues were constrained at this initial docking stage. The best drug positions were subjected to 100 steps of unconstrained molecular mechanics minimization. The final ranking order of structures was based on the resulting energy values.

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