Structural and Mechanistic Studies of Escherichia coli Nitroreductase with the Antibiotic Nitrofurazone

REVERSED BINDING ORIENTATIONS IN DIFFERENT REDOX STATES OF THE ENZYME*

The antibiotics nitrofurazone and nitrofurantoin are used in the treatment of genitourinary infections and as topical antibacterial agents. Their action is dependent upon activation by bacterial nitroreductase enzymes, including the Escherichia coli nitroreductase (NTR). Here we show that the products of reduction of these antibiotics by NTR are the hydroxylamine derivatives. We show that the reduction of nitrosoaromatics is enzyme-catalyzed, with a specificity constant ~10,000-fold greater than that of the starting nitro compounds. This suggests that the reduction of nitro groups proceeds through two successive, enzyme-mediated reactions and explains why the nitroso intermediates are not observed. The global reaction rate for nitrofurazone determined in this study is over 10-fold higher than that previously reported, suggesting that the enzyme is much more active toward nitroaromatics than previously estimated. Surprisingly, in the crystal structure of the oxidized NTR-nitrofurazone complex, nitrofurazone is oriented with its amide group, rather than the nitro group to be reduced, positioned over the reactive N5 of the FMN cofactor. Free acetate, which acts as a competitive inhibitor with respect to NADH, binds in a similar orientation. We infer that the orientation of bound nitrofurazone depends upon the redox state of the enzyme. We propose that the charge distribution on the FMN rings, which alters upon reduction, is an important determinant of substrate binding and reactivity in flavoproteins with broad substrate specificity.

The nitrofuran derivatives nitrofurazone and nitrofurantoin are synthetic antibacterial compounds, which are chemically related (Fig. 1). These two compounds remain the only nitrofuran antibiotics to have become established in clinical use. Both are effective against a wide spectrum of Gram-positive and Gram-negative bacteria (1, 2) and are active against organisms displaying resistance to a broad spectrum of other antibacterial agents. Nitrofurantoin is currently used primarily as an oral antibacterial treatment for genitourinary infections (3), and nitrofurazone is used as an atopical antibacterial agent for the treatment of burns and of skin graft patients (4).

The mechanism of action of both of these antibiotics has yet to be fully elucidated. Studies have indicated that the presence of bacterial enzymes capable of reducing nitrofurantoin and nitrofurazone is critical for their activation (5, 6). Strains of bacteria that are sensitive to the nitrofurans have been shown to express a flavoprotein capable of catalyzing the reduction of these drugs (7, 8). Since this reductase activity is not present in nitrofurazone-resistant strains of bacteria, it has been concluded that it is this enzyme that converts nitrofurazone to a compound capable of damaging cells. In Escherichia coli, the enzymes responsible for nitrofurazone and nitrofurantoin activation are the type 1 oxygen-insensitive nitroreductases, encoded by the nfsA (nfsA) and the nfsB (nfsB) genes, respectively (9). The first step of resistance to nitrofurazone is mutation of nfsA. These mutants retain 40% nitrofurazone sensitivity; however, mutants lacking both nfsA and nfsB are nitrofurazone-insensitive, suggesting an important role of nfsB in nitrofurazone activation (6). The toxic products formed following reduction of nitrofurazone and nitrofurantoin by bacteria remain unclear. Some studies have indicated that the reaction may proceed by reduction of the nitro group of nitrofurazone or nitrofurantoin, forming a hydroxylamine product. This hydroxylamine product could react both with proteins and with DNA, introducing strand breaks in the latter (2). Alternatively it has been suggested that the reaction may involve reduction of the opposite, amide, end of the molecule, generating an amine with liberation of the toxic product, formamide (10).

The nfsB enzyme (NTR)$^1$ is a dimeric protein with 217 amino acids per polypeptide chain and one FMN cofactor per subunit. It is capable of using either NADH or NADPH as reducing equivalents and has a substituted enzyme (“ping-pong”) bi-bi mechanism (11, 12). NTR reduces a broad range of nitroaromat-
motic substrates but reduces quinones more rapidly (11, 12). Whereas the latter involves a two-electron reduction to give a quinol, reduction of nitro groups in general gives hydroxylamines, which requires transfer of four electrons. The two-electron nitroso reduction intermediate has never been observed, and it has not been determined whether the enzyme catalyzes both successive two-electron transfers.

We have previously determined the structure of NTR, complexed with the nucleotide analogue nicotinic acid (13). Structures of the free oxidized and reduced NTR protein (14, 15) and of homologous proteins, both free (16, 17) and complexed to different inhibitors or substrates (15, 16), have also been reported recently. Here we report the structure of oxidized NTR complexed with nitrofurazone and the identity of the reaction products. We show that NTR rapidly reduces nitrosoaromatics, supporting the role of the enzyme in catalysis of two successive two-electron transfers. From our results, we infer that nitrofurazone binds in different orientations to the oxidized and reduced enzyme. The cause of this substrate reorientation is discussed in relation to the enzyme structure and implications for related flavoproteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were purchased from Sigma unless otherwise stated.

**Protein Purification**—Recombinant nitroreductase was purified as described previously (13). The enzyme concentration was determined from its absorbance at 280 nm, using a molar absorbance of 43,000 M cm⁻¹ per subunit, estimated from its amino acid composition and flavin content (18) or from Bradford assays, using bovine serum albumin as a standard (19).

**Crystallization and Substrate Soaks**—Native crystals of NTR were grown as described previously (13) in a buffer containing polyethylene glycol 4000 (10% v/v), ethylene glycol (25% v/v), 15 mM nicotinic acid, and 100 mM sodium acetate, pH 4.6. Crystals of the NTR-nitrofurazone complex were obtained by transferring native crystals to a stabilizing solution containing 15 mM nitrofurazone (solubilized in Me2SO), 50 mM sodium citrate, pH 4.6. Similarly, crystals of NTR-acetate complexes were obtained using a soaking solution containing 100 mM sodium acetate, pH 7.0. In each case, crystals were soaked in the appropriate complex for 24 h before being transferred to the cryo-solution.

**Data Collection and Processing**—Diffraction data were collected at 100 K using beamline ID 14-1 at the European Synchrotron Radiation Facility (Grenoble, France). All data were indexed, integrated, and scaled using MOSFLM (20) and SCALA (21). Full data collection statistics are listed in Table I.

**Structure Determination and Model Refinement**—The crystals of the NTR nitrofurazone complex and of one of the NTR acetate complexes (NTR-acetate 1) were isomorphous with previously reported NTR-nicotinate crystals (13). NTR structures 1uc.pdb (P2₁) and 1cr.pdb (P4₁,2,2₁) were used as the starting structures to refine the structures of these two complexes, respectively. After removing the nicotinic acid and all waters, the starting structures were refined against the appropriate data sets using the rigid body, simulated annealing, positional, and isotropic B-factor refinement protocols, as implemented in CNS (22). After preliminary refinement, difference electron density (Fo−Fc) clearly showed the presence of either NF2 or acetate in each of the active sites. Model adjustment and ligand fitting were done manually using the molecular graphics program Turbo-Frodo (23) using SigmaA-weighted (24) Fo−Fc and 2Fo−Fc difference maps as a guide. Water molecules were fitted with the aid of the water-pick protocol in CNS and inspected using molecular graphics. Finally, the structures were refined using the translation and libration correlation parameters and restrained refinement protocol using Refmac5 (25, 26).

An additional crystal form (P2₁,2,2₁) was observed in the same crystallization conditions (Table I). The structure of a second acetate complex (NTR-acetate 2) was solved from such a crystal by molecular replacement, using the orthorhombic NTR-nicotinate complex (1uc.pdb (P2₁,2,2₁)) (13). The structure was initially refined using the program CNS (22) and then in Refmac5 (25) using the same protocols as for the other complexes. Final refinement statistics for the complexes are shown in Table II.

**Kinetic Studies**—Initial steady state kinetic studies were carried out spectrophotometrically by monitoring the initial rate of disappearance of nitrofurazone at 420 nm (ε₄₂₀ nm = 5,590 M⁻¹ cm⁻¹, based on ε₄₄₀ nm = 12,960 M⁻¹ cm⁻¹ (27)) and nitrofurantoin at 420 nm (ε₄₂₀ nm = 7,970 M⁻¹ cm⁻¹, based on ε₅₇₅ nm = 26,300 M⁻¹ cm⁻¹ (28)). For 2-nitrofuran, the oxidation of NADH was monitored at 340 nm (29). The full kinetic studies on nitrofurazone monitored the reaction at 440 nm, using 3μM 880 M⁻¹ cm⁻¹, allowing for absorbance of the product. For nitrotoluene, nitrobenzene, nitrosotoluene, and nitrosobenzene, the rate of NADH oxidation at 360 nm was measured (ε₃₆₀ nm = 2,420 M⁻¹ cm⁻¹ based on ε₃₄₀ nm = 6,220 M⁻¹ cm⁻¹ (29)). All reactions were performed in quartz cuvettes, with a 0.1–1-cm path length. Assays were performed in 10 mM Tris-HCl, pH 7.0, 4.5% MeSO. The temperature of each reaction was maintained at 25 ± 1 °C using a circulating water bath, and all solutions were thermally equilibrated to 25 ± 1 °C prior to measurement. All nitro and nitroso compounds were dissolved in 90% (v/v) MeSO, 10% (v/v) water containing 10 mM Tris-HCl, pH 7.0. In all cases, the reaction was initiated by the addition of a small amount of cold enzyme solution to the reaction mix (≤40 μM). All data were analyzed by nonlinear regression using Sigma Plot 8.0 (SPSS) with equal weighting of points.

For nitrofurazone and NADH, kinetic data were collected at a range of NADH and nitrofurazone concentrations, and plots of initial velocity (v) versus substrate concentration were fitted globally to an equation of the following form (30),

\[
\frac{v_i}{K_{mA}} + \frac{[A]}{K_{mA}} + [B] = \frac{v}{K_{mB}} + \frac{[B]}{K_{mB}} + [A] = \frac{K_{cat(app)} [A]}{K_{cat(app)} + [A]} (Eq. 1)
\]

where [E] represents the enzyme concentration, [A] and [B] are the concentrations of the two substrates, and KmA and KmB are the Michaelis constants for substrate [A] and [B], respectively. For the remaining substrates, plots for each substrate were fitted individually, to an equation of the following form,

\[
\frac{v_i}{K_{cat(app)} + [A]} = \frac{K_{cat(app)} [B]}{K_{cat(app)} + [B]} (Eq. 2)
\]

and

\[
K_{cat(app)} = \frac{K_{cat} [B]}{K_{mB} + [B]} (Eq. 3)
\]

where [A] is the concentration of the variable substrate. In the absence of inhibitor, the following hold true.

\[
K_{cat(app)} = \frac{K_{cat} [B]}{K_{mB} + [B]} (Eq. 4)
\]

As the kinetic parameters of substrate A vary, depending on those of substrate B, it is not possible to correct for partial saturation, using only a single concentration of substrate B. However, the ratio kcat(app)/KmB is equal to the true kcat/Km for that substrate. At low substrate concentrations, the values of kcat(app)/Km can be determined more precisely than either kcat or Km by fitting the equation with kcat(app) as one independent parameter with either kcat or Km as the other.

**Nonenzymatic Reductions**—The rates of nonenzymatic reduction of nitrosobenzene and 2-nitrosotoluene were measured at 60 μM NADH with up to 50 μM nitro compounds; rates at higher concentrations could not be measured due to fast NADH depletion. The rates obtained were plotted versus the concentration of nitrosoaromatic and fitted to a straight line to obtain the pseudo-first-order rate constants. These were divided by the concentration of NADH to obtain the second-order rate constant. At each concentration of substrate, the rates observed in the absence of enzyme were subtracted from those in its presence, and the differences in rates were fitted to the Michaelis-Menten equation (Equation 2).

**Inhibitor Studies**—Assays were monitored as described above, but with the addition of varying concentrations of sodium acetate, acetan-
TABLE I

Data collection statistics of the acetate and nitrofurazone complexes of nitroreductase

|                      | Nitrofurazone | Acetate 1 | Acetate 2 |
|----------------------|---------------|-----------|-----------|
| Crystal properties   |               |           |           |
| Space group          | P2_1          | P4_22     | P2_12     |
| Cell dimensions (Å)  | 70.08, 56.47, 116.09 β = 103.1 | 57.58, 57.58, 263.18 | 46.94, 53.66, 155.6 |
| Solvent content      | 48%           | 43%       | 38.8%     |
| Copies in ASU        | 4             | 2         | 2         |
| Data collection      |               |           |           |
| No. of observations* | 634,727 (64,518) | 400,959 (45,464) | 78,245 (9,885) |
| No. of unique reflections | 97,127 (14,083) | 50,059 (7,068) | 26,277 (3,743) |
| Completeness (%)     | 99.6 (99.5)   | 99.8 (98.5) | 96.2 (95.7) |
| Redundancy           | 6.5 (4.6)     | 8.0 (6.4)  | 3.0 (2.6)  |
| I(0)                 | 8.9 (5.8)     | 3.6 (1.6)  | 12.9 (5.4) |
| R_{syst} (%)         | 5.6 (11.8)    | 10.7 (40.2) | 7.3 (17.5) |
| Resolution (Å)       | 1.7 (1.79 to 1.70) | 1.7 (1.79 to 1.70) | 2.0 (2.11 to 2.00) |

* Values in parentheses are for the outer shell of data.

R_{syst} = \Sigma |I_i| - I_{ave} / I_{ave}

TABLE II

Final refinement statistics for structures of the NTR ligand: complex determined by X-ray crystallography

|                      | Nitrofurazone | Acetate 1 | Acetate 2 |
|----------------------|---------------|-----------|-----------|
| Refinement           |               |           |           |
| Resolution (Å)       | 100 to 1.7    | 66 to 1.7 | 53 to 2.0 |
| No. of reflections   | 92,255        | 47,465    | 24,904    |
| No. of water molecules | 907          | 653       | 528       |
| No. of water molecules | 907          | 653       | 528       |
| R factor             | 14.4%         | 17.3%     | 14.7%     |
| R_{free}             | 16.6%         | 19.5%     | 20.3%     |
| r.m.s.d. bond angles | 1.25 Å^2     | 1.17 Å^2  | 1.08 Å^2  |
| r.m.s.d. bond lengths | 0.008 Å    | 0.010 Å   | 0.009 Å   |
| Average B factor     | 16.1 Å^2     | 18.6 Å^2  | 14.9 Å^2  |
| Protein              | 13.3 Å^2     | 15.2 Å^2  | 12.0 Å^2  |
| Ligand (FMN and NFZ/Act) | 29.0 Å^2 | 32.5 Å^2  | 28.8 Å^2  |

Crystal Structure

In order to examine the mode of action of NTR with the antibiotic nitrofurazone, we determined the crystal structure of the protein in complex with the antibiotic. Crystals of the complex were obtained by soaking crystals of the NTR-nicotinic acid complex in solution containing nitrofurazone. One molecule of nitrofurazone was clearly observed in each active site of the two dimers in the asymmetric unit. The ligand shows good occupancy with B factors of 17.6–25.4 Å^2 in the four sites.
slightly higher than those for the protein atoms in the active site. The nitrofurazone molecule adopts an essentially planar conformation. The amide group is over the central ring of FMN, the aliphatic moiety stacks over the two polar rings of the FMN, and the nitrofuran ring projects into a loop formed by residues 68–71 and into solution (Fig. 2, A and C). There are extensive van der Waals contacts between the ligand and the FMN, whereas the C2' of the nitrofuran ring is in van der Waals contact with Phe70 C'.

Nitrofurazone makes several hydrogen bonds with the protein, FMN, and ordered water molecules. The amide nitrogen (N4) hydrogen-bonds to the side chain of Glu165; the amide oxygen (O4) bonds with the Thr41 backbone NH and with the FMN ribitol O2*, and N3 (from the aliphatic chain) and O1 (from the nitro group) each hydrogen-bond to a bound water molecule. One of these bound water molecules also hydrogen-bonds to the Phe70 backbone amide, hence bridging between the ligand and the protein.

The active site of the protein shows only small changes from that of the nicotinic acid complex. In our previous study of the nicotinate complex, we suggested that the two phenylalanine residues, Phe124 and Phe70, play a major role in controlling access to the active site of NTR (13). In the nitrofurazone complex, the Phe124 side chain, which stacks on the nicotinate ring and is displaced from its position in the free protein, is in a similar position to that in the free protein. In contrast, the Phe70 side chain, which is mobile in the nicotinate complex, has more limited mobility due to the presence of the furan ring in the pocket. The contact between nitrofurazone amide and

![Figure 2](image-url)
NADH and NADPH support the NTR-catalyzed reduction of Ser40 side chain.

Since the ligand is small, the two crystal forms; however, in the orthorhombic crystal form, the two crystal forms were obtained, with very similar structures; the root mean square deviation for the Cα positions between the two complexes is 0.39 Å. One acetate molecule is found in each active site, with good occupancy, in a very similar position in the two crystal forms; however, in the orthorhombic crystal form, there are two additional acetate molecules bound to each dimer, outside the active site. In the tetrahedral crystal form, the B factor of the bound acetate is 24.01 Å² for chain A and 28.75 Å² for chain B, similar to that of the active site residues. The terminal amide of the nitrofurazone and the acetate molecule occupy the same position over the central ring of the flavin. However, the acetate was modeled with its two oxygen atoms facing the bottom of the binding pocket rather than in the same orientation as the amide group. In the orientation modeled, the methyl group points directly into the ring of Phe^{124} and forms van der Waals contact with Thr^{41} CE2 (Fig. 2B). This leaves one oxygen to hydrogen-bond to the Thr^{41} backbone NH and FMN ribityl O6β, with the other oxygen facing toward bulk solvent. The carboxylate group of acetate is in a similar position to that of the nicotinate in the nicotinic acid complex; however, it is rotated about 10° with respect to the FMN cofactor. The Phe^{124} and Phe^{70} residues in the acetate complex are similar in orientation to those in the free protein, since the ligand is small.

**Kinetic Characterization and Product Isolation**

**Substrate Specificity**—Initial steady-state kinetic assays at constant (750 μM) antibiotic concentration show that both NADH and NADPH support the NTR-catalyzed reduction of nitrofurazone and nitrofurantoin with similar rates and apparent $K_m$ values (Table III). Similarly, at 60 μM NADH or NADPH, nitrofurazone and nitrofurantoin have very similar activities. The simple 2-nitrofur an ring is a poorer substrate for NTR than either antibiotic, whereas nitromethane is not reduced by the enzyme.

To determine the full kinetic parameters for nitrofurazone, its rates of reduction by NTR were measured at a series of NADH and nitrofurazone concentrations (Fig. 3A). As expected for a substituted enzyme reaction, at increasing fixed concentrations of NADH, the apparent $k_{cat}$ and $K_m$ values of nitrofurazone increase; however, the $k_{cat}/K_m$ ratio remains constant, giving the same intercept on the ordinate in a half-reciprocal Hanes-Woolf plot (Fig. 3B). The rates thus can be fitted globally to Equation 1 (Fig. 3C), giving the following estimated parameters for the reduction of nitrofurazone by this enzyme under these conditions: $k_{cat} = 225 ± 34 s^{-1}$, $K_{m,NF2} = 1850 ± 400 μM$, and $K_{m,NADH} = 350 ± 76 μM$. The specificity constants are estimated as $k_{cat}/K_{m,NF2} = 0.15 ± 0.02 μM s^{-1}$ and $k_{cat}/K_{m,NADH} = 0.88 ± 0.13 μM s^{-1}$.

**Inhibition Studies**—Acetate displayed competitive inhibition with respect to NADH and uncompetitive inhibition with respect to both nitrofurazone and nitrofurantoin (Fig. 4, A and B), with a dissociation constant, $K_i$, in the millimolar range. Similarly, the potent flavoenzyme inhibitor, dicoumarol, and the NADH headpiece mimic, nicotinic acid, displayed competitive inhibition with respect to NADH and uncompetitive inhibition with respect to nitrofurazone and nitrofurantoin, with $K_i$ values in the micromolar range (Table IV). Acetate and acetamide show similar degrees of inhibition versus NADH and nitrofurazone, indicating that the observed effect is unlikely to be due to charge or changes in ionic strength. These patterns of inhibition, coupled with the constant $k_{cat}/K_i$ observed for the nitrofurazone as the concentration of NADH is varied, confirm kinetically that the enzyme has a bi-bi substituted (ping-pong) enzyme mechanism and show that acetate binds only to the oxidized enzyme.

**Isolation of Nitrofurazone and Nitrofurantoin Reduction Products**—The products of the enzymic reduction of nitrofurazone (mass 198) and nitrofurantoin (mass 238) were isolated by semipreparative reverse phase HPLC and analyzed by UV-visible spectroscopy and EIMS (Fig. 5). When the HPLC was conducted under nitrogen, the reduction of nitrofurazone by NTR gave a single distinct product, with a parent ion with a mass 184 Da on EIMS. This corresponds to a loss of an oxygen atom and the gain of two hydrogen atoms (i.e., a four-electron reduction to the hydroxylamine species). The hydroxylamine gave dark yellow crystals on recovery from solution and showed two peaks in UV-visible spectra in the range 250–600 nm, at 300 and 380 nm. When the HPLC was conducted in air, however, a different single product was isolated, which gave only one absorbance peak, at 265 nm, in the UV-visible range. EIMS showed that this product had a parent ion of mass 182 Da, consistent with a two-electron reduction to nitrosourea and the loss of an oxygen atom. The hydroxylamine was readily oxidized when exposed to air, forming the nitroso derivative, whereas the nitroso derivative was readily reduced to the hydroxylamine by NADH in solution. Similar behavior was observed with nitrofurantoin, but only one product was analyzed by EIMS, that formed under aerobic conditions. The parent ion of this compound was shown to have a mass of 222 Da, consistent with the two-electron reduced nitroso species. Thus, the
final products of NTR reduction of the antibiotics in the presence of NADH are the hydroxylamine derivatives, which, upon isolation in air, are oxidized to the nitroso compounds.

The reduction of a nitro group to a hydroxylamine is a four-electron reduction, via the nitroso derivative. Thus, each reaction must use two molecules of NADH. To confirm the stoichiometry of the reaction and the identity of the products, reactions at low nitrofurazone concentration were monitored either at 420 nm, observing the nitrofurazone reduction, or at 330 nm, where the nitrofurazone and hydroxylamine product have the same molar absorption coefficient, monitoring NADH oxidation. The rate of oxidation of NADH was 2.1 times that of the reduction of the nitrofurazone.

Reduction of Nitro and Nitroso Aromatic Compounds by NTR—Each FMN group can only perform two-electron reductions, but no nitroso intermediates have been observed for NTR or other homologous enzymes. To determine whether the nitroso group is reduced by the enzyme or nonenzymatically by NADH, we examined the reduction of the simple analogues 2-nitrotoluene, 2-nitrosotoluene, nitrobenzene, and nitrosobenzene in the presence and absence of NTR.

Nitrosobenzene and 2-nitrosotoluene are each readily reduced by NADH with second-order rate constants of $120 \text{ M}^{-1} \text{s}^{-1}$ (Table V). Both nitroso compounds are reduced much more rapidly in the presence of nanomolar concentrations of enzyme, showing that this reduction is also enzyme-catalyzed. For nitrosobenzene, the full Michaelis-Menten curve could be followed, giving values for all three constants, $k_{\text{cat(app)}}$, $K_{M(app)}$, and $k_{\text{cat}}/K_m$. For 2-nitrosotoluene, however, the $K_{M(app)}$ is higher than for nitrosobenzene, and only the initial part of the Michaelis-Menten curve could be studied; at higher substrate concentrations, significant NADH depletion occurred within a few seconds. This allowed determination of $k_{\text{cat}}/K_m$ but not of $k_{\text{cat(app)}}$ or $K_{M(app)}$ separately.

Nitrobenzene and 2-nitrotoluene are much poorer substrates for the enzyme than the nitroso compounds. For nitrobenzene, the $K_{M(app)}$ under these conditions is higher than its solubility limit, so only the $k_{\text{cat}}/K_m$ ratio could be determined, whereas for 2-nitrotoluene all three parameters could be determined. The $k_{\text{cat}}/K_m$ ratio for the enzymic reduction of both nitroso compounds is 20,000–30,000-fold higher than that for the corresponding nitrocompound. It is this ratio, the specificity constant, that determines the relative rates of reaction for two competing substrates. Thus, reduction by NTR of the nitroso intermediates is much faster than that of the nitrocompounds, explaining why it is difficult to observe these intermediates.

DISCUSSION

In this study, we have confirmed kinetically that the reduction of the antibiotic nitrofurazone by E. coli NTR follows a bi-bi substituted enzyme (ping-pong) mechanism, based on the constant $k_{\text{cat}}/K_m$ for nitrofurazone with varying NADH concentration and on the pattern of inhibition with several inhibitors. The $k_{\text{cat(app)}}$ and $K_{M(app)}$ values for nitrofurazone and NADH when measured at low concentrations of substrates (Table III) are similar to those determined previ-
ously for this enzyme: $K_{m(NFZ)} = 64 \mu M$, $K_{m(NADH)} = 6 \mu M$, and $k_{cat} = 10 s^{-1}$ (11) and $K_{m(NFZ)} = 153 \mu M$, $K_{m(NADH)} = 22 \mu M$, and $k_{cat} = 13 s^{-1}$ (12). However, when the concentration of both substrates is increased, the global $K_p$ values obtained for each substrate and $k_{cat}$ values are over 10-fold higher than those estimated previously. Zenno et al. (12) measured the reaction only at low concentrations of substrates, whereas Anlezark et al. used a stopped assay, and substrate depletion may have limited the observed rates (11). Because of the limited solubility of the antibiotic, it is not possible to measure rates above ~2.5 mM nitrofurazone, limiting the precision of the global fit of the parameters. The $K_p$ determined for NADH is below the estimated concentration of NADH in E. coli (~1 mM), so in vivo the enzyme will be ~75% saturated with NADH in this reaction, with the rate being limited by the nitrofurazone concentration. The global rate determined in this study shows that the enzyme is more active toward nitroaromatic substrates than previously estimated.

We have isolated the end product of the reaction with nitrofurazone in the presence of excess NADH and shown this to be the hydroxylamine derivative, which results from four-electron reduction of the nitro group on the furan ring. We have shown that for nitrosoaromatics, the specificity constant of the enzyme, $k_{cat}/K_m$, is over 10,000-fold greater than that for the corresponding nitro compounds and that the reduction of nitrosoaromatics is faster in the presence of enzyme. This suggests that the reduction of nitro aromatics by NTR proceeds via two consecutive two-electron reductions, both of which are enzyme-catalyzed. The much faster rate of the second reaction explains why nitroso intermediates have not been observed.
In all flavoproteins, the substrate binds such that the site of reduction is 3.5 Å from the N5 of the flavin (32). In order to yield the nitroso and hydroxylamine products, in the reduced form of the enzyme the nitro group of the substrate must be close to the N5 of the FMNH₂. Surprisingly, in the crystal structure of the oxidized NTR-nitrofurazone complex, the amide group of the nitrofurazone is bound over the reactive N5 of the flavin, whereas the nitro group to be reduced projects into a loop and is 7.7 Å away from the N5. Thus, in the crystal structure of the oxidized enzyme, nitrofurazone binds in a nonproductive orientation. This strongly suggests that nitrofurazone binds in the opposite orientation to the oxidized and reduced enzymes. The inhibitors acetate and acetamide both give competitive inhibition with respect to nitrofurazone, showing that they only bind to the oxidized enzyme. Thus, amide and carboxylic acid groups appear to bind preferentially over the flavin in the oxidized enzyme, whereas nitro groups bind in this position in the reduced enzyme.

The structures of the oxidized and reduced forms of nitroreductase from Enterobacter cloacae, which is 88% identical to the E. coli enzyme, have been published (16). The oxidized form contained acetate in the active site and is very similar to that presented here. The reduced form, which was collected under identical conditions to the oxidized form, no longer bound acetate. This is in agreement with our kinetic data. The only structural change in the enzyme observed following reduction was a slight increase in the butterfly angle of the isoalloxazine ring system of FMN, from 16 to 25°. Residues interacting with the ring system accommodated this increased bend by a largely rigid movement of nearby structural elements. Recently, the structure of the reduced form of NTR was determined, at lower resolution than that of the Enterobacter cloacae enzyme (15), and again very little structural difference between oxidized and reduced enzyme was observed. However, there is a large change in the electron distribution of the FMN ring system upon reduction, partly due to the addition of electrons and also due to the change in delocalization (33). This is reflected, for example, in the pKₐ values of N5 of the free flavin. In the oxidized form, the electrons are delocalized over all three rings, and the pKₐ of N5 is 8.5, whereas in the reduced form only the two outer rings are aromatic, and the pKₐ of N5 is 6.8. We propose that it is this difference in charge distribution at the active site that leads to the alteration in binding orientation of nitrofurazone in the reduced and oxidized enzyme. This may be particularly important in nitroreductase, since it has a broad substrate specificity, reducing 5- and 6-membered nitroaromatics and also quinones. The substrate is sandwiched between the flavin and a phenylalanine residue (Phe124) and is held primarily by weak van der Waals and dipole-dipole interactions. Hence, the electron distribution of the FMN may play a major role in substrate orientation and reactivity. In addition, the large change in charge distribution on the flavin on reduction would not only orient the correct substrate but could prevent most substrates from binding to the wrong form of the enzyme (substrate inhibition) as well as helping to eliminate the products of the reaction.

Different binding orientations for substrate in the oxidized and reduced enzyme have also been reported in pentaerythritol tetranitrate reductase, another flavoprotein with broad substrate specificity (34). As with NTR, the major consequence of flavin cofactor reduction in pentaerythritol tetranitrate reductase is a change in the butterfly angle of the active site FMN, with minimal structural changes in surrounding residues. The
charge distribution on the reduced flavin cofactor may play the major role in substrate selectivity, binding affinity, and reactivity in many flavoproteins with broad substrate specificity.

Acknowledgments—We acknowledge the European Synchrotron Radiation Facility for access to the facilities and help in data collection. We thank R. A. Parslow for skillful preparation of the enzyme.

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