Kinetic Characterization of Xenobiotic Reductase A from *Pseudomonas putida* 86†

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ABSTRACT: Xenobiotic reductase A (XenA) from *Pseudomonas putida* is a member of the old-yellow-enzyme family of flavin-containing enzymes and catalyzes the NADH/NADPH-dependent reduction of various substrates, including 8-hydroxycoumarin and 2-cyclohexenone. Here we present a kinetic and thermodynamic analysis of XenA. In the reductive half-reaction, complexes of oxidized XenA with NADH or NADPH form charge-transfer (CT) intermediates with increased absorption around 520–560 nm, which occurs with a second-order rate constant of 9.4 × 10^5 M\(^{-1}\) s\(^{-1}\) with NADH and 6.4 × 10^5 M\(^{-1}\) s\(^{-1}\) with NADPH, while its disappearance is controlled by a rate constant of 210–250 s\(^{-1}\) with both substrates. Transfer of hydride from NADPH proceeds 24 times more rapidly than from NADH. This modest kinetic preference of XenA for NADPH is unlike the typical discrimination between NADH and NADPH by binding affinity. Docking studies combined with electrostatic energy calculations indicate that the 2-phosphate group attached to the adenine moiety of NADPH is responsible for this difference. The reductions of 2-cyclohexenone and coumarin in the oxidative half-reaction are both concentration-dependent under the assay conditions and reveal a more than 50-fold larger limiting rate constant for the reduction of 2-cyclohexenone compared to that of coumarin. Our work corroborates the link between XenA and other members of the old-yellow-enzyme family but demonstrates several differences in the reactivity of these enzymes.

Xenobiotic reductases are bacterial enzymes of the old-yellow-enzyme (OYE) family known to catalyze the reduction of the olefinic bond of α,β-unsaturated carbonyl compounds, including ketones and esters with NADH or NADPH as the electron source (1–7). Xenobiotic reductase A (XenA) from *Pseudomonas putida* catalyzes the NAD(P)H-dependent reduction of various biotic and abiotic compounds (8). Recently, it was shown that XenA catalyzes the reduction of the C3=C4 double bond of 8-hydroxycoumarin, indicating its involvement in the degradation of quinoline, a ubiquitous N-heterocyclic pollutant with carcinogenic properties (9), along the 8-hydroxycoumarin pathway in *P. putida* 86 (10).

The crystal structure of XenA has been determined for the enzyme alone and in complex with two different substrates (10). The structure reveals a dimeric arrangement with one (β/α)\(_s\) barrel domain per monomer, which binds a FMN molecule on the solvent-exposed C-terminal side of the barrel. A tryptophan residue from the C-terminal helix of the neighboring monomer protrudes into the active site and forms one wall of the substrate-binding pocket. The active site is further lined by histidine and tyrosine residues, which presumably are needed to bind and orient the substrates, stabilize developing charges during turnover, and donate protons. A feature distinguishing XenA from most members of the OYE family is the presence of a cysteine residue in the active site near the N5 position of the isoalloxazine ring (10). XenA shares its overall arrangement and active site architecture with YqjM, and both have been suggested to form a new subfamily within the OYE family (11).

Genome sequencing projects revealed the OYE-like enzymes to be a rapidly growing family, which in some organisms, such as *Saccharomyces cerevisiae* (12), *Shewanella oneidensis* (13), and *P. putida* KT2440 (14), occur in up to six copies. The modest substrate specificity of OYE-like enzymes has attracted the attention of biotechnologists (15), further motivating more detailed studies of the physiological function and catalytic mechanisms of different variants. Therefore, several members of this enzyme family have been intensely studied, and the structural, kinetic, and thermodynamic characteristics of their interaction with various substrates have been elucidated, which have revealed that despite their similar structures they differ remarkably in their reactivities (11, 16–33). Here, we have investigated the thermodynamic characteristics of XenA and studied its reactivity with various substrates. Our data suggest an electrostatic basis for the kinetic preference of XenA for NADPH over NADH.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. Complex microbial media were purchased from Roth and Otto Nordwald and were prepared as described by Sambrook et al. (35). All chemicals and enzymes were purchased from Fluka and AppliChem. 5-Deaza-10-methyl-3-sulfopropylisoalloxazine was a gift from P. M. H. Kroneck (University of Konstanz, Konstanz, Germany). The extinction

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§Abbreviations: CT, charge transfer; EDTA, ethylenediaminetetraacetate; FMN, flavin mononucleotide; OYE, old yellow enzyme; PDB, Protein Data Bank; XenA, xenobiotic reductase A.
cofactors of XenA (ε_{290} = 71050 M^{-1} cm^{-1}, and ε_{464} = 12200 M^{-1} cm^{-1}), NADH (ε_{530} = 6200 M^{-1} cm^{-1}), and NADPH (ε_{440} = 6220 M^{-1} cm^{-1}) were used to calculate the concentrations of the enzyme, cofactor, and substrates.

**Protein Expression, Purification, and Activity Assay.** The gene of XenA was isolated from *P. putida* 86 and cloned into a pET11a vector as described previously (10). XenA was expressed in *Escherichia coli* Rosetta(DE3)pLysS using LB medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, at 20 °C overnight.

Crude cell extracts were prepared in 50 mM Tris buffer (pH 8.0) for a purification that included three chromatographic steps. The first column was a Q-Sepharose FF column, followed by ion-exclusion chromatography was conducted, the protein was reconstituted on ice with 5 mM FMN overnight. Eight liters of cell culture yielded 600 mg of enzyme with a purity exceeding 95%.

The specific activity of XenA was determined from absorbance changes at 340 nm due to the oxidation of NADPH. The reaction was performed using an Analytik Jena Specord40 spectrophotometer at 25 °C.

**Photoreduction of XenA.** XenA was photoreduced in a glass tonometer with a cuvette attached to a projector (Agfa, Opticus 100) was used. The reduction of XenA-bound FMN in the presence and absence of 1 mM NADPH was determined by multiple nonlinear regression analysis of the measured rates using eq 4 (40):

\[
v = \frac{V_{\text{max}} [A][B]}{K_{\text{mab}}[A] + K_{\text{mab}}[B] + [A][B]}
\]

where [A] is the concentration of 2-cyclohexenone, [B] is the concentration of NADPH, V is the observed rate, V_{\text{max}} is the rate at saturating substrate concentrations, K_{\text{mA}} is the K_m for 2-cyclohexenone at saturating NADPH levels, and K_{\text{mB}} is the K_m for NADPH at saturating 2-cyclohexenone levels.

**Rapid Reaction Techniques.** Measurements on the kinetics of the reductive half-reaction were performed under anaerobic conditions using an Applied Photophysics SX-20MV kinetic spectrophotometer with a 1 cm observation path length cuvette coupled either to a diode array detector or to a monochromator and photomultiplier. The reaction temperature was controlled with a Haake F8/C25 thermostat. Standard reaction conditions were 50 mM Tris (pH 8.0) at 20 °C. In a typical experiment, enzyme at XenA concentration of approximately 10 μM was mixed with an equal volume of substrate solution, the latter at concentrations ranging from 50 to 5000 μM. Each experiment was repeated at least five times for each substrate concentration. The reactions were monitored at 464 and 540 nm over an appropriate time scale. Observed kinetic transients at 464 nm were fit to single exponentials, and transients at 540 nm.
monitoring formation and decay of charge-transfer complexes, were fit with double exponentials using Pro-Data (Applied Photophysics) to yield observed rate constants ($k_{\text{obs}}$).

The reductive half-reaction sequence was modeled as shown in eq 5

$$A + B \quad \xrightarrow{k_1} \quad C \quad \xrightarrow{k_2} \quad D$$

where $A$ is $\text{XenA}_{\text{ox}}$, $B$ is NAD(P)H, $C$ is the $\text{XenA} - \text{NAD(P)H}$ charge-transfer complex, and $D$ is $\text{XenA}$ containing the two-electron-reduced state of FMN and bound NAD(P)$^+$. The oxidative half-reaction sequence was modeled as shown in the general eq 6

$$E + F \quad \xrightarrow{k_1} \quad G \quad \xrightarrow{k_2} \quad H$$

where $E$ is $\text{XenA}_{\text{red}}$, $F$ is 2-cyclohexenone (coumarin), $G$ is the $\text{XenA}_{\text{red}} - 2$-cyclohexenone (coumarin) charge-transfer complex, and $H$ is $\text{XenA}_{\text{red}}$ with bound 2-cyclohexanone (chroman-2-one).

Hyperbolic plots of observed rate constants versus substrate concentration were fitted using eq 7 to yield the limiting rate of reaction at high $[S]$, $k_X$, and the dissociation constant $K_D$ (41).

$$k_{\text{obs}} = k_X[S]/(K_D + [S])$$

Rate constants for the formation of the charge-transfer complex between oxidized XenA and NADH/NADPH were approximated by linear regression analysis with eq 8, where $k_2$ is the limiting rate of reduction of XenA with the respective nicotinamide used.

To relate the individual limiting rate constants of the reductive and oxidative half-reactions to the steady-state catalytic constants, eq 9 has been used:

$$k_{\text{cat}} = k_2k_4/(k_2 + k_4) = k_{\text{red}}k_{\text{ox}}/(k_{\text{red}} + k_{\text{ox}})$$

Solutions of oxidized XenA were made anoxic in a glass tonometer as described above. Solutions of reduced XenA were made anoxic in a tonometer with a cuvette side arm and titrated with NADH to achieve complete flavin reduction. We made all substrate solutions anoxic by flushing them with dinitrogen gas.

Docking. NADPH was docked to the crystal structure of XenA (PDB entry 2H8X) using DOCK 6 (42). The charges were taken from the CHARMM27 force field (43). A "divide and conquer" approach was used to reduce the number of rotatable bonds. The nicotinamide was docked close to FMN using chemical docking, which was used to incorporate information about the chemical complementarity of ligand and receptor moieties into the matching process. In a second step, adenosine was independently docked within a radius of 25 Å from FMN. The remaining parts of NAD(P)H (i.e., the ribose and phosphates) were constructed geometrically and subsequently energetically minimized using CHARMM (44), while the rest of the protein, the nicotinamide ring and the adenine ring, was kept fixed.

Electrostatic Calculations. To study the effects of the phosphate group of NADPH on the hydride-transfer energies between the nicotinamide ring and the isoalloxazine ring, electrostatics were calculated using the program SOLINPROT of the MEAD package (45). We calculated the interaction of the nicotinamide ring and the isoalloxazine ring with the monophosphate group bound to the ribose before and after the electron transfer. The XenA dimer was used to define the dielectric boundaries. For this calculation, only the charges of the nicotinamide ring and the phosphate group of NADPH and the isoalloxazine ring of the FMN were considered. The dielectric constants of protein and of water were set to 4.0 and 80.0, respectively. The ionic strength was set to 0.1 M. The calculation was done in two focusing levels with grids of 181 × 180 × 180 grid points. The grid spacing was set to 1.0 and 0.25 Å for the outer and the inner grid, respectively. The outer grid was centered at the coordinate center of the protein; the inner grid was centered at the N1 atom of FMN.

RESULTS

Photoreduction of XenA. XenA was reduced using the light-mediated generation of electrons by the deazaflavin–EDTA couple in the presence of phenafoxanine as the redox mediator (Figure 1A). This method has been applied to ensure single electron transfer to allow for initial semiquinone formation. Photoreduction proceeds in a two-step mechanism. Directly after each illumination period, we observe a signal increase around 350 nm, which we assign to the formation of the red anionic
The linear fit of the plot of log \( E_{\text{ox}} / E_{\text{red}} \) vs log \( D_{\text{ox}} / D_{\text{red}} \). The solid line displays the linear fit with a slope of \(-1\). The redox potential of XenA was calculated to be \(-263\) mV.

Figure 1B shows the spectrum (dashed line) with a characteristic peak around 400 nm and an increase in signal magnitude between 500 and 550 nm. Three minutes after illumination, the semiquinone signature was not observed any more and the enzyme-bound flavin was converted to the dihydroflavin form as a result of the dismutation of the semiquinone. After complete reduction of the enzyme, the cuvette was exposed to air, allowing XenA to reoxidize, resulting in a spectrum indistinguishable from the starting spectrum.

**Determination of Reduction Potential.** The generation of electrons by the xanthine oxidase–xanthine couple has been used as an alternative method to reduce XenA. The presence of a reference dye (phenosafranine; \( E_m = -252 \) mV) allowed the determination of the reduction potential of XenA-bound FMN. The small amount of xanthine oxidase ensured equilibrium conditions at all times, whereas both enzyme and dye take up two electrons. Reduction of XenA by xanthine oxidase in the absence of phenosafranine showed the slow conversion of FMN from the quinone to the hydroquinone state without any detectable formation of semiquinone species (data not shown).

Spectra recorded during the reaction with phenosafranine show that XenA and phenosafranine are reduced to similar extents (Figure 2). The absorbance values at 464 and 521 nm were used to calculate the concentrations of oxidized XenA and the dye. The inset shows the plot of log \( E_{\text{ox}} / E_{\text{red}} \) vs log \( D_{\text{ox}} / D_{\text{red}} \). The solid line displays the linear fit with a slope of \(-1\). The redox potential of XenA was calculated to be \(-263\) mV.

**Steady-State Kinetics.** Catalytic turnover of XenA with various concentrations of 2-cyclohexenone and NADPH under anaerobic conditions was analyzed to determine the values of \( k_{\text{cat}} \), \( K_{\text{mB}} \), and \( V_{\text{max}} \). The inset in Figure 3 shows the rate dependencies of 2-cyclohexenone for different NADPH concentrations and the corresponding nonlinear fits using the normal Michaelis–Menten equation. The parallel lines in the double-reciprocal plot...
correlation of $1/k_{obs}$ with $1/[\text{NAD(P)H}]$ (Figure 4D, E, insets) indicates that the equilibrium condition for the XenA–NAD(P)H reaction ($k_1 \gg k_2$) holds, as seen experimentally, and that eq 7 can be used to determine the limiting rate constant for the reduction of FMN ($k_2$) and the dissociation constant ($K_D$) of the complex (41). For the reaction of XenA$_{ox}$ with NADH, a rate constant ($k_2$) of $1.50 \pm 0.02 \text{ s}^{-1}$ is obtained, while with NADPH, the rate constant is $24 \text{ times higher, } 35.7 \pm 0.6 \text{ s}^{-1}$. The dissociation constants for the XenA$_{ox}$–NADH complex of $176 \pm 14 \mu\text{M}$ and for the XenA$_{ox}$–NADPH complex of $256 \pm 12 \mu\text{M}$ obtained using the rapid equilibrium model are in good agreement with the ratio between the rate constants for the formation of the CT complexes (XenA$_{ox}$–NADH, $k_1/k_2 = 272 \mu\text{M}$; XenA$_{ox}$–NADPH, $k_1/k_2 = 336 \mu\text{M}$). Appreciable rates for the back reaction would cause the double-reciprocal plot of $1/k_{obs}$ versus $1/[\text{NAD(P)H}]$ to curve down for high substrate concentrations (41), which is not observed (Figure 4D,E, insets). Furthermore, an adaptation of the model to include the rate constant of the back-reaction ($k_{-2}$) did not improve the fit to the observed rates, as judged from testing the goodness of fit. We conclude that $k_{-2}$ is very small and that reduction of XenA by either NADH or NADPH is functionally irreversible.

**Oxidative Half-Reaction.** To examine the reoxidation of XenA, two different substrates have been studied, 2-cyclohexene-none and coumarin. 2-Cyclohexene-none has been used as a substrate in most rapid kinetic studies with members of the OYE family, notably with OYE (19), morphinone reductase (24, 46), PETN reductase (29), and YqjM (34), and it is therefore possible to compare the reactivity of XenA with the reactivities of these enzymes. However, since we have recently shown that XenA participates in the degradation of quinoline along the 8-hydroxycoumarin pathway and is able to reduce the C3–C4 double bonds of heteroaromatic compounds such as coumarin (10), we have also examined the reaction of XenA with this substrate.

Spectral changes are observed directly after reduced XenA reacts with 50 μM 2-cyclohexene-none, as compared to the spectrum of reduced XenA with substrate (Figure 5A). The observed maximum at 424 nm 5 ms after reduced XenA is mixed with 2-cyclohexene-none indicates that it is not due to a fast reaction.
phase in which FMN is oxidized but rather that another reaction
intermediate involving reduced XenA and 2-cyclohexenone is
formed rapidly within the dead time of the instrument. Formation
of a CT complex is discernible with both substrates by an initial
absorption increase around 650 nm (data not shown). The CT
complex forms very rapidly and decays with the same rate as XenA
becomes oxidized. The low absorbance around 464 nm at very
short times indicates that the CT complex involves reduced XenA
and substrate. A larger absorption increase is subsequently ob-
served at 464 nm (Figure 5B). The rate constant observed for the
majority of the absorbance increase at 464 nm shows a hyperbolic
dependence on the concentration of 2-cyclohexenone and cou-
marin (Figure 5C,D). The linear relation between 1/

\[ k_{\text{obs}} \]

and 1/[2-cyclohexenone] or 1/[coumarin] (Figure 5C,D, insets) indi-
cates that it is justified to include the rapid equilibrium condition in
our model (41). A rate constant (\( k_4 \)) of 13.1 (±0.1 s

\[-1 \) and a

\[ K_D \]

of 86 (±2 μM have been determined for 2-cyclohexenone. The
reduction of coumarin is slower than the reduction of 2-cyclohexe-
none by a factor of 50 with a rate constant (\( k_4 \)) of 0.243 (±0.001 s

\[-1 \), and the complex has a \( K_D \) of 19.3 (±0.2 μM.

**Modeling NADH and NADPH in the Active Site of XenA.** We next examined a model for both NADH and NADPH bound to oxidized XenA, generated using the DOCK 6 program. In this model, NADH and NADPH bind both in the same way to XenA. The nicotinamide ring of NAD(P)H docks in a stacked conformation with the isoalloxazine ring of FMN (Figure 6) and is hydrogen-bonded to His178, His181, and Cys25. The diphosphate forms a salt bridge with Lys106 and a hydrogen bond with Tyr183. The distance between the phos-
phorus atom of the 2'-phosphate of NADPH and N1 of the
icotinamide is 6.2 Å. The 2'-phosphate of NADPH is oriented
toward the solvent and does not interact with the protein matrix
but does form a hydrogen bond to the 2'-OH group of the ribose
attached to nicotinamide (Figure 6).

From electrostatic calculations using the Poisson–Boltzmann
equation, we find that the free energy of the transfer of hydride
from the nicotinamide to the isoalloxazine ring of the FMN is
shifted by ~1.83 kcal/mol due to the presence of the 2'-phosphate
on NADPH compared to NADH. Thus, the hydride transfer is
more favorable with NADPH than with NADH. Most of this
difference in free energy manifests itself in a lower activation
energy for NADPH versus NADH as reflected in the relative
limiting rates of reduction (36 s

\[-1 \) vs 1.5 s

\[-1 \), respectively).

**DISCUSSION**

Recently, we have shown that XenA participates in the
degradation of quinoline and reacts with heteroaromatic com-
ponents such as coumarin and 8-hydroxycoumarin using both
NADH and NADPH as electron sources (10). Here we have
examined its reactivity with both reducing and oxidizing sub-
strates, allowing us to compare it to related flavoenzymes.

No stable semiquinone form of XenA was observed in the course
of the reductive titrations (using either the xanthine–xanthine
oxidase or deazaflavin–light couple as the reductant), indicating
that transiently formed red, anionic semiquinone rapidly disproportionated. The hydrogen bonding distance between the amide nitrogen of Cys25 and N5 of FMN (Figure 6) indicates that the amide nitrogen acts as hydrogen bond donor and N5 as hydrogen bond acceptor in the oxidized state. This interaction disfavors the formation of the neutral semiquinone as the hydrogen bond would be broken when N5 becomes protonated. The anionic semiquinone is therefore favored; however, as frequently observed, it is thermodynamically unstable. The protein environment thus does not stabilize the semiquinone state to any significant degree. Morphinone reductase (24), PETN reductase (29), and YqiM (2) also fail to form detectable amounts of semiquinone, although OYE forms 15–20% of the anionic semiquinone species under equilibrium conditions (47).

The rate constants for the reaction of reductases with both reducing and oxidizing substrates are critically dependent on the reduction potential of the flavin cofactor, which determines which reactions are thermodynamically feasible. The reduction potential of the FMN–FMNH\(^{-}\) couple in XenA is \(-263\) mV, substantially lower than found for PETN reductase (\(-193\) mV) (29), OYE (\(-230\) mV) (47), and morphinone reductase (\(-242\) mV) (26). XenA has several structural peculiarities that may be responsible for this, including the presence of an active site cysteine residue [Cys25 (Figure 6)] in place of a conserved threonine residue found in other members of the OYE family. The hydroxyl group of the threonine residue of other family members forms a hydrogen bond with the C4 oxygen atom of the isalloxazine ring, and its replacement with alanine lowered the reduction potential of the FMN–FMNH\(^{-}\) couple from \(-230\) to \(-263\) mV in OYE (21) and from \(-242\) to \(-290\) mV in morphinone reductase (26). It may therefore be that the presence of the cysteine residue in place of the threonine contributes to the low reduction potential of XenA. We have also examined the reductive and oxidative half-reactions to improve our understanding of the reactivity of XenA. The first observed step in the reductive half-reaction with both NADH and NADPH is the formation of a CT complex. The linear relationship between observed rates of CT complex formation and the concentration of reductants is consistent with a simple bimolecular reaction (Figure 7). There is good agreement between the apparent dissociation constants for the complexes between oxidized XenA and NAD(P)H obtained from the hyperbolic plots of \(k_{\text{red}}\) versus [NAD(P)H] and the ratio between on and off rate constants for the formation of the CT complexes (\(k_{\text{2}}\) and \(k_{\text{1}}\), respectively, in Figure 6 and Table 1).

To gain further insight into the interaction of NAD(P)H with XenA, we performed docking simulations. In the docked structure, both NADPH and NADH are kept in position by H-bonds to amino acids like His178 and Tyr183, which are highly conserved within the OYE family. The binding mode of NADPH in complex with XenA, including the short distance between the 2'-phosphate and the nicotinamide ring, has not been observed so far. In most flavoprotein structures, the 2'-phosphate is oriented away from the nicotinamide ring and is stabilized by arginine residues.

The second step in the reaction of XenA\(_{\text{ox}}\) with NAD(P)H is the transfer of hydride from the reduced nicotinamide to the N5 atom of XenA-bound FMN. The rate constants (\(k_{\text{2}}\)) for this step differ by a factor of 24 for NADH and NADPH, with the latter being faster. The main difference in the structure of the docked

**Figure 6:** Docked complex between XenA and NADPH. The stereoview shows residues of the active site in the vicinity of NADPH depicted as stick models with carbon atoms colored cyan. Interactions mentioned in the text are represented by dashed lines. NADPH and FMN are shown as stick models with carbon atoms colored white. NADH binds to XenA in the same way as NADPH. This figure was generated using PyMol (48).

**Figure 7:** Scheme for the reaction cycle catalyzed by XenA.
The limiting rate constant of the reaction of NADH-reduced XenA with 2-cyclohexanone and oxidized XenA occurs. The good agreement of the catalytic constant derived from eq 4 with NADPH and 2-cyclohexanone as substrates in the steady-state assay ($k_{\text{cat}} = 7.2 \text{ s}^{-1}$) with the catalytic constant following from the limiting rate constants of the oxidative and oxidative half-reactions with NADPH and 2-cyclohexanone (eq 9; $k_{\text{cat}} = 5.3 \text{ s}^{-1}$) indicates that the two product release steps, which have not been observed by our transient kinetic analysis, occur rapidly and are not limiting the reaction rate of XenA.

The structures of XenA and other members of the OYE family are very similar, but at the same time, some distinct features in the composition of the active site do exist. This analysis of native XenA revealed further remarkable properties of the enzyme such as its low reduction potential and its only modest specificity for NADPH over NADH.

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**REFERENCES**


