Understanding the Broad Substrate Repertoire of Nitroreductase Based on its Kinetic Mechanism

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Background: Nitroreductase reduces a broad range of nitroaromatics.

Results: Steady-state and pre-steady-state kinetics were combined with tests for aminoaromatic product formation.

Conclusion: Both half reactions occur via a simple mechanism lacking detectable gating steps consistent with the broad substrate repertoire.

Significance: Nitroreductase does not generate \( p \)-aminobenzoic acid and therefore appears not to reduce nitro groups to amines.

ABSTRACT

The oxygen-insensitive nitroreductase from Enterobacter cloacae (NR) catalyzes two-electron reduction of nitroaromatics to the corresponding nitroso compounds and, subsequently, to hydroxylamine products. NR has an unusually broad substrate repertoire, which may be related to protein dynamics (flexibility) and/or a simple non-selective kinetic mechanism. To investigate the possible role of mechanism in NR's broad substrate repertoire, the kinetics of oxidation of NR by \( p \)-nitrobenzoic acid (\( p \)-NBA) were investigated using stopped-flow techniques at 4°C. The results revealed a hyperbolic dependence on the \( p \)-NBA concentration with a limiting rate of \( 1.90 \pm 0.09 \text{ s}^{-1} \), indicating one-step binding prior to the flavin oxidation step. There is no evidence for a distinct binding step in which specificity might be enforced. The reduction of \( p \)-NBA is rate-limiting in steady-state turnover (1.7 \( \pm \) 0.3 \text{ s}^{-1}). The pre-steady-state reduction kinetics of NR by NADH indicate that NADH reduces the enzyme with a rate constant of \( 700 \pm 20 \text{ s}^{-1} \) and a dissociation constant of \( 0.51 \pm 0.04 \text{ mM} \). Thus we demonstrate simple transient kinetics in both the reductive and oxidative half-reactions that help to explain NR's broad substrate repertoire. Finally, we tested NR's ability to reduce \( p \)-hydroxylaminobenzoic acid (\( p \)-HABA), demonstrating that the corresponding amine does not accumulate to significant levels even under anaerobic conditions. Thus the \( E. \) cloacae NR is not a good candidate for enzymatic production of aromatic amines.

NAD(P)H:nitroaromatic reductases (Nitroreductase, EC 1.6.99.7) are flavoenzymes that catalyze the NAD(P)H-dependent reduction of the nitro groups of nitroaromatic and nitroheterocyclic compounds (1) (Figure 1A).

\( E. \) cloacae nitroreductase (NR)\(^1\) was first isolated from bacteria collected at a weapon storage facility by Bryant and co-workers (2,3) based on its ability to degrade explosive compounds such as 2,4,6-trinitrotoluene (TNT). The enzyme exists as a homodimer of 217-residue monomers with two identical active sites in the dimer interface each containing a non-covalently bound FMN that derives H-bonds from one monomer and hydrophobic contacts from both (4) (Figure 1B).

Nitroreductases are widely distributed among bacteria, but NR-like proteins are also found in archaea and eukaryotes including man (5). \( E. \) cloacae NR is able to reduce a variety of nitroaromatics ranging from nitrofurans, nitroarene pollutants such as 1-nitropyrene, nitro-
containing drugs (metronidazole), herbicides (dinoseb), and most famously nitroaromatic and nitramine explosives such as TNT (2,6,7). The E. coli homologue NfsB is being developed for use as a prodrug activator for treatment of cancer (8-10).

The NRs from E. cloacae and E. coli reduce nitroaromatics to the corresponding nitrosoaromatics and then to the corresponding hydroxylaminoaromatics via two successive two-electron reductions (6,11) (Figure 1A). It is difficult to isolate the nitroso intermediates because they react rapidly with NR, and the resulting hydroxylaminoaromatics react with their nitroso precursors to yield stable azoxy compounds (6,12,13). Nonetheless, there are reports of accumulation of a nitroso intermediate during biological reduction of certain nitroaromatics (14).

The hydroxylamine product is a basis for the toxicity of metronidazole and related antibiotics, which are activated in vivo by pathogen NRs (15,16). In anaerobes, other mechanisms reductively eliminate the nitro group producing nitro radicals that initiate oxidative stress (17,18).) Thus NR’s chemistry is being developed for clinical implementation against cancer via GDEPT (gene-directed enzyme prodrug therapy) and ADEPT (antibody-directed enzyme prodrug therapy) wherein an NfsB-antibody fusion would be targeted to tumors where it would then reductively activate the prodrug CB1954 in ADEPT (8,9,19). Thus it is important to know what product NR produces.

Bioremediation and biocatalytic uses of NR have also been envisioned based on NR’s possible production of aromatic amines. Aromatic amines would be valuable synthons, and there are a few instances in which amine products have been detected (12,13,20,21). In particular, the NR-Ms of Mycobacterium smegmatis converts the nitro group of benzothiazinone to an amine (20). The NR-Sal from Salmonella typhimurium displays both nitroreductase and enoate reductase activity, transforming nitrobenzene to nitrosobenzene, phenylhydroxylamine, and even the fully reduced product aniline (13). Similarly, the NR-I of Klebsiella sp. C1 transforms 2,4,6-TNT to 2-amino-4,6-dinitrotoluene (12). However amine products can elude detection due to their reactivity with oxygen. Thus dissolved O₂ can react with reduced products of NR including both the hydroxylamine and putative amine products in a futile cycle that will tend to diminish detected yields and mask formation of reduced products (7). This and the products’ proclivity for reactions among themselves has made it difficult to confirm accumulation of aminoaromatic products, especially given the common practice of conducting assays in air.

There are cases in which production of amines has been ruled out. For E. coli’s NfsB, it was demonstrated that only two equivalents of NADH are oxidized per equivalent of nitroaromatic reduced (11). Moreover Race et al showed that if reaction products were analyzed under aerobic atmosphere, hydroxylamines were converted to nitrosos. Thus full reduction of nitro groups to the corresponding amine is not a general property of the subfamily, and the identity of the substrate may be a major determinant of what final product can be achieved. Therefore we have tested whether E. cloacae NR can produce para-aminobenzoic acid (p-ABA) from para-hydroxylaminobenzoic acid (p-HABA) under anaerobic conditions.

NR’s broad substrate repertoire makes it an attractive enzyme for bioremediation of nitroaromatics and for producing valuable pharmacological reagents because this one enzyme could be used to transform a variety of related compounds (6,13). A number of enzymes are known that transform diverse substrates, including certain cytochrome P450s (22,23), and pentaerythritol tetranitrate reductase (PETN reductase) (24,25). Binding of diverse substrates appears to be associated with relatively weak and non-specific substrate binding (for example (26)). Indeed, NfsB’s substrates have been found to bind via water molecules and to have relatively large $K_m$ values (27). Thus one hypothesis is that NR’s broad specificity is related to its relatively high $K_m$s and large solvent-accessible active site (4).

Nitroreductase’s broad substrate repertoire could also have a mechanistic basis. In several enzymes that are highly discriminating regarding their substrates, the mechanisms have been found to incorporate multiple steps in which the prospective substrate is ‘tested’ on the basis of its ability to elicit a conformation change in the enzyme or to enhance binding of additional substrates needed in order for the reaction to proceed. Thus a second hypothesis is that NR’s
mechanism may lack gating steps that could constrain its use of diverse substrates.

Prior steady-state kinetics studies have shown that NR employs a ping-pong bi-bi mechanism in which the flavin is alternately reduced by a nicotinamide (preferably NADH or NADPH) and then reoxidized by a nitroaromatic (or quinone) substrate (6). Pre-steady-state studies of NfsB were used to identify variants of the enzyme that enhance its activity on CB1954 and tailor the site-specificity of the reduction with respect to the two nitro groups of CB1954 (28). In these, Jarrom et al determined the second order rate constant $k_i/K_d$ for the oxidative half-reaction (in which the flavin is reoxidized) but could not evaluate $K_d$ due to the limited solubility of CB1954, as well as loss of a substantial portion of the kinetic trace in the instrument dead time for the higher CB1954 concentrations. The second-order rate constants were many times faster than the overall rate constant, indicating that the rate-limiting step is slow product release, possibly depending on conformational change (28). Thus the oxidizing substrates used in that study (CB1954 or nitrofurazone) were not able to address the possibility of a binding step preceding the chemical reaction. Nor did this study address the reductive half-reaction due to reaction times within the dead time of the instrument at 25 °C.

To slow down NR’s reaction we have worked at lower temperatures, and performed pre-steady-state as well as steady-state analyses of each of the two half reactions of NR to identify elements of the reaction that could participate in defining NR’s substrate specificity (and lack thereof). We have chosen a poor substrate, $p$-NBA (para-nitrobenzoic acid) in order to allow chemical steps to contribute more to the observed rates, and we have simulated the reactions in terms of all the participating states of the enzyme to test for possible additional steps not explicit in the observed kinetics. $p$-NBA’s higher solubility in water also enabled us to probe binding events. Knowledge of the natures and lifetimes of all the participating states for this simple substrate provides a crucial baseline for understanding the different rates and product distributions displayed by different NR substrates as well as different variants of NR produced by mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Reagents** – NADH (100% purity) was purchased from Roche. $p$-NBA was from Acros Organics. $p$-Nitrosobenzoic acid ($p$-NOBA) was produced as per the procedure of Defoin (29) and validated by $^1$H NMR spectroscopy at 400 MHz. $p$-HABA was the generous gift of K. Ferguson and A. S. Bommarius (School of Chemical & Biomolecular Engineering, Georgia Institute of Technology) and was determined to be 99% pure based on NMR. Concentrations of reagents were determined using the known extinction coefficients at pH 7.0: $\varepsilon_{340} = 6.22 \times 10^3$ M$^1$cm$^{-1}$ or $\varepsilon_{370} = 2.66 \times 10^3$ M$^1$cm$^{-1}$ for NADH and $\varepsilon_{273} = 10.1 \times 10^3$ M$^{-1}$cm$^{-1}$ for $p$-NBA. Wild-type NR was expressed and purified as previously described (1). The concentration of holo-NR was evaluated based on the extinction coefficient of the bound flavin, $\varepsilon_{454} = 14.3 \times 10^3$ M$^{-1}$cm$^{-1}$ and rate constants reported refer to holo-enzyme (apo-FMN enzyme represented less than 10% of the total protein based on the ratio of A$_{454}$ to A$_{280}$ (1).

**Steady-State Kinetic Experiments** - Initial velocities at various concentrations of NADH and $p$-NBA were measured using stopped-flow spectrophotometry at 4°C (TgK Scientific). The reactions were monitored at 370 nm in order to observe conversion of NADH to NAD$^+$. The assays contained 60 nM enzyme, various concentrations of NADH (0.1 mM, 0.2 mM, 0.3 mM and 0.4 mM) and various $p$-NBA concentrations (0.1 mM, 0.2 mM, and 0.4 mM) in 50 mM potassium phosphate pH 7.50. Initial rates of the reaction were calculated using Program A (Generous gift of D. P. Ballou) and steady-state kinetic parameters were extracted using KaleidaGraph Version 3.6.2 to fit the data to the equation for a ping-pong mechanism (Equation 1) (30,31) where $K_{m}^{NADH}$ and $K_{m}^{pNBA}$ are the $K_m$ values for NADH and $p$-NBA, respectively.

$$
\frac{v}{E} = \frac{k_{cat}[NADH][pNBA]}{K_{m}^{NADH}[pNBA]+K_{m}^{pNBA}[NADH]+[NADH][pNBA]}
$$

(Rapid Reaction Experiments) - Pre-steady state reductive and oxidative half-reactions of NR were studied using a stopped-flow spectrophotometer (TgK Scientific). The flow system was made

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anaerobic by rinsing with an anaerobic buffer and incubating overnight with a solution of 50 mM sodium dithionite in 50 mM potassium phosphate pH 7.50. The buffer to be used for the sodium dithionite solution was first sparged with N₂ and then equilibrated overnight to remove oxygen in an anaerobic glove box (M-BRAUN UniLab glovebox with Siemens Corosop 15 controller). Sodium dithionite was added to the anaerobic buffer inside the glove-box and the resulting solution was transferred into tonometers. These were mounted on the stopped-flow apparatus to provide reservoirs of anaerobic solutions and used to flush the flow system, which was then allowed to stand overnight with dithionite solution throughout. Prior to experiments, the instrument was rinsed thoroughly with anaerobic buffer composed of 50 mM potassium phosphate pH 7.50 equilibrated with N₂ gas that had been passed through an oxygen removal column (Labclear).

In general NR was dissolved in 50 mM potassium phosphate pH 7.50 and experiments were performed at 4°C because the reaction rates at higher temperatures were too fast to permit detailed characterization. For single-mixing experiments a solution of ~35 µM NR was loaded in one syringe and a concentration of substrate twice the concentration desired for the reaction, in the same buffer, was loaded in the other syringe. Equal volumes of the two solutions were co-injected into the rapid mixer (75 µl each) and the dead-time was 1 ms.

For each substrate concentration, at least four replicate measurements were made once the instrument had stabilized, and these transients were averaged. Analysis was conducted by fitting the resulting kinetic trace to exponential equations for growth and decay using the Marquardt algorithm in Program A, developed by C. J. Chiu, R. Chung, J. Diverno, and D. P. Ballou at the University of Michigan (Ann Arbor, MI) or with the Kinesyst 3 software provided with the stopped-flow spectrophotometer (TgK Scientific). Plots of observed rate constants ($k_{obs}$) versus substrate concentration were analyzed using the non-linear least-square fitting algorithms in the KaleidaGraph package (Synergy Software).

To test the presumed kinetic mechanism, simulations of reaction transients predicted by various mechanisms, rate constants and binding equilibria were performed using Berkeley Madonna, ver. 8.3 (Macey and Oster, University of California - Berkeley) refined by the Runge-Kutta 4 integration method. In brief, in the reductive half-reaction, the known parameters, which are concentration of oxidized NR, the extinction coefficients of oxidized and reduced NR, and the reduction rate constant were provided to the simulation, then the other parameters which were $k_1$, $k_1$, $k_3$ and the extinction coefficients of $E_{ox}$:NADH, and $E_{red}$:NAD⁺ were adjusted manually to produce the best agreement with experimental results based on the superposition of

$$k_{obs} = \frac{k[\text{Substrate}]}{K_d + [\text{Substrate}]}$$

Rates of background reactions between substrate and dissolved oxygen were measured and constituted less than 1% of the rate observed in the presence of enzyme.

Kinetic traces were collected at a series of wavelengths from 340 to 700 nm at 5 nm intervals to look for the signatures of potential intermediates.

**Reductive half-reaction:** A solution of 26 µM NR was made anaerobic by equilibration in the anaerobic glove-box for 30 min then placed in a tonometer. All buffer and substrate solutions were made anaerobic by bubbling with N₂ gas that had passed through an O₂-removing cartridge. Substrate solutions were loaded in tonometers, which in turn were mounted on the stopped-flow spectrophotometer along with the NR solution. Upon rapid mixing of the enzyme with substrate, flavin reduction was monitored by measuring loss of absorbance at 454 nm. This wavelength is the peak of the oxidized enzyme's absorption and provides the maximum difference between the oxidized and the reduced enzyme. After mixing, the final concentration of the enzyme was 13 µM, whereas the concentration of the substrate was kept at least 5-fold greater than that of the enzyme, so that the pseudo-first-order condition was maintained. Analysis was carried out as described above. Rate constants ($k_{obs}$) were determined from fits of the kinetic traces obtained at 454 nm. Plots of $k_{obs}$ versus substrate concentration were analyzed using the non-linear least-square fitting algorithms in the KaleidaGraph package (Synergy Software).
simulated transients with experimental transients, (see Figure 3). A whole set of transients is treated at once. We applied the same method to the oxidative half-reaction in which the reduced NR concentration, extinction coefficients of oxidized and reduced NR, and the oxidation rate constant from the experiments were provided to the simulation, and allowed $k_d$, $k_a$, and the extinction coefficients of other species to be optimized.

**Oxidative half-reaction:** Reduced enzyme in 50 mM potassium phosphate at pH 7.50 (17.5 µM after mixing) was rapidly mixed with either $p$-NBA or $p$-NOBA in the same buffer. All substrate concentrations were at least 5-fold higher than the enzyme concentration used, to maintain pseudo-first-order conditions. The reduced enzyme solution was prepared by equilibration with the inert atmosphere in the glove box followed by reduction with a slightly sub-stoichiometric amount of dithionite (or NADH) in the anaerobic glove box (with equivalent results). The reduction process was monitored using a spectrophotometer at 4°C. Oxidation of the enzyme by substrate was observed at 454 nm by the stopped-flow spectrometer, revealing formation of oxidized NR-bound flavin. Analysis was carried out as described above.

**Testing for formation of aromatic amine product:** The decomposition of $p$-HABA was monitored by UV-visible spectrophotometry using a CARY-300 spectrophotometer to observe the absorbance of the final product of $p$-HABA oxidation (Figure 6C). $p$-HABA was dissolved to a concentration of 1.3 mM in 50 mM potassium phosphate pH 7.50 that had been made anaerobic, or equilibrated with air as a control. The anaerobic sample of $p$-HABA was produced by weighing $p$-HABA (15.3 mg) and transferring it into the anaerobic glove-box. The $p$-HABA powder was equilibrated in the glove-box for 10 min to ensure that there was no longer oxygen associated with it. A 5 ml stock solution of 20 mM was prepared in the glove-box using anaerobic buffer. For analysis, 1.3 mM $p$-HABA was transferred to a gas-tight anaerobic cuvette before removing it from the glove-box.

The stability and purity of $p$-HABA in anaerobic vs. air-equilibrated buffer was also characterized by $^1$H NMR spectroscopy at 400 MHz. $p$-HABA (13.3 mM) was dissolved in 50 mM potassium phosphate pH 7.50 containing 10% v/v $^2$H$_2$O and 0.1 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as a chemical shift reference ($\delta = 0$ ppm). The two samples were prepared as described above for optical samples but were transferred to NMR tubes instead of cuvettes. Wilmad NMR tubes, 5 mm diameter precision, were used to contain 600 µL of sample. For the anaerobic sample, Wilmad’s gas-tight anaerobic NMR tube (535-TR-7) was used after prior degassing and incubation in the glove box overnight. The Wet1D sequence was used to achieve solvent suppression (32).

The possible formation of aromatic amines was assessed using 3-methyl-2-benzothiazolinone hydrazone (MBTH) which reacts with aromatic amines to produce a strongly-absorbing chromophore (33,34). ($\varepsilon_{550} > 2$ mM$^{-1}$ cm$^{-1}$ in the presence of competing reactions with aromatic hydroxylamines.) All manipulations were performed under inert atmosphere in a glove box ([O$_2$] $\leq$ 10 ppm) at room temperature. With the exception of concentrated enzyme solution, all reagents and materials were equilibrated in the glove-box overnight. Enzyme solutions were rendered anaerobic by 5-10 pump-purge cycles of ~30 seconds followed by equilibration in the glove-box for 1 hour. Enzymatic reactions and controls were run for 0 min, 15 min, 30 min, or 10 hours in 50 mM potassium phosphate pH 7.50 containing 1.25 mM NADH, 17.5 µM NR, 0-1 mM $p$-ABA and/or 0-1 mM $p$-HABA. At the end of the enzymatic reaction, protein was removed by ultrafiltration using a centrifugal filter (EMD-millipore, 10 kDa cutoff) for both experimental samples and controls. Removal of the protein resulted in lower background absorbance. The published protocol for quantification of substituted aromatic compounds using MBTH was optimized to accommodate the NADH. Under anaerobic conditions 1.5 mM MBTH and 6.5 mM Ce(SO$_4$)$_2$ in the presence of 0.5-1 mM $p$-ABA produced the largest increase in absorbance at 550 nm. The total time between termination of the reaction by protein removal and reading of the colorimetric assay results did not exceed 40 minutes.

**RESULTS**

**Steady-State Kinetics of NR** — Although steady-state kinetic analyses of NR have been reported before (6), they were repeated as part of this effort in order to obtain data sets with which to test the
The kinetic mechanism of nitroreductase

The overall steady-state kinetics of NR were characterized using NADH and p-NBA as substrates at 4°C. Either NADH or p-NBA was varied at each of several concentrations of the other substrate as described in the experimental procedures and the previous study (6). The data (Figure 2A) were fit with Equation 1 to give a $K_m^{NADH}$ of $35 \pm 8 \mu M$, $K_m^{p-NBA}$ of $130 \pm 5 \mu M$, and a $k_{cat}$ value of $1.7 \pm 0.3 \text{ s}^{-1}$. The plot of initial velocity versus NADH concentration (0.02 – 0.33 mM) at high p-NBA concentration (0.4 mM) (Figure 2B) and the plot of initial velocity versus p-NBA concentration (0.025 – 0.6 mM) at high NADH concentration (0.25 mM) (Figure 2C) yielded similar parameters to those resulting from double-reciprocal plot (Figure 2A). The complete steady state study was repeated for two different preparations of NR. The results were within error of one another so only the parameters obtained from the larger study are reported.

Reductive half-reaction of oxidized NR with NADH – NR’s reaction with NADH was investigated by stopped-flow spectrophotometry under anaerobic conditions at 4°C. An anaerobic solution of oxidized NR was mixed with various concentrations of NADH to yield final concentrations of 13 µM NR and 0.08 mM, 0.16 mM, 0.32 mM, 0.64 mM, 1.28 mM, 2.56 mM, 5.12 mM, or 10.24 mM NADH (all concentrations are quoted after mixing here and below). Reduction of the enzyme-bound oxidized FMN was monitored via the loss of absorbance at 454 nm (Figures 3A and 3B), and formation and decay of a charge-transfer complex was monitored at 580 nm (Figure 3C).

The results showed that reduction of NR by NADH is fast and the beginning of the kinetic trace (first phase) of all but the lowest concentration of NADH (0.08 mM) was within the dead time (0.001s) of the stopped-flow instrument (Figure 3B). Two rate constants were required to adequately describe the data; the use of only one resulted in systematic residuals that were eliminated by the use of a second rate constant (Figure 3B). The first process (~0.001-0.01s for 0.08 mM NADH) was characterized by decreased absorbance at 454 nm and an increase in absorbance at 580 nm (Figure 3C). Therefore, the first rate constant was attributed to hydride transfer and formation of a charge-transfer complex between reduced FMN and NAD$^+$. The second phase (~0.01-0.1s for 0.08 mM NADH) was attributed to dissociation of NAD$^+$. A plot of the observed rates of hydride transfer for reactions with 2.56 mM NADH and below yielded a hyperbolic dependence on [NADH] and therefore was fit with Equation 2 (Figure 4A) to yield the rate constant $k_2 = 700 \pm 20 \text{ s}^{-1}$ and the dissociation constant ($K_d = k_{-1}/k_1$) of $0.51 \pm 0.04 \text{ mM}$ depicted in Scheme 1.

A rate constant for the second process was estimated from the high-[NADH] asymptote of the observed rate of the second phase ($65 \pm 3 \text{ s}^{-1}$, Figure 4B) and confirmed by simulations of the data using the obtained value of $k_3 = 60 \text{ s}^{-1}$ (Table 1).

Scheme 1. Reductive half-reaction of NR

To determine whether the observed rate constants suffice to fully describe the observed behavior, simulations of the behavior predicted by the model in Scheme 1 with the parameters in Table 1 were performed, and the parameters were refined to obtain the best visual agreement with the experimental observations. The optimized simulation results (red dashed lines in Figures 3A and B) provide a good description of the observed kinetics (solid lines in Figures 3A and B), and yield a 'simulated' dissociation constant $^{sim}K_d$ of 0.48 mM (comparable to the $K_d$ obtained from the experiments of $0.51 \pm 0.04 \text{ mM}$). The simulated reduction rate constant ($^{sim}k_2$) was $700 \text{ s}^{-1}$ (vs. $700 \pm 20 \text{ s}^{-1}$ from experiment) and the rate of dissociation of NAD$^+$ from NR$_{red}$ ($^{sim}k_3$) was found to be $60 \text{ s}^{-1}$ (Table 1). The study of NR reduction kinetics was repeated for two different preparations of NR. The results were again within error of one another so only the parameters obtained from the larger study are reported.

Thus we are able to report the first numerical values for the rate of reduction of NR’s FMN by NADH, which we find to occur very rapidly. Moreover these experiments yield a limiting value for the $K_m$ of NADH that is not complicated by concurrent operation of the other half reaction.
Oxidative half-reaction of reduced NR with p-nitrobenzoic acid (p-NBA) – these reactions were investigated using stopped-flow experiments at 4°C by rapidly mixing the reduced enzyme in 50 mM potassium phosphate pH 7.50 with the same buffer containing different concentrations of p-NBA to yield a final NR concentration of 17.5 µM and p-NBA concentrations of 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, and 1.6 mM (after mixing). The experiment was limited to a maximum p-NBA concentration of 1.6 mM by the modest solubility of p-NBA. The reduced enzyme was prepared by stoichiometric reduction with NADH as described in experimental procedures. Kinetic traces at wavelengths from 340 to 700 nm at 5 nm intervals were collected. The kinetics at all wavelengths were the same, indicating that only formation of oxidized flavin contributes to optical spectrophotometric changes and no transient intermediate was detected (data not shown).

The oxidation of NR’s flavin by p-NBA exhibited monophasic kinetics at 454 nm (Figure 5). The observed rate constant ($k_{obs}$) displayed saturation with respect to the p-NBA concentration as evinced by the hyperbolic curve (inset to Figure 5). Therefore, the plot of $k_{obs}$ versus p-NBA concentrations was fit using Equation 2 yielding the limiting value of $k_5 = 1.90 \pm 0.09 \text{ s}^{-1}$ (Scheme 2). The intercept of the plot ($k_s$) approaches a value of zero, indicating that the reverse reaction rate is negligible (inset to Figure 5). The dissociation constant ($K_d = k_d/k_a$) calculated from the inset in Figure 5 and Equation 2 is $0.33 \pm 0.04$ mM. The hyperbolic dependence on substrate concentration indicates simple one-step binding prior to the flavin oxidation step (Scheme 2). The product of the oxidative half-reaction in this experiment was p-NOBA since no reductant was present to regenerate reduced enzyme and support a second cycle. The product was still bound to the active site at the end of the reaction (30 s) as indicated by a red shift of the final spectrum to 466 nm (from 454 nm for free oxidized enzyme) (data not show). The oxidative rate ($1.90 \pm 0.09 \text{ s}^{-1}$) was close to the turnover value measured in steady-state kinetic experiments ($k_{cat} = 1.7 \pm 0.3 \text{ s}^{-1}$).

**Scheme 2.** Oxidative half-reaction of NR

\[
E_{\text{red}} + S \overset{k_4}{\underset{k_1}{\rightleftharpoons}} E_{\text{red}}:S \overset{k_5}{\rightarrow} E_{\text{ox}}:P \overset{k_6}{\rightarrow} E_{\text{ox}} + P
\]

Kinetic simulation of the model in scheme 2 and optimization of the kinetic parameters (Table 1) produced excellent agreement with the experimental data as shown in Figure 5 (dashed lines vs. solid, respectively). The simulations yielded $K_d = 0.27$ mM and an oxidation rate constant ($k_5$) of 2 s$^{-1}$ that are within error of the values determined directly from the data, indicating that this simple model and the parameters we report suffice to describe the optically perceptible events in the oxidative half-reaction of NR. All rate and equilibrium constants from the experiments and simulations are summarized in Table 1. These results reveal that oxidation of reduced flavin by p-NBA could be the rate-limiting step for overall turnover ($k_s$).

The above results were confirmed using double-mixing stopped-flow spectrophotometry to first produce reduced NR by mixing oxidized NR with 20 µM NADH allowing an aging time of 0.5 s for complete reduction of NR and then combining the result with various concentrations of p-NBA (0.2, 0.4, 0.8, and 1.6 mM p-NBA and 17.5 µM NR were the final concentrations) in the second mix. The reactions were monitored at 454 nm to detect flavin reoxidation. The oxidation rate constant and dissociation constant were observed to be $k_5 = 2.00 \pm 0.06 \text{ s}^{-1}$ with an intercept close to zero and $K_d = 0.20 \pm 0.02$ mM, respectively (not shown). The oxidative half-reaction of enzyme in the absence of NAD$^+$ was studied using single-mixing stopped-flow spectrophotometry by reducing NR using stoichiometric sodium dithionite and rapidly mixing it with various concentrations of p-NBA as above. The observed rate constants were hyperbolically dependent on the p-NBA concentration. The limiting value was $k_5 = 1.90 \pm 0.07 \text{ s}^{-1}$ and the $K_d$ value was $0.30 \pm 0.04$ mM (data not shown). These results confirm that dissociation of NAD$^+$ from the active site of wild-type enzyme is complete within 0.5 s and its presence in the reaction medium does not significantly affect the kinetics of the enzyme's reaction with p-NBA. This supports a simple ping-pong bi-bi mechanism (6).

Oxidative half-reaction with p-nitrosobenzoic acid – The dominant product resulting from reduction of nitroaromatics by NR is the...
corresponding hydroxylamino aromatic (6,11). However a few reports exist of the formation of a nitroso intermediate during biological reduction of certain nitroaromatics (14). It is difficult to isolate nitroso intermediates because they are reactive with hydroxylamines, and rapidly reduced by NR (6). Thus reports of kinetic parameters are scarce.

To characterize the kinetics of nitroso aromatic reduction by NR, p-NOBA was generated by oxidation of p-ABA (see methods section) and used as the substrate in a study of the kinetics of NR oxidation. An anaerobic solution of reduced NR was mixed with p-NOBA in 50 mM potassium phosphate pH 7.5 to yield final concentrations of 17.5 µM NR and 0.1 mM p-NOBA. The kinetic trace was monitored at 454 nm, 4°C using stopped-flow spectrophotometry. Most of the transient occurred during the dead time of the instrument (0.001 s) even with the lowest concentration of substrate (0.1 mM) at 4°C, so the oxidation rate constant could not be determined directly. Based on the portion of the transient we could detect, the observed rate constant was roughly estimated to be \( \approx 650 \text{ s}^{-1} \) (at 0.1 mM p-NOBA) using Program A. This result is consistent with prior work.

A third oxidative half-reaction? tests for accumulation of amino aromatic product – Several groups have shown that NR produces hydroxylamino products in multiple turnover reactions of nitroaromatic substrates (6,11-13) but there are also a few reports of amine production (12,13,20,21). Thus the capacity to produce amines may depend on the specific enzyme and substrate in use, and require testing in each case. We have therefore assessed the extent to which NR can produce p-ABA.

Low yields of anything are very difficult to ascertain, moreover use of multi-turnover assays beginning with a nitroaromatic substrate could produce the amino aromatic in the presence of the corresponding nitroso, which would react with the amine (35,36). In addition, some of the prior studies were performed under air whereas careful work has shown that hydroxylamino aromatic products can react with dissolved O\(_2\) to regenerate more oxidized precursors and establish a futile cycle (7) that would compete with further reduction and suppress formation of amine product. Finally, sample preparation and workup for detection can expose hydroxylamine and labile enzymatic products to O\(_2\). Again, hydroxylamino aromatics would be readily oxidized and the resulting nitroso aromatics could then react with and mask amine products (37,38). Therefore, to detect the possible formation aromatic amines at low concentration we have reacted NR with p-HABA instead of p-NBA, in order to focus directly on the reaction in question, and we have refined a sensitive direct chromogenic probe for aminoaromatic products that can be applied in the presence of hydroxylamines without any workup or exposure to air (33,34).

To assess possible complications due to side-reactions and/or impurities, we first characterized the purity and stability of our p-HABA under the anaerobic conditions of our reaction, by NMR and UV-visible spectrophotometry, and compared the results with those obtained for p-HABA in conventional air-equilibrated solutions.

NMR spectra collected after incubation at room temperature for times ranging from 10 minutes to 24 hours demonstrate that p-HABA is converted to another species in air-equilibrated buffer on a time scale of minutes to hours, but no reaction occurs under inert atmosphere (Figure 6). The p-HABA concentration was chosen to be 1.3 mM, 5-times the concentration of dissolved oxygen in air-saturated buffers (0.26 mM) to obtain pseudo-first order conditions. Within 5 hr, the intensity of signals from 1.3 mM p-HABA had begun to visibly diminish and new signals appeared and increased in successive \(^1\)H NMR spectra (Figure 6A). The new signals at \( \delta = 8.01, 8.03, 8.26, \) and \( 8.28 \) ppm match those of the aromatic protons of authentic p-NOBA indicating that this is one of the oxidation products of p-HABA. Additional features in the spectrum are attributable to a small amount of contaminant (<5%) present in the p-HABA, and a compound that forms with the same time dependence as p-NOBA and which also forms when p-HABA is combined with p-NOBA under inert atmosphere, which we therefore tentatively assign to 4,4'-azoxydibenzoic acid (13). The decrease in the quantity of p-HABA present is fully accounted for by the sum of the increase in p-NOBA and putative azoxy product at all time points (Figure 6B). Thus the optical signature of the proposed azoxy product could be used to monitor decay of p-HABA (Figure 6C).
Azoxy compounds are intensely colored whereas neither p-NOBA nor p-HABA absorb in the visible range so we monitored formation of the putative 4,4’-azoxydibenzoic acid at 400 nm. This was found to increase with a rate constant compatible with the growth of the new peaks in the NMR spectrum after accounting for concentration differences. Thus the initial oxidation rate constant was 3.0 × 10^{-2} hr^{-1} for p-HABA. In contrast, for p-HABA in anaerobic solution the rate constant for appearance of the HABA. In contrast, for p-HABA in anaerobic solution the rate constant for appearance of the azoxy product was ≈ 3.2 × 10^{-5} hr^{-1} (Figure 6C). These results show that our anaerobic solutions of p-HABA do not spontaneously react to form any other products on a time scale extending to a day.

Amine detection – We assayed directly for accumulation of p-ABA as a result of NR-catalyzed reduction of p-HABA in the presence of NADH. Reactions were incubated in the anaerobic glove box at ≈ 28°C and terminated by filtration of the reaction mixture to remove NR (in the glove box). Controls lacking either NR or NADH were treated the same way. The MBTH assay for aromatic amines was performed in-situ avoiding any exposure to air or work-up.

MBTH reacts with aromatic amines forming an intensely coloured adduct which can be detected with high sensitivity via its absorbance at 550 nm in a window between the optical signatures of NADH and FMN. The absorbance at 550 nm responded linearly to p-ABA concentration in the range of 0.05 - 0.3 mM p-ABA with a relatively low constant background, attributable to products of reaction with NADH and/or p-HABA. To simulate the possible outcome of an enzymatic reaction beginning with 0.3 mM p-HABA and converting some amount to p-ABA, a second standard curve was constructed varying the mole fractions of p-ABA and p-HABA contributing to a total of 0.3 mM. The resulting response curve again reveals that the A_{550} increases with increasing amounts of p-ABA and decreasing amounts of p-HABA (Figure 7). High concentrations of p-HABA appear diminish the amount of dye available for reaction with p-ABA, therefore controls were constructed to have the same initial concentration of p-HABA as experimental samples. Based on these data and their standard errors we expect accumulation of more than 0.05 mM p-HABA to produce a significant A_{550} increase above background (Figure 7).

We compared the outcome of MBTH assays on samples in which NR was allowed to react with p-HABA vs. controls wherein NR was omitted, to evaluate the extent to which any amine accumulation was enzyme-catalyzed. Table 2 shows slightly higher absorbance for the reactions initially containing 0.3 mM p-HABA compared to those initially containing 1 mM p-HABA, consistent with Figure 7 and possible diversion of some MBTH by p-HABA. However among reactions begun with equal p-HABA concentrations, no significant differences were found across treatments by one-way ANOVA at 0.3 mM p-HABA (p=0.097) or at 1 mM p-HABA (p=0.097) (Table 2). The fact that omission of NR from the 10 hr controls did not significantly affect absorbance at 550 nm indicates that there was little or no accumulation of aromatic amine due to enzymatic activity.

DISCUSSION

Nitroreductases are best known for their ability to catalyze reduction of the nitro groups of a wide range of aromatic substrates, but the biological function and the natural substrate of most nitroreductases are unknown. The prevalence of NR-family genes among bacteria nonetheless suggests that NRs confer a significant advantage on the organisms that bear them, and recent appreciation of the wide range of reactions conducted by members of the larger superfamily opens even wider the range of possibilities to be considered (5,39). Recent data suggests a role as a dihydropteridine reductase for the NR homologue of Rhodobacter capsulatus (40).

Structural explanations for the broad substrate repertoire of NR homologues have been discussed (4,41). The active site of NR is a large cavity between helices E and F, which extend out from the structural core of one monomer, and helix D and the preceding loop of the other monomer (Figure 1). Diverse substrates bind by stacking on the flavin ring system; many exploit electrostatic interactions with the side chain of Lys14 or employ water-mediated interactions with protein residues that are too far away for direct contact (41). The side chain of Phe70 (helix D) was found to adopt diverse orientations and thereby accommodate different substrates (11,41). Helix F
also moves due to interactions between Phe124 and substrate analogs (4, 27). The generally small number of interactions between substrate analogs and the enzyme is consistent with the generally large (> 0.1 mM) $K_d$s and $K_m$s observed for oxidizing substrates (above and (6, 11)).

In other enzymes with broad substrate repertoires, the active site has been observed to alter its conformation to bind different substrates. The active site of the sulfotransferase SULT1A1 adopts different conformations to bind two molecules of nitrophenol at once vs. estradiol (42) and isopropylmalate isomerase of Pyrococcus horikoshii was proposed to be able to bind either homocitrinate or isopropylmalate due to the flexibility of a loop that replaces the helix that discriminates between similar substrates in the homologous aconitase (43). Similarly, a more flexible and accessible active site was credited with the expanded substrate repertoire of an evolved metallo-β-lactamase (44), and a glutathione-S-transferase (GST) with a broader repertoire was found to be more flexible than a GST with greater substrate selectivity on the basis of H/D exchange as well as fluorescence lifetime analyses (45).

In contrast, mechanistic bases for specificity include conformational changes required as part of the catalytic cycle. For example, $p$-hydroxybenzoate hydroxylase (PHBH) exhibits substrate specificity in the formation of a reaction intermediate. In order for reducing equivalents to pass from NADPH to the tightly-bound FAD's flavin ring the enzyme must undergo a conformational change. This only occurs in the WT enzyme when a substrate is bound, and only when that substrate can be deprotonated (46, 47). α-ketoglutarate-dependent dioxygenases also incorporate a similar 'substrate trigger' (48): the substrate to be oxidized must be bound in order for a coordination site for O2 to become available on the catalytic Fe2+ ion (49). These and many other examples underscore the use of mechanistic intermediates in mediating substrate specificity. We have tested the corollary: that an enzyme with a very broad substrate repertoire might be expected to employ a much simpler mechanism that does not include multiple events which could depend on different features of the substrate.

To test this, we have attempted to elucidate all the rate-contributing steps of NR's mechanism through analysis of the steady-state and pre-steady state kinetics of NR's reductive and oxidative reactions. We find that both NR's reductive and oxidative half-reactions employ simple kinetic mechanisms with one-step binding and concluding with simple product release. There is no evidence for distinct binding steps in which specificity might be enforced. Release of product NAD+ does not appear to be rate-contributing for the simple substrate $p$-NBA, and the re-oxidation rates and dissociation constants of the reduced enzyme prepared by reduction with dithionite (1.90 ± 0.07 s−1, 0.30 ± 0.04 mM, respectively) or NADH (2.00 ± 0.06 s−1 and 0.20 ± 0.02 mM, respectively) were comparable supporting a simple ping-pong bi-bi mechanism.

For NR, we find that oxidation of reduced flavin by $p$-NBA (1.90 ± 0.09 s−1) is likely an important contributor to the overall rate (1.7 ± 0.3 s−1) based on comparison of the steady-state turnover number $k_{cat}$ with the rate constants obtained by transient kinetics. For NfsB, product release (either NAD+ or nitroso aromatic) or steps other than the chemical reaction were found likely to be rate-limiting (28). The difference can be explained by the different substrates used, since the substrates CB1954 and nitrofurazone turn over at $k_{cat}$ ≈ 140 s−1 and 230 s−1 respectively (28), much faster than the $k_{cat}$ for $p$-NBA of 1.7 s−1. We chose the poor substrate, $p$-NBA, in order to be able to look for mechanistic steps that might define the substrate repertoire. Despite the very slow chemical conversion of this substrate we were unable to observe any intermediates or evidence for additional steps. Thus, we have ascertained that the broad substrate repertoire of NR rests not only upon the structure of the enzyme, but also on the very simple mechanism it employs.

NR's relatively high $K_m$ for NADH of 0.51 mM (± 0.04) is in excellent agreement with the results of Koder and Miller (6). The $K_m$ for $p$-NBA of 0.33 ± 0.04 mM is also consistent with the mM values obtained for other substrates in previous work (6). The large magnitudes of both these $K_m$s are compatible with binding via relatively few and water-mediated contacts as indicated by crystal structures of the enzyme complexed with other substrates.

The value of $k_{cat}$ = 1.7 ± 0.3 s−1 obtained from steady-state kinetics agrees well with $k_3$ = 1.90 ± 0.09 s−1 obtained from pre-steady-state kinetics.
Similarly, when a value is calculated for \( K_m^{PNBA} \) from the individual rate constants measured via pre-steady-state kinetics, the resulting \( K_m^{PNBA} = 0.27 \) mM agrees well with the experimental \( K_m^{PNBA} = 0.13 \) mM produced by the double reciprocal plot (Figure 2A) and the 0.25 mM describing the hyperbolic plot (Figure 2C). However the \( K_m^{NADH} = 1.5 \) µM we calculate from our individual rate constants is significantly different from our experimental values of 44 µM from the double reciprocal plot and 35 µM from the hyperbolic plot. This may reflect the fact that genuinely saturating \( p\)-NBA concentrations could not be used due to their inhibitory effect (6). However for \( k_{cat} \) and \( K_m^{PNBA} \) our primary rate constants succeed well in accounting for observed overall behaviour.

Using the substrates nitrobenzene and its two-electron reduced product nitrosobenzene, Koder found that the nitroso substrate was reduced some 720 times faster than was the nitro substrate (6). Race et al confirmed analogous behavior for NfsB and nitro/nitroso toluene or nitro/nitroso benzene (11). For \( p\)-NOBA, we could only estimate the apparent rate of reduction at 0.1 mM to be 650 s\(^{-1}\) whereas the rate of \( p\)-NBA reduction is 0.50 s\(^{-1}\) at the same concentration so we find that reduction of the nitroso is some 1,300 times faster than reduction of the nitro.

One reason for interest in NR is the hope that it might be useful for producing aromatic amines from corresponding nitroaromatics (13). However our current results confirm earlier findings that significant quantities of aromatic amines do not accumulate under the action of \( E.\ cloacae \) NR (6).

Further work is called for to understand the basis for \( M.\ smegmatis \) nitroreductase's ability to reduce benzothiazinone 043 to the corresponding amine (20) and \( Klebsiella \) nitroreductase's, success in fully reducing one nitro group of TNT (12) in contrast with the common reduction of nitroaromatics only as far as the hydroxylamine followed by use a hydroxylamino mutase to produce the corresponding aminophenol (50,51). We find that the \( M.\ smegmatis \) nitroreductase has a flavin midpoint potential very similar to that of NR (Pitsawong and Miller, unpublished), arguing against greater reducing power on the part of the enzyme (53). Instead the substrates in question may be more amenable to full reduction by virtue of additional functionalization of the aromatic ring by a trifluoromethyl group in the case of benzothiazinone 043 and two additional nitro groups that are not reduced in the case of TNT. Our refinement of a sensitive selective chromogenic assay to detect aromatic amines affirms that \( p\)-HABA and thus also \( p\)-NBA is not converted to \( p\)-ABA by \( E.\ cloacae \) NR. However this assay provides a valuable tool for determining whether the same enzyme can produce aromatic amine products from different substrates.
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FOOTNOTES

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1. Abbreviations

ADEPT, antibody-directed enzyme pro-drug therapy; ANOVA, analysis of variance; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; GDEPT, gene-directed enzyme prodrug therapy; GST, glutathione s-transferase; MBTH, 3-methyl-2-benzothiazolinone hydrazone; NfsB, Escherichia coli homologue of Enterobacter cloacae nitroreductase; NR, nitroreductase; NRox, oxidized NR; NRed, reduced NR; p-ABA, para-aminobenzoic acid; PETN, pentaerythritol tetranitrate reductase; p-HABA, para-hydroxylaminobenzoic acid; p-HBH, para-hydroxybenzoate hydroxylase; p-NBA, para-nitrobenzoic acid; p-NOBA, para-nitrosobenzoic acid; TNT, trinitrotoluene; WT, wild-type.
FIGURE LEGENDS

FIGURE 1. (A) The reduction of nitroaromatic substrates catalyzed by nitroreductase, for the example of nitrobenzene. (B) Ribbon structure of NR with lettering of helices indicated. One monomer is in cyan and the other is in magenta, the bound FMN is depicted with yellow sticks and bound substrate analog benzoate is depicted with green sticks. Based on 1KQB.pdb and generated using Pymol (52).

FIGURE 2. Steady-state kinetics of NR with NADH and p-NBA as substrates. (A) Double reciprocal plots of the initial rates of reaction of NR with various concentrations of NADH (0.1 mM, 0.2 mM, 0.3 mM and 0.4 mM after mixing). Upper to lower lines of the primary plot are from the reactions of 0.1 mM, 0.2 mM, and 0.4 mM (after mixing) p-NBA, respectively. The assay reactions were performed using a stopped-flow spectrophotometer at 4°C. The inset shows the plot of ordinate intercepts from the double-reciprocal plot vs. the reciprocal of p-NBA concentrations. The parameters obtained from the fits are provided in Table 1. (B) Plot of initial velocity versus NADH concentration (0.02, 0.042, 0.08, 0.17, 0.25, and 0.34 mM) at a fixed p-NBA concentration (0.4 mM). The results showed hyperbolic dependence on p-NBA concentrations with $K_{m}^{pNBA}$ of 0.25 mM and $k_{cat} = 1.7$ s$^{-1}$. (C) Plot of initial velocity versus p-NBA concentration (0.025, 0.050, 0.1, 0.2, 0.3, 0.4 and 0.6 mM) at a fixed NADH concentration (0.25 mM). The results showed hyperbolic dependent on NADH concentrations with the $K_{m}^{NADH}$ of 44 µM and $k_{cat} = 1.7$ s$^{-1}$.

Figure 3. Reductive half-reaction of NR with NADH. (A) Linear time scale (B) Log time scale for kinetic traces of the reaction of NR (13 µM) with various concentrations of NADH (0.08 mM, 0.16 mM, 0.32 mM, 0.64 mM, 1.28 mM, and 2.56 mM) in 50 mM potassium phosphate pH 7.50 at 4°C. Reactions were monitored in the stopped-flow spectrophotometer under anaerobic conditions. All concentrations are reported as final concentrations after mixing. Dashed red lines are simulated traces obtained using the kinetic constants listed in Table 1 and Scheme 1. Residuals of fitting the reduction of NR with 0.08 mM NADH using one exponential (magenta dots) and two exponentials (green dots) are shown below with a vertical $\Delta A_{454}$ axis on the right. (C) Kinetic traces of the reduction of NR at 454 nm (black line) and formation and decay of the charge-transfer complex observed at 580 nm (blue line) with a concentration of 0.08 mM NADH.

Figure 4. Plot of the observed rate constants of the reduction of NR. (A) Plot of observed rate constants from the first phase; 100 ± 2, 176 ± 2, 264 ± 3, 380 ± 3, 484 ± 5, and 590 ± 9 s$^{-1}$ (from low to high NADH concentrations; 0.08 mM, 0.16 mM, 0.32 mM, 0.64 mM, 1.28 mM, and 2.56 mM) in 50 mM potassium phosphate pH 7.50 at 4°C. The data including their error bars are well described by a rectangular hyperbola, which is the source of the values of $k_2$ and $K_d$. (B) Plot of observed rate constants from the second phase; 42 ± 1, 46 ± 2, 50 ± 2, 55 ± 2, 61 ± 2, and 65 ± 3 s$^{-1}$ (from low to high NADH concentrations as described above). The $k_3$ value was estimated from the NADH asymptote as indicated by the dotted line.

FIGURE 5. Oxidation of reduced NR by p-NBA. A solution of reduced NR (17.5 µM after mixing) was prepared by stoichiometric addition of NADH and this was then mixed with various concentrations of p-NBA to produce 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, and 1.6 mM p-NBA in 50 mM potassium phosphate pH 7.50. The reaction was monitored using the absorbance at 454 nm, at 4°C. The plot of $k_{obs}$ versus p-NBA concentration (inset) was fit with Equation 2 for a rectangular hyperbola. The calculated oxidation rate constant $k_3$ and $K_d$ are 1.90 ± 0.09 s$^{-1}$ and 0.33 ± 0.04 mM, respectively. The $k_{obs}$ values from low to high p-NBA concentrations obtained from the transients are 0.49 ± 0.02, 0.66 ± 0.02, 1.03 ± 0.05, 1.31 ± 0.05, and 1.60 ± 0.06 s$^{-1}$. The vertical line at each data point represents the standard deviation of the fit from the experiments. The 0.2 mM and 1.6 mM p-NBA samples were slightly oxidized at the start of the
transient so a smaller fraction of the sample reacted with \( p \)-NBA. This is manifested by a slight deviation between the \( t=0 \) absorbance of the sample (1.6 mM) and the need to employ a modified vertical axis for the sample containing 0.2 mM \( p \)-NBA. Dashed red lines are simulated traces obtained using the kinetic constants listed in Table 1 and defined in Scheme 2.

**FIGURE 6. Decomposition of \( p \)-HABA under aerobic and anaerobic conditions.** (A) \(^1\)H NMR spectra of 13.3 mM \( p \)-HABA in 50 mM potassium phosphate pH 7.50, 10% D\(_2\)O, 0.1 mM DSS under aerobic and anaerobic conditions after 10, 30, and 60 min. Asterisks indicate signals that increase with time. (B) Total integrated NMR peak area corresponding to \( p \)-HABA (at \( \delta = 7.04, 7.06, 7.81, \) and 7.83, blue circles) or \( p \)-NOBA plus 4,4'-azoxydibenzoic acid (at \( \delta = 7.99, 8.00, 8.01, 8.03, 8.26, \) and 8.28 ppm, red circles) as a function of time. Black circles represent the sum of the area corresponding to \( p \)-HABA and oxidation products. Purple triangles represent the total integrated NMR peak area corresponding to \( p \)-HABA under anaerobic conditions. (C) Kinetic traces of formation of decomposition product of \( p \)-HABA under aerobic (black trace) and anaerobic (red trace) conditions. The reactions were monitored via the absorbance at 400 nm, at 25\(^{\circ}\)C. The inset shows a vertical expansion of the anaerobic reaction of \( p \)-HABA. The observed decomposition rates were calculated to be 3.0 \( \times \) 10\(^{-2}\) hr\(^{-1}\) and 5.4 \( \times \) 10\(^{-4}\) hr\(^{-1}\) for aerobic and anaerobic decomposition, respectively.

**FIGURE 7. MBTH assay response to mole fraction of \( p \)-ABA in 0.3 mM combined concentration of \( p \)-ABA and \( p \)-HABA simulating increasing yields of \( p \)-ABA produced from \( p \)-HABA.** Solutions totaling 0.3 mM \( p \)-ABA plus \( p \)-HABA but varying with respect to the mole fraction of \( p \)-ABA were augmented with 1.25 mM NADH and 17.5 \( \mu \)M NR and incubated for 15 minutes at 29 \(^{\circ}\)C before being assayed for aromatic amine content using MBTH. The mole fraction is expressed as a function of the amine content: \([\text{p-ABA}]/([\text{p-ABA}]+[\text{p-HABA}])\).
The kinetic mechanism of nitroreductase

### TABLES

#### Table 1. Kinetic parameters for NR at 4°C: comparison of steady-state, pre-steady-state and simulation results.\(^a\)

| Chemical species | From experiment | From simulation | Chemical species | From experiment | From simulation |
|------------------|-----------------|-----------------|------------------|-----------------|-----------------|
| \(K_d^{NADH}\) = 0.51 ± 0.04 mM | \(K_d^{NADH}\), \(k_{i}/k_1 = 0.48\) mM | \(K_d^{PNBA}\), \(k_{d}/k_4 = 0.27\) mM |
| - | \(k_1^{b} = 1.3 \times 10^7\) M\(^{-1}\) s\(^{-1}\) | \(E_{ox}\) | 14300 | 14340 |
| \(k_2 = 700 ± 20\) s\(^{-1}\) | \(k_2 = 700\) s\(^{-1}\) | \(E_{ox}:NADH\) | - | 10200 |
| \(k_3 = 65 ± 3\) s\(^{-1}\) | \(k_3 = 60\) s\(^{-1}\) | \(E_{red}:NAD^+\) | - | 1940 |
| \(K_d^{PNBA}\) = 0.33 ± 0.04 mM | \(K_d^{PNBA}\), \(k_{d}/k_4 = 0.27\) mM |
| \(k_5 = 1.90 ± 0.09\) s\(^{-1}\) | \(k_5 = 2\) s\(^{-1}\) |

#### Kinetic parameters - Steady-State kinetics

- \(k_{cat} = 1.7 ± 0.3\) s\(^{-1}\)
- \(K_m^{NADH} = 35 ± 8\) µM
- \(K_m^{PNBA} = 130 ± 5\) µM

\(^a\) The values were obtained from the experimental data performed in 50 mM potassium phosphate pH 7.50 at 4°C, under anaerobic condition, using stopped-flow spectrophotometer or kinetic simulations.

\(^b\) The values provided are not necessarily be unique, but are able to fully explain the data observed in conjunction with the model we provide. The values of \(k_1\) and \(k_{-1}\) are inversely correlated, as are the values of \(k_4\) and \(k_{-4}\).
Table 2. Analysis of reaction of \( p \)-HABA with NADH using MBTH.\(^a\)

| Reaction Time | \( p \)-HABA (mM) | Absorbance (550 nm) |
|---------------|------------------|-------------------|
| 0 min         | 0.3              | 0.111(2)\(^b\)   |
| 10 hrs\(^c\)  | 0.3              | 0.120(8)          |
| 10 hrs control (no NR) | 0.3              | 0.120(3)          |
| 30 min        | 1                | 0.065(2)\(^a\)   |
| 10 hrs        | 1                | 0.080(3)          |
| 10 hrs control (no NR) | 1                | 0.074(11)         |

\(^a\) Samples were held at 29°C in 50 mM potassium phosphate pH 7.50 containing 1.25 mM NADH, 17.5 μM NR, 0.3 or 1 mM \( p \)-HABA. Enzymatic reactions were terminated by protein removal using a centrifugal filter (EMD-millipore, 10 kDa cutoff). Aromatic amines were detected via the absorbance at 550 nm of the product of the reaction with 1.5 mM MBTH and 6.5 mM Ce(SO\(_4\))\(_2\).

\(^b\) Standard deviations based on 3 repetitions are provided in parentheses. No significant differences were found across treatments by one-way ANOVA at 0.3 mM \( p \)-HABA (\( F(2,6)= 3.52, p=0.097 \)) or at 1 mM \( p \)-ABA (\( F(2,6)= 3.53, p=0.097 \)).

\(^c\) Partial enzyme precipitation was observed after 6 hours at 29°C however aliquots of enzyme allowed to stand in buffer under the same conditions for 24 hours retained 76% activity.
Figure 1.
The kinetic mechanism of nitroreductase

Figure 2.
Figure 3.
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Figure 4.
Figure 5.
Figure 6.

A. Anaerobic and Aerobic conditions

B. Peak area of p-HABA and peak area of oxidation products over time

C. Absorbance at 400 nm over time
Figure 7.
Understanding the Broad Substrate Repertoire of Nitroreductase Based on its Kinetic Mechanism
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