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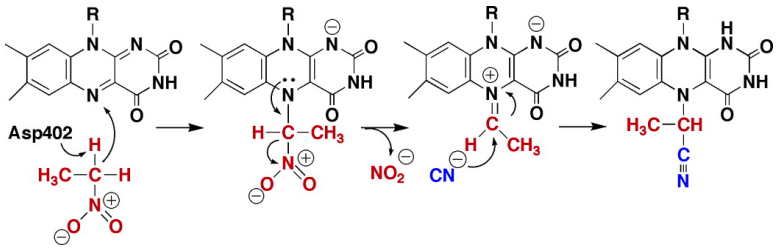

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Establishing the Kinetic Competency of the Cationic Imine Intermediate in Nitroalkane Oxidase

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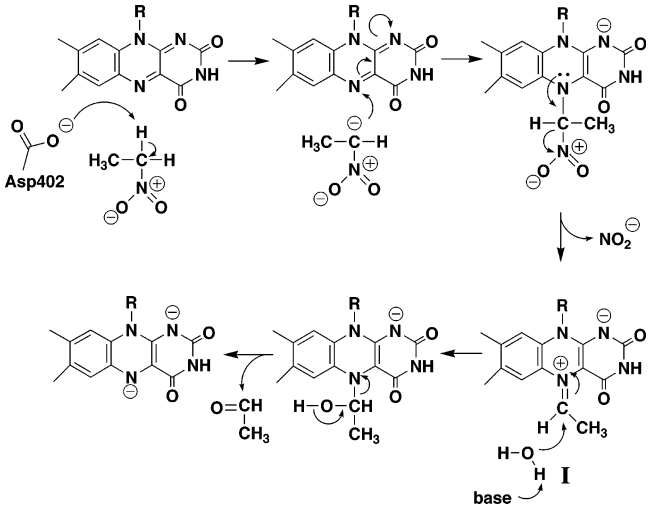
Abstract: The flavoprotein nitroalkane oxidase catalyzes the oxidation of neutral nitroalkanes to the corresponding aldehydes and ketones. Cyanide inactivates the enzyme during turnover in a concentration-dependent fashion. Mass spectrometry of the flavin from enzyme inactivated by cyanide in the presence of nitroethane or nitrohexane shows that a flavin cyanoethyl or cyanohexyl intermediate has formed. At high concentrations of cyanide, inactivation does not consume oxygen. Rapid reaction studies show that formation of the adduct with 2-$^2H_2$-nitroethane shows a kinetic isotope effect of 7.9. These results are consistent with cyanide reacting with a species formed after proton abstraction but before flavin oxidation. The proposed mechanism for nitroalkane oxidase involves removal of a proton from the nitroalkane, forming a carbanion which adds to the flavin N(5). Elimination of nitrite from the resulting adduct would form an electrophilic imine which can be attacked by hydroxide. The present results are consistent with cyanide trapping this electrophilic intermediate.

Introduction

The flavoenzyme nitroalkane oxidase (NAO) catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones with consumption of molecular oxygen and production of nitrite and hydrogen peroxide.[1] Although other flavoenzymes can oxidize nitroalkanes, in those cases the reaction is clearly nonphysiological and requires the anionic substrate.[2] In contrast, NAO preferentially oxidizes neutral nitroalkane substrates,[3] and the expression of NAO can be induced in the fungus *Fusarium oxysporum* upon addition of nitroalkanes.[4] NAO is also unusual in that it catalyzes flavin reduction via carbanion formation rather than the more common mechanism of hydride transfer.[5] The mechanism proposed for the reductive half-reaction of NAO is illustrated in Scheme 1.[6] After binding of the neutral nitroalkane in the active site, aspartate 402[7,8] abstracts the α-proton of the substrate to form the nucleophilic nitroalkane anion. The anion then attacks the N5 position of FAD to form an initial adduct which eliminates nitrite to generate the cationic imine I. This reactive species is subsequently attacked by hydroxide, followed by release of the aldehyde or ketone product to form reduced FAD. In the more typical oxidative half-reaction, the reduced FAD loses two electrons to molecular oxygen to form hydrogen peroxide and regenerate the oxidized cofactor.

Scheme 1



Support for the cationic imine I as a catalytic intermediate derives from the identification of a 5-nitrobutyl-FAD adduct bound to the native enzyme as isolated from *Fusarium oxysporum*.[9] The structure of this modified cofactor has been confirmed by both mass spectrometry[9] and crystallographic analysis (Nagpal, A., Valley, M. P., Fitzpatrick, P. F., and Orville, A. M., manuscript in preparation). This adduct can be formed in vitro by reacting active NAO containing unmodified FAD with a combination of neutral and anionic nitroethane.[9] The inactive

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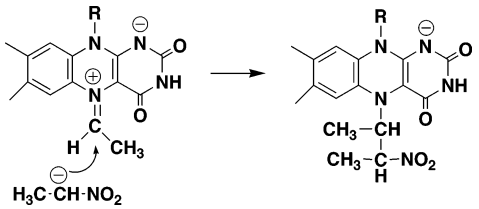
Scheme 2



5-nitrobutyl-FAD adduct is presumably generated in vivo by the attack of a nitroethane anion on intermediate I during nitroethane turnover (Scheme 2). However, formation of the nitrobutyl adduct does not establish intermediate I as being along the catalytic pathway, since the rate of enzyme inactivation by the nitroethane anion is slow and the nitroethane anion acts as both a substrate and an irreversible inhibitor. In the present study, we demonstrate that the cationic imine I is kinetically competent for catalysis.

Experimental Procedures

Materials. Materials were routinely purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Recombinant nitroalkane oxidase was expressed and purified as previously described.[10] Enzyme concentrations were determined using an ϵ_{446} value of 14.2 mM^{-1} cm^{-1}.[11]

Methods. Enzyme activity was measured in 100 mM HEPES, 0.1 mM FAD, 30 °C, by monitoring oxygen consumption with a computer-interfaced Hansatech Clark oxygen electrode (Hansatech Instruments, Pentney King's Lynn, U.K.). Experiments were performed at ambient oxygen concentration since the K_m value for oxygen ranges between 20 and 42 μM with various primary nitroalkanes.[3] To prevent the formation of the anionic form of the substrate, stock solutions of neutral nitroalkanes were prepared in dimethyl sulfoxide and assays were initiated by the addition of substrate. Rapid reaction kinetics were performed with an Applied Photophysics SX-18MV stopped-flow spectrophotometer. To minimize formation of the nitroalkane anion in these experiments, small volumes (e.g., 0.1 mL) of the concentrated substrate were added to buffered solutions (e.g., 3 mL) and used within 15 min.

To synthesize large quantities of the cyanoalkyl adducts, 25 μM NAO was incubated with 50 mM sodium cyanide in 100 mM HEPES, pH 8, at 4 °C. The nitroalkane was added to yield a final concentration of 25 mM, and the sample was incubated for an additional 5 min. The sample was then exchanged into 1.5 mM ammonium acetate, pH 6.8, with an Ultrafree-15 centrifugal filter device (Millipore Co., Bedford, MA). To separate the cofactor from the enzyme, 0.2 mL of concentrated acetic acid was added to 1.6 mL of sample to give a pH value of ~2. After 15 min of incubation at 4 °C, the sample was loaded onto a PD-10 column (Amersham Biosciences Co., Piscataway, NJ) equilibrated with 1.5 mM ammonium acetate, pH 6.8. The same buffer was used to elute the sample, and fractions containing the free cofactor were collected and concentrated with a Savant DNA SpeedVac model DNA100.

Mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectra were recorded in reflected ion mode with an Applied Biosystems Voyager DE-STR mass spectrometer (ABI, Framingham, MA). An overlayer preparation which has been described previously[12] was used with 2,4,6-trihydroxyacetophenone (THAP). A 1:1 mixture of a 1 μM analyte and 10 mg/mL THAP solution in methanol was spotted in 1 μL aliquots on top of a 100 mg/mL THAP bed of matrix. Negative ions were generated by using a nitrogen laser pulse (λ = 337 nm, 20 Hz) and accelerated under 20 kV using delayed extraction (175 ns) before entering the time-of-flight mass spectrometer. Laser strength was adjusted to provide minimal fragmentation and optimal signal-to-noise ratio. An average of 200 laser shots was used for each spectrum, and data were processed with the accompanying Voyager software package.

Data Analysis. Data were fit using the program Kaleidagraph (Adelbeck Software, Reading, PA) and simulated with the program DYNAFIT (www.biokin.com).[13] To determine the rate constants for enzyme inactivation, the concentration of oxygen was measured every 0.5 s, and the rates of oxygen consumption were calculated from the change in oxygen concentration for each 1.5-s interval. These initial velocity values were then fit to eq 1, where R is the measured rate, R_0 is the initial rate, t is time, and k is the rate constant for inactivation. Changes in the absorbance of enzyme-bound FAD at 450 nm from stopped-flow analyses were fit to eq 2, where A is the measured absorbance, A_∞ is the final absorbance, t is time, and A_n and λ_n are the amplitude and rate constant for the nth exponential phase.

$$R = R_0 e^{-kt} \tag{1}$$

$$A = A_\infty + A_1 e^{-\lambda 1 t} + A_2 e^{-\lambda 2 t} + A_3 e^{-\lambda 3 t} \tag{2}$$

Results and Discussion

Inactivation of NAO by Cyanide. To determine the kinetic competency of intermediate I, a nucleophile was required that would react rapidly with the cationic imine intermediate without otherwise inhibiting the enzyme. The flavin adduct formed upon oxidation of the nitroethane anion by D-amino acid oxidase can be trapped using cyanide.[14] Consequently, the ability of cyanide to inactivate NAO by trapping intermediate I was determined. In a typical assay, when the substrate nitroethane is added to an aerobic solution containing NAO, the concentration of oxygen decreases at a constant rate until the concentration of oxygen falls below ~100 μM. In the presence of cyanide, however, the rate of oxygen consumption is not constant and instead decreases to zero over time (Figure 1A). This decrease in rate occurs more rapidly at higher cyanide concentrations, such that at 2 mM cyanide and above there is no longer any detectable consumption of oxygen. This is consistent with inactivation of NAO by cyanide; similar results were observed with 1-nitrobutane, 1-nitrohexane, and phenylnitromethane (results not shown).

To obtain the first-order rate constants for enzyme inactivation at different cyanide concentrations with nitroethane as substrate, the rate of oxygen consumption over time was fit to eq 1 (Figure 1B). The values of the resulting rate constants were linearly dependent on the cyanide concentration, yielding a second-order rate constant of 4.6 ± 0.1 mM^{-1} min^{-1} for enzyme inactivation at pH 8 and 30 °C (Figure 1C). At pH 7, the first-order rate constants for inactivation also exhibit a linear cyanide concentration dependence but give a lower second-order rate constant of 0.6 ± 0.2 mM^{-1} min^{-1} (results not shown). The increase in the rate constant for enzyme inactivation at higher pH is consistent with the cyanide anion as the reactive nucleophile and the HCN pK_a of 9.2. Incubation of NAO with cyanide for different lengths of time prior to the addition of nitroethane had no effect on the rate constants for enzyme inactivation. This suggests that cyanide is specifically reacting with a catalytic intermediate and not the resting form of NAO.

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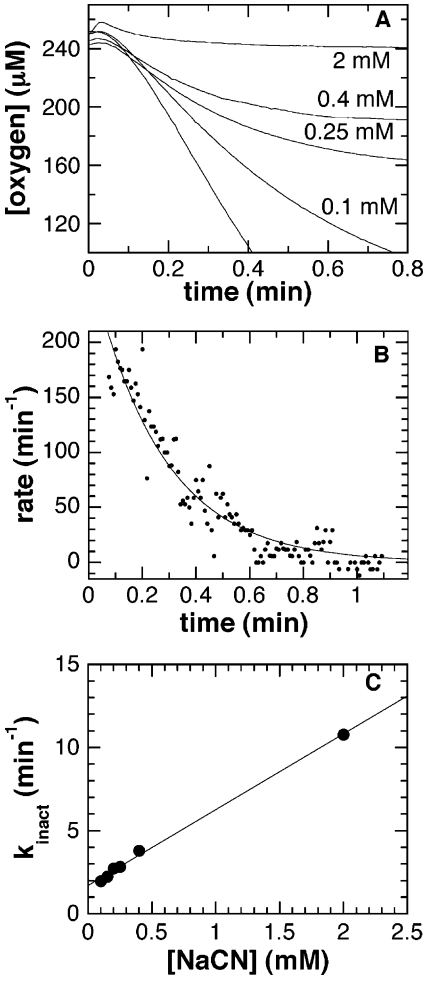


Figure 1. Inactivation of nitroalkane oxidase by cyanide. (A) The concentration of oxygen was monitored after addition of 12.5 mM nitroethane to 0.8 μM NAO in 100 mM HEPES, pH 8, in the absence or the presence of the indicated concentrations of sodium cyanide at 30 °C. (B) The rate of oxygen consumption versus time in the presence of 0.4 mM cyanide. The rates were calculated from the change in oxygen concentration for each 1.5-s interval and then fit to eq 1. (C) The first-order rate constants for enzyme inactivation at different concentrations of cyanide in 100 mM HEPES at pH 8.

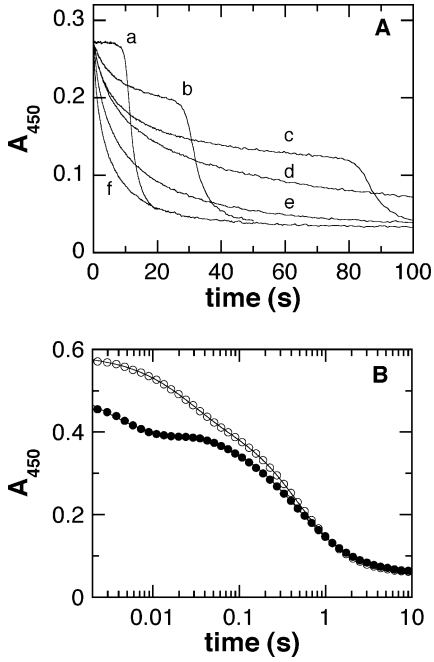


Figure 2. Absorbance traces at 450 nm upon aerobic addition of nitroethane to NAO in the presence of cyanide. (A) Nitroethane (909 μM) was mixed with NAO (41 μM) in 100 mM HEPES, pH 8, containing different concentrations of sodium cyanide at 30 °C. The traces labeled a−f correspond to 0, 0.575, 1.25, 2.5, 5, and 10.8 mM cyanide, respectively. (B) NAO (44 μM) was mixed with 58 mM nitroethane (filled circles) or 58 mM [1,1-$^2H_2$]-nitroethane (open circles) in 100 mM HEPES, pH 8, in the presence of 50 mM sodium cyanide at 30 °C. Each trace is an average of four experiments, with only 5% of the data points shown for clarity, and the lines represent fits to eq 2.

To define the locus of cyanide action in the catalytic mechanism of NAO, the visible absorbance spectrum of the enzyme was monitored during nitroethane turnover. In the absence of cyanide, rapid aerobic mixing of NAO and nitroethane initially results in little to no decrease in absorbance of the bound flavin at 450 nm (*a*, Figure 2A). The flavin is in the oxidized state under steady-state conditions because flavin oxidation is faster than flavin reduction with nitroethane as substrate, and the rate-limiting step in catalysis is product release or a conformational change involving the oxidized enzyme.[7] As the concentration of oxygen decreases, the rate of oxidation becomes slower and the absorbance of the enzyme decreases until all of the oxygen is consumed and the flavin becomes completely reduced. In the presence of cyanide, a longer period of time is required to consume the same amount of oxygen and the steady-state absorbance of the enzyme is lower (b−d, Figure 2A). This is not only consistent with inactivation of NAO, but it also indicates that the flavin cofactor of the inactivated enzyme is in a reduced state. As the concentration of cyanide increases, the absorbance drops more rapidly and the enzyme eventually becomes fully inactivated prior to oxygen depletion (e−f, Figure 2A). This trend is such that at 50 mM cyanide, the change in absorbance becomes similar to that observed upon anaerobically reducing NAO with nitroethane. At a saturating concentration of nitroethane, the decrease in absorbance at 450 nm in the presence of oxygen and 50 mM cyanide occurs in three phases with rate constants of 341 ± 18 s^{-1}, 2.4 ± 0.1 s^{-1}, and 0.64 ± 0.05 s^{-1} (Figure 2B). These rate constants are comparable to those previously observed upon mixing NAO and nitroethane in the absence of oxygen and cyanide (247 ± 5 s^{-1}, 2.3 ± 0.2 s^{-1}, and 0.46 ± 0.09 s^{-1}).[7] The second and third phases of the absorbance change are not catalytically relevant since they are slower than turnover, but the first phase is faster than turnover and corresponds to flavin reduction. Moreover, only the rate constant for the first phase in the presence of oxygen and 50 mM cyanide is perturbed with [1,1-$^2H_2$]-nitroethane as substrate (43 ± 3 s^{-1}, 3.0 ± 0.2 s^{-1}, and 0.68 ± 0.06 s^{-1}) (Figure 2B). This yields a deuterium kinetic isotope effect of 7.9 ± 0.7 and specifically demonstrates that inactivation by cyanide takes place after proton abstraction and before reaction with oxygen.

Characterization of the Flavin in the Inactive Enzyme. To determine the product of the reaction with cyanide, 5 mL of 25 μM NAO was reacted with 25 mM nitroethane in 100 mM HEPES, pH 8, in the presence of 50 mM sodium cyanide. After 5 min at 4 °C, the enzyme was exchanged into 1.5 mM ammonium acetate, pH 6.8, and concentrated acetic acid was added to decrease the pH to ~2 and release the flavin cofactor from NAO. The cofactor was then isolated by gel filtration and concentrated. The spectrum of the isolated cofactor has an absorbance maximum at 330 nm and is featureless above 400

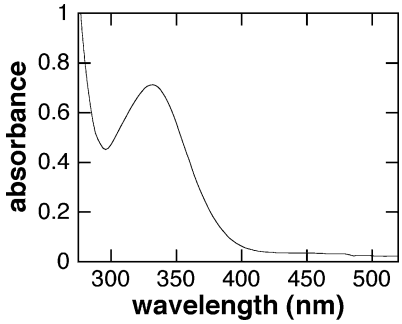


Figure 3. Absorbance spectrum of the isolated flavin produced upon the reaction of NAO with nitroethane and cyanide. Conditions: 1.5 mM ammonium acetate, pH 6.8.

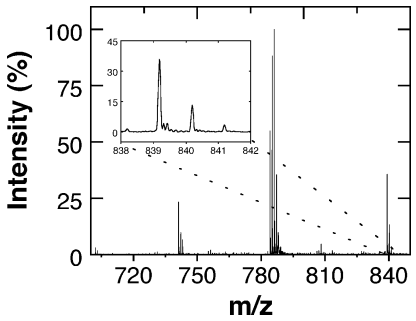
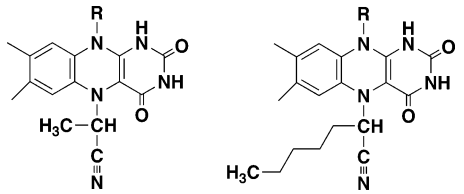


Figure 4. Negative ion MALDI-TOF mass spectrum of the isolated flavin cofactor. The modified flavin produced from reaction with nitroethane and cyanide was prepared and the mass spectrum was acquired as described under Experimental Procedures. The peaks at *m*/*z* values of 785 and 839 correspond to the $(M-H)^-$ ions of unmodified FAD and the 5-(1-cyanoethyl)-FAD, respectively. Inset, expansion of the spectrum in the region at *m*/*z* 839.

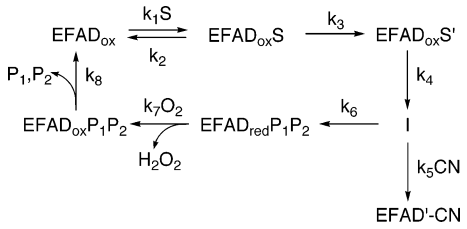
nm (Figure 3). This spectrum is similar to that observed for the cyanide adduct formed by D-amino acid oxidase in the presence of nitroethane anion.[14] The protocol in the present study differs somewhat from the protocol for purification of the 5-nitrobutyl-FAD adduct formed by NAO with anionic nitroethane as the attacking nucleophile.[9] Acidic conditions are required for isolation of the nitrobutyl adduct since it is only stable at low pH and readily decays at pH 8. In contrast, the adduct formed with cyanide as the attacking nucleophile is much more stable; it shows no signs of decay at pH 8 and requires more basic conditions (e.g., pH 12) before any increase in absorbance is observed at 450 nm (results not shown).

The structure of the modified flavin formed by NAO with nitroethane and cyanide was analyzed by mass spectrometry (Figure 4). The negative ion MALDI-TOF mass spectrum exhibits a peak with an *m*/*z* value of 839.1861, which is 54 mass units greater than the *m*/*z* value of 785.1572 for the predominant species, unmodified FAD formed during the laser desorption ionization process. This mass difference represents the addition of C_3H_4N to FAD and is consistent with formation of 5-(1-cyanoethyl)-FAD adduct II (Scheme 3). The calculated molecular mass for this species is 839.1837, corresponding to an *m*/*z* difference of 0.0024 and a mass accuracy of 2.9 ppm. For comparison, the stable adduct formed by NAO with 1-nitrohexane and cyanide was also isolated. Its negative ion MALDI-TOF mass spectrum exhibits a peak with an *m*/*z* value of 895.2580, a difference of 110 mass units compared to the unmodified cofactor that represents the addition of $C_7H_{12}N$ to FAD. This is consistent with formation of 5-(1-cyanohexyl)-FAD adduct III, and compared to the calculated molecular mass

Scheme 3



Scheme 4



of 895.2541, corresponds to an *m*/*z* difference of 0.0039 and a mass accuracy of 5.5 ppm.

The Locus of Cyanide Action and Kinetic Competence of Intermediate I. In this study, we have explicitly localized the catalytic intermediate that reacts with cyanide to inactivate NAO. The intermediate forms prior to formation of the reduced flavin because the reduced flavin should react with oxygen yet no oxygen consumption is observed above 2 mM cyanide. This is further supported by the inability of NAO, as monitored at 450 nm, to progress beyond the reductive half-reaction in the presence of oxygen and 50 mM cyanide. The cyanide-sensitive intermediate must also form after formation of the nitroethane anion, because the rate constant determined from the first phase of the absorbance change in the latter experiment matches the rate constant for reduction of the enzyme by nitroethane and exhibits a kinetic isotope effect with [1,1-$^2H_2$]-nitroethane. This establishes that the abstraction of the α-proton from nitroethane limits the rate constant for cyanide inactivation, and thus formation of the intermediate occurs somewhere between proton abstraction and oxygen attack. This locus can be further defined on the basis of the structures of the modified flavins formed in the presence of cyanide as analyzed by mass spectrometry. The results with both nitroethane and 1-nitrohexane as substrates are consistent with the loss of nitrite, the addition of cyanide, and the retention of the ethyl and hexyl moieties, respectively, in the flavin adducts. Therefore, the cyanide-sensitive intermediate must be formed after formation of nitrite yet before formation of the aldehyde product. This is entirely consistent with the attack of cyanide on the cation imine intermediate I.

Is the cationic imine I a kinetically competent intermediate? Since the rate constant for cyanide inactivation is limited by the rate constant for nitroethane deprotonation, the rate constants for formation of intermediate I and attack by cyanide must be at least greater than 250 s^{-1}.[7] This is much larger than the value of 3.8 s^{-1} calculated from the second-order rate constant for enzyme inactivation as determined from changes in the rate of oxygen consumption at pH 8 and 50 mM cyanide. However, this latter value is a gross underestimate because of the very low concentration of intermediate I present during catalysis. To illustrate that formation of intermediate I and attack by cyanide occur very rapidly, the mechanism shown in Scheme 4 was simulated with the program DYNAFIT.[13] The reaction condi-

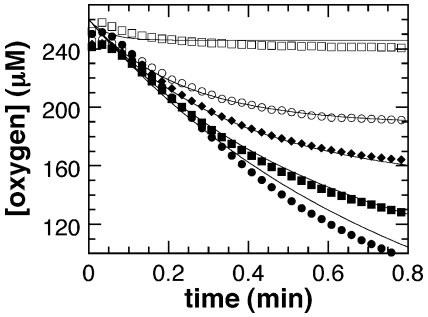


Figure 5. Simulation of enzyme inactivation by cyanide. Data from Figure 1A are shown for 0.1 (filled circles), 0.15 (filled squares), 0.25 (diamonds), 0.4 (open circles), and 2 mM cyanide (open squares). The solid lines represent simulated data at the same cyanide concentrations generated with DYNAFIT.[13] The simulation used the mechanism shown in Scheme 3 and the following concentrations and kinetic constants: $EFAD_{ox}$, 0.5 μM; S, 12.5 mM; O_2, 260 μM; k_1, 1×10^6 M^{-1} s^{-1}; k_2, 1.4×10^4 s^{-1}; k_3, 250 s^{-1}; k_4, 2500 s^{-1}; k_5, 4.4×10^4 M^{-1} s^{-1}; k_6, 2500 s^{-1}; k_7, 2×10^5 M^{-1} s^{-1}; and k_8, 15 s^{-1}. P_1 and P_2 represent the products nitrite and acetaldehyde.

tions were set to match those in Figure 1A, and a majority of the rate constants were assigned on the basis of previously determined values.[7] The rate constant for nitroethane binding (k_1) was set at 1×10^6 M^{-1} s^{-1}, and the rate constant for nitroethane dissociation (k_2) of 1.4×10^4 s^{-1} was determined from the measured K_D value of 14 mM. The rate constants for formation of intermediate I (k_4) and formation of reduced flavin (k_6), each 2500 s^{-1}, were estimated to be 10-fold greater than the rate constant for proton abstraction (k_3) of 250 s^{-1}, consistent with the lack of a reverse commitment.[7,15] The rate of oxygen attack (k_7) was set at 2×10^5 M^{-1} s^{-1} to match the rate of flavin oxidation measured in the absence of products, and the rate of release of both products (k_8) corresponds to the k_{cat} value of 15 s^{-1}. The rate constant for the reaction between intermediate I and cyanide (k_5) was then varied until a reasonable simulation of the data over a range of cyanide concentrations was achieved with a k_5 value of 4.4×10^4 M^{-1} s^{-1} (Figure 5). This demonstrates that the mechanism shown in Scheme 4 and the kinetic constants measured in previous studies provide a valid description of catalysis by NAO. Moreover, since the rate constants for formation of intermediate I (2500 s^{-1}) and its reaction with 50 mM cyanide (2200 s^{-1}) greatly exceed both the rate constant for proton abstraction (250 s^{-1}) and the overall rate constant for catalysis (15 s^{-1}), the cationic imine I is most certainly a kinetically competent intermediate in the mechanism of NAO.

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