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Kinetic evidence for an anion binding pocket in the active site of nitronate monooxygenase ☆

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ABSTRACT

A series of monovalent, inorganic anions and aliphatic aldehydes were tested as inhibitors for *Hansenula mrakii* and *Neurospora crassa* nitronate monooxygenase, formerly known as 2-nitropropane dioxygenase, to investigate the structural features that contribute to the binding of the anionic nitronate substrates to the enzymes. A linear correlation between the volumes of the inorganic anions and their effectiveness as competitive inhibitors of the enzymes was observed in a plot of pK_{is} *versus* the ionic volume of the anion with slopes of 0.041 ± 0.001 mM/Å^3 and 0.027 ± 0.001 mM/Å^3 for the *H. mrakii* and *N. crassa* enzymes, respectively. Aliphatic aldehydes were weak competitive inhibitors of the enzymes, with inhibition constants that are independent of their alkyl chain lengths. The reductive half reactions of *H. mrakii* nitronate monooxygenase with primary nitronates containing two to four carbon atoms all showed apparent K_d values of ~5 mM. These results are consistent with the presence of an anion binding pocket in the active site of nitronate monooxygenase that interacts with the nitro group of the substrate, and suggest a minimal contribution of the hydrocarbon chain of the nitronates to the binding of the ligands to the enzyme.

1. Introduction

Nitronate monooxygenase (E.C. 1.13.11.32; NMO), formerly known as 2-nitropropane dioxygenase [1] is a flavin mononucleotide-dependent (FMN) enzyme that catalyzes the oxidative denitrification of alkyl nitronates to their corresponding aldehyde and keto compounds and nitrite [2,3]. The most extensively characterized NMOs studied to date are those from *Neurospora crassa* [2,4–6] and *Hansenula mrakii* [3,7], although the X-ray crystallographic structure of the enzyme from *Pseudomonas aeruginosa* has also been reported [8]. Detailed mechanistic studies have been carried out only for *N. crassa* NMO where it was shown that a transient anionic flavosemiquinone is formed during oxidative catalytic turnover of the enzyme through a single electron transfer reaction between an enzyme-bound nitronate and the flavin cofactor (Scheme 1) [2,4]. The formation of an anionic flavosemiquinone intermediate during oxidative catalysis is a characteristic feature of NMO that distinguishes it from the well characterized nitroalkane oxidase [9], which catalyzes a similar oxidation reaction through a different mechanism that involves the formation of a covalent flavin N(5)-adduct [10–13].

Recombinant NMO from *H. mrakii* was recently cloned and expressed in *Escherichia coli* cells and the resulting purified enzyme was characterized in its biochemical and kinetic properties [3]. The enzyme is similar to that from *N. crassa* in that it contains a single non-covalently bound FMN per monomer of enzyme and is devoid of metal cofactors [2,3]. Moreover, an anionic flavosemiquinone was observed upon anaerobic mixing of *H. mrakii* NMO with alkyl nitronates with chain lengths ranging from two to six carbon atoms [3]. Neither hydrogen peroxide nor superoxide is released during turnover of the enzyme with primary alkyl nitronates as evident from the absence of superoxide dismutase or catalase effects on the rates of oxygen consumption in activity assays of *H. mrakii* NMO [3], a result that was also obtained with the *N. crassa* enzyme [2].

Both *H. mrakii* and *N. crassa* NMOs are able to effectively oxidize a number of alkyl nitronates into their corresponding carbonyl compounds and nitrite. Such an enzymatic oxidation is of considerable interest given that many alkyl nitronates are known to be toxic or mutagenic [14–17]. Ingestion of propyl-2-nitronate, for example, has been demonstrated to result in the formation of 8-aminodeoxyguanosine and 8-oxodeoxyguanosine through a

Abbreviations: NMO, nitronate monooxygenase; FMN, flavin mononucleotide.

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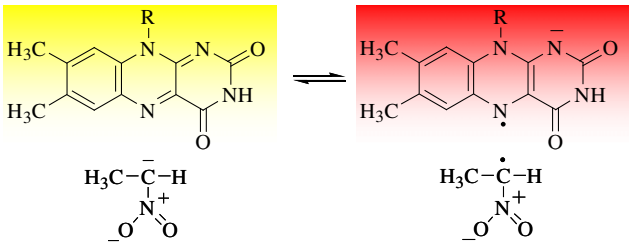

Scheme 1. The one-electron oxidation of ethylnitronate catalyzed by nitronate monooxygenase.

phenol sulfotransferase-mediated metabolic pathway in both human and rat cell lines [18,19]. Despite their toxicity, alkyl nitronates are widely used in chemical industry because they provide a quick and efficient route for the synthesis of a wide range of commercially useful compounds [20–22]. An investigation of the substrate specificity of NMO can therefore provide the basis for use of the enzyme in bioremediation applications to detoxify waste generated from industrial uses of alkyl nitronates.

In the current study, the contributions of both the nitro and hydrocarbon moieties of the alkyl nitronate substrate of NMO for binding and specificity were investigated through inhibition and rapid kinetics studies. The results are consistent with the presence of an anion binding pocket in both the *H. mrakii* and *N. crassa* enzymes, which interacts with the nitro group of the substrate. These interactions are key determinants for binding and recognition of the substrate by the enzyme, rather than the hydrophobic interactions that could occur between the enzyme and the alkyl moiety of the substrate, as in the case of nitroalkane oxidase [23,24].

2. Materials and methods

2.1. Materials

NMOs from *H. mrakii* and *N. crassa* were obtained through the expression and purification protocols described previously [3,5]. Nitroethane, 1-nitropropane and 1-nitrobutane were from Sigma–Aldrich (St. Louis, MO). All other reagents were of the highest purity commercially available.

2.2. Steady state kinetics

Enzymatic activity was measured in 50 mM potassium phosphate at pH 7.4 and 30 °C with the method of initial rates [25] by monitoring the rate of oxygen consumption with a computer interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument Ltd.). Enzyme concentrations were expressed per bound FMN content using experimentally determined values of 13,100 M^{-1} cm^{-1} ($\varepsilon_{446\ nm}$) for the *H. mrakii* enzyme [3] and of 11,850 M^{-1} cm^{-1} ($\varepsilon_{444\ nm}$) for the *N. crassa* enzyme [2]. The final concentration of enzyme used in each assay was between 25 and 65 nM, whereas substrate concentrations ranged from 0.5 to 20 mM. The nitronate form of the substrate was prepared in 100% ethanol by incubating the corresponding nitroalkane with 1.2 M excess of potassium hydroxide for at least 24 h at room temperature. Since the second-order rate constant for the protonation of ethylnitronate is 15 M^{-1} s^{-1} [26], enzymatic activity assays were initiated by the addition of substrate to the reaction mixture to ensure that a negligible amount of the neutral form of the nitronate is formed during the time required to determine initial rates of reaction (typically ~30 s).

2.3. Pre-steady state kinetics

The pre-steady state kinetic parameters of *H. mrakii* NMO were determined in 50 mM potassium phosphate at pH 7.4 and 30 °C using a TgK Scientific SF-61 stopped-flow spectrophotometer. Rates of flavin reduction were measured by monitoring the increase in absorbance at 372 nm that results from anaerobic mixing of the enzyme with substrate as previously described for *N. crassa* NMO [4]. Nitronate solutions (100 mM) were prepared in water by incubating the nitroalkane in a 1.2 M excess of potassium hydroxide for at least 24 h and were diluted in water prior to use. The final enzyme concentration in each assay was between 9 and 20 μM, whereas the substrate concentrations used ranged from 0.1 to 50 mM, thereby ensuring that the enzymatic reaction follows pseudo first-order kinetics.

2.4. UV–visible absorbance spectra of NMO in the presence of sodium nitrite

Changes in the UV–visible absorbance spectrum of NMO upon addition of sodium nitrite were monitored using an Agilent Technologies diode-array spectrophotometer Model HP 8453, thermostated at 15 °C. Spectra of the *H. mrakii* and *N. crassa* enzymes (at concentrations of ~80 μM) were recorded before and after addition of 1 mM sodium nitrite. Difference spectra were then constructed by subtracting the final absorbance spectrum of the enzyme in the presence of sodium nitrite from that of the free enzyme.

2.5. Data analysis

Steady state kinetic data were fit with either Enzfitter (Biosoft, Cambridge, UK) or KaleidaGraph software (Synergy Software, Reading, PA). Stopped-flow traces monitoring the reductive half reaction of *H. mrakii* NMO were fit with Eq. (1), which describes a single exponential process where k_{obs} is the observed first-order rate for the increase in absorbance at 372 nm, A_t is the absorbance at time t, and A is the final absorbance. Pre-steady state kinetic parameters were determined using Eq. (2), where k_{obs} is the observed rate of flavin reduction, k_{red} is the limiting rate constant for flavin reduction at saturating substrate concentrations, and K_d is the apparent dissociation constant of the substrate (S). Inhibition data were fit with Eq. (3), which describes a competitive inhibition pattern where K_{is} is the dissociation constant for the inhibitor (I).

$$A_{total} = A_t e^{-k_{obs} t} + A \quad (1)$$

$$k_{obs} = \frac{k_{red} S}{K_d + S} \quad (2)$$

$$\frac{v_o}{e} = \frac{k_{cat} S}{K_m [1 + (\frac{I}{K_{is}})] + S} \quad (3)$$

3. Results

3.1. Nitrite inhibition of NMO with respect to ethylnitronate as substrate

In order to establish if the nitro group of the akyl nitronate substrates of *H. mrakii* and *N. crassa* NMO contributes to binding and specificity, nitrite was used as a mimic of the substrate to establish whether it inhibits the enzymes in 50 mM potassium phosphate pH 7.4 and 30 °C. As shown in Fig. 1A for the case of the *H. mrakii* enzyme, sodium nitrite behaved as a competitive inhibitor with respect to ethylnitronate as substrate for both enzymes as indicated