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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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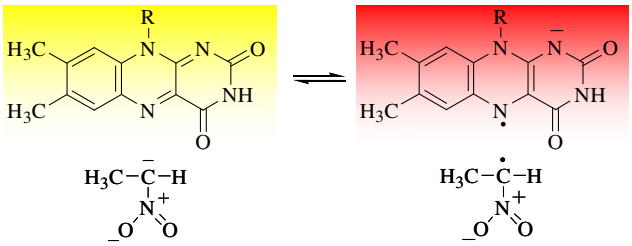

Scheme 1. The one-electron oxidation of ethylnitronate catalyzed by nitronate monooxygenase.

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

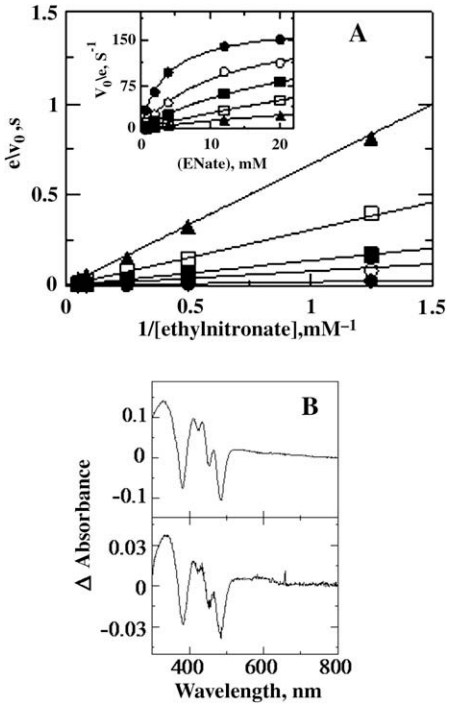

Fig. 1. Inhibition of *H. mrakii* NMO by sodium nitrite with respect to ethylnitronate as substrate. *Panel A:* Inhibition of *H. mrakii* NMO. Enzymatic activity was measured at varying concentrations of ethylnitronate in the presence of 0 mM (•); 5 mM (o); 10 mM (■); 20 mM (□) and 50 mM (▲) sodium nitrite in 50 mM potassium phosphate at pH 7.4 and 30 °C. The lines are from fits of the data to Eq. (3). *Panel B:* UV–visible absorbance spectral changes induced upon addition of 1 mM sodium nitrite to *H. mrakii* (top) of *N. crassa* (bottom) NMO in 50 mM potassium phosphate pH 7.4 at 15 °C. Difference spectra were constructed by subtracting the final absorbance spectrum of the enzyme in the presence of nitrite from that of the free enzyme.

by the pattern of lines that intersect on the *y*-axis in double reciprocal plot of the initial rate of oxygen consumption *versus* ethylnitronate concentration at different fixed concentrations of inhibitor[1]. The corresponding inhibition constants (K_{is}) were 1.7 ± 0.1 mM for the *H. mrakii* enzyme and 9.8 ± 0.6 mM for the *N. crassa* enzyme. The effect of the nitrite on the UV–visible absorbance spectra of the FMN cofactor of the *H. mrakii* and *N. crassa* NMOs was also determined. As shown in Fig. 1B, binding of sodium nitrite to both enzymes induced spectral changes in the FMN cofactor bound at the active sites of the enzymes as seen from the increase in the absorbance intensities at 333, 414, 436 and 465 nm along with the concomitant decrease in absorbance intensities at 381 and 485 nm. All taken together, the inhibition and spectroscopic studies are consistent with binding of nitrite occurring at the active site of the enzyme.

3.2. Inorganic anion inhibition of NMO with respect to ethylnitronate as substrate

A series of monovalent, inorganic anions were tested as inhibitors for NMO in 50 mM potassium phosphate pH 7.4 and 30 °C to determine if the enzyme contains an anion binding site for recognition of the nitro group of the substrate. As shown in Table 1, inorganic anions ranging in size from 25 to 64 Å^3 were competitive inhibitors for the *H. mrakii* enzyme, with inhibition constants ranging from ~80 mM with potassium fluoride to ~1 mM with potassium iodide. Similar results were obtained for the *N. crassa* NMO, with inhibition constants ranging from ~125 mM for potassium fluoride inhibition to ~5 mM for potassium iodide inhibition. Neither enzyme was inhibited by potassium phosphate at concentrations as high as 100 mM, suggesting that either dianions or anions with an ionic volume of 90 Å^3 [27] are unable to bind at the active site of both the NMOs tested.

Table 1
Inorganic anion inhibition of NMO with respect to ethylnitronate as substrate[a].

Inhibitor	Ionic volume (Å^3)[b]	*H. mrakii*, K_{is} (mM)[c]	*N. crassa*, K_{is} (mM)[d]
Sodium nitrite	55	1.7 ± 0.1	10 ± 1
Potassium fluoride	25	77 ± 2	125 ± 7
Potassium chloride	47	11.3 ± 0.3	30 ± 2
Potassium bromide	56	4.0 ± 0.3	20 ± 1
Sodium nitrate	64	1.9 ± 0.1	12 ± 1
Potassium iodide	72	0.95 ± 0.06	6.0 ± 0.4

[a] Enzymatic activity was measured at varying concentrations of ethylnitronate and several fixed concentrations of inhibitor in 50 mM potassium phosphate pH 7.4 at 30 °C. Data were fit with Eq. (3).
[b] From [27].
[c] The steady state kinetic parameters had the following average values: k_{cat} = 180 ± 15 s^{-1}; K_m = 3.6 ± 0.2 mM and k_{cat}/K_m = 48,500 ± 6500 M^{-1} s^{-1}.
[d] The steady state kinetic parameters had the following average values: k_{cat} = 68 ± 1 s^{-1}; K_m = 1.6 ± 0.1 mM and k_{cat}/K_m = 42,700 ± 3300 M^{-1} s^{-1}.

A plot of the pK_{is} values as a function of the ionic volume of the inorganic anions tested as inhibitors in the range from 25 to 64 Å^3 (Fig. 2) yielded a straight line with both enzymes, with the exception of sodium nitrite. The experimentally determined inhibition constants for sodium nitrite (Table 1) were between two- to three-times lower than the values that can be predicted from the size of the inorganic anion by lines of Fig. 2 of 4.7 mM for the *H. mrakii* and 19 mM for the *N. crassa* enzymes. Inhibition of the *H. mrakii* enzyme was more sensitive to the volume of the inorganic anion as indicated by the slope of 0.041 ± 0.001 mM/Å^3 in the plot of pK_{is} *versus* ionic volume of the anion used as a competitive inhibitor for the enzyme, as compared to 0.027 ± 0.001 mM/Å^3 for the *N. crassa* NMO (Fig. 2). The *y*-intercepts determined from the linear fitting of the data as shown in Fig. 2 were similar to one another irrespective of the enzyme used, with a value of ~ −2.8 mM. No correlation between the electron affinities of the inorganic anions tested and the inhibition constants of NMO was found, as illustrated in Fig. 2. The counterions of the inhibitors tested had no effect on the inhibition of either enzyme as indicated by the inhibition constants of sodium nitrate, which conformed to the linear relationship between the ionic volumes of the anions tested using potassium salts with the inhibition constants of the NMO.

3.3. Contributions of the alkyl chain length to substrate binding in NMO

In order to establish whether the hydrocarbon chain of the alkyl nitronate substrate is a determinant for binding to the active site of the enzymes, the dissociation constants for a number of nitronate substrates and aliphatic aldehydes inhibitors with hydrocarbon chains of various lengths were determined in 50 mM potassium phosphate at pH 7.4 and 30 °C. As shown in Fig. 3 for the case of ethylnitronate as substrate, stopped-flow measurements demonstrated that the reductive half reaction of the *H. mrakii* NMO involves the formation of an anionic flavosemiquinone as evident from the peaks centered at ~372 and 490 nm in the UV–visible absorbance spectrum obtained after anaerobic mixing of the enzyme with the substrate. The observed rates of flavin reduction in-

[1] All of the inhibition data reported in this study were also fit with equations describing noncompetitive and uncompetitive inhibition patterns. Since the data were all best fit with a model describing competitive inhibition, only these fits are shown.

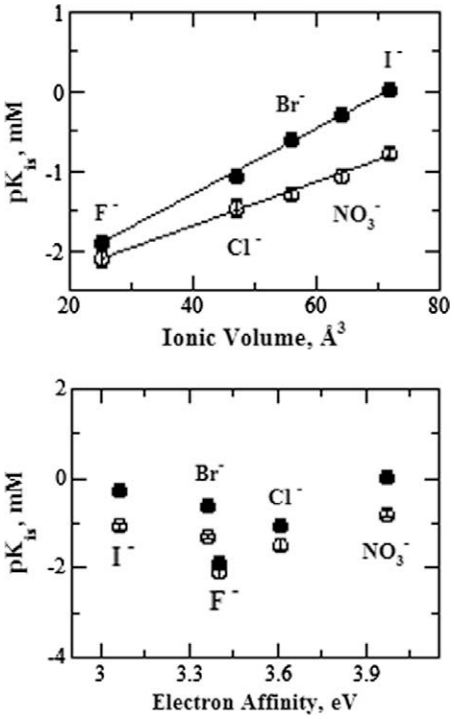

Fig. 2. Anion inhibition of NMO with respect to ethylnitronate as substrate. The inhibition constants (K_{is}) for a series of monovalent anions were determined by measuring enzymatic activity of *H. mrakii* (•) or *N. crassa* (○) NMO at varying concentrations of ethylnitronate in the presence of several fixed concentrations of inhibitor. All assays were carried out in 50 mM potassium phosphate at pH 7.4 and 30 °C. *Top panel:* plots of pK_{is} *versus* ionic volume. Values for the ionic volumes of the anions used were taken from [27]. *Bottom panel:* plots of pK_{is} *versus* electron affinity. Values for the electron affinity were taken from [31].

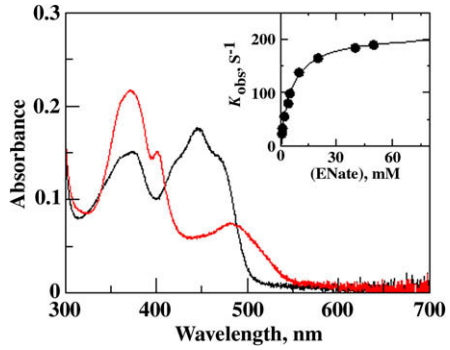

Fig. 3. Pre-steady state kinetics of *H. mrakii* NMO with ethylnitronate as substrate in 50 mM potassium phosphate pH 7.4 and 30 °C. Flavin absorbance at 372 nm was monitored over time after anaerobic mixing of the enzyme with ethylnitronate to give final concentrations of 13 μM enzyme and 0.5 mM ethylnitronate. The UV–visible absorbance spectra before (black) and ~30 s after mixing (red) are shown. *Inset*: Rates of flavin reduction determined from the fits of the stopped-flow traces at 372 nm to Eq. (1) were plotted as function of ethylnitronate concentration (0.5 to 50 mM) and were fit with Eq. (2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

creased hyperbolically with the concentration of nitronate and showed similar apparent K_d values of ~5 mM with ethylnitronate, propyl-1-nitronate and butyl-1-nitronate, suggesting that the length of the alkyl chain of the substrate is not a determinant factor for binding of the substrate to the enzyme. The kinetic parameters for the reductive half reaction of *H. mrakii* NMO are summarized in Table 2.

Table 2
Reductive half reaction of *H. mrakii* NMO at pH 7.4 at 30 °C[a].

Substrate	k_{red} (s^{-1})	K_d (mM)
Ethylnitronate	210 ± 5	5.9 ± 0.3
Propyl-1-nitronate	200 ± 5	5.3 ± 0.3
Butyl-1-nitronate	350 ± 5	5.7 ± 0.2

[a] *H. mrakii* NMO was anaerobically mixed with substrate in 50 mM potassium phosphate in a stopped-flow spectrophotometer and the absorbance changes at 372 nm were monitored over time.

Each aldehyde tested was a competitive inhibitor with respect to ethylnitronate for the *H. mrakii* enzyme with inhibition constants of ~60 mM, which further suggests that the alkyl chain length of the substrate contributes only minimally with respect to the nitro moiety ($K_{is} \sim 2$ mM) for binding of the substrate to the enzyme (Table 3). Similar, results were obtained with the *N. crassa* enzyme with propanal, butanal, pentanal or hexanal, in that the inhibition constants for aldehyde inhibition were large (i.e., ⩾70 mM), though accurate determinations could not be attained due to limited solubility of the inhibitors in aqueous solution.

4. Discussion

Binding of alkyl nitronates to NMO is primarily mediated through interactions of an anion binding pocket within the active site of the enzyme and the nitro moiety of the substrate. Evidence supporting this conclusion comes from inhibition studies of the *H. mrakii* and *N. crassa* enzymes with a series of monovalent, inorganic anions of varying ionic volumes. Binding of the anions likely occurs at the active site of the enzymes as evident from the spectroscopic changes of the FMN cofactor induced upon incubation of the enzyme with nitrite, which resemble those induced by binding of anthranilate to the active site of L-amino acid oxidase [28], and the competitive inhibition pattern observed with respect to ethylnitronate as substrate. The linear correlation between the ionic volumes of the inorganic anions used as inhibitors and the inhibition constants that describe their binding to the enzymes suggests the presence of a discrete binding pocket with a well-defined size, where the interactions at the anion binding site of the two enzymes between the ligand and the enzyme are maximized as the volume of the inorganic anion increases. The lack of inhibition of either enzyme by phosphate suggests that the anion binding pocket can accommodate either monovalent ligands, but not divalent ones, or only those with an ionic volume smaller than 90 Å^3 [27] which, by assuming a spherical geometry of the inorganic anion, corresponds to a diameter of the monovalent anion that is smaller than 5.6 Å. In agreement with the estimate of the binding pocket deriving from our kinetic analysis, the available three dimensional structure of NMO from *P. aeruginosa* clearly indicates that an anion binding site is present at the active site of

Table 3
Aldehyde inhibition of *H. mrakii* NMO with respect to ethylnitronate as substrate[a].

Inhibitor	K_{is} (mM)
Propanal	58 ± 3
Butanal	60 ± 1
Pentanal	58 ± 3
Hexanal	60 ± 2

[a] Enzymatic activity was measured at varying concentrations of ethylnitronate and several fixed concentrations of inhibitor in 50 mM potassium phosphate pH 7.4 at 30 °C. Data were fit with Eq. (3). The steady state kinetic parameters had the following average values: k_{cat} = 185 ± 3 s^{-1}; K_m = 3.3 ± 0.2 mM; k_{cat}/K_m = 55,000 ± 2200 M^{-1} s^{-1}.

the bacterial enzyme in proximity of the nitro moiety of the substrate [8], as illustrated in Fig. 4. In that enzyme, the anion binding pocket is comprised mainly of the side chain of His_{152}, the peptidyl nitrogen of Gly_{151} and the side chain of Ser_{288}. Although the crystal structures of the enzymes studied in this report are not yet available, the alignment of the amino acid sequences of the three enzymes show that these residues are conserved in all three NMOs [1]. In the *H. mrakii* enzyme, the amino acid residues corresponding to the anion binding pocket of the bacterial enzyme are His_{197}, Gly_{196} and Ser_{351}. The equivalent residues in the *N. crassa* enzyme are His_{196}, Gly_{195} and Ser_{342} (or Thr_{344}).

A comparison of the slopes in the plots of pK_{is} *versus* ionic volumes for the inorganic anions acting as inhibitors of the *H. mrakii* and *N. crassa* NMOs is consistent with the anion binding pocket of the *H. mrakii* enzyme being slightly smaller than that of the *N. crassa* enzyme. In this respect, the slopes of the lines that fit the data in the plots of Fig. 2 depend on either the values of the inhibition constants that describe the binding of the inorganic anions to the enzyme, the ionic volumes of the inorganic anions, or both. In principle, the binding of the inorganic anions to the binding pockets in the two enzymes can be affected by a number of factors including geometric, steric, or electrostatic effects. However, the observation that the fits of the data in Fig. 2 yields values for the y-intercepts for the two enzymes that are similar to one another is consistent with the slope effect that is experimentally observed being due primarily to the volume of the anion binding pockets of each enzyme rather than other factors. The accurate determination of the sizes and geometries of the anion binding pockets at the active sites of the enzymes from *H. mrakii* and *N. crassa* will have to await the elucidation of the X-ray crystallographic structures of the two enzymes, which is currently ongoing in collaboration with Weber's group at Georgia State University.

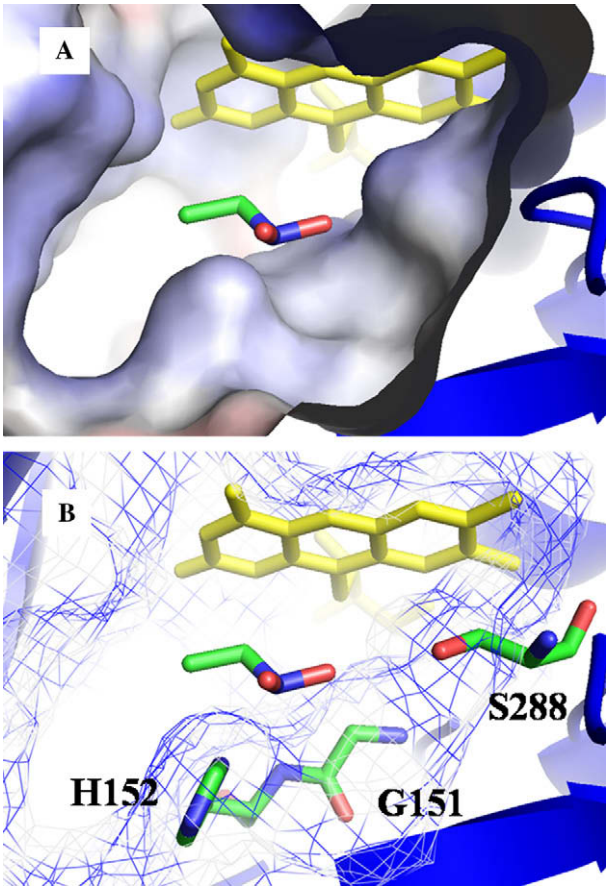

Fig. 4. Anion binding site in *P. aeruginosa* NMO. An electrostatic potential map was generated for the X-ray crystallographic structure of *P. aeruginosa* NMO in complex with 2-nitropropane (PDB ID: 2GJN) using an Adaptive Poisson–Boltzmann Solver [32] and visualized using Pymol. *Panel A*: Electrostatic potential surface of the active site of *P. aeruginosa* NMO. *Panel B*: Active site amino acid residues that comprise the anion binding site in *P. aeruginosa* NMO.

Substrate recognition by NMO is predominantly determined by the interactions occurring at the active site binding pocket of the enzyme with the nitro group of the alkyl nitronate substrate with minimal, if any, hydrophobic interactions of the enzyme with the alkyl chain of the substrate. Evidence supporting this conclusion comes from the comparison of the K_{is} values for aldehyde inhibition with respect to nitrite inhibition, which shows that the former are at least 10-times smaller than the latter with both the enzymes tested. Lack of interaction of the alkyl chain of the ligand with the enzyme is independently supported by the similar values for the dissociation constants for substrate binding (K_d) determined for the *H. mrakii* enzyme with alkyl nitronates of varying chain lengths of between two and four carbon atoms. Indeed, one would expect a progressive decrease in the K_d values for the substrate with increasing lengths of the alkyl chain of the substrate if hydrophobic interactions played a significant role for substrate binding. The results suggesting that NMO does not discriminate its ligands by exploiting the organic moiety of the substrate are consistent with previous studies of the enzyme from *N. crassa* that established that *m*-nitrobenzoate effectively binds to the enzyme (i.e., K_{is} value of 9.1 mM at pH 7.4), despite its large size and aromatic character [4]. Further in agreement with minimal contribution of the alkyl chain of the substrate to binding are previous studies of the *H. mrakii* and *N. crassa* enzymes showing that the k_{cat}/K_m values for nitronates ranging from two to six carbon atoms are independent of the alkyl chain length of the substrate [2,3]. As illustrated in Fig. 4, the three dimensional structure of the bacterial enzyme from *P. aeruginosa* with 2-nitropropane bound at the active site shows the presence of a wide cavity in the active site of the enzyme, which is large enough to accommodate substrates of various lengths or different structures [8].

5. Conclusions

In conclusion, the results presented herein demonstrate that the active site of yeast NMO contains an anion binding pocket, which participates in the binding of the nitro group of the alkyl nitronate substrates. Thus, the nitro group of the substrate is the key determinant for binding of the substrate at the active site of the enzyme as opposed to the hydrocarbon chain of the nitronate molecule acting as substrate, which plays a minimal role, if any, in binding by the enzyme. These results contrast those previously reported for nitroalkane oxidase, whose ability to bind substrates at the active site increases with increasing lengths of the alkyl chain of the substrate and reaches a maximum value with substrates containing four or more carbon atoms [24]. A study of the pH and kinetic isotope effects on nitroalkane oxidase revealed that each methylene group of the substrate provides approximately 2.6 kcal/mol of binding energy [23], which was recently explained through structural studies of the enzyme that demonstrated a hydrophobic channel leading to the active site of the enzyme [12,29,30]. Although structural studies have yet to be reported for the *H. mrakii* and *N. crassa* enzymes, the X-ray crystallographic structure of *P. aeruginosa* NMO shows a solvent accessible active site that lacks a hydrophobic channel like that seen in nitroalkane oxidase [8]. The elucidation of binding pockets for anionic ligands in the active site of enzymes through inhibition studies with inorganic anions of various ionic volumes demonstrates a kinetic method that should be generally applicable to any enzyme whose crystallographic structure is not yet available.

Acknowledgments

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