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ORIGINAL PAPER

The critical role of a conserved lysine residue in periplasmic nitrate reductase catalyzed reactions

Nitai C. Giri[1] · Breeanna Mintmier[1] · Manohar Radhakrishnan[1] · Jonathan W. Mielke[2] · Jarett Wilcoxen[2] · Partha Basu[1]

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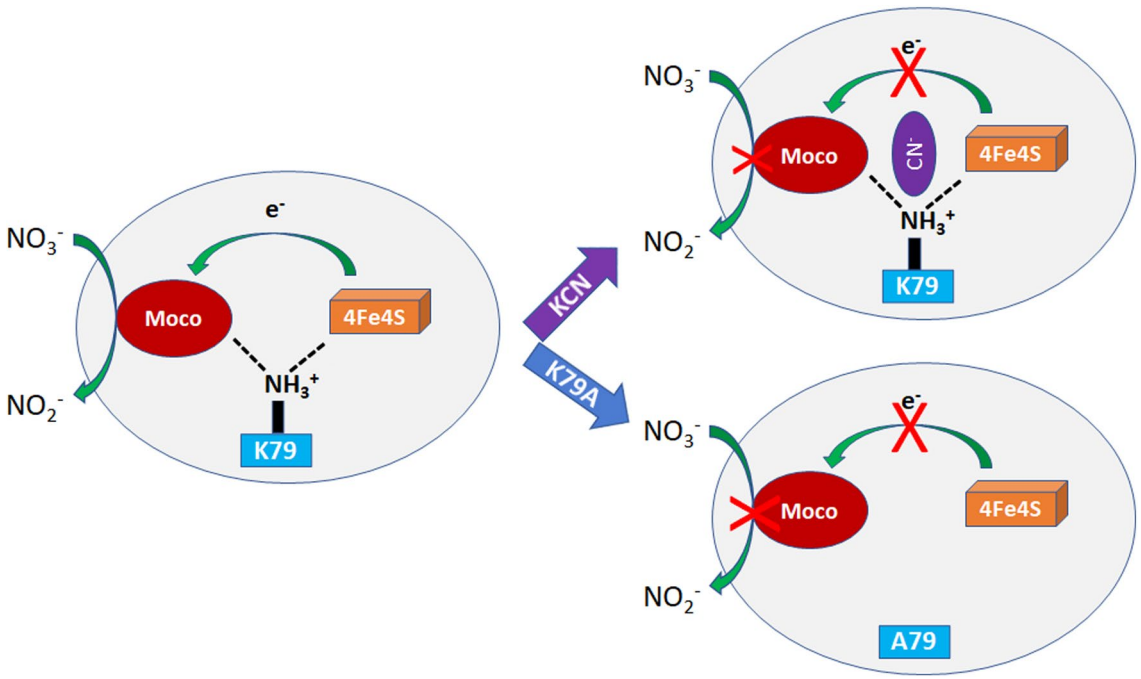
Abstract

Periplasmic nitrate reductase NapA from *Campylobacter jejuni* (*C. jejuni*) contains a molybdenum cofactor (Moco) and a 4Fe–4S cluster and catalyzes the reduction of nitrate to nitrite. The reducing equivalent required for the catalysis is transferred from NapC → NapB → NapA. The electron transfer from NapB to NapA occurs through the 4Fe–4S cluster in NapA. *C. jejuni* NapA has a conserved lysine (K79) between the Mo-cofactor and the 4Fe–4S cluster. K79 forms H-bonding interactions with the 4Fe–4S cluster and connects the latter with the Moco via an H-bonding network. Thus, it is conceivable that K79 could play an important role in the intramolecular electron transfer and the catalytic activity of NapA. In the present study, we show that the mutation of K79 to Ala leads to an almost complete loss of activity, suggesting its role in catalytic activity. The inhibition of *C. jejuni* NapA by cyanide, thiocyanate, and azide has also been investigated. The inhibition studies indicate that cyanide inhibits NapA in a non-competitive manner, while thiocyanate and azide inhibit NapA in an uncompetitive manner. Neither inhibition mechanism involves direct binding of the inhibitor to the Mo-center. These results have been discussed in the context of the loss of catalytic activity of NapA K79A variant and a possible anion binding site in NapA has been proposed.

Graphical abstract



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Keywords Nitrate reductase · Molybdenum · 4Fe–4S cluster · *Campylobacter jejuni*

Introduction

The catalytic subunit of periplasmic nitrate reductase, NapA, from *C. jejuni* contains a molybdenum cofactor (Moco) and a 4Fe–4S cluster like *E. coli* periplasmic nitrate reductase (Fig. 1). It catalyzes the reduction of nitrate to nitrite (Fig. 2) [1, 2]. The reducing equivalents required for the catalysis are transferred from electron-transferring subunits, such as NapB and NapC, following the sequence NapC → NapB → NapA [3]. The electron transfer from NapB to NapA occurs through the 4Fe–4S cluster in NapA. *C. jejuni* NapA has a lysine (K79) between the Mo-cofactor and the 4Fe–4S cluster that is conserved in several members of the DMSO reductase family of enzymes (Fig. 3) [4–6]. For example, this conserved lysine is also found in formate dehydrogenase (Fdh) [7–9] , ethylbenzene dehydrogenase (Edh) [10], and perchlorate reductase (Prc) (Fig. 3) [11]. However, its precise role in catalysis is not clear. Analysis of the crystal structure of NapA (*E. coli* NapA, for example) indicates that this conserved Lys sits in the middle of the edge of Mo-cofactor and the 4Fe–4S cluster (Fig. 1) and connects these two prosthetic groups via H-bonding interactions. Therefore, replacing K79 with another residue that leads to the loss of these H-bonding interactions (as in the case with Ala, for example) might interfere with the intramolecular electron transfer and consequent catalytic activity of NapA. In the present study, we have replaced the conserved Lys to Ala and showed that the K79A NapA variant is essentially inactive (has ~ 0.2% of the WT activity), confirming its role in catalytic activity. NapA is sensitive to inhibition by various anions, such as cyanide, thiocyanate, and azide [12, 13]. However, the mechanism of inhibition could differ depending on the anion. For example, the anions could inhibit NapA by directly binding to the Moco [12, 13]. It is worth noting that the proposed mechanism of nitrate reduction by NapA also involves the direct binding of nitrate to the Mo center of NapA (Fig. 2D) [14–16]. Thus, this type of inhibition will be regarded as competitive inhibition. The anions, e.g., perchlorate, can also inhibit NapA by blocking the access of the substrate (i.e., nitrate) to the Mo center without directly binding to the Mo center of NapA [17]. In this case, the inhibition will be regarded as non-competitive inhibition. It is also possible that the non-competitive inhibitors can bind in a site in between the Mo center and the 4Fe–4S cluster and exert their inhibitory effect by interfering with the electron transfer between the 4Fe–4S cluster and the Mo center of NapA. However, in some cases, the inhibitor binds only to the enzyme–substrate complex and not to the free enzyme. It is possible that a substrate-induced conformational change of the enzyme allows the binding of the inhibitor, enabling the latter to exert its inhibitory effect. Such an inhibition is regarded as uncompetitive inhibition.

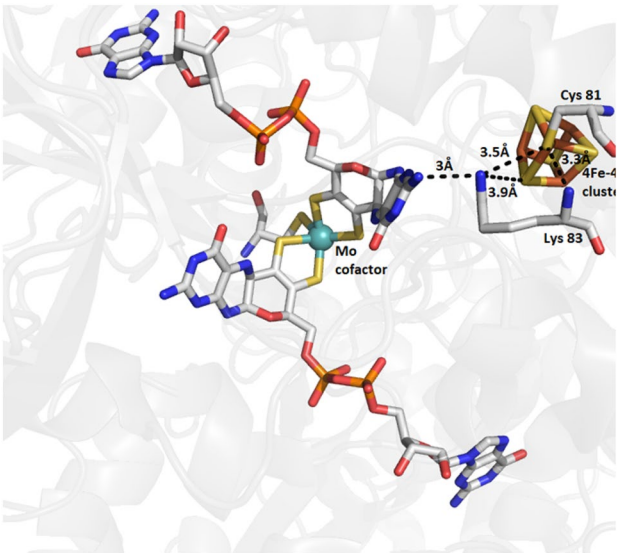

Fig. 1 The Moco, the 4Fe–4S cluster and the conserved Lys in *E. coli* NapA (PDB ID: 2NYA). H-bonding interactions of this conserved Lys with the Mo-cofactor and the 4Fe–4S cluster are indicated by dashed line

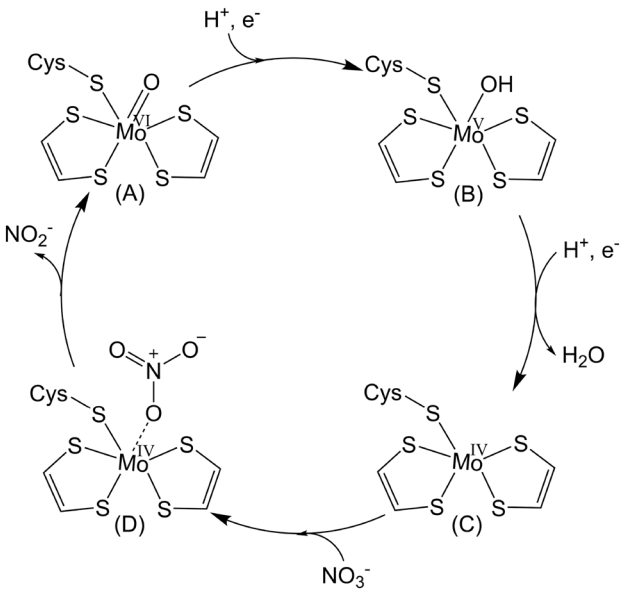

Fig. 2 Proposed mechanism of NapA catalyzed nitrate reduction(14)

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Nap_C. jejuni          NRGLNCIKGYFNAKIM
Nap_C. necator         NKGLNCVKGYFLSKIM
Nap_D. desulfaricans   NAGLLCLKGSLLIPVL
Nap_E. coli            NRGLNCIKGYFLPKIM
Nap_R. sphaeroides     NRGLNCVKGYFLSKIM
Fdh_D. gigas           NEGSLCAKGASTWQLA
Fdh_E. coli            NQGTLCLKGYYGWDFI
Edh_A. aromaticum      YNPLGCQKGSAFNNNL
Pcr_A. oryzae          YNPRGCNKGECGHDYM
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Fig. 3 Conserved Lys (in bold) in periplasmic nitrate reductase (Nap) from different organisms. This Lys is also conserved in formate dehydrogenase (Fdh), ethylbenzene dehydrogenase (Edh) and perchlorate reductase (Pcr). Multiple sequence alignment was performed using Clustal Omega

In the present study, the inhibition of *C. jejuni* NapA by cyanide, thiocyanate and azide has been investigated. These results are discussed in the context of the loss of activity in NapA K79A variant and a possible anion binding site in NapA has been proposed.

Experimental section

Expression and purification of NapA and its variant. Expression and purification of *C. jejuni* NapA were performed following the protocol reported previously [18]. Mutagenesis of *C. jejuni* NapA was performed using the QuickChange II Site-Directed Mutagenesis Kit (Qiagen) as described previously [18].The PCR product was sequenced at ACGT Inc. The resulting plasmid K79A-NapA was expressed and purified using the same procedure as the WT NapA. Mo- and Fe-contents were measured by ICP-MS using the previously reported procedure [18]. Nitrate reductase activity for both the WT and the K79A variant was measured spectrophotometrically by monitoring the oxidation of reduced methyl viologen ($MV^{\cdot+}$) at 600 nm as previously described [14, 18]. For the inhibition experiments, the enzyme (0.125 µM) was incubated with $MV^{\cdot+}$ and different concentrations of KCN (1 µM, 2 µM, or 10 µM), KSCN (10 µM, 50 M, or 100 µM) or NaN_3 (200 µM, 500 µM or 1000 µM) for 10 min prior to the addition of the substrate.

Electron paramagnetic resonance spectroscopy. Samples for X-band (~9.4 GHz) EPR spectroscopy were prepared by titrating to reduction ~200 µM NapA with electrochemically reduced methyl viologen (50 mM stock) under anoxic conditions (< 0.5 ppm O_2) in a LC-100 (LC Technology Solutions Inc) glove box. The protein was allowed to incubate for up to 5 min to ensure complete reduction with or without 200 µM to 2 mM KCN (1:1 to 10:1 KCN to NapA). 190 µL of the protein was then added to a 4 mm OD quartz sample tubes (Wilmad) containing 10 µL of 10 mM nitrate (500 µM final concentration) and immediately frozen in the glovebox in an ethanol/dry ice bath and immediately stored in liquid nitrogen. Continuous wave (CW) spectra were collected using a Magnettech MS5000 spectrometer (Freiburg Instruments) equipped with a liquid nitrogen cryostat with temperature and gas-flow controller. Samples were measured under non-saturating conditions at 150 K using 9.4 GHz microwave frequency, 2 mW microwave power, 120 s sweep time 5G modulation amplitude. Spectra analysis and plotting were performed using Easyspin (Easyspin.org) in Matlab R2019a (Mathworks inc).

Results

Activity of NapA K79A variant. Activity measurements performed with $MV^{\cdot+}$ as an electron donor show that the *C. jejuni* NapA K79A variant is almost inactive (has only 0.2% active compared to the WT *C. jejuni* NapA, Fig. 4). However, the *C. jejuni* NapA K79A variant had almost the same amount of Mo and Fe (~0.8 eq of Mo and ~4 eq of Fe per NapA monomer, respectively) as WT *C. jejuni* NapA, indicating that the loss of activity is not due the loss of the cofactor.

Inhibition of NapA by KCN. NapA is inhibited by KCN. The double reciprocal plot (1/v vs. 1/[S]) for cyanide inhibition shows a decrease in V_{max} but no significant change in

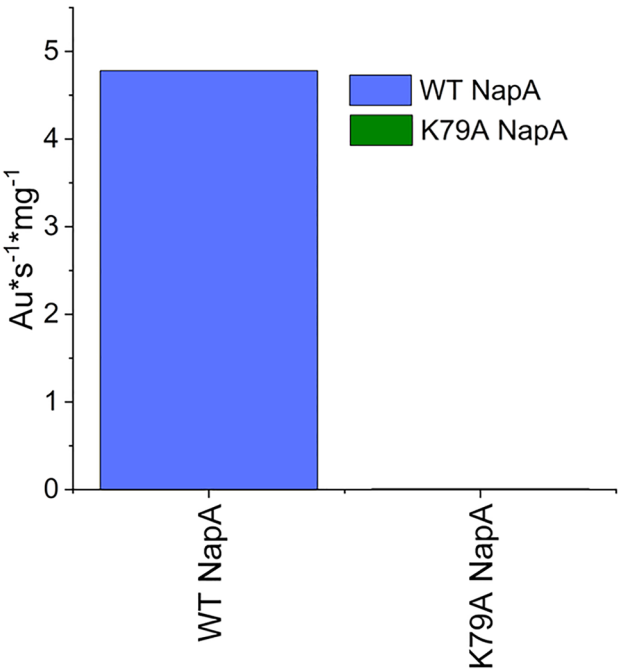

Fig. 4 Activity of WT *C. jejuni* NapA and the K79A variant of *C. jejuni* NapA

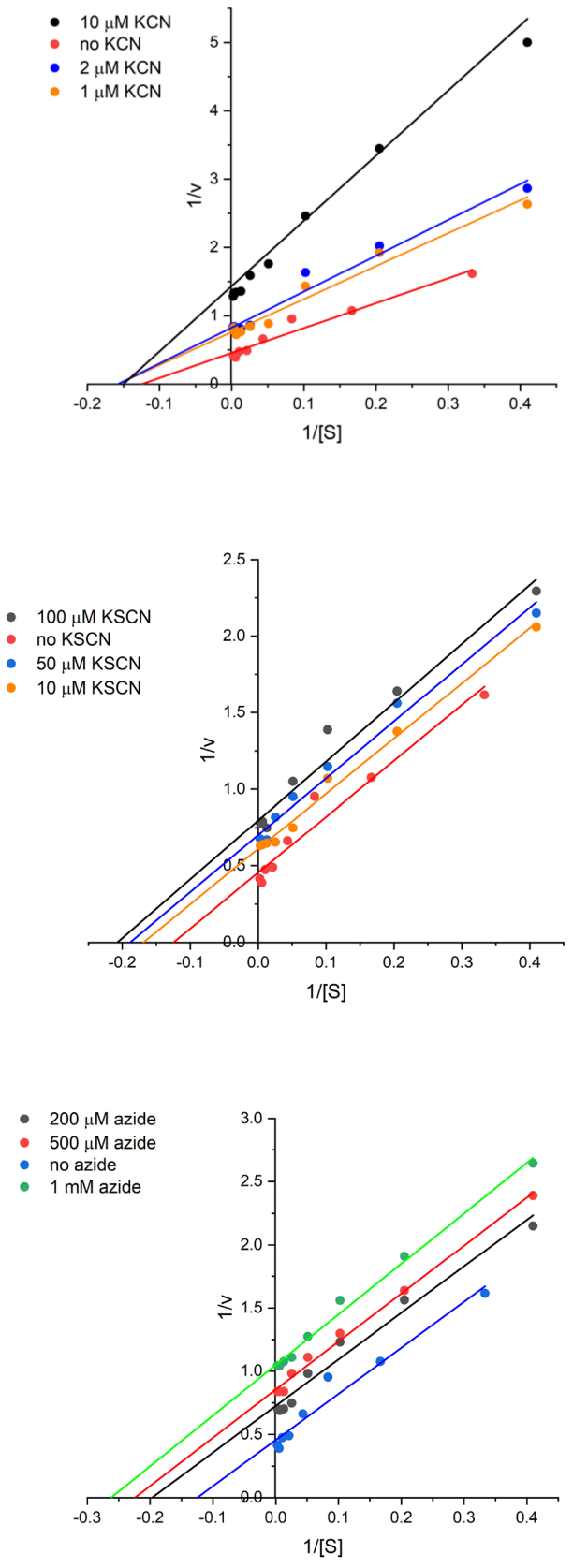

Fig. 5 Inhibition of NapA by different inhibitors: cyanide (top), thiocyanate (middle) and azide (bottom)

K_m with increasing concentration of KCN (Fig. 5, top). This result indicates that KCN inhibits NapA in a non-competitive manner where the $^{-}$CN does not bind to the same site as the substrate nitrate. From these data, the apparent K_i for $^{-}$CN was found to be 3 µM.

EPR of NapA with and without KCN. EPR samples of WT NapA prepared under turnover conditions revealed typical EPR species, SI Fig. 1, of protein in the presence of nitrate [19]. Under these conditions, the enzyme is reduced by three electrons, two at Mo, one at the 4Fe–4S cluster, then oxidized by two reducing equivalents by nitrate, leaving one electron in equilibrium between the Mo center and 4Fe–4S cluster. In the presence of 200 µM to 2 mM KCN, the EPR signal was absent, even after longer incubation times and across several enzyme preparations.

Inhibition of NapA by KSCN and NaN_3. NapA is also inhibited by KSCN and NaN_3. The double reciprocal plot (1/v vs. 1/[S]) for KSCN (Fig. 5, middle) and NaN_3 (Fig. 5, bottom) inhibition shows a decrease in V_{max} and K_m with increasing concentration of inhibitors. These results indicate that KSCN and NaN_3 inhibit NapA in an uncompetitive manner where the inhibitors bind to the enzyme–substrate complex but not to the free enzyme. From these results, the apparent K_i for $^{-}$SCN and N_3^- were found to be 86 µM and 565 µM, respectively. The K_i value for thiocyanate in the present case is much lower than 4 mM, which had been reported for *Paracoccus pantotrophus* (formerly known as *Thiospora pantotrophus*) NapA [13]. The K_i value for azide obtained here is also much lower than 11 mM, which has been reported for *Paracoccus denitrificans* (formerly known as *Thiosphaera pantotropha*) NapA [12].

Discussion

Replacement of K79 in C. jejuni NapA with Ala leads to loss of activity. Previous activity measurements indicated that WT NapA has a k_{cat} of 5.9 s^{-1} and a K_m (for nitrate) of 3.4 µM, suggesting that NapA has a high affinity for nitrate. These results give a k_{cat}/K_m value of 1.7×10^6 $M^{-1}s^{-1}$, suggesting that NapA is very efficient in reducing nitrate. In the present study, we show that the K79A variant of NapA is catalytically impaired (has only 0.2% of WT activity), confirming its crucial role in catalysis. It is worth mentioning that the *C. jejuni* NapA K79A variant had almost the same amount of Mo and Fe (~0.8 eq of Mo and ~4 eq of Fe per NapA monomer, respectively) as WT *C. jejuni* NapA, indicating that the loss of activity is not due the loss of the cofactor. The important role of K79 is supported by the highly conserved nature of this lysine in periplasmic nitrate reductase (Nap), formate dehydrogenase (Fdh), ethylbenzene dehydrogenase (Edh), and perchlorate

reductase (Prc) as mentioned before [4–11]. However, its precise role in catalysis is not clear.

Role of the conserved lysine in NapA. Lysine plays an important role in electron transfer and proton transfer in proteins [20, 21]. The positively charged side chain of lysine can readily accept an electron and form a hypervalent radical, which could lead to the cleavage of the N–H bond and release of a formal H-atom [22]. The conserved water molecule present in the crystal structure of NapA, as well as Fdh, can stabilize this H-atom. Eventually, this H-atom could be transferred to the Mo-center via chemical and H-bonds, and reduce Mo(VI) to Mo(IV) via Mo(V). Considering this H-atom as a proton and electron, this process can be regarded as a net proton-coupled electron transfer (PCET). Thus, the conserved lysine in NapA could play an important role in PCET necessary to complete the catalytic cycle. Analysis of the crystal structure of NapA (Fig. 1) shows that this lysine sits in the middle of the edge of the Mo-cofactor and the 4Fe–4S cluster and connects these two prosthetic groups via H-bonding interactions. Therefore, mutating K79 to other residues will interfere with the H-bonding network and thus interfere with this putative PCET and consequent catalytic activity. Indeed, the mutation of the conserved lysine (K72) in a cyanobacterial NapA to Arg and Gln showed that both K72R and K72Q are catalytically inactive [23] . However, it is worth mentioning that a loss/degradation of cofactor was observed in these variants. The mutation of this conserved lysine (K85) in *C. necator* NapA showed that the K85R variant retained 23% of the WT NapA activity [4]. This is not surprising considering the presence of an Arg at the same position in membrane-bound nitrate reductases (Nar) [24, 25], arsenite oxidase (Aio) [26, 27] and polysulfide reductase (Psr) [28]. However, the K85M variant of *C. necator* NapA is completely inactive. The present work indicates that the mutation of K79 in *C. jejuni* NapA to Ala leads to an almost complete loss of activity. Of course, mutation of Lys to Ala will lead to the loss of a positive charge near the 4Fe-4S cluster. Also, lysine forms weak H-bonding interactions with the 4Fe–4S cluster, as shown in(Fig. 1). Overall, the mutation of Lys to Ala will impair the H-bonding network. This impairment could modulate the redox potential of the 4Fe–4S cluster [29, 30]. This idea is in line with a previous report where the substitution of this conserved lysine in *Cereibacter sphaeroides (formerly known as Rhodobacter sphaeroides)* with His or Met decreases the redox potential of the 4Fe–4S cluster as well as catalytic efficiency [31] . The mutation of Lys to Ala could also interfere with the proposed PCET in NapA, leading to the loss of catalytic activity. Overall, the loss of activity of K79A of *C. jejuni* NapA can be attributed to electrostatic (loss of positive change in K79A variant) as well as H-bonding (loss of H-bond in K79A variant) factors.

Inhibition of NapA by cyanide. The double reciprocal (1/v vs. 1/[S]) plot for cyanide (Fig. 5, top) shows a decrease in V_{max} but no significant change in K_m with increasing concentration of KCN, indicating a non-competitive inhibition. For this type of inhibition, the inhibitor (i.e., cyanide) and the substrate (i.e., nitrate) do not bind to the same site. The proposed mechanism for nitrate reduction involves the direct binding of nitrate to the Mo center of NapA (Fig. 2D) [14–16]. Thus, this result implies that the cyanide inhibition of NapA is not due to the direct binding of cyanide to the Mo center. The lack of cyanide binding to Mo is supported by the absence of cyanide binding to the Mo center of NapA in the EXAFS analysis of cyanide-treated NapA [12]. However, the crystal structure of NapA obtained in the presence of cyanide shows cyanide binding to the Mo center (PDB ID: 2JIR). The cyanide binding, in this case, could be due to the large concentration of cyanide (10 mM, which is at least three orders of magnitude higher than the concentrations used in the present study) used during the crystallization process. Finally, EPR experiments performed here reveal no signal under turnover conditions in the presence of KCN. This is in contrast to previous work where dithionite-reduced protein (non-turnover condition) in the presence of KCN did yield a Mo(V) species but did not show hyperfine splitting associated with either ^{13}C or ^{15}N, suggesting no direct binding between cyanide and Mo in the EPR active species [19]. In this work, the authors note a slight shift in the spectrum of the 4Fe–4S cluster, suggesting a KCN interaction with the cluster. An important distinction between the reductants used is size and accessibility to the Mo-center. In the turnover reaction, $MV^{\cdot+}$ is thought to reduce the 4Fe–4S cluster and not the Mo-center directly, where dithionite is small enough potentially to navigate the substrate access channel and interact directly with Mo. The EPR species under turnover conditions rely on Mo(V) being generated via reduction by the 4Fe–4S cluster following oxidation by substrate. Considering the observed kinetics and behavior of the lack of EPR species under turnover, we propose that cyanide binds near the active site (Fig. 6) and exerts its inhibitory effect, most likely by interfering with the electron transfer between the Mo-cofactor and the 4Fe–4S cluster. In this proposal, cyanide would bind to a site between the Mo-cofactor and the 4Fe–4S cluster. Another possible way of inhibition could be by blocking the access of substrate to the Mo center, which has been proposed for perchlorate ion [17]. However, this type of blockage is unlikely since cyanide is a small diatomic anion compared to tetrahedral perchlorate ion. Also, this type of blockage is likely to increase the K_m, which is not the case here (no significant change in K_m was observed).

Inhibition of NapA by KSCN and NaN_3. The double reciprocal (1/v vs. 1/[S]) plots for thiocyanate and azide (Fig. 5, middle and bottom) indicate an uncompetitive

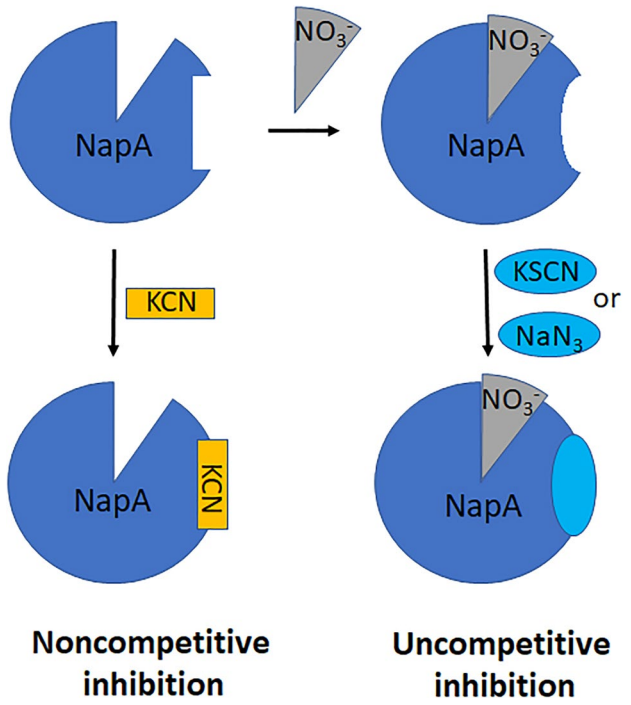

Fig. 6 Proposed mechanism of inhibition of NapA by different inhibitors

inhibition. For this type of inhibition, the inhibitor (i.e., thiocyanate or azide) binds to the enzyme-substrate complex but not to the free enzyme. Competitive inhibition of NapA by thiocyanate and azide has also been reported [12, 13]. In this case, the inhibition involved the direct binding of thiocyanate or azide to the Mo center of NapA. However, it is worth mentioning that the concentrations of thiocyanate (1 mM and 10 mM) or azide (40 mM and 80 mM) used are much higher than those used in the present experiments. Also, the crystal structure obtained with lower concentrations of azide (10 mM) does not show azide binding to the Mo center in the crystal (PDB ID: 2JIM). Finally, EPR spectra of as-isolated proteins under reducing conditions did not show any significant change upon the addition of azide, suggesting no direct binding of azide to the Mo center [19]. We think that thiocyanate and azide bind to a site similar to the cyanide binding site (Fig. 6). However, due to the different sizes of thiocyanate and azide compared to cyanide, the previous two inhibitors cannot bind to the free enzyme, as is the case for cyanide. Thus, the binding of thiocyanate and azide to NapA requires a conformational change that could occur due to the substrate binding to NapA. A conformational change has been attributed to explain the slight shift in the EPR signal of the 4Fe–4S cluster upon cyanide binding to NapA, although EPR experiments did not find any evidence for cyanide coordination either to Mo or the 4Fe–4S cluster [19]. Also, we have previously reported the stabilization of the enzyme-substrate (ES) complex (lower Gibbs free energy) compared to the enzyme (E) only [14]. This stabilization can also be attributed to a conformational change upon the substrate binding to NapA.

Possible anion binding site in NapA. Our inhibition studies indicate that the inhibition of NapA by all the anions does not involve direct binding of anion to the Mo center. This is supported by our EPR experiments as well as previously reported EPR studies [19]. So, the other possibilities include a) binding to the substrate channel and blocking the access of the substrate to the catalytic center, and b) binding to a site between the Mo center and the 4Fe–4S cluster and interfering with the intramolecular electron transfer by disrupting the H-bonding network. The inhibition of NapA by perchlorate has been attributed to the first possibility. This idea is supported by the crystal structure where the perchlorate binds to the putative substrate binding channel [17]. However, this is unlikely for the anions in the present case since—a) they are linear and not tetrahedral like perchlorate and b) this type of blockage will likely increase the K_m for the substrate, which is not observed in the present case. The inhibition of NapA by these anions occurs more likely due to possibility 2, where the anion binds to a site in between Moco and the 4Fe–4S cluster and interferes with the intramolecular electron transfer by disrupting the H-bonding network. Our EPR experiments performed with KCN support the role of $^{-}$CN in the interference of intramolecular electron transfer. In the absence of KCN, Mo(VI) produced after turnover undergoes reduction by accepting electrons from the 4Fe–4S cluster. However, in the presence of 200 µM to 2 mM KCN, the Mo(VI) produced after turnover is not able to accept electrons from the 4Fe–4S cluster and thus, the EPR signal was absent, even after longer incubation times. Previously, a slight shift in the EPR signal of the 4Fe–4S cluster upon cyanide binding to NapA has been observed, although EPR experiments did not find any evidence for cyanide coordination either to Mo or the 4Fe–4S cluster [19]. The slight shift of the EPR signal can be explained by this type of $^{-}$CN binding, which can also explain the inhibition of NapA by cyanide. Analysis of the crystal structures of NapA (except *C. sphaeroides* NapAB, which has a resolution of 3.2 Å) indicates that there is a conserved water molecule in between the Moco and the 4Fe–4S cluster (Fig. 7). It is worth mentioning that this water molecule is also present in Fdh (Fig. 7: bottom right). This water molecule not only forms weak H-bonding interactions with the 4Fe–4S cluster but also forms an H-bonding network between the 4Fe–4S cluster, K79 and the pyranopterin of the Mo-cofactor. Thus, one possible mechanism of the inhibition of NapA by these anions could be the substitution of this water molecule by these anions. This substitution will disrupt the H-bonding

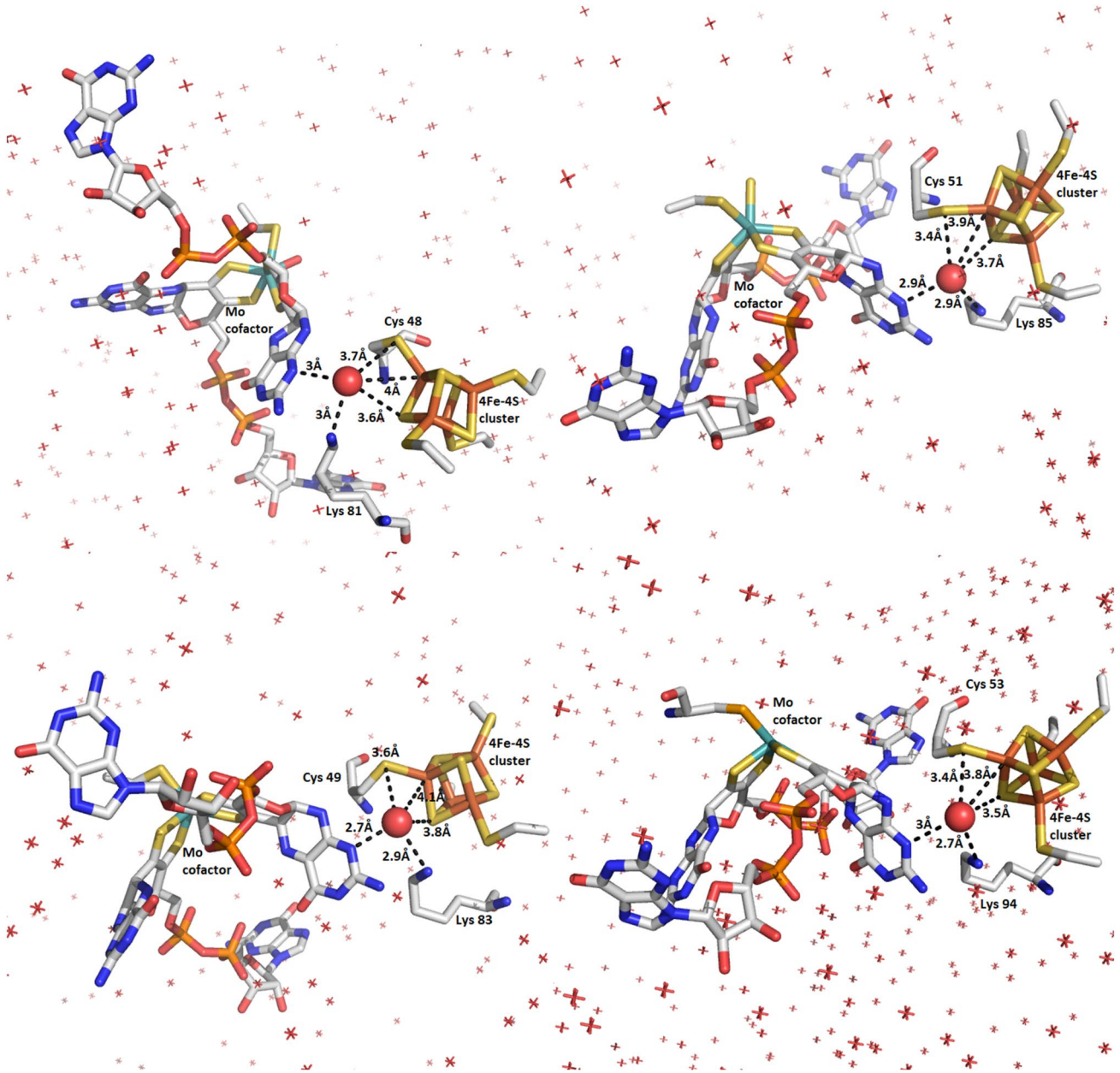

Fig. 7 Conserved water molecule in *D. desulfuricans* (top left, PDB ID: 2NAP), *C. necator* (top right, PDB ID: 3ML1) and *E. coli* (bottom left, PDB ID: 2NYA) NapA and Fdh (bottom right, PDB ID: 1KQF) is shown as a red sphere. Other water molecules are shown as cross. H-bonding interactions of this conserved water with the Mo-cofactor, conserved Lys and the 4Fe–4S cluster are indicated by dashed line

network involving the conserved Lys, the Mo-cofactor and the 4Fe–4S cluster. This disruption could slow down the intramolecular electron transfer and lead to the decrease in V_{max}. Also, placing a negative charge near Lys will increase its pK_a and thus will slow down the proposed proton transfer to the catalytic center. The reduced efficiency of proton transfer could cause a decrease in V_{max}.

Proposed role of water molecule. Electron transfer between two distant metal centers or cofactors via water molecules has been demonstrated in many systems. For example, the electron transfer between two Cu-centers separated by 11 Å in peptidoglycine-α-hydroxylating monooxygenase via water has been demonstrated [32, 33]. In this case, protein structure does not change much upon the binding of a substrate analog. The enzyme also lacks any flexible motif that will allow the Cu-centers to come close to each other during catalysis. Theoretical study performed on cytochrome b_5 suggests that this type

of electron transfer process is highly efficient, especially when the donor–acceptor distances are between 9 and 12 Å. [34] It is worth mentioning that the distance between the Mo center and the nearest Fe in the 4Fe–4S cluster in NapA is ~ 11.9–12.2 Å in different crystal structures [5, 6, 15, 16]. This "through water" electron transfer involves water molecules and a number of hydrogen and chemical bonds. Water molecules also play an important role in proton transfer [35–37]. For example, the protons required for the reduction of water by cytochrome c oxidase are transported via the side chains of polar amino acids and conserved water molecules [38–40]. Water is also involved in PCET [41, 42]. Several PCET steps are involved in RNR-catalyzed reduction [43]. In some of these steps, water plays an important role. Water also plays an important role in the PCET in cytochrome c oxidase [43, 44]. The role of the H-bonding network in PCET has been shown in RNR [45, 46]. Considering that the water molecule is conserved in all available structures of NapA (except *C. sphaeroides* NapA, where no water molecule was described) as well as closely related formate dehydrogenase (Fig. 7), it is very likely that it plays an important role. Based on the location of the conserved water molecule, its connectivity with the Mo-cofactor and the 4Fe–4S cluster, and the role of conserved water in electron transfer, proton transfer and PCET mentioned above, we propose that this conserved water in NapA can mediate one of these processes. In this context, it is worth noting that the reduction of Mo(VI) to Mo(IV) requires protons and electrons (Fig. 2). The transfer of these protons and/or electrons could be mediated by the water molecule. Thus, the displacement of the conserved water molecule by anions will slow the production of Mo(IV) and, thus, reduce the V_{max}. Future experiments will focus on testing this proposal.

Potential implications of NapA inhibition. High concentrations of nitrate in groundwater have long been associated with excessive fertilizer use and discharge of insufficiently treated industrial wastewater. There is global concern about the adverse effects of this nitrate on the environment and public health [47] . Consumption of water with high concentrations of nitrate negatively impacts human health, including birth defects, respiratory problems, etc [48–52]. Nitrate can also lead to the formation of carcinogenic N-nitrosamines, which alter DNA bases leading to cancer [53, 54]. Nitrate poisoning has also been reported in animals [55]. The US EPA has set a maximum allowable limit of 10 ppm nitrate in drinking water. Conventional purification of nitrate-contaminated water uses approaches such as adsorption, ion exchange, and reverse osmosis. Wastewater treatment accounts for ~ 3–4% of electrical energy load in the US [56]. Bioremediation of nitrate has advantages such as no sludge production, harmless end product, etc., over conventional approaches. Microbial denitrification has also been used for wastewater treatment and water purification [57, 58]. Recently, Nap enzymes from *Achromobacter* sp. have been used for bioremediation of nitrate [59]. However, biological denitrification has challenges due to the presence of various anions in contaminated water. Anions inhibit nitrate reduction by NapA, which is the first step of denitrification, as shown in the current work. Therefore, the presence of various anions (oxyanions, Cl^-, etc.) should be considered for the successful implementation of nitrate bioremediation.

Nitrate is present in the mammalian host intestinal environment. Studies show that *Salmonella enterica* serovar Typhimurium uses nitrate reduction via the Nap system to boost colonization [60]. Nitrate reduction is also an important factor during *C. jejuni* host colonization [61]. *C. jejuni* induces the expression of *napAGHBLD* operon during the colonization in chicken [62], while the expression of NapA is increased when infecting mammalian cells [63]. Deletion of *napA* resulted in a reduced ability of *C. jejuni* to infect host cells, signifying the influence of NapA in pathogenesis [61].Thus, in principle, the selective inhibition of NapA could lead to the development of new antibiotics against pathogenic bacteria such as Salmonella, Campylobacter, etc. Rusmana et al. demonstrated the selective inhibition of NarG over NapA by chlorate using pure cultures of *C. testosteroni* and *K. pneumoniae* [64]. More recently, it has been shown that procyanidins inhibit biological denitrification by specifically inhibiting NarG [65]. Thus, the differences in localization, structure, and function among nitrate reductases can be exploited to inhibit nitrate reductase activity.

Bacterial denitrification, carried out using NapA or NarG, is the main form of nitrogen loss in most soil [66–68]. Nitrogen, a macronutrient for plants, is mainly assimilated from nitrate. Thus, plants are in direct competition with microorganisms for nitrogen acquisition [69]. Some plants have developed a strategy to inhibit microbial denitrification in soil. This strategy, which utilizes procyanidins, leads to a six-fold increase of nitrate in soil compared to the untreated soil [70]. This strategy also reduces N_2O (a greenhouse gas) emission by up to 95% [71]. In the European agroecosystem, N_2O emission accounts for 59% of nitrogen loss from the system [72]. Inhibition of assimilatory nitrate reductase activity by glutamine in soil has also been reported [73, 74]. Thus, the inhibition of nitrate reduction could potentially lead to the development of environmentally friendly (by reducing N_2O emission) agriculture by limiting nitrogen loss from the soil and thus reducing fertilizer input while increasing plant growth and productivity.

Conclusion

The present work shows that the substitution of conserved Lys79 in *C. jejuni* NapA by Ala leads to an almost complete loss of activity, confirming its role in catalytic activity. The inhibition studies suggest that *C. jejuni* NapA is inhibited by different anions (e.g., cyanide, thiocyanate, and azide). Cyanide inhibits NapA in a non-competitive manner, while thiocyanate and azide inhibit NapA in an uncompetitive manner. Thus, the inhibition of NapA by these anions does not involve direct binding of these anions to the Mo center. Based on these results and the previous literature reports, we suggest that the inhibition of NapA by these anions is due to the binding of the anions in the place of a conserved water molecule observed in the crystal structures of NapA. This water molecule connects Lys79, the Moco and the 4Fe–4S cluster via an H-bonding network. Thus, we believe the reason for the loss of activity of K79A variant and the inhibition of NapA lies in the disruption of this H-bonding network and the consequent impairment of electron transfer and/or proton transfer. The potential broader impact of this research includes the development of nitrate bioremediation strategies considering the presence of anions.

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Declarations

Conflict of interest The authors have no financial or non-financial interests to disclose.

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Authors and Affiliations

Nitai C. Giri[1] · Breeanna Mintmier[1] · Manohar Radhakrishnan[1] · Jonathan W. Mielke[2] · Jarett Wilcoxen[2] · Partha Basu[1]

✉ Jarett Wilcoxen
jarettw@uwm.edu

✉ Partha Basu
basup@iu.edu

[1] Department of Chemistry and Chemical Biology, Indiana University Indianapolis, Indianapolis, IN, USA

[2] Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA