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Oxyl and Hydroxyl Radical Transfer in mARC Catalyzed Nitrite Reduction

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Abstract

A combination of EPR spectroscopy and computational approaches has provided insight in to the nature of the reaction coordinate for the one-electron reduction of nitrite by the mitochondrial amidoxime reductase component (mARC) enzyme. The results show that a paramagnetic Mo (V) species is generated when reduced enzyme is exposed to nitrite, and an analysis of the resulting EPR hyperfine parameters confirms that mARC is remarkably similar to the low pH form of sulfite oxidase. Two mechanisms for nitrite reduction have been considered. The first shows a modest reaction barrier of 14 kcal/mol for the formation of •NO from unprotonated nitrite substrate. In marked contrast, protonation of the proximal substrate oxygen to Mo in the Mo(IV)-O-N-O substrate bound species results in barrierless conversion to products. A fragment orbital analysis reveals a high degree of Mo-O(H)-N-O covalency that provides a π orbital pathway for one-electron transfer to the substrate and defines orbital constraints on the Mo-substrate geometry for productive catalysis in mARC and other pyranopterin molybdenum enzymes that catalyze this one-electron transformation.

Graphical abstract

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Supporting Information

Experimental and computational details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

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Keywords

Electron paramagnetic resonance; radical; mARC; MOSC; mechanism; molybdenum enzyme
Supporting Information Placeholder

The mitochondrial amidoxime reductase component (mARC) enzymes are members of the molybdenum cofactor (Moco) sulfurase C-terminal (MOSC) domain superfamily of pyranopterin Mo proteins.^{1,2} The mARC enzymes (mARC-1 and mARC-2) possess a single MOSC/MOSC-N domain with Moco as the only redox chromophore. The first coordination sphere for the oxidized Mo site was recently revealed by EXAFS and the coordination geometry is depicted in Fig. 1 along with putative structures for the reduced and Mo(V) enzyme forms.³ The general active site structures (Fig. 1) postulated for mARC enzymes are similar to those of other sulfite oxidase (SO) family enzymes, where the oxidized form possesses a Mo(VI) ion coordinated by two terminal oxo ligands, a pyranopterin dithiolene (pdt) chelate, and a coordinated cysteine.

The mARC enzyme possesses broad substrate specificity,⁴⁻⁶ which is unusual for sulfite oxidase (SO) family enzymes. Amidoxime and *N*-hydroxylated guanidine prodrugs are readily absorbed in the gastrointestinal tract and reduced by mARC to their active forms.⁶ Although the actual physiological substrates for mARC are not known, mARC can efficiently reduce *N*-hydroxy-cytosine to cytosine. This suggests a physiological role for mARC in the detoxification of base analogues that would otherwise be mis-incorporated into DNA with an increased frequency of mutations.⁷ Human mARC has also been suggested to function as a regulator of intracellular •NO concentrations since it can reduce *N*^ω-hydroxy-L-arginine,⁸ which is an intermediate in the conversion of L-arginine to NO by NO synthase. Remarkably, mARC has recently been shown to catalyze the reduction of nitrite (NO₂⁻) to •NO, indicating a potential biosynthetic pathway for •NO production in humans.⁹ This NO₂⁻ → •NO conversion has also been observed for xanthine oxidase,¹⁰ nitrate reductase,¹¹ and human SO.¹² Although mARC can catalyze transformations of a wide variety of substrates, the mechanistic details of these catalytic reductions and the electronic structure basis for mARC reactivity are largely unknown. In general, the mechanistic details of pyranopterin molybdenum enzyme one-electron catalysis, widely known for first row transition metals in

bioinorganic catalysis, are not understood for pyranopterin molybdenum enzymes. This results from the fact that these molybdenum enzymes are known to redox cycle between a Mo(IV) site with a d^2 electron configuration and a two-electron oxidized Mo(VI) center with a d^0 configuration.^{13,14} The vast majority of two-electron catalytic transformations performed by pyranopterin molybdenum enzymes are coupled to the formal transfer of an oxygen atom between the Mo center and the substrate. Here we focus on the one-electron chemistry being catalyzed by mARC in the reduction of NO_2^- to $\bullet\text{NO}$.

Plant mARC isoform 2 (pmARC-2) from *A. thaliana* (740 μM , phosphate buffer, pH = 7.8; 300mM NaCl) was treated with an anaerobic solution containing 10 $\times$ sodium dithionite and 50 $\times$ NO_2^- in order to reduce the enzyme and initiate that catalytic reduction of NO_2^- . The procedure is similar to prior studies that showed NO_2^- /dithionite elicited the evolution of $\bullet\text{NO}$.⁹ It is well known that nitrous acid (HNO_2) can disproportionate according to:

If mARC catalyzed the disproportionation of HNO_2 , this would not involve net Mo redox cycling. However, the catalytic reduction of NO_2^- to $\bullet\text{NO}$ is a one-electron reduction, and the reaction conditions employed⁹ should result in a build-up of enzyme in the one electron oxidized paramagnetic Mo(V) state that is detectable by EPR spectroscopy (Fig. 1). After 10 minutes incubation with dithionite/nitrite, a well-resolved EPR spectrum characteristic of the paramagnetic low pH SO Mo(V) enzyme form arises (Fig. 2). Spin quantitation of the signal shows nearly quantitative conversion to the Mo(V) species (76%). Although the EPR spectra of pmARC and low pH forms of SO are similar, pmARC-2 does not possess the SO (SUOX) fold,¹⁵ indicating that there are distinct differences between mARC and other SO family enzymes. This EPR spectrum is identical to that generated using human mARC that was partially reduced with NADH/cyt *b5R*/cyt *b5*.^{16,17} To our knowledge this is the first report of the ^{95,97}Mo hyperfine anisotropy for the mARC Mo(V) enzyme form. The combined nature of the ^{95,97}Mo hyperfine tensor and the g-tensor clearly indicates that the Mo(V) form of the enzyme is a mono-oxo species with the single unpaired electron occupying a Mo(xy) redox orbital that is oriented orthogonal to the Mo $\equiv$ O bond.^{18–20} Thus, the observation of a Mo(V) EPR signal under these conditions provides strong evidence for the mARC catalyzed one-electron reduction of nitrite to $\bullet\text{NO}$, allowing for an investigation of the reaction coordinate and the nature of the frontier molecular orbitals in order to assess symmetry restricted substrate binding at the Mo center and orbital control of the one-electron transfer chemistry.

In Fig. 3, we show putative mechanisms for the mARC catalyzed formation of $\bullet\text{NO}$ from nitrite. The EPR spectrum generated under reaction conditions clearly shows evidence for strong coupling to a proton, indicating that the Mo(V) species that accumulates under turnover conditions is an oxo-molybdenum (pdt)MoO(SR_{Cys})(OH) site, and not a dioxo-molybdenum (pdt)MoO₂(SR_{Cys}) site. The observation of an equatorial hydroxyl proton (i.e. the protonated equatorial oxo) supports a mechanism that involves one electron reduction of the substrate that is coupled with a protonation step. More importantly, however, this leads to the question of exactly when this proton enters into the reaction sequence.

The reaction coordinate for mARC catalysed NO_2^- reduction was investigated using a combination of closed-shell singlet, open shell broken symmetry singlet, and spin triplet calculations at the DFT level of theory.^{21,22} The computed reaction coordinate for the oxyl radical transfer is depicted in Fig. 4 (top). Although the computed reaction barrier ($H^\ddagger$) for oxyl radical transfer is only 14 kcal/mol, the reaction is essentially thermoneutral. This results from the Mo(V) dioxo species being inherently unstable to protonation.²³ Thus, protonation of the (pdt)MoO₂(SR_{Cys}) equatorial oxo will contribute to an increase in the overall exergonic nature of the reaction. In contrast, protonation of the O_a oxygen of NO_2^- that is bound to the Mo(IV) ion leads to a hydroxyl radical transfer and barrierless conversion to the (pdt)Mo^VO(SR_{Cys})(OH) and •NO products (Fig. 4, bottom). Interestingly, substrate protonation leads to an electronic structure that approximates the transition state for the conversion of the non-protonated substrate to products. This occurs through a polarization of the NO_2^- bonds (i.e. H-O-N=O; Fig. 3 bottom), and a weakening the N-O bond that is proximal to the Mo ion. In the spin unrestricted broken symmetry approximation, this results in a large exchange splitting of the Mo(xy) α ($\uparrow$) and β ($\downarrow$) spin orbitals (Fig. 5, left). This large exchange splitting results in a strong polarization of the Mo(xy) α ($\uparrow$) and β ($\downarrow$) spin orbitals that energetically drives a $\text{Mo}^{\text{IV}} \rightarrow \text{HNO}_2$ one electron transfer to yield (pdt)Mo^VO(SR_{Cys})(OH) and •NO.

The fragment orbital analysis^{21,22} of the spin unrestricted broken symmetry calculation reveals the key donor-acceptor orbital interactions that result in the barrierless conversion to product. Namely, the spin-down β ($\downarrow$) electron originally localized in the Mo(IV) fragment HOMO has been delocalized onto the substrate HNO_2 LUMO. The HNO_2 fragment β LUMO (Fig. 5, right) now comprises a significant portion of the “reactant” MO with appreciable diatomic •NO product π^* character. This orbital description is essentially identical to that obtained at the transition state for the conversion of unprotonated NO_2^- to •NO. Importantly, it is the Mo-substrate d-p π interaction that is key to providing a highly efficient pathway for the $\text{Mo}(\text{IV}) \rightarrow \text{HNO}_2$ single electron transfer. Since the Mo(xy) redox orbital is oriented orthogonal to the $\text{Mo}\equiv\text{O}$ bond, this allows one to postulate that the catalytically productive geometry for NO_2^- reduction must be one where the $\text{Mo}\equiv\text{O}$ bond is oriented in the same plane as the (H)O-N-O atoms of the substrate (Fig. 3).

In conclusion, the observation of barrierless one-electron transfer between Mo(IV) and HNO_2 and conversion to products favors a hydroxyl radical transfer mechanism for mARC upon substrate protonation. Oxyl radical transfer occurs with a modest reaction barrier (14 kcal/mol) when the substrate is not protonated. Thus, a picture begins to emerge whereby mARC is a SO-type protein that can catalyze both two electron (e.g. amidoxime) and one-electron (NO_2^-) substrate reductions. The latter requires a key α -protonation step to facilitate homolytic N-O bond scission and barrierless hydroxyl radical transfer to Mo. The frontier orbital analysis of the reaction coordinate also defines key aspects of substrate attack relative to the active site geometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Putative and experimentally derived structures for mARC in the Mo(IV), Mo(V), and Mo(VI) oxidation states.

Fig. 2.

X-band (9.37 GHz, 77K) EPR spectrum (red) of dithionite reduced mARC in the presence of excess NO_2^- . The spectral simulation (blue) yields spin Hamiltonian parameters: $g_{1,2,3} = 2.002, 1.967, 1.963$; $g_{\text{ave}} = 1.977$; $A_{1,2,3}(^{95,97}\text{Mo}) = 168, 70, 60$ MHz (Euler angles $\alpha = 0, \beta = 16, \gamma = 0$); $A_{\text{ave}}(^{95,97}\text{Mo}) = 99$ MHz; $A_{1,2,3}(^1\text{H}) = 26, 40, 20$ MHz (Euler angles $\alpha = 0, \beta = 0, \gamma = 0$); $A_{\text{ave}}(^1\text{H}) = 28.7$ MHz. The $I=1/2$ ^1H splitting on g_1 (3344 G) is highlighted.

Fig. 3. Oxyl radical transfer mechanism (top) and hydroxyl radical transfer mechanism (bottom) for mARC catalyzed $\bullet\text{NO}$ formation from NO_2^- . HR is a general acid that protonates the coordinated NO_2^- .

Fig. 4. Reaction coordinate for oxyl (top) and hydroxyl (bottom) radical transfer. Note that in 4 (bottom) the reaction proceeds smoothly from high energy (Mo(IV) + substrate) to low energy (Mo(V) + product) without going over a transition state barrier.

Fig. 5.

Spin unrestricted broken symmetry description of mARC catalyzed N-O bond cleavage for $r_{\text{N-O}} = 1.5 \text{ \AA}$ (α spin orbitals are in black and β spin orbitals in red). Note the delocalization of the (pdt)MoOS_{Cys} β ($\downarrow$) electron (red arrow) into the HNO₂ LUMO. There is a large spin polarization in the broken symmetry mARC frontier MOs, with the β ($\downarrow$) HOMO possessing Mo(xy) and $\bullet\text{NO}$ π^* character and the α ($\uparrow$) HOMO (not shown) possessing Mo(xy) and essentially no $\bullet\text{NO}$ π^* character. The description is similar for the transition state using NO_2^- as substrate ($r_{\text{N-O}} = 1.7 \text{ \AA}$).