Pyranopterin Coordination Controls Molybdenum Electrochemistry in Escherichia coli Nitrate Reductase*

Sheng-Yi Wu, Richard A. Rothery, and Joel H. Weiner

From the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Background: The role of the pyranopterin component of the mononuclear molybdenum cofactor is largely unknown.

Results: Variants of pyranopterin-coordinating amino acid residues were generated, and their effects on electrochemistry/catalysis investigated.

Conclusion: The pyranopterin environment modulates molybdenum electrochemistry.

Significance: The pyranopterin coordination environment enables redox-tuning of the molybdenum atom, and facilitates molybdoenzyme reactivity toward a broad range of substrates.

Enzymes containing the mononuclear molybdenum cofactor (molybdenum-enzymes) support an extraordinary diversity of redox transitions spanning a reduction potential range of approximately 1 volt (1, 2). Their active sites comprise a molybdenum atom coordinated by one or two pyranopterin dithiolene chelates, with additional metal coordination being provided by oxo/sulfido groups and/or oxygen/sulfur atoms derived from amino acid side chains (3, 4). Molybdenum-enzymes fall into three major families, each of which has a distinct protein-fold: the sulfite oxidases and plant nitrate reductases (the SUOX family) (5); the xanthine dehydrogenases and carbon-monoxide dehydrogenases (the XDH family (6)); and enzymes related to the bacterial DMSO reductases (the DMSOR family (6, 7)). The precise function of the pyranopterin component of the molybdenum cofactor remains unclear: it clearly functions as part of the molecular scaffold holding the molybdenum atom in an optimum position for catalysis (10, 11); and it has recently been proposed that it may also modulate molybdenum reduction potentials and hence reactivity via alternate oxidation states (e.g. dihydro- versus tetrahydro- oxidation states) (8, 12–16). Evolutionary analyses have established the presence of the DMSOR protein-fold in the “last universal common ancestor” from which all extant species evolved (17, 18). An important consequence of this is that the fold has evolved to support catalytic functions encompassing those necessary prior to and after the evolution of oxygenic photosynthetic water oxidation (19). This process likely encompassed substitutions of the protein-metal ligands, modulation of the metal coordination sphere via the bis-dithiolene chelate, and modification of the pyranopterin coordination environment. The DMSOR family is able to support a broad range of redox transitions including the following: oxidation of formate, arsenite, ethylenzene, and dimethyl sulfide; and the reduction of polysulfide, nitrate, chlo-

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1 To whom correspondence should be addressed: Membrane Protein Disease Research Group, Dept. of Biochemistry, 474 Medical Sciences Building, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-2761; Fax: 780-492-0886; E-mail: joel.weiner@ualberta.ca.

2 The abbreviations used are: SUOX, sulfite oxidase; DmsABC, E. coli Mo-bisPGD reductase; DMSOR, Mo-bisPGD reductase; EbdABC, A. aromaticum ethylenzene dehydrogenase; Mo-bisPGD, molybdo-bis(pyranopterin guanine dinucleotide); NarGHI, E. coli nitrate reductase A; XDH, xanthine dehydrogenase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; $E_m$ midpoint potential.
rate, trimethylamine-N-oxide, and dimethyl sulfoxide (1, 6–8). A plausible hypothesis explaining the presence of the second pyranopterin in the DMSOR family is that it adds additional facets of fine tuning to facilitate reactivity toward the observed broad range of substrates. The pyranopterin coordination environments may play a critical role in modulating dithiolene chelate electrostatics via differences in hydrogen bonding environment, conformation, and oxidation state (8, 12–15, 20, 21). Although much attention has been paid to the role of the immediate metal environment in defining catalysis (10, 22–24), little is known about the role of the pyranopterin coordination environment.

The molybdenum cofactor found in the DMSOR family is a molybdo-bis(pyranopterin guanine dinucleotide) (Mo-bisPGD) (1, 6–8). The pyranopterins are referred to as proximal and distal based on their positions relative to a conserved [4Fe-4S] cluster, known as F50, which is present in almost every member of the DMSOR enzyme family. In enzymes for which high-resolution structures are available, the proximal pyranopterin has a more distorted conformation than that of the distal pyranopterin, consistent with the former being in a fully reduced tetrahydro-form; and with the latter having accessibility to the partially oxidized 10,10a-dihydro form (15). In two members of the family, *Escherichia coli* respiratory nitrate reductase (NarGHI) (25) and *Aromatoleum aromaticum* ethylbenzene dehydrogenase (EbdABC) (26), high-resolution protein structure reveals that the distal pyranopterin has a bicyclic structure with an open pyran ring. These observations raise the question of the role of the alternate open pyran ring structure in defining active site redox chemistry and catalysis. In the case of NarGHI, protein crystallography reveals that the open pyran ring oxygen participates in hydrogen bonding interactions with two conserved residues in NarG: NarG-His1163 and NarG-Ser719 (3) (Fig. 1A). The distances between the Ser719 OG proton and the pyran oxygen and between the His1163 NE2 proton and the pyran oxygen are both ~2 Å. Closer inspection reveals the presence of a second conserved His residue (NarG-His1184) that completes a charge-transfer relay connecting the pyran oxygen with three structurally conserved water molecules at its distal end (Fig. 1). It would therefore be of interest to generate variants of NarG-His1192 and His1198 and determine their effects on molybdenum electrochemistry and catalysis.

We recently examined the coordination environment of the pyranopterin piperazine nitrogens in members of the DMSOR family for which high-resolution structures are available (8, 15), revealing that all members of the family contain a His or Arg residue that bridges the two pyran N-5 atoms. With the exception of the *Thermus thermophilus* polysulfide reductase (27), all DMSOR-fold enzyme structures obtained to date contain an additional residue (a His/Ser/Gln) that stabilizes the N-5 of the proximal pyranopterin in an sp² hybridized state. Fig. 2A illustrates pyranopterin nitrogen coordination of the NarGHI pyranopterins. The N-10 atoms of both piperazines are hydrogen-bond donors to backbone amide oxygens (of NarG-Thr559 and NarG-Ser720). The N-5 atoms are bridged by the imidazole of NarG-His1092, with the N-5 atom of the proximal pyranopterin participating in an additional H-bond with the NE2 nitrogen of NarG-His1098. These two residues are referred to as the “bridging” and “stabilizing” histidines, respectively, and their H-bonding contacts are summarized in Fig. 2B. That almost all of the proximal N-5 atoms in the DMSOR family have two hydrogen bonding contacts favors a tetrahydro oxidation state for the proximal pyranopterin. The single H-bonding contact observed for the N-5 atoms of all the distal pyranopterins provides further support for the suggestion that these have accessibility to the 10,10a-dihydro oxidation state (8, 15). In some members of the DMSOR family (e.g. *E. coli* formate dehydrogenase N (FdnGHI (28)) and the periplasmic nitrate reductase from *Cupriavidus necator* (NapAB (29))), the bridging ligand is an Arg residue. It would therefore be of interest to generate variants of His1092 and His1098 and determine their effects on molybdenum electrochemistry and catalysis.

In this paper, we tested the hypothesis that the pyranopterins of the DMSOR family of molybdoenzymes have a non-innocent role in defining molybdenum electrochemistry and catalysis. If the role of the pyranopterin dithiolene chelates is merely to provide further support for the suggestion that these have accessibility to the 10,10a-dihydro oxidation state (8, 15). In some members of the DMSOR family (e.g. *E. coli* formate dehydrogenase N (FdnGHI (28)) and the periplasmic nitrate reductase from *Cupriavidus necator* (NapAB (29))), the bridging ligand is an Arg residue. It would therefore be of interest to generate variants of His1092 and His1098 and determine their effects on molybdenum electrochemistry and catalysis.

**Experimental Procedures**

**Bacterial Strains and Plasmids—** *E. coli* LCB79 (araD139 Δ(laclPOZYA-argF) rpsL, thi OT79(nar-lac)) (62) was used as the host for all the experiments described herein. NarGHI was expressed from the plasmid pVA700 (63).

**Site-directed Mutagenesis—** Mutant plasmids were generated using the QuikChange site-directed mutagenesis kit from Stratagene. DpnI was purchased from Invitrogen, and DNA purification kits were purchased from Qiagen. Mutants were verified by DNA sequencing (DNA Core Facility, Department of Biochemistry, University of Alberta). Preparation of competent cells and their transformations with plasmids were carried out as previously described (30).

**Generation of a NarG-S719A Variant—** A 4.5-kbp SacI-AvaI fragment of pVA700 was subcloned into pTZ18R and the resultant plasmid was used as the host for the site-directed mutagenesis. A 3.3-kbp SacI-NcoI fragment was cloned back into the pVA700 expression vector, creating pVA700/S719A.

**Generation of a NarG-H1092A Variant—** A 4.6-kbp EcoRI-SacII fragment of pVA700 was subcloned into pBlueScript to generate a template for site-directed mutagenesis. A 2.1-kbp Ncol-SacII fragment was cloned back into the pVA700, creating pVA700/H1092A or pVA700/H1184A.

**Generation of NarG-H1092A, NarG-H1092R, and NarG-H1098A Variants—** The EcoRI-SacII fragment cloned into pBlueScript was used as a site-directed mutagenesis template, after which a 1.2-kbp Ncol/BstBI fragment was cloned back

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3 The bicyclic form is therefore formally a pterin rather than a pyranopterin. However, for the sake of brevity, it is referred to as a pyranopterin herein.
into the pVA700, creating pVA700/H1092A, pVA700/H1092R, or pVA700/H1098A.

Growth of Cells—Cells were grown microaerobically in 2-liter batch cultures of Terrific Broth (30) at 30 °C in the presence of 100 μg ml⁻¹ streptomycin and ampicillin. A 10% inoculum of a stationary phase culture was used and NarGHI overexpression was induced by addition of 0.2 mM isopropyl 1-thio-β-D-galactopyranoside. Following addition of the inoculum and isopropyl 1-thio-β-D-galactopyranoside, cells were grown overnight with gentle shaking, and were harvested by centrifugation, and subsequently washed in a buffer containing 100 mM MOPS and 5 mM EDTA (pH 7.0). Cells were resuspended in buffer, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.2 mM. Cell lysis was achieved by three passages through an Emulsiflex C3 microfluidizer (Avestin) at a pressure of 17,000 p.s.i. Crude membranes were prepared by differential centrifugation as previously described (31). These were resuspended in buffer and layered on top of a 55% (w/v) sucrose step (made up in buffer). Following ultracentrifugation at 40,000 rpm for 1.5 h, the floating band enriched in cytoplasmic membrane vesicles was collected. Excess sucrose was removed by two dilution and ultracentrifugation steps. The final membrane pellet was resuspended in buffer to a concentration of ~30 mg ml⁻¹, and flash frozen in liquid nitrogen prior to being stored at −70 °C until use.

Bacterial Growth on Glycerol-Nitrate Minimal Medium—Anaerobic growth of E. coli harboring LCB79/pVA700 and mutant derivatives was assessed in a glycerol-nitrate minimal medium essentially as previously described (33, 34). Nitrate was added (as KNO₃) to a final concentration of 40 mM. Growth was evaluated at 37 °C, and culture turbidity was measured using a Klett-Summerson spectrophotometer equipped with a number 66 filter. Maximal growth rates were calculated as described by Zwietering et al. (35), and were expressed as the parameter μₘ in units of (Klett units) h⁻¹.

Form A Fluorescence of the Pyranopterin Cofactor—Pyranopterin was assayed by generation of its Form A fluorescent derivative following protein denaturation (36, 37), essentially as previously described (33). As starting material, 10 mg of membrane protein was used.

Redox Potentiometry and EPR Spectroscopy—Redox titrations were carried out under argon at 25 °C as previously described (38, 39) in 100 mM Tricine and 5 mM EDTA (pH 8.0). The protein concentration used was ~30 mg ml⁻¹. The following redox mediators were used at a concentration of 25 μM: quinhydrone, 2,6-dichloroindophenol, 1,2-naphthoquinone, tolune blue, phenazine methosulfate, thionine, methylene blue, resorufin, indigotrisulfonate, indigocarmine, anthraquinone-2-sulfonic acid, phenosafranine, and neutral red. All samples were prepared in quartz EPR tubes with a 3-mm internal diameter, and were rapidly frozen in liquid nitrogen-chilled ethanol and stored at 77 K until use. EPR spectra were recorded at 150 K using a Bruker Elexsys E500 spectrometer equipped...
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Results and Discussion

Residues Targeted for Site-directed Mutagenesis—During nitrate reduction, the molybdenum atom of the Mo-bisPGD cycles through the IV, V, and VI oxidation states to catalyze the two-electron reduction of nitrate to nitrite (45). To investigate the effect of pyranopterin coordination on molybdenum electrochemistry, we generated variants of two sets of pyranopterin-coordinating residues in NarG: (i) those interacting directly or indirectly with the oxygen of the open pyran ring of the distal pyranopterin (NarG-Ser719, NarG-His1163, and NarG-His1184; Fig. 1); and (ii) those involved in bridging the proximal and distal pyranopterins and stabilizing the oxidation state of the proximal pyranopterin (NarG-His1092 and NarG-His1098; Fig. 2). As judged by SDS-PAGE and Form A pyranopterin assays, each variant was expressed in a Mo-bisPGD-containing form at levels comparable with that of the wild-type. We used these variants to test the hypothesis that residues coordinating the pyranopterin moiety of the Mo-bisPGD cofactor play a critical role in defining molybdenum electrochemistry and substrate reactivity.

Impact of the Variants on the NarG Mo(V) EPR Spectrum—Of the three accessible molybdenum oxidation states, only the intermediate Mo(V) state is paramagnetic and EPR visible. It can be observed in spectra recorded of samples poised at appropriate reduction potentials. Because the NarGHI Mo(V) signal is sensitive to pH in an anion-dependent way (46, 47), we recorded spectra of samples poised at pH 8.0, wherein the so-called “high pH” form is dominant. This form exhibits a simple rhombic EPR spectrum with an absence of resolved hyperfine (1H) couplings (46), simplifying subsequent analyses of potentiometric data (see below).

Fig. 3A shows spectra of redox-poised samples containing the wild-type enzyme and variants of residues implicated in coordinating the open pyran oxygen of the distal pyranopterin (S719A, H1163A, and H1184A). These spectra are similar to those previously observed for the Mo(V) species of NarGHI (46, 47), wherein a rhombic Mo(V) species is observed with $g_{1.2.3}$ values of ~1.988, 1.982, and 1.962. As was the case in previous studies, features consistent with the presence of a small amount of the “low pH” form of the Mo(V) are observed immediately downfield from the high-pH $g_1$ (46, 47), but these are inconsistent in surveys of samples studied herein. Also in agreement with previous work (46–48), no hyperfine coupling was resolved with the isotopes of molybdenum having a nuclear spin of 5/2 ($^{95}$Mo and $^{97}$Mo, with natural abundances of ~16 and 10%, respectively). These data indicate that the S719A, H1163A, and H1184A variants do not significantly impact the molybdenum coordination sphere.
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**Fig. 3.** Mo(V) EPR spectra of redox-poised NarGHI variants of residues involved in pyranopterin coordination. A, Ala variants of NarG-Ser719, NarG-His1163, and NarG-His1184; and B, Ala variants of His1092 and His1098, and an Arg variant of His1098. Spectra were recorded at 150 K using a microwave power of 20 milliwatts (at a frequency of −9.332 GHz), a modulation amplitude of 4 Gpp at a frequency of 100 kHz. Approximate g<sub>1</sub>,<sub>2</sub>,<sub>3</sub> values and potentials at which the samples are poised are: 1.988, 1.982, 1.962 (wild-type, Em<sub>stab</sub> = 146 mV); 1.992, 1.985, 1.964 (H1163A, Em<sub>stab</sub> = 48 mV); 1.986, 1.980, 1.962 (H1184A, Em<sub>stab</sub> = 103 mV); 1.978, 1.962, 1.945 (H1092A, Em<sub>stab</sub> = −5 mV); 1.989, 1.984, 1.965 (His1092Arg, Em<sub>stab</sub> = 146 mV); 1.992, 1.985, 1.951 (H1098A, Em<sub>stab</sub> = 28 mV).

Fig. 3B shows spectra of redox-poised samples of variants of residues implicated in bridging the two pyranopterins (H1092A and H1092R), or stabilizing the proximal pyranopterin in its tetrahydro form (H1098A). Of these, the H1092A variant has the most dramatic effect on the Mo(V) EPR spectrum, shifting g<sub>2</sub> upfield from approximately g = 1.982 (wild-type) to approximately g = 1.962 (H1092A). The H1092R variant exhibits an EPR spectrum wherein heterogeneity is observed, especially in the g<sub>3</sub> feature. Finally, the H1098A variant exhibits a spectrum with clearly defined g<sub>1</sub>,g<sub>2</sub>,g<sub>3</sub> values, with greater rhombicity than that of the wild-type. Overall, Mo(V) species are clearly visible in all of the NarGHI variants studied herein, with the H1092A variant having the greatest effect on the Mo(V) EPR spectrum (see below).

**Influence of the NarGHI Variants on Molybdenum Electrochemistry**—Molybdenum redox cycling is defined by two reduction potentials, E<sub>1</sub> and E<sub>2</sub>, corresponding to the Mo(VI/V) and Mo(V/IV) reduction potentials, with the average of these being defined as the overall reduction potential (E<sub>m</sub>). The difference between E<sub>1</sub> and E<sub>2</sub> is a reflection of the stability of the intermediate Mo(V) species, and can be used to calculate its stability constant (K<sub>stab</sub>) (41). We performed potentiometric titrations on all the variants of NarGHI studied herein, and in each case titrations were carried out on samples generated from 3 to 4 independent biological replicates. Fig. 4A shows representative titrations for the variants of residues interacting with the O-1 pyran oxygen (Fig. 1). Fig. 4B shows plots of the residuals between the experimental data and the fits. For the wild-type enzyme, we estimate values of E<sub>m</sub> and K<sub>stab</sub> of 142 mV and 28, respectively (summarized in Fig. 4C). These values are in reasonable agreement with those previously obtained (38, 49−51). The S719A variant has a statistically insignificant effect on the E<sub>m</sub> (ΔE<sub>m</sub> = 4 mV, p = 0.57) and decreases the K<sub>stab</sub> from 28 to 8, whereas the H1163A variant elicits a large decrease in the E<sub>m</sub> (ΔE<sub>m</sub> = −88 mV, Table 1) and a concomitant decrease in the K<sub>stab</sub> from 28 to 1. The H1184A variant has an intermediate effect, eliciting a ΔE<sub>m</sub> of −36 mV, and decreases the K<sub>stab</sub> again from 28 to 1. Thus, the charge-transfer relay depicted in Fig. 1B appears to play two critical roles: (i) modulating the molybdenum reduction potentials (see below for further discussion of this); and (ii) stabilizing the Mo(V) intermediate.

Fig. 5A shows representative potentiometric titrations of the H1092A, H1092R, and H1098A variants. The H1092R variant replaces the bridging His residue with an Arg, and mimics the pyranopterin-coordination environment observed in the formate dehydrogenases and periplasmic nitrate reductases (e.g. FdnGHI and NapAB, respectively (8)). The H1092R variant elicits a modest increase of marginal statistical significance in the overall molybdenum E<sub>m</sub> (ΔE<sub>m</sub> = 18 mV, p = 0.07; Table 1), consistent with there being no change in overall charge of the bridging residue when it is changed from a His to an Arg (the pK<sub>a</sub> of the Arg guanidinium group is ~12.5). This suggests that the imidazole pK<sub>a</sub> is significantly increased from ~6.0 in aqueous solution to >8.0 in the bridging environment between the two pyranopterins. The H1092A variant elicits a ΔE<sub>m</sub> on the molybdenum center of approximately −143 mV along with a
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modest decrease in its $K_{stab}$ (from 28 to 19, Table 1). A possible explanation for the large negative $\Delta E_m$ is that the void between the two pyranopterins contains waters in the H1092A variant, and that these waters lack an overall positive charge, thus decreasing the overall molybdenum reduction potential. Alternatively, given that the H1092A variant has the most severe effect on the Mo(V) EPR spectrum, loss of “scaffolding” by the bridging imidazole moiety may result in a significant alteration of the geometry of dithiolene coordination that may cause the large observed $\Delta E_m$.

The H1098A variant elicits a $\Delta E_m$ of $-101$ mV, along with a significant increase in Mo(V) stability, with the $K_{stab}$ increasing from 28 to 1822 (Fig. 5, A and C, Table 1). Fig. 2B shows a model for how His$^{1092}$ and His$^{1098}$ define the H-bonding environment of the proximal pyranopterin piperazine N-5 atom. As suggested above, formal protonation of the imidazole of His$^{1092}$ is supported by the relative lack of effect of the H1092R variant on the overall molybdenum $E_m$. One of the H-bonds is eliminated in the H1098A variant, which may result in transfer of the proton from the ND1 atom to the piperazine N-5, resulting in loss of a positive charge and the observed decrease in the overall molybdenum $E_m$.

We proposed that in the DMSOR family of enzymes, the role of the stabilizing residue (His$^{1098}$ in NarG) is to maintain the N-5 atom of the proximal pyranopterin in an $sp^3$-hybridized form, thus stabilizing the tetrahydro oxidation state (8, 15). This suggests a possible explanation for the observed increase in Mo(V) stability in the H1098A variant. Removal of the hydrogen-bonding contact provided by the His$^{1098}$ imidazole may result in the 10,10a-dihydro oxidation state becoming available to the proximal pyranopterin. If the 10,10a-dihydro to tetrahydro reduction potential occurs within the range in which Mo(V) is extant, then $E_p$ (the Mo(VI/V) reduction potential) could be shifted to the observed value of 139 mV (Table 1; see Ref. 15).

Correlation between Enzyme Activity, Cell Growth, and Molybdenum Electrochemistry—How do changes in molybdenum electrochemistry impact enzyme activity and anaerobic respiratory growth on nitrate? Specific activities in enriched membranes for the variants studied herein range from 1 to 54 $\mu$mol min$^{-1}$ mg$^{-1}$ for the H1092A and S719A variants, respectively (Table 1). With the exception of the H1092R variant, a strong correlation exists between overall molybdenum reduction potential and enzyme activity ($r = 0.99$, Fig. 6). A correlation also exists between respiratory growth rate and molybdenum reduction potential across all variants ($r = 0.95$, inset to Fig. 6). Thus, catalysis and growth are convincingly correlated with increased molybdenum $E_m$. A possible explanation for this is that catalytic turnover depends on the energetics of electron transfer from FS0 ($E_m = -55$ mV (49)) to the molybdenum.

It is notable that the NarGHI catalytic activity appears to be dependent on the overall molybdenum $E_m$, but not on the Mo(V) $K_{stab}$ (Table 1). Protein film voltammetry studies of NarGHI (45) and two other members of the DMSOR family (the periplasmic nitrate reductase from Rhodobacter sphaeroides, NapAB (52), and the Me$_2$SO reductase from E. coli (53)) demonstrated that enzyme turnover decreases below a reduction potential referred to as $E_{switch}$. It was therefore proposed that observation of an $E_{switch}$ arises because substrate binds preferentially to the Mo(V) form of the cofactor (45, 52, 53). In the case of NarGHI, this is corroborated by the observation of a stable nitrate adduct to the Mo(V) form of the cofactor (45, 52, 53). As previously noted (15), an alternative explanation for the observed $E_{switch}$ is that it controls a reduction of the distal pyranopterin from an oxidation state equivalent to the 10,10a-dihydro form to one equivalent to the tetrahydro form. This provides a possible explanation for why the H1092R variant supports respiratory growth on nitrate, but has decreased enzyme activity in our in vitro assay (Fig. 6). It is possible that a significant positive $\Delta E_m$ is elicited on the $E_{switch}$, resulting in...
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**TABLE 1**

| Variant      | $E_{m}^{a}$ | $\Delta E_{m}^{b}$ | $E_{4}^{c}$ | $E_{2}^{d}$ | $K_{stab}$ | Maximal growth rate | BV$^{-}$:NO$_{3}^{-}$ activity |
|--------------|-------------|--------------------|-------------|-------------|------------|--------------------|-------------------------------|
| Wild-type    | 142 ± 12 (n = 4) | 0 | 184 ± 10 | 99 ± 16 | 28 | 15.4 ± 0.7 (n = 3) | 48 ± 14 (n = 3) |
| S719A        | 146 ± 7 (n = 3) | 4 | 170 ± 19 | 122 ± 7 | 8 | 15.5 ± 1.0 (n = 3) | 54 ± 11 (n = 3) |
| H1163A       | 54 ± 3 (n = 4) | −88 | 53 ± 5 | 54 ± 9 | 1 | 6 ± 0.2 (n = 3) | 23 ± 4 (n = 3) |
| H1184A       | 106 ± 5 (n = 3) | −36 | 106 ± 14 | 106 ± 5 | 1 | 11.4 ± 0.6 (n = 3) | 36 ± 1 (n = 3) |
| H1092A       | −1 ± 3 (n = 3) | −143 | 35 ± 9 | −38 ± 11 | 19 | 4.4 ± 0.5 (n = 2) | 1 ± 0 (n = 3) |
| H1092R       | 160 ± 7 (n = 3) | 18 | 225 ± 15 | 95 ± 2 | 171 | 12.8 ± 0.4 (n = 2) | 8.6 ± 1 (n = 3) |
| H1098A       | 41 ± 4 (n = 4) | −101 | 139 ± 7 | −57 ± 2 | 1822 | 4.8 ± 0.5 (n = 2) | 16 ± 2 (n = 3) |

$^{a}$ Overall Mo(VI/V/IV) reduction potential at pH 8.0.

$^{b}$ $E_{m}$ (variant) minus $E_{m}$ (wild-type). Where appropriate, $p$ values indicating the statistical significance of each $\Delta E_{m}$ are given in the text.

$^{c}$ Mo(V/IV) reduction potential.

$^{d}$ Mo(V/IV) reduction potential.

**FIGURE 5.** Potentiometric titrations of membranes containing variants of His$^{1092}$ and His$^{1098}$. A, comparison of representative titrations of wild-type (■), H1092R (▲), H1098A (●), and H1092A (●) enzymes. Data were fit to the following parameters: wild-type, $E_{m} = 140$ mV ($E_{1} = 183$ mV, $E_{2} = 97$ mV); H1092R, $E_{m} = 155$ mV ($E_{1} = 213$ mV, $E_{2} = 97$ mV); H1098A, $E_{m} = 35$ mV ($E_{1} = 129$ mV, $E_{2} = 60$ mV); H1092A, $E_{m} = −2$ mV ($E_{1} = 24$ mV, $E_{2} = −29$ mV). B, plot of residuals. C, summary of $E_{m}$, $E_{1}$, and $E_{2}$ values for the variants based on the data presented in Table 1. Colored squares indicate the mean $E_{m}$ values and the horizontal lines indicate the $E_{1}$ and $E_{2}$ values.

lower activity measured using benzyl viologen as electron donor ($E_{m} = −374$ mV (54)). NarGHI is able to function in vivo with either ubiquinol or menaquinol as electron donor (55), and these have reduction potentials of +110 and −80 mV, respectively (2), rendering them both less likely to reduce the distal pyranopterin than the artificial electron donor benzyl viologen.

**FIGURE 6.** Correlation between enzyme activity and overall molybdenum reduction potential. Excluding the NarG-H1092R variant, a correlation exists between enzyme activity and overall molybdenum reduction potential ($r = 0.99$). Inset, correlation between growth rate and overall molybdenum reduction potential. Including the NarG-H1092R variant, a correlation exists between the maximal rate of respiratory growth on nitrate and overall molybdenum reduction potential ($r = 0.95$). Growth rates were calculated using the method of Zwietering et al. (35).

**Role of the His$^{1163}$/His$^{1184}$ Charge-transfer Relay in NarGHI Maturation and in Modulation of Molybdenum Electrochemistry**—NarGHI maturation is a carefully orchestrated process involving the NarJ system-specific chaperone (56) and the twin arginine translocase (57). The process ensures assembly of correctly folded enzyme with a complete complement of iron-sulfur clusters and hemes (56, 58). Insertion of the Mo-bisPGD cofactor depends on the presence of the FS0 [4Fe-4S] cluster (56, 58). With the exception of EbdABC (26), NarGHI is the only enzyme examined to date that has a bicyclic distal pyranopterin (25). Closer examination of the charge-transfer relay comprising His$^{1163}$ and His$^{1184}$ (Fig. 1) suggests a possible mechanism for pyran ring opening (Fig. 7), which proceeds as follows. The His$^{1163}$/His$^{1184}$ charge-transfer relay catalyzes elimination of the C-4a proton (Fig. 7A), generating an open ring-form with a piperazine ring in a form equivalent to the 5,10-dihydro form (Fig. 7B) in a mechanism essentially identi-
This form rearranges to the lowest energy tautomer (equivalent to the 10,10a-dihydro form (15, 59)) shown in Fig. 7C. A mechanism essentially identical to that presented in Fig. 7, A–C, can be proposed for ring-opening of the distal pyranopterin in EbdABC, with the distinction that ring opening would be catalyzed by a guanidinium side chain rather than by an imidazole. The equilibrium between the structures shown in Fig. 7, C and D, illustrates how the formal change on the pyran oxygen (and the overall molybdenum reduction potential) can be modulated by the His1163/His1184 charge-transfer relay. Stabilization of a the pyran oxygen in its alkoxide form would decrease the overall molybdenum reduction potential, whereas formal protonation to the hydroxyl form would increase the overall molybdenum reduction potential.

In the context of the model presented in Fig. 7, C and D, for charge-transfer-induced molybdenum reduction potential modulation, it is notable that the H1163A variant elicits a large \( \Delta E_{\text{mo}} \) that is approximately double that elicited by the H1184A variant. This is consistent with the entire charge-transfer relay being involved in molybdenum reduction potential modulation. It is possible to speculate that the H1163A variant may impact an equilibrium between the open and closed pyran ring forms of the distal pyranopterin, and we are currently addressing this issue using protein crystallography.

**Conclusions**—We have demonstrated the importance of the pyranopterin coordination environment in defining molybdenum electrochemistry and substrate reactivity, with enzyme activity correlating with increasing overall molybdenum reduction potential. Our studies demonstrate the importance of factors beyond the immediate molybdenum coordination sphere in defining enzyme activity.

**Author Contributions**—S. W. designed, performed, and analyzed the experiments presented in the manuscript. R. A. R. wrote the paper and contributed to the data analyses. J. H. W. conceived and coordinated the study.

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