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Insights into the Mechanism of N_2O Reduction by Reductively Activated N_2O Reductase from Kinetics and Spectroscopic Studies of pH Effects

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Nitrous oxide reductase (N_2OR) from *Achromobacter cycloclastes* (*Ac*) can be reductively activated with reduced methyl viologen over a broad range of pH. Activated *Ac* N_2OR displays a complex activity profile as a function of the pH at which catalytic turnover is measured. Spectroscopic and steady-state kinetics data suggest that [H^+] has multiple effects on both the activation and the catalytic reactions. These experimental results are in good agreement with previous theoretical studies, which suggested that the transition state is stabilized by H-bonding interactions between the active site and an N_2O-derived intermediate bound to the catalytic Cu_Z cluster (*J. Am. Chem. Soc.* **2006**, *128*, 278–290).

Denitrification is the reductive cascade from nitrate (NO_3^-) → nitrite (NO_2^-) → nitric oxide (NO) → nitrous oxide (N_2O) → dinitrogen (N_2) and is an intrinsic part of the global nitrogen cycle.[1−3] Moreover, N_2O is kinetically inert and 300 times more potent as a greenhouse gas than carbon dioxide. Thus, it is important to achieve a comprehensive understanding of N_2O metabolism in the biosphere. N_2O reductase (N_2-OR) catalyzes the two-electron reduction of N_2O to N_2 and H_2O, which is the terminal step of denitrification.[4] Crystallographic studies on N_2ORs from *Pseudomonas nautica*[5,6] and *Paracoccus denitrificans* (*Pd*)[6,7] revealed that the enzyme contains two copper centers, designated Cu_A and Cu_Z. The dinuclear Cu_A site in N_2OR closely resembles Cu_A in cytochrome c oxidase (CcO) and is implicated in electron transfer to the catalytic site, Cu_Z.[8,9] Cu_Z is a unique structural motif consisting of μ_4-sulfide bridged tetranuclear Cu cluster.[6,10,11] Although Cu_Z undoubtedly functions as the catalytic center, the mechanism of N_2O reduction is not well understood.[12−16] Recently, we demonstrated that N_2OR is significantly activated by reduced methyl viologen (MV).[17] Our results and those of Solomon and co-workers indicated that the catalytically active state of Cu_Z in N_2OR is the fully reduced 4[Cu(I)] form.[18] Herein, we present the novel pH dependences of both the activation process and catalysis by activated *Achromobacter cycloclastes* (*Ac*) N_2OR, which indicate that the enzyme may utilize multiple mechanisms for N_2O reduction. These data also provide valuable new constraints for evaluating previously proposed mechanisms.

Figure 1 depicts three representative pH profiles for catalytic turnover by *Ac* N_2OR following activation with reduced MV at pH 5.7, 7.1, and 9.4, as well as the three-dimensional profile of specific activity as functions of the pH of activation (pH_{act}) and the pH of measurement for turnover (pH_t).[19] *Remarkably, the shape of the pH profile for turnover depends upon the pH at which the enzyme is activated.* Whereas the shape of the pH profile following activation at pH 5.7 is approximately similar to that at pH 7.1, the magnitudes of the specific activities, the positions of the maxima, and the pH range over which the activity is at least 50% of the maximum are different: the profile for

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(19) The redox potentials of methyl and benzyl viologens are largely independent of pH over the ranges employed (ref 27).

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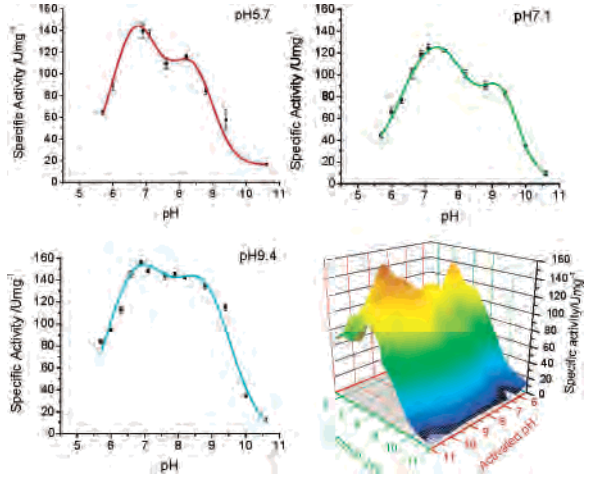

Figure 1. pH dependences of reductively activated *Ac* N_2OR with methyl viologen at pH 5.7 (A, top left), pH 7.1 (B, top right), pH 9.4 (C, bottom left), and the whole pH range surface (D, bottom right). Specific activity measurements of *Ac* N_2OR were monitored by the decrease in absorbance at 600 nm during N_2O reduction. N_2OR (1 mg/mL) was activated at 25 °C for 180 min under an Ar atmosphere.

$pH_{act} = 5.7$ displays a maximum (139.8 ± 7.1 U/mg) at pH 6.9 and a secondary shoulder at pH 8.2; the profile for $pH_{act} = 7.1$ displays a maximum (124.0 ± 3.5 U/mg) at pH 7.1 and a secondary shoulder at pH 8.8. Moreover, the shape of the $pH_{act} = 9.4$ profile is significantly altered, and the specific activities are relatively higher than those for activation at acidic and neutral conditions: for $pH_{act} = 9.4$, an activity maximum (145.6 ± 1.8 U/mg) is observed at pH 7.9, with a secondary shoulder at pH 8.2. The pH profiles in Figure 1 can be deconvoluted into two "bell-shaped" curves characterized by four apparent pK_a's: for activation at pH 7.1, these are 6.3, 8.4, 8.8, and 9.8 (see Supporting Information for a complete compilation). Collectively, our measurements of N_2OR activity as a function of both pH_{act} and the pH_t (represented as a 3D profile in Figure 1D) indicate that $[H^+]$ has multiple effects on the reaction and that its effects on activation and N_2O reduction are distinct. The widths of the pH_t profiles and their dependence on pH_{act} suggest that a requirement for N_2OR to function as an effective catalyst under a variety of environmental conditions may have been an evolutionary constraint.

DFT calculations suggested that the charge on coordinated N_2O in the transition state for reduction is higher than the reactant state; consequently, H-bonding interactions that stabilize the increased charge may lower the activation energy for N−O bond cleavage.[20] These calculations further indicated that the transition state stabilization correlates with the acidity of the proton donor and that the activation energy can fall to zero in the limit of tight coupling between N−O bond cleavage and H^+ transfer. Gorelsky et al. concluded that at $4 \leq pH \leq 8$ the rate-limiting step is likely to be electron transfer to Cu_Z, rather than N−O bond cleavage.[20] Our data are broadly consistent with such considerations: the complexity of the pH profiles could reflect pH-dependent changes in the rate-limiting step and the differential efficacies

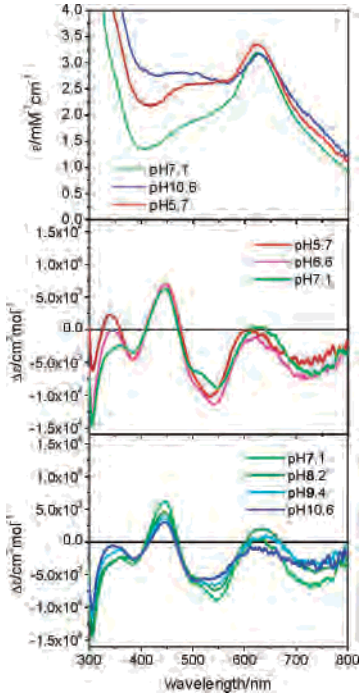

Figure 2. Absorption spectra (top) and circular dichroism spectra (middle and bottom) of resting-state *Ac* N_2OR under several pH conditions and Ar atmosphere. All spectra were normalized. pH conditions were maintained with 50 mM MES (pH 5.7), MOPSO (pH 6.6), phosphate (pH 7.1), TAPS (pH 8.2), CHES (pH 9.4), and CAPS (pH 10.6).

of multiple proton donors. For example, the apparent pK_a's at ~8 could reflect the ionization of coordinated solvent (where the pK_a may depend on the coordination structure). The decrease in activity correlated with $pK_a \approx 9.8$ is consistent with ionization of an active site lysine, implicated in the DFT calculations as involved in transition state stabilization.[20]

Additional evidence for multiple pH effects is provided by electronic and circular dichroism (CD) spectra of the resting state of *Ac* N_2OR, where the Cu_Z center has a [3Cu(I)/Cu(II)] configuration, and Cu_A is oxidized [Cu(I)/Cu(II)].[21,22] Figure 2 shows absorption and CD spectra at various pH values. On the basis of the current assignments, the spectra indicate that both Cu_A and Cu_Z are affected by changes in pH. Specifically, variations in electronic transitions in the 340−430 nm range (apparent in both the absorption and the CD spectra) reflect perturbation of the Cu_A center.[11,24] Perturbation of the Cu_Z site is more evident in the CD, where the band at 447 nm (from an imidazole → Cu(II) charge-transfer transition in Cu_Z) is pH dependent only under basic conditions.[22] Although the 546 nm band is also dependent on pH, this feature is composed of a d−d transition in the Cu_Z and a thiolato → Cu(II) charge transfer in Cu_A.[22] Therefore, these data suggest that the Cu_A site is

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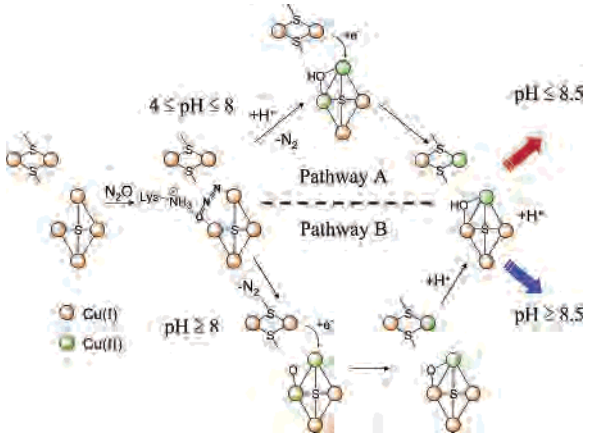
broadly affected by pH but that pH-related perturbation of the Cu_Z site occurs predominately under basic pH conditions.

Given the core Cu_2S_2 structure of Cu_A, the magnitude of the electronic effects induced by pH variations is somewhat surprising. Our hypothesis is that pH variations perturb the active-site conformation around the Cu_A center and these structural changes influence or modulate the reactivity of the Cu_A site in inter- and intramolecular electron transfer. Structural data reinforce this hypothesis because the Cu_A center is located close to the surface of N_2OR and is solvent accessible.[5−7] Further, similar pH effects on the spectroscopic and kinetics properties of the Cu_A domain in *Pd* CcO have been documented.[23,24] In N_2OR, the Cu_A site from one subunit and the Cu_Z site on the other appear to be positioned and structured to facilitate facile intramolecular electron transfer. Thus, conformational changes of the Cu_A center may affect electron transfer from the physiological electron donor and the electron-transfer rate from Cu_A to Cu_Z. Both experimental and theoretical studies have indicated that electron transfer may be rate limiting in N_2O reduction, at least under some conditions.[20,25] Therefore pH effects on electron-transfer could well contribute to the complex pH-dependent behavior of N_2OR.

The data in Figure 1, particularly the dependence of the pH_t profile on pH_{act}, and the requirement of at least four pK_a's to fit the pH_t profiles, indicate that additional factors beyond perturbation of the Cu_A site and electron transfer must be involved. H-bonding to the transition state or H^+ transfer is likely to be affected by pH. Solomon and co-workers have also suggested that protonation of the $[Cu_4S(im)_7OH]^{2+}$ species to $[Cu_4S(im)_7OH_2]^{3+}$ is mechanistically important and have assigned a p$K_a \approx 8.5$ to this ionization.[20,22] Again, we emphasize that the spectra in Figure 2 are consistent with electronic perturbations of the Cu_Z center at basic pH values, which may be correlated to the activation process, that is, the data suggest that the structure of the Cu_Z center is also pH dependent.

Our results clearly indicate that $[H^+]$ influences both the enzymatic reactivity and the activation process of N_2OR. Scheme 1 summarizes selected mechanistic possibilities for H^+ effects on N_2O reduction. During the N_2O-association step, charge is delocalized onto a bent N_2O from the fully reduced Cu_Z site via back-bonding interactions; the increased negative charge on N_2O is stabilized by adjacent positively charged amino acid residues.[20] At $4 \leq pH \leq 8$ (pathway A), proton transfer coupled with N−O bond cleavage could occur, and the subsequent intramolecular electron transfer (ET) would be rate limiting. Above pH 8 (pathway B), proton transfer is less favorable because of the reduced availability of H^+. In this case, since the N−O bond cleavage is still

Scheme 1



energetically feasible,[20] both the ET step and protonation are potentially rate limiting. At basic pH, the second protonation step (the last step in Scheme 1) may affect the rate depending on the pK_a of coordinated hydroxide in the Cu_Z site. In addition, pH-dependent perturbation of the Cu_A site (as suggested by the data in Figure 2) could influence both the activation and the catalytic processes. It is conceivable that dissociation of coordinated solvent from the Cu_Z site influences turnover at basic pH, since recent structural studies have identified H_2O and hydroxide ion coordinated on the edge line of the Cu_Z center.[26] Collectively, multiple $[H^+]$-related effects might well contribute to the complexity of pH dependence for the reaction of reductively activated N_2-OR with substrate and electron donors.

Finally, the present results establish that, despite perturbations of both the Cu_A and Cu_Z sites, N_2OR is an effective catalyst across a broad pH range *and that reductive activation at selected pH values enhances catalysis in that pH range*, which may be physiologically important. The detailed structural changes induced in the Cu_A and Cu_Z sites by pH variations warrant further investigation, as do the specific chemical ionizations that control the pH dependence of this remarkable enzyme.

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Supporting Information Available: Experimental procedures and additional data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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