Reductively Activated Nitrous Oxide Reductase Reacts Directly with Substrate

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Nitrous oxide reductase (N$_2$OR) catalyzes the two-electron reduction of nitrous oxide to dinitrogen and water in the terminal step of dissimilatory denitrification.[1] Recent X-ray crystal structures of the homodimeric enzymes from *Pseudomonas nautica*[2,3] and *Paracoccus denitrificans*[3,4] confirmed the presence of two Cu centers per monomer, the Cu$_A$ site and the Cu$_Z$ site. The closely similar Cu$_A$ sites of N$_2$OR and cytochrome *c* oxidase[5,6] are thought to mediate electron transfer to the active sites. The Cu$_Z$ center has been shown to be a novel μ_4-sulfide[3,7,8] bridged tetranuclear Cu cluster ligated by seven His ligands and is the site of N$_2$O reduction. Notwithstanding several detailed structural and spectral investigations of N$_2$OR, little is understood about the mechanism of action of the enzyme. N$_2$OR can be activated by incubation with reduced methyl or benzyl viologen (MV or BV);[9–13] however, this high-activity form of the enzyme has not been further analyzed. The characterization of reductively activated recombinant N$_2$OR from *Achromobacter cycloclastes* (*Ac*) is reported herein, and for the first time, direct evidence is presented for the reaction of this state with N$_2$O.

The specific activity of anaerobically purified *Ac* N$_2$OR was determined to be 7.89 ± 0.54 U/mg. In contrast, when *Ac* N$_2$OR was preincubated in 2 mM reduced BV, the specific activity increased to 61.7 ± 2.8 U/mg, similar to values reported for activated N$_2$OR from other organisms.[12,13] To evaluate possible effects of the midpoint potential (E_m) of the reductant on enzyme activation, *Ac* N$_2$OR was also incubated with 2 mM reduced MV and with 1 mM dithionite for comparison to activation with BV ($E_m = -360$ mV[14]). Incubation with MV ($E_m = -441$ mV[14]) increased the specific activity to 124 ± 6 U/mg, indicating that a more reducing environment produces higher enzyme activation. With a 1 mM dithionite solution, pH 7.1 ($E_m \approx -470$ mV[15]), however, the *Ac* N$_2$OR specific activity was only 34.4 ± 2.0 U/mg. This is in contrast to early reports that dithionite inactivates *Ac* N$_2$OR,[16] although the extent of activation is clearly lower than expected for a reductant with a potential similar to MV. Charge differences between dithionite and the viologens,[17,18] binding of dithionite or its oxidized products, and bisulfite contamination of the dithionite solution[15] may reduce the efficacy of dithionite activation.

The spectrum of *Ac* N$_2$OR following incubation with reduced MV and subsequent removal of the reductant is shown in Figure 1A, trace a. As suggested previously,[11] the spectrum is similar to dithionite-reduced N$_2$OR (Figure 1A, trace c). When directly compared, the apparent extinction coefficient of MV-reduced *Ac* N$_2$OR is lower. Both reduced spectra appear to have two S → Cu(II) charge-transfer bands centered near 640 and 680 nm that may arise from distinct forms of the enzyme or from multiple electronic transitions within the active site. Further analysis is underway. Upon the addition of N$_2$O-saturated buffer to the reduced, reductant-free enzyme, substantial changes were observed in the electronic absorption spectrum (Figure 1A, trace b). Absorbance
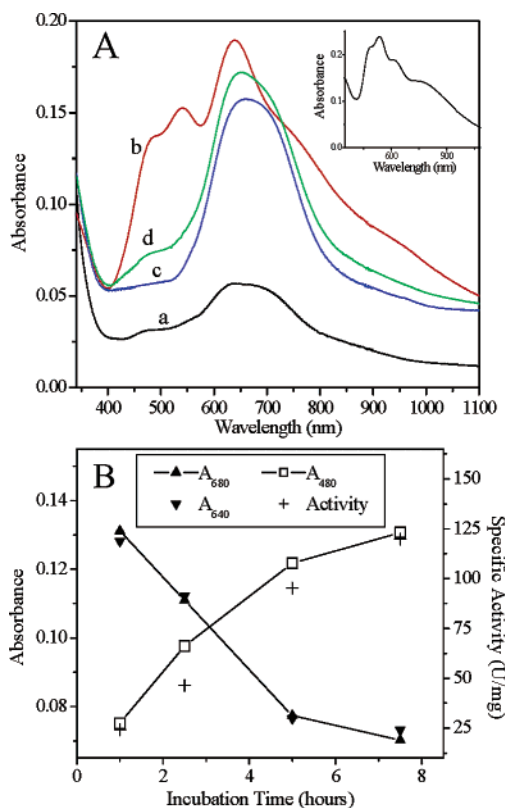

Figure 1. (A) Visible absorption spectra of (a) methyl viologen (MV)-reduced N$_2$OR, (b) MV-reduced N$_2$OR + N$_2$O, (c) dithionite-reduced N$_2$OR, and (d) dithionite-reduced N$_2$OR + N$_2$O. Inset: ferricyanide-oxidized N$_2$OR. All spectra have been normalized to 30 μM *Ac* N$_2$OR. (B) MV incubation time dependence of the absorbance at 640 and 680 nm prior to addition of N$_2$O, the absorbance at 480 nm after addition of N$_2$O, and the specific activity of *Ac* N$_2$OR.

increases at 480, 540, and 790 nm are indicative of one-electron oxidized Cu$_A$,[19] whereas the absorption band at 640 nm is attributed to Cu$_Z$ in a [3Cu(I)1Cu(II)] state.[20,21] In addition, a new broad feature appeared at approximately 970 nm, which does not appear in the spectrum of fully oxidized *Ac* N$_2$OR. Notably, dithionite-reduced *Ac* N$_2$OR, which has a lower specific activity, had significantly smaller changes in its spectrum (Figure 1A, compare traces c and d) when N$_2$O was added.

As shown in Figure 1B, the absorbance at 640 and 680 nm decreased as a function of incubation time with reduced MV, suggesting a time-dependent conversion of the Cu$_Z$ center to a state different from the [3Cu(I)1Cu(II)] state. This decrease in absorbance correlated with both an increase in N$_2$OR specific activity and an increase in the magnitude of the absorption spectral change accompanying the addition of N$_2$O-saturated buffer, as monitored at 480 nm. These results strongly indicate that the reductively activated state reacts with N$_2$O.
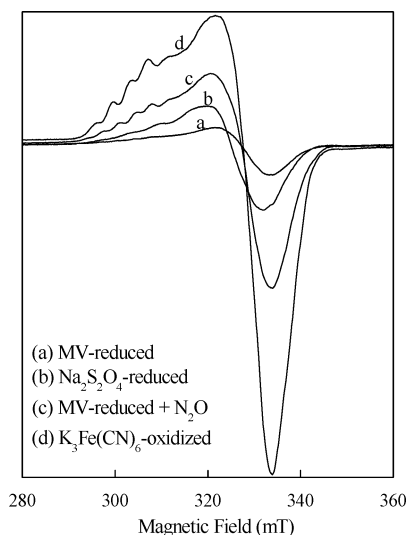
Figure 2. X-band EPR spectra of *Ac* N$_2$OR (80 μM) at 9.37 GHz, 15 K.

X-band EPR spectra of reductively activated N$_2$OR were collected at 15 K (Figure 2). The spectrum of the MV-reduced enzyme was compared to the dithionite-reduced enzyme, and integration of the signals revealed that the spin quantitation of MV-reduced N$_2$OR ($\cong$0.1 spins/monomer) is approximately one-third that of the dithionite-reduced enzyme ($\cong$0.3 spins/monomer). This, taken together with the lower apparent extinction coefficient of MV-reduced N$_2$OR, establishes that the MV-reduced enzyme has a higher proportion of Cu(I) present. Hence, these data argue that the fully reduced, all-Cu(I) state of Cu$_Z$ is the catalytically relevant redox state of N$_2$OR. Upon addition of N$_2$O-saturated buffer to the MV-reduced sample, a distinct spectral change was observed. The line shape of the EPR signal showed that some of the Cu$_A$ sites oxidized to a [Cu(1.5)·Cu(1.5)] redox state,[22] and the spin integration increased to $\cong$0.7 spins/monomer, indicating partial oxidation upon addition of substrate.

Using GC-MS detection of ^{30}N$_2$, we determined that reductively activated N$_2$OR is able to reduce ^{15}N-labeled N$_2$O to N$_2$, in the absence of any chemical reductants. The amount of N$_2$ produced was directly correlated with the specific activity. The as-purified, reductant-free enzyme was not able to reduce N$_2$O. Dithionite-reduced N$_2$OR produced 0.01 N$_2$ per N$_2$OR monomer. In contrast, N$_2$ was detected at a level about 10 times higher for the MV-reduced enzyme with a value of 0.13 N$_2$ per N$_2$OR monomer. The visible and EPR spectroscopy suggest that one electron comes from Cu$_A$ and one from Cu$_Z$. The substoichiometric production of N$_2$ perhaps suggests that interactions with physiological electron donors or coupling of the electron-transfer and chemical reduction steps, or both, may be important in turnover.

In summary, the current studies suggest that the reductively activated *Ac* N$_2$OR Cu$_Z$ center is in a more reduced redox state than that primarily induced by dithionite, which has been assigned as [3Cu(I)1Cu(II)].[20,21] Therefore, the catalytically relevant state of Cu$_Z$ is [4Cu(I)]. This activated form of *Ac* N$_2$OR converted N$_2$O to product and represents the first report of substrate reduction in the absence of steady-state turnover conditions. Similar conclusions have been reached by Ghosh et al. on the basis of studies of N$_2$OR from *P. nautica*.[23] Reduction by dithionite appears to produce significantly less [4Cu(I)] in *Ac* N$_2$OR than MV or BV reduction. As mentioned above, the reaction with dithionite is more complex than that with MV, which may account for differences in reactivity.

A new absorption band was also detected following reaction of activated *Ac* N$_2$OR with N$_2$O, and additional studies will be conducted to determine if the signal arises from an intermediate state of Cu$_Z$. Although reduction by MV is not physiologically relevant, in particular considering that full activation takes several hours, *Ac* N$_2$OR may still reach the [4Cu(I)] state in vivo during the catalytic cycle. Physiological generation of the reductively activated state could require interaction with and electron transfer from the physiological electron donor, which may induce conformational changes within N$_2$OR that facilitate substrate reduction.[24,25] In addition, the MV-reduced state of N$_2$OR was susceptible to oxidation upon removal of the reductant in vitro, implying that this highly reactive state may exist only transiently during catalysis. Future studies of reductively activated N$_2$OR are expected to reveal new insights into the mechanism of N$_2$O reduction at Cu$_Z$.

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

References

(1) Richardson, D. J.; Watmough, N. J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 207.
(2) Brown, K.; Tegoni, M.; Prudencio, M.; Pereira, A. S.; Besson, S.; Moura, J. J.; Moura, I.; Cambillau, C. *Nat. Struct. Biol.* **2000**, *7*, 191.
(3) Brown, K.; Djinovic-Carugo, K.; Haltia, T.; Cabrito, I.; Saraste, M.; Moura, J. J. G.; Moura, I.; Tegoni, M.; Cambillau, C. *J. Biol. Chem.* **2000**, *275*, 41133.
(4) Haltia, T.; Brown, K.; Tegoni, M.; Cambillau, C.; Saraste, M.; Mattila, K.; Djinovic-Carugo, K. *Biochem. J.* **2003**, *369*, 77.
(5) Kroneck, P. M. H.; Antholine, W. A.; Riester, J.; Zumft, W. G. *FEBS Lett.* **1989**, *248*, 212.
(6) Scott, R. A.; Zumft, W. G.; Coyle, C. L.; Dooley, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4082.
(7) Rasmussen, T.; Berks, B. C.; Sanders-Loehr, J.; Dooley, D. M.; Zumft, W. G.; Thomson, A. J. *Biochemistry* **2000**, *39*, 12753.
(8) Alvarez, M. L.; Ai, J. Y.; Zumft, W.; Sanders-Loehr, J.; Dooley, D. M. *J. Am. Chem. Soc.* **2001**, *123*, 576.
(9) Kristjansson, J. K.; Hollocher, T. C. *Curr. Microbiol.* **1981**, *6*, 247.
(10) Snyder, S. W.; Hollocher, T. C. *J. Biol. Chem.* **1987**, *262*, 6515.
(11) Berks, B. C.; Baratta, D.; Richardson, D. J.; Ferguson, S. J. *Eur. J. Biochem.* **1993**, *212*, 467.
(12) Prudêncio, M.; Pereira, A. S.; Tavares, P.; Besson, S.; Cabrito, I.; Brown, K.; Samyn, B.; Devreese, B.; Van Beeumen, J.; Rusnak, F.; Fauque, G.; Moura, J. J. G.; Tegoni, M.; Cambillau, C.; Moura, I. *Biochemistry* **2000**, *39*, 3899.
(13) Sato, K.; Okubo, A.; Yamazaki, S. *J. Biochem. (Tokyo)* **1999**, *125*, 864.
(14) Watt, G. D.; Burns, A. *Biochem. J.* **1975**, *152*, 33.
(15) Mayhew, S. G. *Eur. J. Biochem.* **1978**, *85*, 535.
(16) Hulse, C. L.; Averill, B. A. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 729.
(17) Cheddar, G.; Meyer, T. E.; Cusanovich, M. A.; Stout, C. D.; Tollin, G. *Biochemistry* **1989**, *28*, 6318.
(18) Furukawa, Y.; Ishimori, K.; Morishima, I. *Biochemistry* **2000**, *39*, 10996.
(19) Riester, J.; Zumft, W. G.; Kroneck, P. M. H. *Eur. J. Biochem.* **1989**, *178*, 751.
(20) Chen, P.; DeBeers, G. S.; Cabrito, I.; Antholine, W.; Moura, J. J.; Moura, I.; Hedman, B.; Hodgson, K. O.; Soloman, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 744.
(21) Chen, P.; Cabrito, I.; Moura, J. J. G.; Moura, I.; Solomon, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 10497.
(22) Kroneck, P. M. H.; Antholine, W. A.; Riester, J.; Zumft, W. G. *FEBS Lett.* **1988**, *242*, 70.
(23) Ghosh, S.; Gorelsky, S. I.; Chen, P.; Cabrito, I.; Moura, J. J. G.; Moura, I. *J. Am. Chem Soc.* **2003**, *125*, 15708.
(24) Gray, K. A.; Davidson, V. L.; Knaff, D. B. *J. Biol. Chem.* **1988**, *263*, 13987.
(25) Prudencio, M.; Eady, R. R.; Sawers, G. *Biochem. J.* **2001**, *353*, 259.

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