Enzyme Models

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Spectroscopic Characterization of Reaction Intermediates in a Model for Copper Nitrite Reductase**

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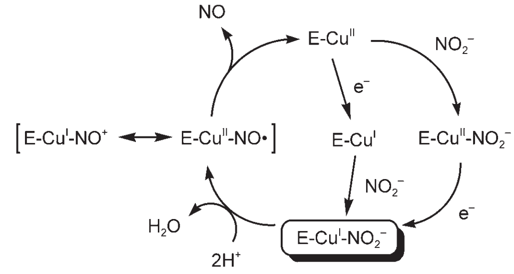
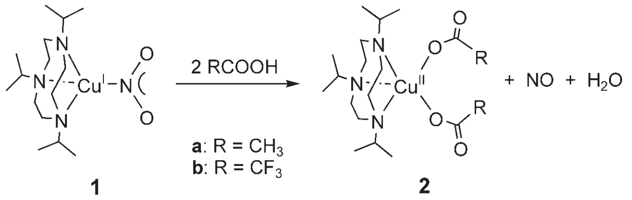
The reduction of nitrite (NO_2^-) to gaseous nitric oxide (NO) is a key process in the global nitrogen cycle and is achieved catalytically by the copper-containing nitrite reductases (NiR) of certain types of bacteria.[1] The reaction mechanism of NiR has been proposed on the basis of the results of a number of biochemical, spectroscopic, and crystallographic studies.[1–4] The reaction involves the formation of a copper(I) nitrite complex by binding of nitrite to oxidized NiR followed by reduction, or by the reduction of oxidized NiR followed by nitrite binding. The copper-bound nitrite is then reduced to NO by protons from protein residues and by one electron from the copper(I) center via a copper nitrosyl intermediate (Scheme 1).[2–4] In this mechanism, the copper(I) nitrite complex is a key intermediate in the reduction by NiR. While there are numerous reports of copper nitrite complexes,[5] the first and only structurally characterized model of the copper(I) nitrite complex of NiR, namely, [(iPr$_3$tacn)Cu^I(NO$_2$)] (**1**, iPr$_3$tacn = 1,4,7-triisopropyl-1,4,7-triazacyclononane), was reported by Tolman et al.[6,7] Compound **1** is a good functional model of NiR and reacts with two equivalents of acetic acid to yield NO and a copper(II) acetate complex, [(iPr$_3$tacn)Cu^{II}(O$_2$CCH$_3$)$_2$] (**2a**, Scheme 2). The structure of the nitrite complex of reduced NiR was recently determined by incubating nitrite with crystals of reduced NiR at low temperature.[4] On the other hand, the mechanism of reduction of copper-bound nitrite has not yet been elucidated. While protonation of the copper(I) nitrite complex appears to be a key process in nitrite reduction, the protonation step has not been observed in either enzymatic or synthetic model systems. Herein, we report the first observation of reaction intermediates in the reduction of nitrite in a copper(I) nitrite complex by rapid mixing of **1** and trifluoroacetic acid (TFA) with stopped flow at low temperature. The characterization of the observed intermediate suggests the formation of a unique copper(I)/nitrous acid structure. It also suggests a reaction mechanism in which two protons are transferred in a stepwise manner to copper-bound nitrite, followed by intramolecular electron transfer from copper to the nitrite and cleavage of the N–O bond of the copper-bound nitrite ligand in the second protonation step.



Scheme 1. Proposed mechanism for nitrite reduction by NiR. E = enzyme.



Scheme 2. Reaction of **1** with acetic acid and trifluoroacetic acid (TFA).

The reaction of **1** with acetic acid is sufficiently slow that intermediates could be detectable, but none were observed (Figure 1 a). The clear isosbestic points suggest that protonation of **1** is the rate-limiting step of the overall reaction. Therefore, it seems likely that a reaction intermediate might be detected if a much stronger acid were used. To examine this, we monitored the reaction of **1** with TFA, which is a stronger acid than acetic acid, at low temperature.[8] As shown in Figure 1 b, the change in the absorption spectrum after mixing **1** with an excess of TFA at 203 K was completely different from that in the presence of acetic acid. The absorption spectrum of **1** immediately transformed into a new spectrum with absorption maxima at 350 and 423 nm during mixing (less than 10 ms) in stopped flow. The absorption band at 423 nm then rapidly decreased and those at 290 and 680 nm increased in intensity. Finally, the absorption spectrum corresponded to that of copper(II) trifluoroacetate complex [(iPr$_3$tacn)Cu^{II}(O$_2$CCF$_3$)$_2$] (**2b**).[9] A previous study reported that the reaction of **1** with trimethylsilyl triflate (Me$_3$SiO$_3$SCF$_3$), a proton equivalent, initially yielded a mixed-valent dinuclear copper(I,II) nitrite complex as the result of a side reaction of **1** and the copper(II) reactant,[7] but the absorption spectrum of the new species is different from that of the dinuclear copper(I,II) nitrite complex. The time course for the reaction, monitored at 290, 423, and 680 nm, is poorly described by a single-exponential function, but better fitted by a biexponential function with k_{fast} and k_{slow} (Figure 1 and Supporting Information). These results indicate that at least two reaction intermediates are produced in this reaction [Eq. (1)]. From the absorbance coefficient of intermediate **B**, calculated from

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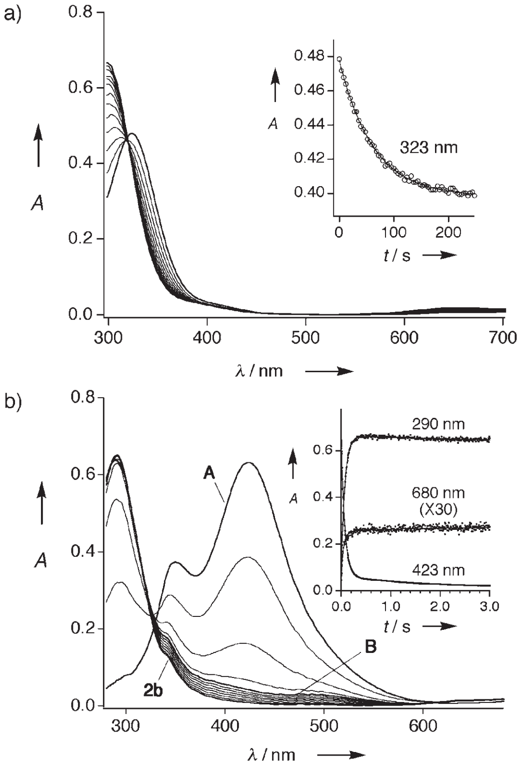


Figure 1. Change in the absorption spectrum after the rapid mixing of **1** (0.125 mM) in CH_2Cl_2 with a) acetic acid (12.5 mM) at 233 K, 0–250 s and b) with TFA (2.5 mM) at 203 K, 0–4.0 s. Typical spectra of intermediates **A** and **B** and of **2b** are shown in bold lines in (b). Insets: kinetic profiles for the reactions. a) Single-exponential curve fit at 323 nm, $k_{obs} = 1.62\times10^{-2}\ s^{-1}$. b) Biexponential curve fits at 290, 423, and 680 nm, $k_{fast} = 13.7\ s^{-1}$ and $k_{slow} = 0.67\ s^{-1}$.

the time-course curve fit, it is reasonable to assume that the second step (**A**→**B**) is a fast process and the third step (**B**→**2b**) slow.[10]

$$\mathbf{1} \rightarrow \text{intermediate } \mathbf{A} \rightarrow \text{intermediate } \mathbf{B} \rightarrow \mathbf{2b} \qquad (1)$$

To gain further understanding of the reaction mechanism, we examined the dependence of the rate constants k_{fast} and k_{slow} on the concentration of excess TFA. The rate constant for the second step k_{fast} is linearly dependent on the concentration of excess TFA (Figure 2 and Supporting Information). From the slope of k_{fast}, the second-order rate constant for the second step is $k_{second} = 6.54\times10^3\ M^{-1}s^{-1}$ at 203 K. This also indicates that one equivalent of TFA reacts in this step. On the other hand, the rate constant for the third step k_{slow} is independent of the concentration of TFA ($k_{third} = 6.82\times10^{-1}\ s^{-1}$). Since intermediate **A** is produced by the reaction of **1** and TFA, the rate constant for the first step (**1**→**A**) would be expected to be correlated with the concentration of TFA. However, the first step is too fast to permit the reaction rate constant to be estimated, even at a low concentration of TFA. All of these results support a scenario in which two equivalents of TFA react in the first and second steps.

To further investigate proton transfer in the reaction, the kinetic isotope effect was determined for each step. Even when deuterium-labeled TFA (CF_3COOD, [D]TFA) was mixed with **1** at 203 K, the first step could not be observed, and the overall absorption spectral change after mixing **1** with [D]TFA was the same as that with TFA. On the other hand, the reaction rate for the second step with [D]TFA ($k_{second} = 4.84\times10^3\ M^{-1}s^{-1}$) was slightly smaller than that for TFA (Figure 2 and Supporting Information). The observed kinetic isotope effect for the second step was $k_H/k_D \approx 1.4$ at 203 K. As expected, no significant deuterium isotope effect could be observed for the third step ($k = 6.98\times10^{-1}\ s^{-1}$, $k_H/k_D = 1.0$).

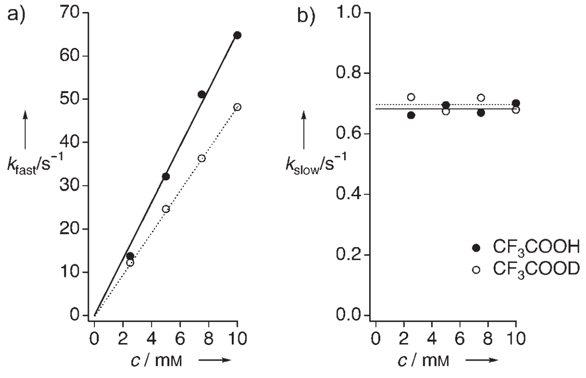
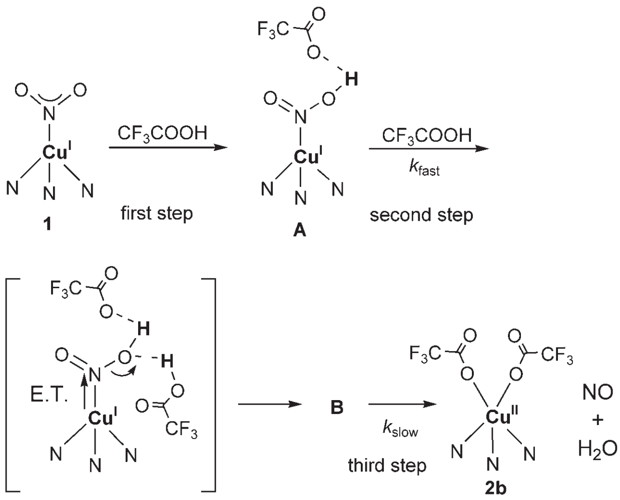


Figure 2. Plots of observed pseudo-first-order rate constants for a) the second step and b) the third step in the reaction of **1** (0.125 mM) in CH_2Cl_2 at 203 K at various TFA concentrations, *c*. Filled circles: TFA, empty circles: [D]TFA.

To further characterize the reaction steps, we determined the activation parameters for the second and third steps from Eyring plots over the temperature range $T = 193$–223 K (Supporting Information). The enthalpy of activation $\Delta H^{\neq}$ and the entropy of activation $\Delta S^{\neq}$ for the second step were found to be $28.4\pm3.2\ kJ\,mol^{-1}$ and $-27.1\pm2.4\ J\,mol^{-1}K^{-1}$, and those for the third step were $18.9\pm1.5\ kJ\,mol^{-1}$ and $-151.0\pm24.7\ J\,mol^{-1}K^{-1}$, respectively. The negative $\Delta S^{\neq}$ values suggest associative transient states in the second and third steps.

To identify the oxidation state of copper in the intermediates, we examined the reaction with O_2 by means of a double-mixing stopped-flow technique (see Supporting Information). It has been reported that the copper(I) complex of iPr$_3$tacn reacts with O_2 to form a μ-η_2:η_2-peroxo dicopper(II) complex which shows a strong absorption peak at 365 nm in CH_2Cl_2 at low temperature, but the copper(II) complex of iPr$_3$tacn does not react with O_2.[11] When O_2 in CH_2Cl_2/10% THF was mixed with intermediate **A** in CH_2Cl_2 at 203 K, the absorption bands of intermediate **A** immediately disappeared within the mixing time, and the absorption band at 368 nm, resulting from the μ-η_2:η_2-peroxo dicopper(II) complex, then appeared within a few seconds.[12] However, when O_2 in CH_2Cl_2/10% THF was mixed with intermediate **B** in CH_2Cl_2 at 203 K, the μ-η_2:η_2-peroxo dicopper(II) complex was not detected. These results show that the intermediates **A** and **B** have copper(I) and copper(II) oxidation states, respectively, and that one electron from the copper(I) center is utilized for nitrite reduction in the second step.

A possible structure of intermediate **A** and a reaction mechanism are proposed, based on the above results (Scheme 3). Complex **1** rapidly reacts with one equivalent



Scheme 3. Proposed mechanism for nitrite reduction of **1**. E.T. = electron transfer.

of TFA to give intermediate **A**. Intermediate **A** gives rise to strong absorption peaks at 350 and 423 nm (see Figure 1). The absence of a d–d transition band at 680 nm in the absorption spectrum and the double-mixing experiment clearly indicate an oxidation state +I of copper in intermediate **A**. Thus, the strong absorption band at 423 nm is assigned to the MLCT band of intermediate **A**, which shows a strong red shift from the MLCT band of **1** (308 nm). Intermediate **A** can not be regarded as a copper nitrosyl intermediate because it is still a copper(I) compound, and one additional proton is required for the subsequent reaction. Rather, intermediate **A** is a copper(I) nitrous acid complex formed by protonation of **1**. The red shift in the MLCT band is reasonable for protonation of the copper-bound nitrite, because the protonation of copper-bound nitrite stabilizes its π* orbital. In fact, the MLCT band of **1** shifts to lower energy on binding of metal ions to the copper-bound nitrite.[6,7] To our knowledge this is the first example of a metal complex of nitrous acid. The strong MLCT band of intermediate **A** would be a useful marker for identifying a similar intermediate in the reaction of NiR. Intermediate **A** subsequently reacts with a second equivalent of TFA to form intermediate **B**. This is assisted by the first-order dependence of the reaction rate k_{fast} on the concentration of TFA. The appearance of a d–d transition band at 680 nm and the double-mixing experiment are consistent with intramolecular electron transfer from copper(I) to the copper-bound nitrite in this step. The significant deuterium isotope effect in the second step also supports the second protonation of the copper bound nitrite. The negative $\Delta S^{\neq}$ value in this step is also consistent with the associative transient state shown in Scheme 3. The second protonation of the copper-bound nitrite would further stabilize the energy level of its π* orbital and result in intramolecular electron transfer. Donation of one electron and two protons to the copper-bound nitrite result in breakage of the N–O bond, and would lead to the copper nitrosyl intermediate $\mathrm{Cu^{II}{-}NO^{\bullet}}\leftrightarrow\mathrm{Cu^{I}{-}NO^{+}}$ and water. Finally, two trifluoroacetate counteranions bind to the copper(II) ion with release of NO to give **2b**. Although we were able to observe intermediate **B** after protonation of intermediate **A**, the structure of intermediate **B** is in doubt, because its absorption spectrum (see Figure 1, $\lambda_{\max} = 290$, 490, ca. 680 nm) is close to that of **2b**, and the spectroscopic properties of the copper nitrosyl intermediate are not known with certainty. Intermediate **B** could be the copper nitrosyl intermediate or a copper(II) complex that has released NO. Further characterization will be required to identify intermediate **B**. More details of the reaction mechanism and the structures of the reaction intermediates are currently being investigated in our laboratory.

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[8] The spectroscopic titration of **1** with TFA showed that, as in the case of acetic acid[6,7], 2 equivalents of TFA are required to complete the reaction. According to EPR analysis, about 70 % of

NO gas is trapped by iron(II) octaethylporphyrin complex in this reaction.

[9] Data for **2b** prepared from **1** and 2 equiv of TFA: ESI-MS: m/z (%): 431.2 (100) $[\mathbf{2b}-CF_3CO_2^-]^+$; UV/Vis ($CH_2Cl_2$): λ_{max} (ε) = 289 (6000), 692 nm (110 $M^{-1}cm^{-1}$); EPR (CH_2Cl_2, 4 K): $g = 2.06$, $g_\parallel = 2.28$, $A_\parallel = 15.5$ mT. C,H,N analysis (%) calcd for $C_{19}H_{33}N_3O_4F_6Cu$: C 41.87, H 6.10, N 7.71; found: C 41.62, H 5.99, N 7.63.

[10] If the second step were the fast process, the absorption coefficient at 423 nm of intermediate **B** would be approximately 500 $M^{-1}cm^{-1}$. On the other hand, if the second step were the slow process, the absorption coefficient at 423 nm of intermediate **B** would have a large negative value (approximately −90 000 $M^{-1}cm^{-1}$).

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[12] The small red shift (3 nm) of the absorption peak may be induced by THF or TFA in the solvent.