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Mechanisms of Electron Transfer in Catalysis by Copper Zinc Superoxide Dismutase

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Understanding the origins of oxidation−reduction catalysis by metalloenzymes requires detailed information concerning the mechanisms of electron transfer (ET). Copper zinc superoxide dismutase (SOD) is perhaps the most thoroughly studied member of a family of exceptionally well-tuned catalysts for the dismutation of superoxide: $2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$.[1] Under physiological conditions, the rate of the enzymatic reaction is ~10^4 times faster than that of the spontaneous disproportionation of $O_2^{\bullet-}$ in solution. The origin of enzyme catalysis with respect to the near barrierless nature of ET has not been understood, yet this enhanced reactivity is the basis of SOD's protective function against physiological oxidative damage.[1]

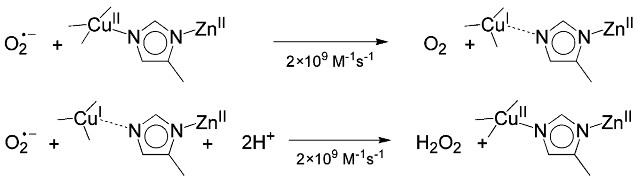
While there is a wealth of structural and mechanistic data on SOD, there is little evidence to suggest whether ET occurs by an inner-sphere or outer-sphere mechanism.[2] In inner-sphere ET, a bond exists between the reactants in the transition state, whereas in outer-sphere ET, there is no bond between the reactants which interact only by weak electronic coupling. Kinetic studies of SOD have indicated that the copper ion cycles between the +1 and +2 oxidation states in two irreversible reactions each of which is first-order with respect to $O_2^{\bullet-}$ (Scheme 1).[3]

The timing of proton transfer steps during SOD catalysis has remained unclear.[1] Steady-state rates are pH independent (from pH 5 to 9.5),[3c] an observation that has been difficult to reconcile with the requirement for protons in the reaction where $O_2^{\bullet-}$ is reduced. In the following studies conducted at pH 10, where rates are only slightly lower than at physiological pH, it is assumed that the imidazolate in the active site is deprotonated and bridges the copper and zinc ions during enzyme turnover.[4]

The experiments described below provide what is to our knowledge the first direct kinetic evidence for intermediates in SOD catalysis. Reactions were examined using a technique developed to measure competitive oxygen (^{18}O) kinetic isotope effects (KIEs) on reactions of natural abundance O_2.[5] We have adapted this technique, which employs isotope ratio mass spectrometry (IRMS), to study reactions of non-enriched $O_2^{\bullet-}$ with synthetic copper compounds.[6] Here ^{18}O KIEs upon SOD-catalyzed $O_2^{\bullet-}$ dismutation are analyzed together with the ^{18}O KIE upon the reaction of reduced SOD with O_2.

Experiments were conducted as previously described[7] by reacting potassium superoxide (KO_2) in dimethyl sulfoxide (DMSO) with a carbonate-buffered SOD solution at pH 10.[8] Under these conditions, the yield of O_2, based on the initial concentration of $O_2^{\bullet-}$, was 53 ± 4%. Care was taken to ensure that the SOD reaction occurred more rapidly than the spontaneous disproportionation of $O_2^{\bullet-}$ in enzyme-free solutions. Evidence is provided by competition experiments where $O_2^{\bullet-}$ was oxidized to O_2 in a solution containing both SOD and Fe^{III}−cytochrome *c*. In this reaction, O_2 was formed in 61 ± 6 versus 90 ± 7% yield when the solution contained only Fe^{III}−cytochrome *c*.[8b]

Scheme 1



Competitive ^{18}O KIEs are typically determined by analyzing the change in the $^{18}O/^{16}O$ of unreacted starting material at varying fractional conversions (see below).[5] The SOD reaction, with its rate approaching the diffusion limit, is not amenable to this type of experiment. Instead, an approach analogous to one described by Taube[9] was used to obtain a ratio of fractionation factors from the isotope composition of the products. The ratio defined here as β reflects the KIEs for the reactions of oxidized and reduced SOD with $O_2^{\bullet-}$ (eq 1);[3] β can be expressed in terms of the $^{18}O/^{16}O$ in the initial, unreacted $O_2^{\bullet-}$ (R_0) and the $^{18}O/^{16}O$ in the O_2 produced (R_{O_2}).[8b] The $^{18}O/^{16}O$ in the H_2O_2 ($R_{H_2O_2}$) is equal to ($2R_0 - R_{O_2}$).

$$\beta = \mathrm{KIE}(\mathrm{Cu^I} + \mathrm{O_2}^{\bullet-})/\mathrm{KIE}(\mathrm{Cu^{II}} + \mathrm{O_2}^{\bullet-}) \cong R_{O_2}/(2R_0 - R_{O_2}) \quad (1)$$

Oxygen isotope ratios relative to a known standard were measured by IRMS and used to calculate β. $R_0 = 1.0166 \pm 0.0001$ versus standard mean ocean water was determined from the O_2 formed in 101 ± 1% yield upon reacting $O_2^{\bullet-}$ with $K_3Fe^{III}(CN)_6$.[8b] R_{O_2} was determined from the O_2 isolated after the enzymatic reaction with excess $O_2^{\bullet-}$. Values of $\beta = 1.0104(12)$ and 1.0069(11) were determined in H_2O and D_2O, respectively.[8b] The small yet statistically significant solvent isotope effect is consistent with proton transfer in or before the first kinetically irreversible step of reaction where $O_2^{\bullet-}$ is reduced to H_2O_2.[4,10]

The reaction of homodimeric (Cu^I,Zn)SOD with O_2 was examined at pH 10 to resolve the contributions to β from the individual reactions in Scheme 1.[8b,11,12] Stoichiometric reaction of the reduced enzyme with O_2 affords 2 equiv of Cu^{II} and 1 equiv of H_2O_2.[13] Kinetic studies have indicated rate-limiting 1e$^-$ reduction of O_2 (k_{O_2} = 19.4 M^{-1} s^{-1}, 22 °C, pH 10) followed by rapid $O_2^{\bullet-}$ dismutation at another copper site.[11,14] Disappearance of O_2 is, therefore, characterized by an apparent isotope effect (^{18}O KIEap) corresponding to the product of the kinetic isotope effect upon k_{O_2} {i.e., ^{18}O KIE($Cu^I + O_2$)} and β.[8b]

The ^{18}O KIEap = 1.0148(10) was determined by a standard approach which involves analysis of the unreacted O_2 according to the Rayleigh equation: $R_f/R_0 = f^{(1/\mathrm{KIEap}-1)}$ (Figure 1).[5,8b] R_0 and R_f are the $^{18}O/^{16}O$ in the initial O_2 and remaining O_2, respectively, and *f* is the fraction of O_2 left in solution. The KIE($Cu^I + O_2$) = 1.0044(16) was obtained by dividing KIEap by β which was determined independently for the SOD reaction with $O_2^{\bullet-}$.

The KIE($Cu^I + O_2$) is almost an order of magnitude smaller than ^{18}O KIEs of ~1.028 previously reported for outer-sphere ET to O_2 at driving forces ($\Delta G°$) close to zero.[15] To evaluate whether the KIE($Cu^I + O_2$) could reflect outer-sphere ET from reduced SOD to O_2, the dependence of ^{18}O KIE on $\Delta G°$ was simulated following

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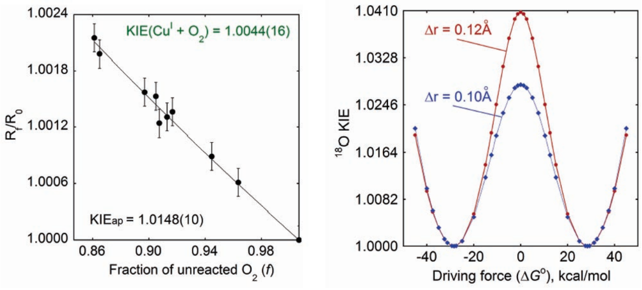

Figure 1. Left: ^{18}O fractionation due to oxidation of (Cu^I,Zn)SOD by O_2; data are fitted to $R_f/R_0 = f^{(1/\mathrm{KIEap}-1)}$.[8b] Right: simulation of ^{18}O KIEs as a function of $\Delta G°$ for outer-sphere ET to O_2 (see text for details).

an approach outlined by Jortner and co-workers (Figure 1).[16] This treatment, originally derived for ET reactions of transition metal compounds, expresses KIEs in terms of Franck−Condon factors describing the probability of nuclear overlap at the time of ET. Variations with $\Delta G°$ can be solved numerically within the saddle-point approximation.[8b,16] Parameters used in the present analysis include $\Delta G° = 10.4$ kcal mol^{-1} estimated from the redox potentials of O_2 and SOD[17] and $\lambda = 28.4$ kcal mol^{-1} estimated from k_{O_2} using an adiabatic expression for ET.[14b] Average O−O stretching frequencies ($^{16,16}\nu = 1264.5$ cm^{-1} and $^{16,18}\nu = 1228.8$ cm^{-1}) along with the difference in O−O bond lengths of O_2 and $O_2^{\bullet-}$ ($\Delta r = 0.1$ or 0.12 Å) were used to calculate nuclear overlap factors as previously described.[15]

The simulations indicate that, at the $\Delta G°$ estimated for an outer-sphere ET from reduced SOD to O_2, the ^{18}O KIE may be as low as ∼1.02. Since this estimate is much larger than the observed KIE(Cu^I + O_2), reduction of O_2 by inner-sphere ET is indicated. Further, a $Cu^{II}-O_2^{-I}$ intermediate is suggested by the closeness of the KIE(Cu^I + O_2) to equilibrium isotope effects (EIEs) of 1.0039−1.0054 determined for end-on superoxo structures in reversible O_2 binding reactions.[5] The analysis does not indicate the position of the transition state, however, and either O_2 binding to reduced SOD or $O_2^{\bullet-}$ dissociation from the CuO_2 may be rate-limiting.

The principle of microscopic reversibility implicates the same transition state for oxidation of $O_2^{\bullet-}$ by (Cu^{II},Zn)SOD and reduction of O_2 by (Cu^I,Zn)SOD. The active site is deprotonated in both enzyme forms at pH 10,[11a] and the bridging histidine is assumed to remain intact (cf. Scheme 1). It follows from the principle of microscopic reversibility that the KIE(Cu^I + O_2) divided by the theoretical EIE for converting O_2 to $O_2^{\bullet-}$ (1.0331)[5] gives the KIE(Cu^{II} + $O_2^{\bullet-}$) = 0.9722(16). The latter value, together with the definition of β in eq 1, allows calculation of the KIE(Cu^I + $O_2^{\bullet-}$) = 0.9823(10).

The KIE(Cu^{II} + $O_2^{\bullet-}$) (0.972) is more inverse than the ^{18}O KIEs of ∼0.986 which we have reported for the oxidation of $O_2^{\bullet-}$ by synthetic copper complexes.[6] These reactions were also proposed to occur by inner-sphere ET, and in one case, a CuO_2 intermediate was spectroscopically observed.[6] In a simple view where the KIE arises from a change in O−O force constant,[18] the more inverse KIE observed for the enzyme reaction suggests a transition state that is more $Cu^I-O_2^0$-like than $Cu^{II}-O_2^{-I}$-like.

The inverse KIE(Cu^I + $O_2^{\bullet-}$) (0.982) is in contrast to the normal isotope effects expected for reduction of $O_2^{\bullet-}$ by ET. From the stretching frequencies of reactants and products, an ^{18}O EIE of 1.016 is calculated for converting $O_2^{\bullet-}$ to O_2^{2-} and an EIE of 1.001 is calculated for converting $O_2^{\bullet-}$ to HO_2^-.[5] The observed KIE(Cu^I + $O_2^{\bullet-}$) is close to the EIE of 0.978 calculated for simple protonation of $O_2^{\bullet-}$ to $HO_2^{\bullet}$.[5] This observation, together with the solvent isotope effect upon the β for $O_2^{\bullet-}$ dismutation, suggests that initial proton transfer is rate-limiting in the reaction where $O_2^{\bullet-}$ is reduced,[4,7] at least under the high pH experimental conditions used in this study.

In summary, we have determined competitive oxygen kinetic isotope effects upon SOD-catalyzed $O_2^{\bullet-}$ dismutation as well as the reaction of reduced SOD with O_2. The results obtained for the latter reaction are interpreted as evidence for a CuO_2 intermediate during the oxidation of $O_2^{\bullet-}$ by (Cu^{II},Zn)SOD. This result supports an earlier proposal of an inner-sphere reaction based largely on crystallographic data.[2] In contrast, reduction of $O_2^{\bullet-}$ by (Cu^I,Zn)-SOD has been proposed to occur by an outer-sphere mechanism[1,2] with hydrogen bonds stabilizing the bound $O_2^{\bullet-}$ and possibly facilitating coupled electron−proton transfer.[1a] The oxygen kinetic isotope effect determined for this reaction is inverse and, therefore, inconsistent with electron transfer in the first irreversible step. This is an unusual observation as it predicts formation of $HO_2^{\bullet}$ as an intermediate in the SOD active site. These results concerning potential intermediates in SOD will be relevant to future studies of other superoxide-scavenging enzymes as well as oxygen-utilizing copper enzymes where the identities of the reactive oxidants during catalysis are subject to debate.[19]

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Supporting Information Available: Full descriptions of the experiments and calculations of isotope effects. This material is available free of charge via the Internet at http://pubs.acs.org.

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