Characterization of the Product-inhibited Complex in Catalysis by Human Manganese Superoxide Dismutase*

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The reduction with excess H2O2 of human Mn(III) superoxide dismutase (SOD) and the active-site mutant Y34F Mn(III)/SOD was measured by scanning stopped-flow spectrophotometry and revealed the presence of an intermediate in the reduction of the manganese. The visible absorption spectrum of this intermediate closely resembled that of the enzyme in the inhibited, zero-order phase of the catalyzed disproportionation of superoxide. The decay of the visible spectrum of this intermediate was 2-fold faster for the wild-type compared with the mutant Y34F Mn-SOD. This correlates with the enhanced product inhibition of Y34F during the catalysis of O2 dismutation. The visible spectrum of the product-inhibited complex resembles that of the azide-Mn-SOD complex, suggesting that the inhibited complex has expanded geometry about the metal to octahedral. This study shows that the inhibited complex responsible for the zero-order phase in the catalysis by Mn-SOD of superoxide dismutation can be reached through both the forward (O2) and reverse (H2O2) reactions, supporting a mechanism in which the zero-order phase results from product inhibition.

Superoxide dismutases are enzymes that catalyze the disproportionation of superoxide to yield oxygen and hydrogen peroxide. To date, there have been four different classes of superoxide dismutases identified based on the metal involved in the catalysis: CuZn-SOD,1 Fe-SOD, Mn-SOD, and most recently Ni-SOD. Among these Mn-SOD and Fe-SOD are homologous based on amino acid sequences and crystal structures (1), whereas CuZn-SOD and Ni-SOD are each clearly distinct and unique from the other forms. The manganese superoxide dismutases function through sequential redox processes in which the metal cycles between oxidized and reduced states (1).

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P\text{-Mn}^{3+} + \text{O}_2^- + 2\text{H}^+ &\xrightarrow{k_1} [P\text{-Mn}^{3+}\text{-O}_2^-] &\xrightarrow{k_2} P\text{-Mn}^{4+} + \text{O}_2 \quad (\text{Eq. 1})
\]

Here P-Mn\textsuperscript{3+} represents the protein bound to metal through three histidines and one aspartate as ligands; there is also one aqueous ligand in this trigonal bipyramidal geometry (2). Steady-state constants for catalysis of superoxide decay by manganese superoxide dismutase (Mn-SOD) from Thermus thermophilus and for the human Mn-SOD are similar to \(k_{\text{cat}}\) near \(10^4\) s\textsuperscript{-1} and \(k_{\text{cat}}/K_m\) between \(10^6\) and \(10^9\) M\textsuperscript{-1} s\textsuperscript{-1} (3, 4). Initial studies of Mn-SOD from Bacillus stea thermophilus used pulse radiolysis to determine that there is an initial burst of activity followed by an extended region of zero-order decay of superoxide. The zero-order region was explained as being due to the presence of a reversibly inhibited form of the enzyme that can interconvert to an active form (5). Bull et al. (3) observed the visible absorption spectrum of the enzyme during the zero-order phase of catalysis and, on the basis of comparison with visible absorption spectra of inorganic complexes, suggested that the zero-order phase results from product inhibition by peroxide. More specifically, they suggested a side-on peroxo complex of Mn(III)/SOD resulting from the oxidative addition of O\textsuperscript{2-} to Mn(II)/SOD. This inactive enzyme is represented as P-Mn\textsuperscript{3+}-X in Equation 3. The zero-order rate is determined, in part, by the rate constant \(k_{\text{cat}}\) of Equation 2 for the return of the inactive form to a species in the catalytic cycle. Another possibility is that inhibition occurs by the conversion of an outer to an inner sphere complex of product peroxide with the manganese (6). Both CuZn-SOD and Fe-SOD are irreversibly inactivated by hydroxyl radicals produced by the Fenton reactions of H\textsubscript{2}O\textsubscript{2} with their metal ions (6); Mn-SOD is not irreversibly inactivated in this manner. Thus, in this report product inhibition refers to the transitory inhibited complex of the zero-order phase observed during catalysis by Mn-SOD, and not to the irreversible type of inactivation that occurs in Fe-SOD and CuZn-SOD.

Tyr-34, which is a conserved residue in Mn-SOD and Fe-SOD, is part of an extensive hydrogen bond network that includes Gln-143 and the metal-ligand cluster and may connect the properties of this tyrosine to catalysis at the metal (2, 7). The crystal structure of Y34F Mn-SOD shows that its active-site structure is nearly exactly superimposable with that of the wild type except for the absence of the side-chain hydroxyl of residue 34 (8). The mutants Y34F Mn-SOD and Y34F Fe-SOD have been observed in a number of studies comparing their catalytic and spectral properties to the wild type (7–10). The catalytic activity of these mutants is decreased as much as 10-fold. Y34F Mn-SOD is more product-inhibited during catalysis than wild type, demonstrated by the smaller decrease in [O2] during the initial burst of catalysis and by a 4-fold slower
rate in the zero-order inhibited region for Y34F compared with wild type (8).

We have used scanning stopped-flow spectrophotometry to measure both the appearance of the inhibited enzyme in the zero-order phase of the dismutation of O$_2^*$ catalyzed by Mn-SOD and the reduction of Mn(III)SOD by excess H$_2$O$_2$; these measurements were extended to Y34F Mn-SOD as well. An immediate in the reduction by H$_2$O$_2$ was observed that has a visible spectrum nearly identical to that of the inhibited enzyme during the zero-order phase of superoxide dismutation. This observation confirms the suggestion that the zero-order phase during catalysis represents a product-inhibited enzyme rather than alternative explanations such as a conformational change involving an inactive state.

**MATERIALS AND METHODS**

Preparation and Purification of Enzymes—The polymerase chain reaction was used to amplify human Mn-SOD cDNA as described by Hsu et al. (4), and polymerase chain reaction-based site-directed mutagenesis was used to obtain cDNA for Y34F human Mn-SOD as described by Guan et al. (8). These sequences along with the mutation and the remainder of the coding regions were verified by DNA sequencing. These polymerase chain reaction products were cloned into the TA-cloning vector (pCRII) and subsequently subcloned into the expression vector, pTrec 99A (Amersham Pharmacia Biotech). The constructs expressed human Mn-SOD in the mutant Sod$^-$/A$^+$Sod$^-$/B Escherichia coli (strain QC 774) as a mature protein tagged with an extra Met at the amino terminus. Culture conditions included additional supplementation by 5 mM MnCl$_2$. Yields of Mn-SOD and mutant protein were on average 53 mg of protein/50 g of bacterial pellet. Human Mn-SOD and the mutant Y34F human Mn-SOD were purified from E. coli using a combination of heat treatment (60 °C) and ion exchange chromatography (DE52 and CM52) according to the procedures of Beck et al. (11). The purity of the resulting samples was determined on SDS-polyacrylamide gels that showed one intense band. The purified enzyme was dialyzed extensively against EDTA, and a portion of the resulting protein was digested with nitric acid for manganese analysis using atomic absorption spectrometry (Perkin Elmer 5100PC). These measurements were used to determine the concentration of enzyme.

Scanning Stopped-flow—Properties of the enzyme in its reaction with product hydrogen peroxide were monitored by observation of the visible absorption spectrum of Mn-SOD and Y34F Mn-SOD using a rapid scanning stopped-flow spectrophotometer (On-Line Instrument Systems, Inc., Bogart, Georgia or Applied Photophysics, Ltd. SX.18MV). One syringe contained a buffered solution of human Mn-SOD and a second syringe contained a buffered solution of H$_2$O$_2$; final concentrations after mixing are given in the figure legends.

The visible spectra of Mn-SOD and Y34F Mn-SOD during catalysis of the decay of O$_2^*$ were observed by scanning stopped-flow spectrophotometry (Applied Photophysics Ltd.) in which K$_2$O$_2$ in a solution of dimethyl sulfoxide and 18-crown-6 (7 mM) (12) was diluted in a sequential mixing experiment. To attain a sufficiently large final concentration of O$_2^*$, this initial solution of K$_2$O$_2$ was not greatly diluted; in the first mixing, the solution containing K$_2$O$_2$ was diluted 1.3.5 with 2 mM Caps and 1 mM EDTA at pH 12.5. This solution was allowed to age 1 s before the second mixing, which was 1:1 with the buffered enzyme solution. The final concentrations after mixing are given in the figure legends.

**RESULTS**

Using scanning stopped-flow spectrophotometry and mixing O$_2^*$ with Y34F Mn-SOD, and in separate experiments mixing H$_2$O$_2$ with Y34F Mn-SOD, we observed similar spectra characterized by an absorption with a maximum at 420 nm (Fig. 1). The same experiments mixing H$_2$O$_2$ and wild-type Mn-SOD also showed similar spectra with maximum at 420 nm and a weak absorption near 630 nm (Fig. 2); the spectrum for mixing O$_2^*$ and wild-type Mn-SOD also showed an absorption at 420 nm but of lesser intensity (data not shown). These spectra are very similar to that of the complex that characterizes the zero-order phase in the O$_2^*$ dismutation direction measured by Bull et al. (3) for T. thermophilus, which has a maximum at 410 nm and a weak band at 650 nm. Moreover, the extinction coefficient at 420 nm for the complex derived from the spectrum of human Mn-SOD after mixing with H$_2$O$_2$ (Fig. 2) is 500 M$^{-1}$ cm$^{-1}$, assuming complete conversion of enzyme to complex. This is an underestimate because it neglects the intermediate lost during the dead time of the instrument (~1.4 ms). The value of $\epsilon_{410 \sim 700}$ M$^{-1}$ cm$^{-1}$ was found for this complex by Bull et al. (3) measuring catalysis in the forward dismutation direction.

The visible absorption spectrum of wild-type human Mn(III)-SOD is dominated by a broad peak with a maximum at 480 nm.
and with a inset curve 2 concentrations was varied from 1:1 to 200:1. Interestingly, the showing a maximum absorbance near 420 nm. presence of excess hydrogen peroxide (Figs. 1 and 2), both absorption at 480 nm. Mn(III)SOD and Mn(II)SOD, the latter of which has a very weak of Mn(III)SOD. It represents a sample of mixed oxidation states et al. introduction of H2O2 and manganese superoxide dismutase. This corresponds to a rate constant of 0.1M
\text{e}^{-0.01 s^{-1}} \text{ and 0.66 } \epsilon = 0.01 s^{-1}. \\
(\epsilon_{420} \approx 600 \text{ m}^{-1} \text{ cm}^{-1})^2 \text{ (Fig. 2, inset curve 1); this is the predominant redox form of purified Mn-SOD (3). The reduced enzyme Mn(II)SOD has an absorption spectrum that is very weak with broad absorption (\epsilon < 50 \text{ m}^{-1} \text{ cm}^{-1}) and no peak in the range of 450–600 nm (3, 13). The binding of azide to the wild-type human Mn-SOD (0.25 nm) was also investigated. The ratio of azide to enzyme concentrations was varied from 1:1 to 200:1. Interestingly, the spectrum of the enzyme fully complexed with azide (Fig. 2, inset curve 2) closely resembled that of the enzyme in the presence of excess hydrogen peroxide (Figs. 1 and 2), both showing a maximum absorbance near 420 nm. Because it took large concentrations of H2O2 to observe these spectra, their rate of appearance was too rapid to be measured within the dead time of the stopped-flow process. However, we did observe the disappearance of these spectra to a very weak absorption consistent with the formation of Mn(II)SOD (Figs. 2 and 3). The decrease of the absorption at 420 nm was adequately fit by a second-order process in wild-type enzyme with a rate constant of 55.5 \pm 0.5 \text{ (absorbance units)}^{-1} \text{ s}^{-1} \text{ (Fig. 3). This corresponds to a rate constant of 0.1 M}^{-1} \text{s}^{-1} \text{ assuming } \\
\epsilon_{420} = 500 \text{ m}^{-1} \text{ cm}^{-1} \text{ for the complex. This result is similar to the value of 40 (absorbance units)}^{-1} \text{ s}^{-1} \text{ obtained from the experiment of Bull et al.} \text{ (3) observing the decay at 480 nm after the introduction of H2O2 to wild-type Mn-SOD. The decrease of the absorption at 420 nm (Fig. 3) appeared equally well fit by the sum of two first-order processes with rate constants 7.0 \pm 0.1 \text{ s}^{-1} \text{ and 0.66 } \epsilon = 0.01 s^{-1}. \\
\text{The disappearance of the 420 nm absorption after mixing H2O2 with Y34F Mn-SOD was also observed. The second-order rate constant for decrease of the maximum at 420 nm was 23.7 \pm 0.2 \text{ (absorbance units)}^{-1} \text{ s}^{-1}. When fit to the sum of two exponentials, the rate constants were 4.1 \pm 0.1 \text{ s}^{-1} \text{ and 0.47 } \epsilon = 0.01 s^{-1}. \\
\text{In parallel experiments, human Mn-SOD was exposed briefly (10 s) to 100-fold molar excess of H2O2, which was then rapidly diluted. Subsequent removal of H2O2 by dialysis resulted in Mn-SOD that was fully active as measured by stopped-flow. Unlike its analog Fe-SOD, which is inactivated in the presence of H2O2 through the Fenton-type chemistry in which H2O2 reacts with iron (14), Mn-SOD is not a redox catalyst for Fenton chemistry. The result that Mn-SOD is not irreversibly inactivated with H2O2 is consistent with earlier reports of McAdam et al. (15) and more recent studies of Yamakura et al. (16). DISCUSSION}

The rapid mixing of H2O2 with human Mn(III)SOD and subsequent scanning stopped-flow spectrophotometry has resulted in the appearance of a visible absorption spectrum (Fig. 2) with a maximum and extinction coefficient very similar to that obtained by Bull et al. (3) for the inhibited complex in the dismutation direction, obtained after mixing of O2 and T. thermophilus Mn-SOD. Moreover, the spectra of Fig. 2 are also similar to the spectrum of the inhibited complex of Y34F Mn-SOD in the dismutation direction and, in a separate experiment, after mixing with H2O2 (Fig. 1). Each spectrum shows a predominant absorbance near 420 nm and often a weak absorbance near 630 nm. This is the first reported observation of this complex obtained by the opposing reaction of H2O2 with Mn-SOD. It is significant in showing the nature of the zero-order phase of catalysis, supporting the interpretation of this phase as a form of product inhibition and providing another means by which this inhibition can be investigated. This result renders much less likely the attribution of this zero-order phase to a conformational change not related to product binding.

At the rather large concentrations of both H2O2 and Mn(III)-SOD that were used, we do not observe the build-up of the concentration of the intermediate, which must occur within the dead time (near 1.4 ms) of the instrument. Instead, we have observed the decay of this intermediate complex to the featureless visible spectrum characteristic of Mn(II)SOD (Fig. 2). The decrease of the absorption at 420 nm characteristic of the intermediate form (Fig. 3) was adequately fit either by a second-order process in enzyme or by the sum of two exponentials. At this time, we cannot determine whether the second-order or the sum of two first-order processes is the best representation of the reaction. The larger of the first-order rate constants, 7 s^{-1}, is close to k_{-2} = 10 s^{-1} determined by Bull et al. (3) by fitting progress curves (2 °C) of the dismutation catalysis, although it is smaller than the value of 130 s^{-1} (20 °C) determined for k_{-2} by Hsu et al. (4). The slower rate constant of these two exponentials, 0.7 s^{-1}, is less readily explicable but could describe a long-lived intermediate in the decay of P-MnX or one of the many reactivities of O2 and H2O2 (17). Bull et al. (3) carried out experiments observing the rate of decrease of the absorbance at 480 nm following mixing by stopped-flow of T. thermophilus Mn-SOD and excess H2O2. The decay of the 480 nm absorbance was best fit by second-order kinetics, which they ascribe to the slow reduction of Mn(II)SOD by H2O2 resulting in Mn(II)-SOD and O2 and then the subsequent reaction of O2 with a second Mn(II)SOD. The work reported here differs in that it addresses the 420 nm absorption characteristic of the inhibited form of the enzyme.

Regardless of whether the decay of the peroxide-enzyme complex is best described by a second-order or two first-order processes, it was observed that the visible absorption spectrum of the intermediate formed upon mixing H2O2 and Mn(III)SOD changed to that consistent with Mn(II)SOD about twice as fast for wild type as for Y34F Mn-SOD. This smaller rate of decay of the inhibited complex for Y34F Mn-SOD probably contributes to the enhanced product inhibition of this mutant compared to the wild-type Mn-SOD.
with wild type. The models for catalysis of both McAdam et al. (5) and Bull et al. (3) relate the magnitude of inhibition and the zero-order rate constant of the inhibited phase to this decay rate designated $k_{-5}$ of Equation 2. However, it is important to point out that many of the rate constants of Equations 1 and 2 besides $k_{-5}$ could also possibly contribute to the greater product inhibition of Y34F Mn-SOD observed during the catalyzed dismutation of $O_2$; the observation of a smaller rate of decay for the spectrum of the complex formed on mixing $H_2O_2$ and Y34F Mn-SOD is suggestive rather than sufficient for greater product inhibition in the dismutation direction.

We observed a similarity in visible absorption spectra of Mn-SOD in the presence of excess hydrogen peroxide and Mn-SOD complexed with azide (Fig. 2, inset curve 2). The crystal structure of the azide complex of Mn(III)SOD from T. thermophilus shows a six-coordinate species with the azide bound to the metal and forming a hydrogen bond with the hydroxyl of Tyr-34 (18). This suggests that the complex we observed in the presence of hydrogen peroxide, like the azide complex, may have expanded coordination geometry, which might occur if the peroxide is a direct ligand in its complex with Mn(III)SOD and does not displace the aqueous ligand, or the peroxy ligand displaces the aqueous ligand by forming a side-on peroxo (bidentate) complex as proposed by Bull et al. (3). The similarity of anion complexes of E. coli Mn-SOD with the product-inhibited complex has been discussed by Whittaker and Whittaker (19).

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