Protein Coordination to Manganese Determines the High Catalytic Rate of Dimanganese Catalases. Comparison to Functional Catalase Mimics†

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ABSTRACT: Catalysis of hydrogen peroxide dismutation by the dimanganese catalase from *Thermus thermophilus* has been measured and found to obey Michaelis−Menton kinetics with no evidence for substrate inhibition at concentrations up to 0.45 M H_2O_2. Comparison among three dimanganese catalases (*Thermus thermophilus, Thermoleophilium album,* and *Lactobacillus plantarum*) reveals that their apparent second-order rate constants, k_{cat}/K_m, differ by at most a factor of 5, even though the individual kinetic constants differ by as much as a factor of 20. This similarity suggests that all three enzymes may have the same rate-determining step. For *T. thermophilus* catalase we find that $k_{cat}/K_m \sim k_{bi}$, the bimolecular rate constant at limiting substrate concentrations. Thus, the rate of the rate-determining step is unaltered over the entire range of substrate concentrations, unlike *T. album* and *L. plantarum* catalases where substrate inhibition has been reported. Comparison to structurally characterized dimanganese complexes and dimetalloproteins (arginase, hemerythrin), which are functional, albeit kinetically slow, catalase mimics, reveals that high catalase activity correlates with a greater number of stronger σ-ligand donors like anionic carboxylatos vs neutral histidines that stabilize the oxidized Mn_2(III,III) state over reduced Mn_2(II,II). A critical feature for enzymatic functionality *in vivo* is suppression of one-electron chemistry leading to formation of the mixed-valence forms, Mn_2(III,IV) and Mn_2(II,III), which are kinetically inactive or precursors to inactive species, respectively. Evidence is presented from model compounds suggesting that the μ-carboxylato bridge between Mn ions in catalase may play the key role in suppressing formation of these detrimental oxidation states through destabilization of these one-electron redox processes.

The principal method by which cells protect themselves against oxidative damage is by recruiting specific enzymes capable of rapidly deactivating the initial toxic products, primarily superoxide (O_2^-) and hydrogen peroxide (H_2O_2). All aerobic cells appear to contain one or more examples of catalase, the enzyme responsible for dismutation of H_2O_2, eq 1a (pH = 7, vs NHE).

$$2H_2O_2 \rightarrow 2H_2O_2 + O_2 \qquad E_0 = 1.1\ V \qquad (1a)$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \qquad E_0 = 1.35\ V \qquad (1b)$$

$$H_2O_2 \rightarrow O_2 + 2e^- + 2H^+ \qquad E_0 = -0.26\ V \qquad (1c)$$

The dimanganese catalases are rare enzymes, being found in a small number of thermophilic or lactate-requiring hemeless bacteria, and thus are less well understood than the widely distributed heme catalases (Dismukes, 1992; Penner-Hahn, 1992). Nevertheless, their obligate role in protection against oxidative stress has been firmly established, both from studies of deletion strains (Kono & Fridovich, 1983a,b) and from the observation of induction of enzyme synthesis by addition of agents which produce intracellular H_2O_2 (Allgood & Perry, 1986).

Herein we report determination of the steady-state kinetics parameters for *Thermus thermophilus* catalase and provide a structural/redox rationale for the rapid rates found for the three best characterized dimanganese catalases, versus the slow rates found in dimanganese proteins (arginase and hemerythrin) and dimanganese complexes proposed as catalase mimics.

MATERIALS AND METHODS

T. thermophilus catalase was isolated as previously described from cultures grown at 74 °C (Barynin & Grebenko, 1986). The protein exists as a hexamer of equivalent subunits with molecular mass 35 kDa. Catalase activity was measured by O_2 polarography (Pessiki & Dismukes, 1994). Manganese oxidation states were established by EPR and electronic spectroscopies as previously described (Khangulov et al., 1990a,b). The enzyme was prereduced with hydroxylamine followed by dialysis at the chosen pH in citrate buffer (20 mM).

RESULTS

The reduced enzyme, oxidation state Mn_2(II,II), exhibits substrate saturation kinetics with no evidence for inhibition by peroxide at concentrations as high as 0.45 M. A Lineweaver−Burk plot of the results is given in Figure 1. From the intercepts the Michaelis constant (K_m= 83 ± 8 mM) and the catalytic turnover constant (k_{cat} = 2.6 × 10^5 s^{-1} (subunit)$^{-1}$) were determined. The oxidized enzyme, Mn_2(III,III), exhibits steady-state kinetics identical to those of the reduced enzyme. As previously reported, the superoxidized enzyme, Mn_2(III,IV), is inactive (Khangulov et al., 1990c; Penner-Hahn, 1992). It forms quantitatively in the

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Table 1: Steady-State Kinetic Parameters and Mn Coordination of Dimanganese Proteins and Complexes

catalase	k_{cat} (s^{-1})	K_m (mM)	Mn ligands,[a] Mn···Mn, Å	ref
T. thermophilus	2.6×10^5	83 ± 8	Mn_2(III,IV), O_6 or O_5N, Mn_2(II,II), 3.5–3.6 Å	this work; Barynin et al., 1986; Barynin, 1991; Khangulov et al., 1993; Zheng et al., 1994; Khangulov et al., in press
L. plantarum	2.0×10^5	350	Mn_2(II,II), $(N/O)_4His_2$, Mn_2(III,IV), 2.67 Å	Penner-Hahn, 1992; Waldo et al., 1992
T. album	2.6×10^4	15	nd	Allgood and Perry, 1986
liver arginase	30	2750 ± 250	Mn_2(II,II)O_xN_y, 3.59 Å	Reczkowski & Ash, 1992; Cavalli et al., 1994; Khangulov et al., in press
Mn_2–hemerythrin	no activity observed		O′,O″$_2$,His_{2-3}, Co_2(II,II), 3.54 Å	Zhang et al., 1991, 1992; Zhang & Kurtz, unpublished
$[Mn^{III}$(2-OH-5-Cl-salpn)$]_2$[b] (**1**)	16	47 ± 10	O‴$_4$,N_2, 3.23 Å	Gelasco & Pecoraro, 1993
Mn_2^{II}(^{1}L) ($MeCO_2$)] (ClO_4)$_2$[c] (**2**)	k_{bi} = 0.23 M^{-1} s^{-1} [d]	nd	O‴,O″,N_3, 3.54 Å	Mathur et al., 1987; Pessiki & Dismukes, 1994; Pessiki et al., 1994
Mn_2^{II}(^{2}L)($PhCO_2$)$_2$ (N CS)[e] (**3**)	k_{obs} = 0.33 M^{-1} s^{-1} [e]	nd	0‴,O′$_2$,N,N′, 3.33 Å	Sakiyama et al., 1993a,b

[a] O′ = carboxylate; O″ = hydroxide; O‴ = phenoxide or alkoxide. [b] Mn^{III}(1,3-bis(salicylideneimino)-2-propanol). [c] ^{1}HL = *N,N,N′,N′*-tetrakis(2-methylenebenzimidazolyl)-1,3-diaminopropan-2-ol. [d] Value represents the observed second-order rate constant. [e] ^{2}L = 2,6-bis[*N*-((2-dimethylamino)ethyl)amino]methyl 4-methylphenolate (1−); saturation kinetics were not demonstrated, nor was the molecularity of the reaction established. The number given represents the reported rate divided by the concentration of catalyst and peroxide.

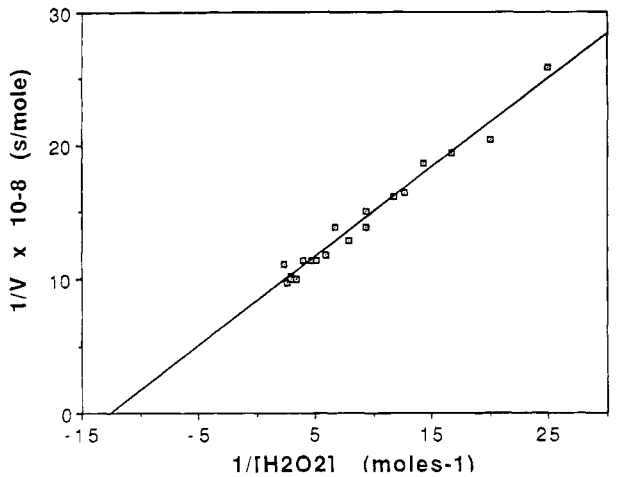


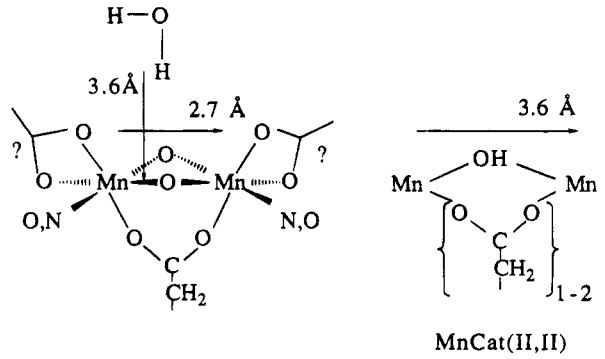
FIGURE 1: Lineweaver–Burk plot of the inverse of the O_2 evolution rate versus the inverse of the substrate concentration for *Thermus thermophilus* manganese catalase.

presence of peroxide and a variety of one-electron reductants, including hydroxylamine, hydrazine, and iodide, via intermediacy of the mixed-valence Mn_2(II,III) oxidation state.

Comparison among three dimanganese catalases reveals a 20-fold difference in K_m and a 10-fold difference in k_{cat} between *T. album* catalase and *T. thermophilus* or *L. plantarum* catalase (Table 1). However, the apparent second-order rate constants, k_{cat}/K_m, for peroxide dismutation by these enzymes differ by only 5-fold. Hence, the slowest bimolecular steps along the forward reaction pathway for these three enzymes proceed at comparable rates, i.e., within a factor of 5. Such similar rates would be expected if the rate-limiting step were the same in each enzyme. The true bimolecular rate constant for dioxygen evolution has been determined at peroxide concentrations well below saturation for *T. thermophilus* catalase and found to be $k_{bi} = 1 \times 10^7$ (unfrozen enzyme) or 2×10^6 $M^{-1}s^{-1}$ (frozen/thawed enzyme) (Khangulov et al., 1990c), compared to $k_{cat}/K_m = 3.1 \times 10^6$ M^{-1} s^{-1} (frozen/thawed enzyme) (Figure 1). Since theory requires that $k_{cat}/K_m \leq k_{bi}$, we see that the two approaches essentially agree. Therefore, at all concentrations of substrate the rate of the rate-limiting step is essentially constant. By contrast, although k_{bi} has not been reported for either *T. album* or *L. plantarum* catalase, both have been reported to exhibit substrate inhibition above peroxide concentrations of 20 mM and 0.1 M, respectively (Kono & Fridovich, 1983b; Allgood & Perry, 1986), in contrast to *T. thermophilus* where peroxide does not inhibit below at least 0.45 M (Figure 1). Since cellular peroxide concentrations are expected to be much lower even under conditions of stress, there is no obvious case where this inhibition could have a physiological impact. Hence operationally, all three dimanganese catalases are kinetically similar and exhibit high catalytic rates comparable to those of the heme catalases.

By contrast, Table 1 reveals that k_{cat}/K_m for dimanganese catalases is at least 10^5 larger than the catalase activity of arginase, a dimanganese(II,II) protein ($k_{cat}/K_m = 11$ s^{-1} M^{-1}) (Reczkowski & Ash, 1992; Sossong et al., 1994). This enzyme functions in the liver to hydrolyze the guanidinium group of L-arginine, by catalysis at the dimanganese site. Similarly, the dimanganese-substituted form of hemerythrin has been characterized and found to lack both dioxygen binding capacity and catalase activity (Zhang et al., 1991; J.-H. Zhang and D. M. Kurtz, personal communication). The absence of catalase activity was surprising because Mn_2–hemerythrin was anticipated to have tertiary and secondary protein structure identical to hemerythrin, which like Mn–catalase folds into a four-helix bundle motif with both metals bound to the inner surface of the four helices (Barynin et al., 1986). However, it is now known that the metal ligands are different in terms of both charge and σ-ligand field strength (Table 1) (Khangulov et al., 1993, 1994). The coordination environment of Fe_2–hemerythrin has been established by X-ray crystallography (Table 1) (Stenkamp et al., 1985). Five neutral histidine ligands and a (μ-OH)-(μ-RCO_2)$_2$ bridge stabilize the Mn_2(II,II) oxidation state relative to Mn_2(III,III), resulting in a reduced protein which is inert to O_2 and exogenous oxidants (J.-H. Zhang and D. M. Kurtz, personal communication). Less is known about the protein coordination to manganese in arginase; mutagenesis and preliminary ^{14}N ENDOR studies have revealed at least two potential histidine ligands to Mn ions (Cavalli et al., 1994; S. V. Khangulov, D. E. Ash, and G. C. Dismukes, unpublished results). The resting oxidation state is Mn_2(II,-II) even in the presence of various oxidants including O_2, H_2O_2, KIO_4, $KMnO_4$, and others.

Chart 1: Current Structural Models for the Dimanganese Site of Mn Catalase(II,II)[a] and Mn Catalase(III,IV)[b]



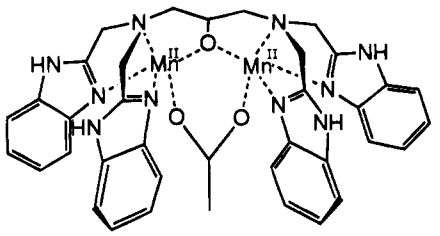
MnCat(II,II)

[a] Barynin et al. (1986); Khangulov et al. (in press). [b] Dismukes (1992); Penner-Hahn (1992); Waldo et al. (1992); Khangulov et al. (1993).

By contrast, the superoxidized state of Mn—catalase possesses a di-μ-oxo-polycarboxylato coordination site with at most one His/Mn ligand and a water (hydroxide) molecule H-bonded to one or more of the oxo bridges (Chart 1). Although less is known about the structure of the reduced enzyme, EPR data suggest a (μ-hydroxo)(μ-carboxylato)1—2 bridge (Chart 1). The resting oxidation state of Mn—catalase, Mn_2(III,III), forms by spontaneous oxidation of the reduced enzyme in air and is believed to possess a single μ-oxo—μ-carboxylato bridge on the basis of the similarity of electronic absorption spectra to those of model compounds (Sheats et al., 1987). The structural origin of the large difference in catalase activities of the (II,II) and (III,III) states vs the (III,IV) state can be understood to originate from the slow kinetics of either peroxide binding by exchange with the μ-oxo(s) bridges or reduction of the (III,IV) state by outer sphere peroxide (Waldo et al., 1992; Khangulov et al., 1993). It is not known which of these two steps is rate-limiting in the reactivation process.

The 10^5-fold lower catalase rate for both hemerythrin and arginase appears not to be due to inaccessibility of peroxide to the active sites, as both enzymes have binding sites for small molecules. We suggest that the large rate enhancement in catalases is due to a much smaller thermodynamic barrier to oxidation of Mn_2(II,II) to Mn_2(III,III), and that this more facile oxidation originates from a stronger σ-ligand field potential at the Mn ions provided by the combined electrostatic and σ-bonding potentials from anionic carboxylatos instead of neutral histidine ligands. Because efficient catalase activity also requires both proton uptake, eq 1b, and proton release, eq 1c, the much higher barrier to turnover in arginase and hemerythrin may have a contribution from the unavailability of ionizable residues or water molecules in the active sites to serve these roles.

Although several dimanganese complexes including complexes **1**, **2**, and **3** (Table 1) have been proposed as functional



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catalase mimics, all have bimolecular rate constants that are 10^5—10^7 smaller than for catalase (Mathur et al., 1987; Gelasco & Pecoraro, 1993; Sakiyama et al., 1993a,b; Nagata et al., 1994; Pessiki & Dismukes, 1994). Support for the proposal that the Mn redox potential plays a major role in this large rate difference comes from electrochemical studies of complex **2** (Table 1). **2** exhibits a reduction potential of 0.81 V for the two-electron process Mn_2(III,III) ↔ Mn_2(II,-II). This highly favorable reduction potential is attributed to a weak ligand field from nitrogen atoms (Pessiki et al., 1994). During catalysis the corresponding oxidation process is coupled to peroxide reduction, eq 1b. Peroxide reduction occurs at a potential of 0.93 V at pH 0 in water (lower in non-protic solvents), and thus oxidation of manganese should be capable of limiting turnover when proton acceptors are unavailable. Like the enzyme, both complexes **1** and **2** utilize the Mn_2(III,III) ↔ Mn_2(II,II) couple for catalyzing eq 1a, while indirect evidence suggests that complex **3** may utilize the Mn_2(IV,IV) ↔ Mn_2(III,III) couple.

μ-Carboxylate Conundrum? Comparison of spectroscopic data on dimanganese catalases and complexes has been interpreted to mean that one or two μ-carboxylato ligands bridge between the Mn ions in the enzymes (Sheats et al., 1987; Khangulov et al., 1993, 1994; Gamelin et al., 1994). A possible functional role for the μ-carboxylato(s) other than as a mere passive structural bridge to separate the Mn ions has not been suggested, but may be critical for proper enzymatic functioning on the basis of the following related observations. Electrochemical data on complex **2** and its isostructural μ-chloroacetato derivative versus the derivatives LMn_2Cl_3 (**4**) and $LMn_2(OH)Br_2$ (**5**), which have the μ-carboxylato replaced by μ-Cl^- and μ-OH^-, respectively, reveal that only the μ-carboxylato derivatives fail to exhibit the one-electron oxidation pathway via the mixed-valence species Mn_2(II,III) (E_0 = 0.49 (**4**) and 0.54 V (**5**)) (Mathur et al., 1987; Pessiki et al., 1994). The μ-carboxylato increases the potential by 0.3 V, thereby favoring the simultaneous two-electron process Mn_2(III,III) ↔ Mn_2(II,II) over sequential one-electron steps at resolved potentials. For catalases *in vivo*, suppression of the mixed-valence Mn_2(II,III) state is absolutely critical for proper functioning and presumably cell viability. The mixed-valence state does not form during enzymatic turnover, but can be induced artificially as an intermediate during reduction with hydroxylamine, iodide, and other one-electron reductants (Khangulov et al., 1987). If this is allowed to occur in the presence of peroxide, the intermediate is quantitatively oxidized to the catalytically inactive di-μ-oxo Mn_2(III,IV) species (Khangulov et al., 1990c; Penner-Hahn, 1992). Although this "superoxidized" state can be fully reactivated, it occurs so slowly (half-time ~ 30 min with peroxide or exogenous reductants) as to render the enzyme effectively unavailable during the cell's lifetime. Consequently, redox chemistry which forms either of the mixed-valence species must be strongly selected against. Nature's solution to this challenge could be to utilize μ-carboxylato bridges to separate and decouple the redox centers, thereby enabling two-electron redox changes to be favored over one-electron changes. This would ensure minimal formation of the harmful mixed-valence species.

μ-Carboxylato bridges appear to be widespread in dimetalloproteins where two-electron redox chemistry prevails (hemerythrin, methane monooxygenase, ribonucleotide reductase), suggesting that Nature may be employing the redox

control endowed by μ-carboxylato coordination to favor two-electron chemistry in these metalloproteins also.

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