Thermally Triggered Metal Binding by Recombinant
Thermus thermophilus Manganese Superoxide Dismutase,
Expressed as the Apo-enzyme*

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Manganese superoxide dismutase from the extremely thermophilic eubacterium Thermus thermophilus has been cloned and expressed at high levels in a mesophilic host (Escherichia coli) as a soluble tetrameric protein mainly present as the metal-free apo-enzyme. Incubation of the purified apo-enzyme with manganese salts at ambient temperature did not restore superoxide dismutase activity, but reactivation could be achieved by heating the protein with Mn(II) at higher temperatures, approaching the physiological growth temperature for T. thermophilus. Heat annealing followed by incubation with manganese at lower temperature fails to reactivate the enzyme, demonstrating that a simple misfolding of the protein is not responsible for the observed behavior. The in vitro metal uptake is nonspecific, and manganese, iron, and vanadium all bind, but only manganese restores catalytic activity. Bound metal ions do not exchange during heat treatment, indicating that the formation of the metal complex is effectively irreversible under these conditions. The metallation process is strongly temperature-dependent, suggesting that substantial activation barriers to metal uptake at ambient temperature are overcome by a thermal transition in the apo-protein structure. A mechanism for SOD metalation is proposed, focusing on interactions at the domain interface.

Antioxidant enzymes are essential survival gear for living cells, providing protection from the reactive oxygen species (including superoxide \(O_2^-\) (1–3), hydrogen peroxide \(H_2O_2\) (4), and organic peroxides \(ROOH\) (5)) that are responsible for oxidative damage to essential elements of cell structure. Superoxide dismutases (1–3) are a ubiquitous class of antioxidant defense metalloenzymes that disproportionate superoxide radical ion into dioxygen and hydrogen peroxide.

\[ 2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \]  

(Eq. 1)

The reaction is catalyzed by a redox-active metal ion in the enzyme active site, and four distinct varieties of SOD have been found, differing in the identity of the functional metal (manganese (6, 7), iron (8), copper (9), or nickel (10)). Although manganese and iron superoxide dismutases (MnSOD and FeSOD) share significant homology, they are structurally distinct from copper and nickel enzymes (11–14).

Manganese superoxide dismutases from Escherichia coli and Thermus thermophilus have been extensively characterized by structural (x-ray crystallography (14–17)) and spectroscopic ( optical absorption, EPR, MCD (6, 18–20)) methods. The active site manganese ion is complexed by histidine and aspartic amino acid side chains forming a trigonal pyramidal coordination polyhedron (Fig. 1). Studies on the closely related FeSODs suggest that this arrangement of protein ligands is independent of the presence of the metal ion, being nearly identical for holo- and apo-enzymes (21). Despite very similar three-dimensional structures (16, 17), mesophilic and thermophilic MnSODs exhibit differences in thermal stability and have distinct temperature dependence for exogenous ligand interactions (22, 23), corresponding to a “thermophilic shift” of properties for the thermophilic MnSOD. To investigate the structural determinants underlying thermophily and other thermally activated protein processes, we have cloned a semisynthetic gene for T. thermophilus MnSOD (TTSOD) and succeeded in expressing the recombinant protein at high levels in E. coli. The heterologous TTSOD is isolated primarily as the apo-protein, a surprising contrast to the homologous E. coli enzyme, whose apo-protein readily binds manganese or other metals present in solution at ambient temperature (24, 25). The abundant supply of purified apo-enzyme from the recombinant expression system provides a unique opportunity to explore the metallation of a thermophilic MnSOD in vitro.

MATERIALS AND METHODS

Biological Materials—T. thermophilus HB8 (ATCC 27634) was grown in culture medium containing 8 g/liter polypeptone, 4 g/liter yeast extract, and 3 g/liter NaCl adjusted to pH 7.5 with NaOH (20) supplemented with 2 mg/liter of MnSO_4\cdotH_2O. Fermentation cultures of T. thermophilus for protein biomass preparation were grown at 65 °C in a New Brunswick Scientific BioFlo 3000 Bioreactor wrapped in a quilted insulating jacket to reduce heat loss. E. coli sodA mutant strain QC781 (26) was obtained from the E. coli Genetic Stock Center of Yale University. Competent E. coli QC781 sodA cells were prepared according to standard procedures.

Purification of MnSOD from T. thermophilus—Manganese superoxide dismutase was purified from T. thermophilus as described previously (20). Protein concentration was determined by optical absorption measurements, using the previously reported extinction coefficients (\(E_{280}^\text{MnSOD} = 15.8(20)\)).

Isolation of Genomic DNA—T. thermophilus HB8 grown overnight in 100 ml of polypeptone medium in stationary culture at 65 °C. The

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1 The abbreviations used are: MnSOD, manganese superoxide dismutase; FeSOD, iron superoxide dismutase; EPR, electron paramagnetic resonance; MOPS, 3-[N-morpholino]-propanesulfonic acid; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; TTSOD, T. thermophilus manganese superoxide dismutase.

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centrifuged cell pellet was subjected to Qiagen genomic Midiprep procedure according to the manufacturer’s instructions.

**Primer Design**—Primers for amplification of the MnSOD structural gene from T. thermophilus HB8 genomic DNA were designed by backtranslations of the reported amino acid sequence (27) using standard Thermus codon usage (28). The forward primer, a 34-mer oligonucleotide, was 5’-CACCGGATATCGGCTACCTGTACGCTACG-3’. The reverse primer, a 2-fold degenerate 44-mer oligonucleotide, was 5’-GCTGAGACGAGAATTAGGCTTGAACTCCTCGGCSACG 3’ (5 = G + C).

**Genomic Amplification**—Genomic DNA isolated as described above was probed with the TTSOD primer set using the proofreading Pfu polymerase (Stratagene) to amplify the MnSOD structural gene. A single major product band was extracted from 1% agarose gel electrophoresis. The resulting amplicons were purified and screened for inserts, and the miniprep DNA from a positive clone transformed into Stratagene Epicurian Coli XL-1 ultracompetent cells by vectorial ligation of the gel-purified products. Ligation products were digested with NdeI/BamHI double digestion of both pQGB1 and pTTSDPCR followed by a NdeI/BamHI/EcoRI cassette to produce the derivative pQGBTTSD. The expression vector pQGBTTSD was constructed by replacing the original expression vector pQGB1. The expression vector pQGBTTSD was constructed by digestion of miniprep DNA. The nucleotide sequence of the pTTSDPCR insert was determined by DNA sequence analysis (Molecular Biology Core Facility, Oregon Regional Primate Research Center, Beaverton, OR).

**Expression Vector**—The pGB1 expression vector containing the oxygen-inducible Promoter 29 was a generous gift of Dr. Joe McCord (Webb-Waring Institute for Biomedical Research, University of Colorado Health Sciences Center). To facilitate subcloning the semisynthetic TTSOD gene for expression, the pGB1 vector was subjected to three rounds of in vitro site-directed mutagenesis using the Stratagene QuickChange™ procedure to eliminate BamHI restriction sites in the flanking regions of the vector and to replace the original NsiI/SacI cloning site with an NdeI/BamHI/EcoRI cassette to produce the derivative pQGB1. The expression vector pQGBTSD was constructed by NdeI/BamHI double digestion of both pQGB1 and pTTSDPCR followed by vectorial ligation of the gel-purified products. Ligation products were transformed into Stratagene Epicurian Coli XL-1 ultracompetent cells and screened for inserts, and the miniprep DNA from a positive clone was used to transform competent E. coli QC781 sodA cells for protein expression.

**Heterologous Expression of Thermus MnSOD**—E. coli cultures for protein biomass were grown in 2× LB medium supplemented with 1% glucose with or without addition of manganese salt up to 2 mM and 125 μg/ml ampicillin (30). Further additions of ampicillin (150 mg/liter) were made hourly after the optical density reached 0.3 at 600 nm, and a second addition of 1% glucose was made at midlog phase. Fermentations were routinely performed in a 10-liter New Brunswick Scientific BioFlo 3000 Bioreactor. Cultures were grown at 37°C with vigorous agitation and O2 purging.

**Purification of Recombinant Thermus MnSOD from E. coli**—Superoxide dismutase was purified as described previously (30) with an additional Chromatofocusing chromatography step using PBE-94 polyanion exchanger and Polybuffer-74 ampholyte but without heat treatment or CM-52 ion exchange chromatography. The enzyme was detected in chromatographic elution profiles by polycrylamide gel electrophoresis.

**Manganese Reactivation**—For time course experiments, 50-μl samples prepared by combining purified apo-enzyme (0.5 mM active sites in 20 mM MOPS, pH 7.0) with 10 mM MnCl2·4H2O in 500-μl thin walled polypropylene tubes were heated in a thermal minicycler (MJ Research) and stored at 4°C. Temperature dependence studies were performed using 10 mM MnCl2·4H2O for 30 min at various temperatures followed by rapid quenching at 4°C.

**Protein Characterization**—Molecular mass determination was made by size exclusion chromatography using a Sephacryl S-200-HR column (1.5 × 99 cm) developed in 50 mM potassium phosphate buffer, pH 7.0. The column was calibrated by chromatographing molecular mass standards (blue dextran, 200 kDa; native Thermus MnSOD, 92.6 kDa; E. coli MnSOD, 46 kDa; horse heart cytochrome c, 12.4 kDa) (1.5–2 mg in each sample), and the relative molecular mass of the eluted fractions for recombinant Thermus MnSOD were estimated by evaluating the effective distribution coefficient Ke = (Vf – Ve)/(Ve – V0), where Ve is the elution volume for blue dextran, Vf is the elution volume for the protein sample, and V0 is the total column volume.

**Enzyme Assays**—SOD activity was measured using the xanthine oxidase/cytochrome c inhibition assay (6) at room temperature. Bromoperoxidase activity was measured by following the bromination of monochlorodimedone at 290 nm (31).

**Spectroscopic Measurements**—Optical absorption spectra were recorded using a Varian Instruments Cary 5 UV-visible near infrared absorption spectrometer interfaced with a microcomputer for data acquisition. Electron paramagnetic resonance spectra were recorded on a Varian E-109 X-Band EPR spectrometer equipped with an Air Products helium cryostat. First derivative powder EPR spectra were simulated using the program sim15 (Quantum Chemistry Program Exchange QCPE265). Samples of Mn/SOD for EPR spectroscopy were prepared by addition the equivalent amount of dithionite under argon. Vanadium-substituted enzyme was prepared for EPR spectroscopy by addition of dithionite (to 25 mM) and incubation under argon at room temperature for 30 min. Metal ion analyses were performed using a Varian Instruments SpectraAA atomic absorption spectrometer equipped with a GTA 96 graphite furnace for high sensitivity metal determinations.

**RESULTS**

Amplification of the sodA structural gene for T. thermophilus HB8 MnSOD was achieved using primers backtranslated from the N-terminal and C-terminal protein sequence data (27) using the established pattern of Thermus codon usage (28). The 3’-primer includes an efficient tetranucleotide termination sequence (TAAT) suitable for high level expression of recombinant protein in E. coli (32). This primer set allowed selective amplification of the SODA structural gene from purified T. thermophilus HB8 genomic DNA as the starting point for vector construction. Because both 5’ and 3’ end sequences are determined by synthetic oligonucleotide primers based on protein rather than nucleotide sequence data specifically designed to facilitate subcloning and expression, we describe the product as a semisynthetic gene. The nucleotide sequence of the amplified product is identical to that reported for T. thermophilus HB27 (GenBank™ accession number 3062846) and preserves 95% identity with the SodA gene from Thermus aquaticus YT-1 (GenBank™ accession number 217183), implying identity and near-identity of the corresponding conceptual protein sequences.

For expression in E. coli, the semisynthetic gene was transferred into a vector (pQGB1) derived from the oxygen-regulated expression vector pGB1 (29) by elimination of superfluous restriction sites and modification of the linker sequence by site-directed mutagenesis. The alteration allows insertion of any...
isolated as metal-free apo-protein, even when the culture medium does not lead to reactivation (Fig. 3, top panel). The time course of reactivation, traced out a sigmoidal activation profile spanning a 20° C transition range with an effective midpoint temperature of 62 °C (335 K). However, the full activity of the enzyme can be restored by incubating the protein in the presence of Mn(II) at 45 °C, yet above that temperature there is an abrupt increase in the reactivation rate, reflecting a change toward more axial electronic symmetry at higher pH (from rhombicity E/D = 0.22, pH 5.0, to E/D = 0.1, pH 8.0). Aside from a majority signal near g = 4.3 associated with adventitiously bound Fe(III), the spectra are clean, reflecting a unique coordination environment for the metal ion. Azide binding results in a complex exhibiting nearly maximal rhombicity (E/D = 0.28) (Fig. 7C).

The apo-protein will also bind vanadate present in solution during the heat treatment, with approximately the same occupancy as observed for manganese reconstitution (0.6 g atom vanadate/active site) (Table I, sample 9). The vanadium-substituted enzyme is colorless, lacking visible absorption and EPR spectra. However, following treatment with 25 mM dithionite, a V(IV) EPR spectrum is observed (Fig. 8, inset). The vanadium content of iron-substituted, reconstituted TTSOD is relatively high, with approximately 80% of the sites occupied (Table I, samples 4 and 7). The manganese content of a fraction of rTTSOD containing relatively high Mn (Table I, sample 5) is unchanged during reconstitution with iron and the extent of conversion to the iron complex is proportional lower for that sample (Table I, sample 6), indicating that iron does not displace manganese already bound to the protein and that the two metal ions compete for the same binding site. The optical spectrum of the iron-substituted TTSOD at low pH (Fig. 6, spectrum 1) exhibits an intense nearly UV absorption (ε350 = 1500 M⁻¹ cm⁻¹), which is sensitive to pH, bleaching as the pH of the solution is raised above 6 (Fig. 6, spectra 1–9), with an effective pHKₐ = 6.8 (Fig. 6, inset). Azide binds to the Fe(III) enzyme (Kₐ = 0.1 mM) producing a complex with optical spectra (data not shown) similar to those observed for the corresponding derivative of iron-substituted E. coli MnSOD (ε390 = 1300 M⁻¹ cm⁻¹) (33, 34). The EPR spectrum of the Fe(III) complex at low pH (Fig. 7A) and high pH (Fig. 7B) are also distinct, reflecting a change toward more axial electronic symmetry at higher pH (from rhombicity E/D = 0.22, pH 5.0, to E/D = 0.1, pH 8.0). Azide from a minority signal near g = 4.3 associated with adventitiously bound Fe(III), the spectra are clean, reflecting a unique coordination environment for the metal ion. Azide binding results in a complex exhibiting nearly maximal rhombicity (E/D = 0.28) (Fig. 7C).

DISCUSSION

The recent rapid expansion of biotechnological applications of thermostable enzymes has stimulated widespread interest in the biochemistry and molecular biology of thermophilic and hyperthermophilic organisms (35). However, the extreme conditions required for growth and maintenance of these organisms presents a problem for large scale isolation of proteins from their native sources (36). Although cloning may permit high level expression of the recombinant thermophilic protein in a mesophilic host, the protein is sometimes inactive at ambient temperatures, regaining activity on heating to near the physiological growth temperature of the source organism (35, 36). The requirement for a degree of thermal excitation for biological function implies a dynamical dimension to the archi-
The architecture of thermophilic proteins that is only beginning to be explored.

This type of temperature-dependent behavior can be modeled as a progressive melting of protein structure as the temperature is raised. A local melting model has been applied to understanding a surprising temperature dependence of active site ligand interactions in both mesophilic and thermophilic MnSODs (37) and a thermophilic shift in enzyme properties (23). We have cloned the MnSOD from *T. thermophilus* to investigate the molecular origins of these effects through recombinant expression and mutagenesis studies. Although the primary structure of the *T. thermophilus* HB8 MnSOD has been known for more than a decade (27) and the three-dimensional structure of the enzyme has been solved (15), the SodA structural gene has not been identified in this organism. Using the published protein sequence, we have isolated the coding sequence from genomic DNA and constructed a vector for high level expression in *E. coli*.

The recombinant protein is soluble but is nearly colorless and separates during chromatography into fractions with SOD activity ranging from low to insignificant. The fractions exhibiting low activity represent a small fraction of the protein containing substoichiometric amounts of the catalytic manganese ion, whereas the inactive fractions appear to be devoid of metals and specifically lack significant amounts of manganese, iron, or zinc. The lack of metal in recombinant TTSOD contrasts with the metal binding avidity exhibited by the homologous *E. coli* MnSOD apo-protein, which makes that form difficult to isolate (24, 25). Similar results have been reported for the mesophilic MnSOD from *Deinococcus radiodurans* (38). A previous study describing the *T. aquaticus* MnSOD apo-protein reported denaturation is required for reconstitution at 4 °C (39). This behavior was later interpreted, in light of results for the *E. coli* enzyme, as a consequence of competitive complexation by a nonchromophoric metal (25). However, we find that the most common nonchromophoric metalloprotein contaminant (zinc) is not present in our preparations and that bound metal ions do not exchange with ions in solution during reconstitution, indicating that the recombinant protein is expressed without a metal cofactor.

A small fraction of the recombinant enzyme (less than 10% of all active sites) does contain manganese when isolated even though the protein, once folded, is quite resistant to metalla
tion (Fig. 3). This fraction may reflect the continuous, slow

| Samples (source) | Metal content |
|-----------------|---------------|
|                 | Manganese     | Iron | Vanadate | Zinc |
| 1 Native TTSOD (*Thermus*) | 0.50 |       |       | 0.02 |
| 2 rTTSOD (*E. coli*) | 0.03 | 0.02 |       |     |
| 3 2, after manganese reconstitution | 0.60 |       |       |     |
| 4 2, after iron reconstitution | 0.03 | 0.80 |       |     |
| 5 rTTSOD (*E. coli*) | 0.27 | 0.05 |       |     |
| 6 5, after iron reconstitution | 0.25 | 0.65 |       |     |
| 7 rTTSOD (*E. coli*) | 0.00 | 0.01 | 0.80  |     |
| 8 7, after iron reconstitution | 0.80 |       |       |     |
| 9 7, after vanadate reconstitution |       |       | 0.60  |     |

**Fig. 3. Reactivation of recombinant *Thermus* MnSOD.** *Top panel,* solid line, time course for reactivation of apo-protein by heating in the presence of MnCl₂. The experimental conditions were 0.5 mM apo-protein, 10 mM MnCl₂, 20 mM MOPS, pH 7.0, heated at 65 °C for the indicated time before cooling to 4 °C. An exponential fit to the data is shown. *Dotted line,* apo-protein pretreated by heating at 65 °C for the indicated time followed by cooling to 4 °C and incubation with 10 mM MnCl₂, 20 mM MOPS, pH 7.0, for 2 h at ambient temperature. Aliquots of these reactions were assayed for SOD activity as described under “Materials and Methods.” *Bottom panel,* dependence of the reactivation on Mn(II) concentration. The experimental conditions were 0.5 mM apo-protein with variable MnCl₂, 20 mM MOPS, pH 7.0, heated at 65 °C for 20 min before cooling to 4 °C. A linear fit to the data is shown.

**Fig. 4. Temperature dependence of reactivation of recombinant *Thermus* MnSOD with MnCl₂.** The experimental conditions were 0.5 mM apo-protein, 10 mM MnCl₂, 20 mM MOPS, pH 7.0, heated at the indicated temperature for 30 min. Aliquots of these reactions were assayed for SOD activity.
binding of manganese during growth of the bacterial culture or might arise from metal binding during the initial folding of newly synthesized protein, when the metal binding site may be more accessible. The unexpected resistance of the majority of the recombinant apo-protein to metal binding could be the result of misfolding during expression far below the physiological growth temperature for Thermus leading to the protein being trapped in a non-native state. However, the recombinant TTSOD is soluble and correctly assembles to form a tetrameric complex essentially identical to that found for the native TT-SOD (Fig. 2). Also, if heating serves to anneal misfolded protein, allowing it to attain its native structure, metallation of apoTTSOD should be facilitated by prior heat treatment in the absence of metal ions. However, we find that activation of the apo-enzyme requires the metal ion to be present during the heat step (Fig. 3). We thus conclude that in vitro metallation of TTSOD involves a reversible transition in the structure of the folded protein that makes the metal binding site accessible to metal ions in solution at higher temperatures.

The temperature dependence of metallation (Fig. 4) indicates that this transition occurs near 60 °C for the isolated apo-

![Graph](image1)

**Fig. 5.** Electronic spectra for manganese-reconstituted recombinant Thermus MnSOD. Top panel, optical absorption spectra for native TTSOD isolated from T. thermophilus (dotted line) and recombinant TTSOD reconstituted with manganese (solid line). (0.9 mmol active sites in 50 mM K$_2$HPO$_4$ buffer, pH 7.0). Bottom panel, EPR spectra for native TTSOD isolated from T. thermophilus (upper spectrum) and recombinant TTSOD reconstituted with manganese (lower spectrum). (1.5 mmol active sites in 50 mM K$_2$HPO$_4$ buffer, pH 7.0). The instrumental parameters were: temperature, 9 K; microwave frequency, 9.34 GHz; microwave power, 2 mW; modulation amplitude, 10 G; gain, $1.25 \times 10^4$.

![Graph](image2)

**Fig. 6.** pH dependence of the optical spectrum of Fe-substituted Thermus MnSOD. Reconstituted protein (0.3 mmol active site) was prepared in 100 mM potassium phosphate at the following pH levels: 5.1 (spectrum 1), 5.73 (spectrum 2), 6.2 (spectrum 3), 6.7 (spectrum 4), 6.95 (spectrum 5), 7.17 (spectrum 6), 7.6 (spectrum 7), and 8.04 (spectrum 8) or 100 mM CAPS-KOH at pH 9.16 (spectrum 9). All samples were adjusted to ionic strength $\mu = 1$ with Na$_2$SO$_4$. The inset shows the absorption data fitted by a theoretical titration curve.

![Graph](image3)

**Fig. 7.** EPR spectra of iron-substituted recombinant Thermus MnSOD. Iron-reconstituted TTSOD (0.5 mmol active sites, in 50 mM KH$_2$PO$_4$ buffer) at pH 5.0 (A), 8.0 (B), or 5.0 (C) in the presence of 10 mM NaN$_3$ is shown. The instrumental parameters were: temperature, 10 K; microwave frequency, 9.34 GHz, microwave power, 2 mW; modulation amplitude, 10 G; gain, $1 \times 10^4$ (A), $5 \times 10^4$ (B), and $1.25 \times 10^4$ (C).

The temperature dependence of metallation indicates that this transition occurs near 60 °C for the isolated apo-

![Equation](image4)

**(Eq. 2)**

$$K_{eq} \left( \frac{\text{Apo}_{i\text{r}}}{\text{Mn(II)}} \right) \downarrow \text{(slow)} \quad \text{Apo}_{i\text{r}} \quad \text{HoloTTSOD} \downarrow \text{(fast)}$$
Thermally Triggered Metal Binding

The model illustrates trigonal coordination of the oxovanadium(IV) [VO$^+$] ion, accommodating the second oxo ligand of dioxovanadium(V) (gray) in the substrate funnel.

reorganization of the protein during metal binding, either within the solvent/substrate access channel or (perhaps more likely) at the domain interface, where a relatively small displacement between N- and C-terminal domains exposes all four metal binding residues. Formation of a metal complex results in four covalent cross-links between the domains, blocking metal exchange.

Thermally triggered metal binding is a convenient method for preparing metal-substituted derivatives TTSOD, including manganese, iron, and vanadium forms. Incorporation of iron into the active site results in a complex that lacks SOD activity at neutral pH. The optical spectrum of the Fe derivative is pH-dependent (Fig. 6), and responds to variation in pH in the same manner as iron-substituted E. coli MnSOD (33, 34). For that enzyme, crystallographic data demonstrate that the high pH form contains an additional solvent ligand (most probably hydroxide) at the base of the substrate funnel, blocking access to the metal center (40). Azide binding converts the enzyme to a complex exhibiting an intense near UV absorption (azide-to-Fe(III) ligand-to-metal charge transfer) associated with the coordinated anion and a change in ground state parameters (Fig. 7) that reflect perturbation by the exogenous ligand.

Although a variety of non-native metal ions (nickel, cobalt, copper, zinc, etc.) have been incorporated into E. coli MnSOD active site using the conventional denaturation-reconstitution approach (41), a complex containing vanadium has not yet been reported. A vanadium complex is of interest because of the similar metal environments in MnSOD and vanadium haloperoxidases (42–45). The vanadium complex of TTSOD prepared by thermally triggered reconstitution has the same metal stoichiometry as the native Mn ion, and vanadium appears to associate with a unique binding site that is likely the enzyme active site. Fig. 9 shows how the EPR-silent dioxovanadium(V) ion ($d^2$, $S = 0$) could be accommodated in the TTSOD active site, preserving the preferred cis-dioxo coordination by direct- ing one o xo group into the base of the substrate funnel and the other into the solvent pocket that normally binds the proton-coupling water (hydroxide). The EPR spectrum of the reduced V(IV) complex ($d^3$, $S = \frac{3}{2}$) (Fig. 8) is similar to the corresponding complex of vanadium bromoperoxidase, for which crystallographic studies (44, 45) have demonstrated a nearly exact trigonal bipyramidal coordination geometry with trans-axial oxo and histidine ligands. However, vanadium-substituted TTSOD lacks haloperoxidase activity, perhaps because of restricted access to the metal center in that complex interferes with peroxide activation.

It is difficult to determine the generality of the requirement for heating to produce the functional form of a thermophilic enzyme because heat treatment is widely used in the purification of recombinant thermophilic proteins, taking advantage of their unusual stability as an aid to purification. For example, the recombinant hyperthermophilic FeSOD from Aquifex pyrophilus (46) is isolated (following heat treatment of the crude E. coli lysate at 80 °C for 1 h) with 0.75 atom iron/active site.

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Here Apo$_{\text{TTSOD}}$ and Apo$_{\text{HoloTTSOD}}$ represent, respectively, low and high temperature forms of the apo-protein. Analyzing the data in terms of the Van’t Hoff equation (Equation 3) provides an estimate of the thermochemical parameters associated with the transition.

$$-\ln K = \frac{\Delta H}{RT} - \frac{\Delta S}{R}$$

(Eq. 3)

The transition curve shown in Fig. 4, representing the theoretical fit (with Van’t Hoff enthalpy, $\Delta H_{\text{TH}} = 54$ kcal/mol, and entropy, $\Delta S_{\text{TH}} = 160$ cal/mol·K), occurs over a narrow temperature range, implying a relatively cooperative transition that could be described as a melting of protein structure. Thus, an essential (metal-binding) function of the protein depends on the excitation of specific molecular motions that are active only near the physiological growth temperature for Thermus. Similar behavior has been implicated in other thermal processes in Thermus with independent reactivity of the four TTSOD active sites (15). The lack of exchange of bound metals in the crystal structure is interpreted in terms of full occupancy of the active site, preserving the preferred cis-dioxo coordination by direct- ing one o xo group into the base of the substrate funnel and the other into the solvent pocket that normally binds the proton-coupling water (hydroxide). The EPR spectrum of the reduced V(IV) complex ($d^3$, $S = \frac{3}{2}$) (Fig. 8) is similar to the corresponding complex of vanadium bromoperoxidase, for which crystallographic studies (44, 45) have demonstrated a nearly exact trigonal bipyramidal coordination geometry with trans-axial oxo and histidine ligands. However, vanadium-substituted TTSOD lacks haloperoxidase activity, perhaps because of restricted access to the metal center in that complex interferes with peroxide activation.

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**Fig. 8.** EPR spectra of vanadium-substituted recombinant Thermus MnSOD. Solid line, experimental spectrum for V-reconstituted TTSOD (5 mM active sites, in 50 mM K$_2$HPO$_4$ buffer, pH 7.0) following reduction by 25 mM Na$_2$S$_2$O$_6$ for 30 min at room temperature. The instrumental parameters were: temperature, 8 K; microwave frequency, 9.54 GHz; microwave power, 10 $\mu$W; modulation amplitude, 10 G; gain, 5 $\times$ 10$^4$. Dotted line, simulated spectrum ($^5$V, $I = \frac{3}{2}$, $g = 1.9465$, $a = 183$ G, $a = 63$ G, $\Gamma_{FWHM} = 14$ G, $\Gamma_{FWHM} = 16$ G).

**Fig. 9.** Proposed structure for vanadium complexes of manganese superoxide dismutase. The model illustrates trigonal coordination of the oxovanadium(IV) [VO$^+$] ion, accommodating the second oxo ligand of dioxovanadium(V) (gray) in the substrate funnel.
However, the apo-protein, prepared by denaturation of the holoenzyme and chelation of the active site metal ion, appears to be stable against metal binding in non-denaturing solution (47), just as we have observed for Thermus MnSOD. In another case, heat treatment of E. coli cell extracts results in a 10-fold increase in specific activity for recombinant T. thermophilus NADH oxidase (a flavoenzyme) (48), but the origin of this effect has not been investigated.

The metallation rate is surprisingly slow on the time scale of microbial growth. Even at the physiological growth temperature, full metallation of the folded protein requires 30 min or more at elevated concentrations of Mn(II) (10 mM), and the rate at physiological Mn(II) concentrations will be even slower. It is possible that a metallochaperone (49–51) delivers metal to the active site in vivo, although no manganese chaperone has yet been identified in any organism, and the relatively nonspecific in vivo metallation of MnSOD in E. coli (25) would appear to be more consistent with random metal binding in that organism. Also, the high degree of structural homology between E. coli and Thermus MnSODs would suggest that both would be recognized by the endogenous chaperone in the expression host, whereas very little metallation of TTSOD is actually observed. Therefore, the intrinsic factors that facilitate metal binding.

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