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A Single Outer Sphere Mutation Stabilizes apo-Mn Superoxide Dismutase by 35 °C and Disfavors Mn Binding

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Abstract

The catalytic active site of Mn-specific SOD (MnSOD) is organized around a redox-active Mn ion. The most highly-conserved difference between MnSODs and the homologous FeSODs is the origin of a Gln in the second coordination sphere. In MnSODs it derives from the C-terminal domain whereas in FeSODs it derives from the N-terminal domain, yet its side chain occupies almost superimposable positions in the two types of SODs' active sites. Mutation of this Gln69 to Glu in *E. coli* FeSOD increased the Fe^{3+/2+} reduction midpoint potential by > 0.6 V without disrupting the structure or Fe binding [E. Yikilmaz, D. W. Rodgers and A.-F. Miller (2006) *Biochemistry* 45(4) 1151–1161]. We now describe the analogous Q146E mutant of MnSOD, explaining its low Mn content in terms increased stability of the apo-Mn protein. In 0.8 M guanidinium HCl, the Q146E-apoMnSOD displays an apparent melting midpoint temperature (T_m) 35 °C *higher* than that of WT-apoMnSOD, whereas the T_m of WT-holoMnSOD is only 20 °C higher than that of WT-apoMnSOD. In contrast, the T_m attributed to Q146E-holoMnSOD is 40 °C *lower* than that of Q146E-apoMnSOD. Thus our data refute the notion that the WT residues optimize structural stability of the protein, being instead consistent with conservation on the basis of enzyme function and therefore ability to bind metal ion. We propose that the WT-MnSOD protein conserves a destabilizing amino acid at position 146 as part of a strategy for favoring metal ion binding.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of both authors. Both authors have given approval to the final version of the manuscript.

SUPPORTING INFORMATION

A table of thermodynamic parameters from fits of the major transitions observed, a ribbon diagram of the MnSOD monomer, a figure of CD spectra, three figures of CD-monitored melting curves. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Protein Stability; Metal Ion Binding; Hydrogen Bond Network; Superoxide Dismutase

Introduction

Four different types of superoxide dismutases (SODs) are distinguished based on the identity of their redox-active metal ion cofactor: the manganese-specific SODs (MnSODs), the iron-specific SODs (FeSODs), the copper and zinc-containing SODs (CuZnSODs) and the nickel-containing SODs (NiSODs).¹ Although encoded by different genes in *Escherichia coli*, FeSOD and MnSOD are believed to have evolved from a common ancestor because they display homologous structures and amino acid sequences.^{1–5} Amino acid conservation is particularly strong in the active site with all four ligands to the metal ion being identically conserved in all FeSODs and MnSODs described so far.^{1, 2} Thus it is not surprising that MnSODs can be prepared with Fe bound instead of Mn^{6–9}, and *vice versa*.^{10, 11} In each case native-like coordination geometry is retained,^{12–14} with the major difference being Fe-substituted MnSOD's higher affinity for small anions including OH[–], consistent with Fe³⁺'s higher Lewis acidity than Mn³⁺.^{8, 12, 14–18} While a few so-called 'cambialistic' Fe/MnSODs have been identified that display activity under physiological conditions regardless of whether Fe or Mn is bound,^{19, 20} the canonical Fe- and MnSODs each require that their cognate metal ion be bound in order to perform with optimal enzymatic activity.^{7, 21, 22} Thus FeSODs and MnSODs provide a unique vantage point for understanding the crucial interface between protein and metal ion, wherein the protein tunes the metal ion reactivity and the metal ion gives rise to the enzyme's signature activity.

FeSODs and MnSODs employ the same coordination sphere: three histidines (His26, His81 and His171), an aspartate (Asp167, *E. coli* MnSOD numbering), and a coordinated solvent molecule (interpreted as a water or hydroxide depending on whether the metal ion is Mn²⁺ or Mn³⁺, respectively).^{23–25} The coordinated solvent molecule is central to an active site H-bond network that connects it to bulk solvent via a second-sphere glutamine (Gln146 in *E. coli* MnSOD or Gln69 in *E. coli* FeSOD) which H-bonds with the hydroxyl of conserved Tyr34 which in turn H-bonds with a solvent molecule in the channel connecting the active site to bulk solvent (Figure 1).^{15, 26–30} The most highly-conserved difference between FeSODs and MnSODs is the origin of the Gln residue (or in some cases His)^{1, 3} that H-bonds to coordinated solvent.^{5, 31–33} MnSODs contribute the conserved Gln146 from a position between a beta strands in the C-terminal domain (Figure 1) whereas FeSODs contribute Gln69 from an alpha helix in the N-terminal domain (Supplemental Figure S1).^{5, 31–33}

The coordinated solvent participates in enzyme turnover by acquiring a proton in conjunction with Mn reduction (1a),^{25, 36} then contributing a proton essential to reaction (1b) in which superoxide becomes reduced to peroxide and the metal ion gets reoxidized.^{25, 37}

where E stands for the MnSOD protein, Mn indicates the active site Mn ion and the OH⁻ or H₂O indicates the state of the solvent molecule coordinated to it.¹ Additional formation and decay of an inhibited complex becomes significant at higher superoxide concentrations.^{38, 39}

The capacity of the enzyme to both oxidize and reduce the same substrate places lower and upper bounds on its reduction midpoint potential, E^o,⁴⁰ but the E^o of hexaaquo Mn^{3+/2+} differs from that of Fe^{3+/2+} by some 0.7 V. Hence the proteins of *E. coli* MnSOD and FeSOD have been shown to exert very different redox tuning on their respective metal ions to achieve similar enzyme E^os, and the relative inactivity of metal-substituted SODs has been explained on the basis of E^os that are too-high (Mn-substituted FeSOD) or too-low (Fe-substituted MnSOD).^{6, 11, 41} Consistent with a correlation between the different redox tuning and the different placement of the active site Gln, mutation of FeSOD's Gln69 resulted in large changes in E^o, with the Q69H and Q69E mutant FeSODs displaying E^os elevated by 250 mV and more than 600 mV respectively.^{42, 43} The active site Gln was proposed to exert its effect on the E^o by altering the energies associated with redox-coupled proton transfers in the active site.^{41, 44} This is consistent with the fact that the H-bond with Gln connects the coordinated solvent to the SOD protein beyond the active site, and thus enables the protein to modulate the coordinated solvent's pK_a.

In human and *E. coli* MnSOD the corresponding Gln146 has been replaced by several different residues. Replacements with His and Leu decreased activity to 8% and 5%, respectively, and altered the metal ion spectral signatures and substrate analog (azide) binding in *E. coli* MnSOD.²⁶ In human MnSOD, mutation of the Gln also had a large effect on catalytic activity, the contribution of the inhibited complex formed in parallel with turnover, and the major unfolding temperature.^{27, 45} The effects of mutating the Gln to Glu have been difficult to study because the resulting protein was produced as the apo-Mn protein: Q146E-apoMnSOD, for *E. coli* and human MnSOD.^{26, 27} Alterations in metal ion binding were also observed upon mutation to Glu among mycobacterial SODs, where metal ion selectivity is less differentiated.⁴⁶ The Fe-specific *M. tuberculosis* SOD has His at the position corresponding to Gln146, but mutation of this His to Glu caused the protein to acquire some Mn instead of Fe, and to display Mn-supported activity.⁴⁶ Thus the conserved Gln 146 appears to be important for metal ion binding. This is consistent with the redox

tuning activity of the corresponding Gln of FeSOD, because the reduction potential represents the difference between the free energy of binding the 2+ metal ion vs. that of binding the 3+ ion. However absence of bound Mn could also represent failure of co- or post-translational Mn insertion steps in maturation of the enzyme.⁴⁷

To learn whether the predominance of the apo-Mn form of the Q146E variant represents the result of thermodynamic destabilization of the holo-Mn form or stabilization of the apo-Mn form, we have compared the thermal stabilities of both the apo proteins, Q146E-apoMnSOD and WT-apoMnSOD, as well as those of the corresponding metal containing forms using far-UV circular dichroism (CD) to quantify secondary structure as a function of temperature. Our data reveal that the Q146E substitution results in an apo-Mn protein that is much more stable than the WT-apoMnSOD, and moreover confers greater stabilization than does Mn binding. We find that Mn or Fe binding greatly stabilizes the WT-SOD but not the Q146E variant. Therefore we propose that the conservation of Gln over Glu at position 146 represents selection for high-affinity acquisition of metal ions by MnSOD, at the cost of the stability of the protein fold. The natural conservation of the destabilizing amino acid, Gln, at position 146 is superficially counterintuitive but can be understood as part of a strategy favoring metal ion binding and thereby function.

Materials and Methods

Bacterial strain and culture

The double deletion *E. coli* strain Ox326-A (sodAsodB) was the generous gift of Prof. H. Steinman, and is unable to produce either FeSOD or MnSOD.⁴⁸ The WT-MnSOD gene from *E. coli* on the pET21 derivative pAK0⁴⁹ was transformed into the Ox326-A cell line which was then stored as a 20% glycerol stock in *Luria-Bertani* (LB) medium with 50 µg/mL kanamycin and 50 µg/mL ampicillin at -80 °C. All growth media used were supplemented with 50 µg/mL kanamycin and 50 µg/mL ampicillin in order to select for the pAK0/Ox326-A strain.

2 L cultures of pAK0/OX326-A in M9 medium were cultured at 37 °C, shaking at 220 rpm. Once the optical density reached 0.7 – 0.8, Mn nitrilotriacetate (Mn-NTA) and IPTG (isopropyl thiogalactopyranoside) were added to final concentrations of 10 µM and 50 µg/mL, respectively, to induce MnSOD overexpression. Cultures were grown for another 12–16 hours at 37 °C with continued shaking at 220 rpm until the cell density reached an OD600 of ~ 2.3, at which point cells were harvested by centrifugation for 20 min at 8000 rpm 4 °C.

Protein purification

Because considerable quantities of MnSOD were exported into the growth medium during extended bacterial culture, a simplified purification method directed at the secreted protein was developed. After the cell pellet had been harvested by centrifugation, the supernatant was concentrated using a pressurized membrane filtration assembly (Advantec MFS Inc.) with a Millipore 30 kDa NMWL (Normal Molecular Weight Limit) cutoff membrane (ultracel regenerated cellulose ultrafiltration discs) by applying air pressure at ~ 50 psi until

the volume of the supernatant was reduced from ~2 L to ~50 mL. Remaining cell debris and precipitate in the retentate were separated from soluble proteins by centrifugation at 63,000 x g for 30 min at 4 °C. Further concentration to 15–20 mL was achieved using an Amicon® ultra-30 centrifugal filter device, by centrifuging at 4000 x g at 4 °C for 20 min intervals. EDTA was added to the retentate to a final concentration of 1 mM to chelate excess metal ions and the concentrated protein solution was then subjected to dialysis at 4 °C overnight against 12 L of 5 mM potassium phosphate, pH 7.4 to remove the EDTA and complexed metal ions, and to effect transfer to the pH 7.4 phosphate buffer. The protein solution was further concentrated to 3–5 mL using an Amicon® ultra-30 centrifugal filter device with a 30,000 NMWL. Concentrated protein solution was loaded on a sephadex G-25 desalting column pre-equilibrated with 2–3 column volumes of 5 mM potassium phosphate buffer, pH 7.4. The protein was eluted with 5 mM potassium phosphate buffer, pH 7.4.

Fractions were characterized with respect to absorbance at 280 nm. Protein concentrations were determined using extinction coefficients of 86600 M⁻¹ cm⁻¹, 91900 M⁻¹ cm⁻¹, 89500 M⁻¹ cm⁻¹ and 83200 M⁻¹ cm⁻¹ per dimer for WT-holoMnSOD, WT Fe-substituted MnSOD (WT-FesubMnSOD), WT-apoMnSOD and Q146E-apoMnSOD, respectively, depending which metal ion (if any) had been used to supplement the growth medium.^{21, 50} The ratio of absorbance at 260 nm to that at 280 nm was used to assess any contamination by nucleic acids.⁵¹ The peak fractions were further analyzed by SDS-PAGE to learn whether the protein present was consistent with MnSOD and to assess purity. Optical spectra were collected for comparison with the known signatures of MnSOD and WT-FesubMnSOD. Fractions containing MnSOD but no other proteins were pooled, concentrated to ~1 mM using an Amicon® ultra -30 centrifugal filter device and stored at 4 °C in the refrigerator.

Activity assays

Superoxide dismutase specific activity was assayed by the method of McCord and Fridovich.⁵² For qualitative assessment of activity and attribution of activity to specific forms of MnSOD, activity was visualized in non-denaturing polyacrylamide electrophoretic gels by the method of Beauchamp and Fridovich.⁵³ Nondenaturing PAGE was carried out with 4 – 12 % polyacrylamide gels in a Tris-HCl/glycine buffer at pH 8.3. Approximately 50 µg of protein was loaded into each lane of the gel and electrophoresed for 2–3 hours at 100 V. The assay was conducted by soaking the gel in 0.3 mM NBT (nitroblue tetrazolium) for 30 min and then in 28 µM riboflavin for 30 min in darkness before irradiating with UV light for 20–30 min. In this assay, SOD activity manifests itself as a local absence of blue formazan produced by the reaction of photochemically-generated superoxide with NBT. As a control, to permit assessment of the relative amounts of different forms of MnSOD, a duplicate gel was stained using Coomassie brilliant blue R250 to reveal the locations of all protein bands.

Metal ion content determination

Metal ion content was determined by ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometry) at the Environmental Research and Testing Lab at the University of Kentucky. In order to account for contamination from the water as well as human error, triplicate samples of each protein, a water blank and a series of duplicate standardized Mn

solutions of different concentrations were prepared in parallel. 1 ml 0.1 M Nitric acid (HNO_3) was slowly added to each 1 ml protein sample (a 1:1 volume ratio). After incubation on ice for 10–15 min, samples were centrifuged at 13,000 rpm for 2 min to eliminate precipitated protein. The supernatants were diluted by addition of 8 ml of distilled water before being fed into the instrument. The light emitted by excited Mn atoms was detected at the characteristic wavelengths of 257.61 nm, 259.37 nm and 260.568 nm by the UV-visible spectrometer. The light emitted by Fe atoms was similarly detected at the characteristic wavelengths of 238.204 nm, 259.94 nm and 261.187 nm. A standard curve was constructed based on the duplicate standard samples and used to determine the concentrations of Mn and Fe in the protein samples.

Metal ion removal and reconstitution

A solution of Q146E-apoMnSOD or WT-SOD was dialyzed against 3.5 M guanidinium hydrochloride (GdmCl), 20 mM Tris-HCl, and 10 mM EDTA at pH 3.1 overnight at 4 °C until the pH inside the dialysis bag was pH 3.1. Freshly prepared dialysis buffer (2.5 M GdmCl and 20 mM Tris-HCl at pH 8.0) was deoxygenated by sparging with nitrogen N_2 for an hour, and then used to dialyze the solution of MnSOD protein for another 8 h to remove EDTA and chelated metal ions. MnCl_2 (or FeCl_2) was then added into the dialysis bag to produce a final concentration of 1 mM. The dialysis bag containing unfolded Q146E-apoMnSOD and MnCl_2 (or FeCl_2) was transferred to fresh dialysis buffer of 25 mM Tris-HCl pH 7.5, which has been deoxygenated by sparging with nitrogen N_2 for at least 15 min, and incubated at 45 °C for 30 min (a range of times were tried, 30 min proved best). To reduce Fe^{3+} and increase the solubility of Fe ion, 5 mM ascorbic acid was added to the dialysis buffer when Fe was used. SOD protein was allowed to refold by dialyzing against 25 mM K_2HPO_4 , pH 7.4 for 8–12 h at 4 °C, during which time the dialysis solution was kept anaerobic by continuous sparging with N_2 . EDTA was then added to the protein to a final concentration of 1 mM to chelate excess Fe ions. After 20–30 min incubation, the protein was separated from the EDTA by chromatography over DEAE-Sephadex G-25 (5 cm $\times$ 30 cm) pre-equilibrated and eluted with 5 mM phosphate pH 7.4.

Characterization of protein by circular dichroism

Circular dichroism (CD) was measured using a Jasco J-810 circular dichroism spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier temperature controller and multicell holder. Wavelength scans were performed between 180 and 250 nm in 5 mM potassium phosphate buffer pH 7.4, with 6 μM protein samples in 1-mm quartz cuvettes at room temperature. The bandwidth was set to 1 nm and at least 4 scans were averaged per spectrum. A baseline was obtained by collecting the CD spectrum of the buffer solution alone. Data were analyzed using the SELCON3 component of the CDpro software package in conjunction with the SP29 protein data base.⁵⁴ For samples in 0.8 M GdmCl (below), it was necessary to limit scans to 250 to 210 nm because GdmCl absorbs strongly at wavelengths < 205 nm. Scans were recorded at a scanning rate of 100 nm/min with a wavelength step of 0.2 nm.

CD-monitored thermal denaturation scans

To characterize protein stability, ellipticity at 222 nm (θ_{222}) was measured as a function of temperature from 20 °C to 100 °C. To mitigate protein aggregation and attendant irreversibility, the buffer contained 0.8 M GdmCl in addition to 5 mM potassium phosphate (pH 7.4).⁵⁵ The temperature of the protein solution was controlled to an accuracy of 0.1 °C using a Peltier thermoelectric device coupled to the cell holder. The temperature was increased at a speed of 1 °C /min and data points were recorded at 5 °C or 1 °C temperature intervals. Due to the duration of the experiments and the nature of the apparatus, the holoMnSOD samples generally contained a mixture of Mn³⁺ and Mn²⁺ containing sites, even when the experiment was begun with homogeneous material.

The apparent denaturation midpoint temperature (T_m) describing each of the unfolding events was extracted from the high-resolution data sets using CDpro to fit the data to the two-state model with unconstrained linear baselines above and below the transition, using least-squared minimization. Van't Hoff analysis was implemented by CDpro to extract the enthalpy and entropy of unfolding at the T_m for each transition (ΔH and ΔS).⁵⁶

Reversibility assay by Circular Dichroism

Protein samples were warmed to a temperature approaching their respective T_m s (70 °C for WT-holoMnSOD, 60 °C for WT-apoMnSOD, and 85 °C for Q146E-apoMnSOD), in CD sample cells in a Peltier multicell holder. Protein samples in 5 mM potassium phosphate buffer (pH 7.4) containing 0.8 M GdmCl with or without 100 μ M Mn were held at the foregoing temperature for 30 min and then cooled gradually to 20 °C at a rate of 1 °C / min while measuring θ_{222} every 5 °C. Samples were then held in a refrigerator at 4 °C for 8 – 12 h for consistency with samples that had not been warmed and re-cooled. Then the warmed and re-cooled samples were characterized again with respect to temperature-induced unfolding, as above.

Metal ion uptake assay

Fluorimetry was used to monitor Co²⁺ uptake by WT-apoMnSOD using a Cary spectrofluorimeter equipped with a Peltier cell holder and temperature controller.⁵⁷ A 6 mM stock solution of CoCl₂ and a 50 μ g/ml sample of apoMnSOD in 20 mM MOPS at pH 7.0 were pre-incubated at 45 °C separately for ~ 5 min. Co²⁺ solution was then added to the protein sample to a final concentration of 10 μ M, at time-point zero. Protein tryptophan fluorescence was monitored at an emission wavelength of 355 nm based on excitation at 280 nm with 5 nm excitation and emission slit widths. Emission intensity was recorded every second for a total of 30 min.

Results

Catalytic activity and metal ion content

Q146E-SOD was isolated predominantly as Q146E-apoMnSOD (0.5 % Mn per site) as also noted in prior work,^{26, 27} regardless of whether or not Mn was provided as a supplement to the bacterial growth medium (Table 1). This could not be explained by occupancy of the active site by Zn,²¹ as ICP-OES revealed a bound Zn stoichiometry of 0.03 per subunit.

Absence of bound metal ion suffices to explain the very low catalytic activity of the as-isolated Q146E-SOD (Table 1), but to learn whether the Q146E substitution also affects activity, we reconstituted Q146E-apoMnSOD with each of Mn and Fe using the method of Yamakura as modified by Vance and Miller.^{6, 58} The metal ion content of Q146E-SOD only increased to 13 % when reconstituted with Mn, or 14 % when reconstituted with Fe (Table 2) although application of the same method to WT-apoMnSOD reconstituted Mn to >90 % of active sites and 70% of activity. Metal ion reconstituted Q146E-SODs displayed activities well below what could be explained by their low metal ion contents alone. Based on reconstituted WT-holoMnSOD's specific activity of 15,200 u/mg protein corrected for Mn content, we calculate that a specific activity of 2,000 u/mg protein would constitute 100% activity on a per-Mn ion basis for SOD containing Mn in 13% of active sites. However our 13% Q146E-holoMnSOD / 87% Q146E-apoMnSOD preparation (Q146E-MnrecSOD) displayed activity of 5 ± 3 u/mg protein, representing a per-Mn ion adjusted activity of 0.3% WT activity. The analogous Fe-substituted version (Q146E-FesubSOD) had 0.8 % per-metal-ion WT activity (16/2000) comparable to that reported at this pH for WT-FesubMnSOD.^{6, 8} We conclude that under standard assay conditions, Q146E-MnrecSOD and Q146E-FesubSOD have negligible activity compared to the WT-holoMnSOD. Therefore the Q146E mutation impairs catalytic function even when metal ion is present, in addition to preventing its acquisition. Moreover this was true for Fe as well as Mn, although Fe's low midpoint potential might be predicted to compensate for the effect of the Q146E substitution considering that in FeSOD the analogous substitution raised the midpoint potential of the enzyme and $\text{Fe}^{3+/2+}$ has a lower intrinsic E° than $\text{Mn}^{3+/2+}$.⁴²

Bases for Q146E-SOD's low metal ion incorporation - stability of the apoprotein

The low yield of our reconstitution experiments indicate that Q146E-apoMnSOD has a lower thermodynamic affinity for metal ions or that Q146E-apoMnSOD presents a higher barrier to formation of the 'open' state competent to bind metal ion⁵⁷ and therefore altered kinetics of metal ion incorporation. Thermodynamic possibilities are that (1) the metal-bound 'holo' form could be relatively less stable and/or (2) the apo form could be more stable, than the corresponding forms of WT-SOD. Indeed, prior measurements have revealed that mutations of Gln146 modify the calorimetric T_m s of human MnSOD and the Q146E mutation raises the T_m believed to correspond to protein unfolding,²⁷ however information was not available as to the metallation status of the species responsible for the T_m . To test the stability of our *E. coli* Q146E-apoMnSOD protein and compare it with those of the WT-apoMnSOD and WT-holoMnSOD, far-UV CD was used to monitor protein secondary structure as a function of temperature, in the presence of 0.8 M GdmCl. This concentration of GdmCl has been used in studies of MnSOD denaturation to prevent protein aggregation and thereby create conditions where the protein unfolding is more reversible and amenable to Van't Hoff analysis.⁵⁵ Indistinguishable far-UV CD spectra were obtained for Q146E-apoMnSOD, WT-apoMnSOD and WT-holoMnSOD in this medium (Supplemental Figure S2), consistent with the crystal structures of WT-apoMnSOD and the Q146H-holoMnSOD which were found to be superimposable on that of WT-holoMnSOD.^{26, 47} These data indicate that the GdmCl does not produce differences between the SODs and that Q146E-SOD's failure to acquire Mn ion and its lack of catalytic activity do not represent gross disruption of the SOD structure by the Q146E substitution.

The ellipticity at 222 nm (θ_{222}) reflects contributions from both alpha helices and beta sheets and therefore provides a good probe of the amount of secondary structure retained by the protein as a function of temperature.^{59, 60} For WT-holoMnSOD (0.95 Mn/site) θ_{222} changed only slightly between 20 °C and 60 °C (Figure 2). However above 60 °C, θ_{222} diminished sharply with approximately 80 % of the ellipticity loss occurring between 55 °C and 80 °C. In contrast, WT-apoMnSOD (0.13 Mn / site) lost 87 % of its ellipticity between 40 °C and 60 °C, with a T_m of 52 ± 1 °C (Table 3). These results are consistent with calorimetric findings of T_m s of 71 °C and 53 °C for *E. coli* WT-MnSOD in holoMn and apoMn form, respectively.⁵⁵ The somewhat ‘lumpy’ shapes of many of our WT-holoMnSOD curves are consistent with the distinct T_m s measured for reduced (Mn^{2+} -containing) and oxidized (Mn^{3+} -containing) WT-holoMnSOD of 69 °C and 90 °C respectively,⁵⁵ in conjunction with the redox heterogeneity of our samples. Our data yield T_m s of 69 ± 2 °C and 81 ± 2 °C which we attribute to Mn^{2+} SOD and Mn^{3+} SOD, respectively (see supplemental Figure S3). However both T_m s associated with WT-holoMnSOD are well separated from the T_m of WT-apoMnSOD, so these two forms can readily be distinguished and we adopt the use of ‘high-temperature’ (69 and 81 °C) vs. ‘low-temperature’ (52 °C) transition to focus on the presence/absence of the metal ion rather than its oxidation state.

Because as-isolated Q146E-MnSOD contained Mn in only a small fraction of the active sites (Table 1), it is most appropriately compared with WT-apoMnSOD. However unlike WT-apoMnSOD, melting of Q146E-apoMnSOD required heating to over 80 °C, and a transition complete between 75 °C and 95 °C accounted for 72 % of the ellipticity change. The melting temperature of $T_m = 88 \pm 1$ °C is more than 35 °C higher than that of WT-apoMnSOD indicating that replacement of Gln146 by Glu stabilizes the secondary structure of this protein. Thus, for the apo-proteins, the WT is *not* the most stable variant, contrary to naive expectation. This makes sense, as the form that is subject to natural selection is not the apoMn form but the holoMn form. However even WT-holoMnSOD is less stable than Q146E-apoMnSOD under our conditions. Thus mutation of Gln146 to Glu confers greater stabilization than does binding of Mn, and the surprising stability of Q146E-apoMnSOD is consistent with its low metal ion occupancy (thermodynamic possibility #1).

Bases for Q146E-SOD’s low metal ion incorporation - instability of the holoprotein

To address thermodynamic possibility 2, that Mn-bound Q146E-SOD might be less stable than the WT analog, we compared the CD-monitored thermal melting curves of samples of Q146E-SOD containing different amounts of Mn. All contents were low, with a maximum incorporation of Mn into only 13 % of sites, nonetheless this was sufficient to reveal an additional thermal transition, near 50 °C (Figure 3, blue curve).

To learn whether the low-temperature transition in Q146E-SOD could be attributed to Mn-containing form, we tested for a correlation between transition amplitude and proportion of active sites containing metal ion. Figure 4 compares the thermal melt curves for WT-SOD samples with a wider range of Mn (or Fe) contents. Low and high-temperature transitions are evident and well resolved in samples containing significant populations of both holoMn and apoMnSOD. The inset shows that the percent amplitude of the transitions above 60 °C correlate well with the percent of sites containing metal ion, despite variations in the value

of the T_m of the high-temperature transition attributable to the identity of the metal ion as Mn^{2+} , Mn^{3+} or Fe^{3+} (69, 81 and 87 °C, respectively⁵⁵).

Application of the same analysis to melting curves of Q146E-SOD in Figure 3 produced the correlations presented in the inset, wherein the amplitude of the high temperature transition (above 60 °C) accounted for ~ 96 %, ~ 90 %, and ~ 72 % of total ellipticity loss, while the fraction of active sites occupied by metal ions was 0.02, 0.03 and 0.13 ± 0.02 respectively. It is evident that the fraction of Q146E-SOD melting at high temperature (above 60 °C) does not correlate positively with the proportion of Q146E-holoMnSOD. However the percentage of ellipticity lost in the minor transition near 50 °C is in better agreement with the percentage of active sites containing metal ion. A plot of % ellipticity loss associated with the lower-T transition vs. % metal ion occupancy suggests that these values could indeed be correlated, although the low metal occupancies attainable limited the amplitude of the plot and the accuracy of its slope. In contrast, the percent ellipticity lost in the high-temperature transition correlates well with the proportion of *apo*MnSOD in these samples. Therefore our data are best explained by attribution of the high-temperature transition to Q146E-*apo*MnSOD and the low-temperature transition to Q146E-holoMnSOD.

Table 3 summarizes the melting transitions observed and their assignments. Our finding that Q146E-holoMnSOD has a lower T_m than Q146E-*apo*MnSOD contrasts with the conclusion reached for WT-SOD. Melting curves collected with θ_{222} measurements at 1 °C intervals were used as the bases for Van't Hoff analysis and the obtained parameters are reported (Supplementary Figure S4 and Table S1). Thus we find that Q146E-holoMnSOD is considerably less stable than the WT-holoMnSOD, consistent with the depressed metal ion incorporation in Q146E-SOD and conservation of Gln (or His) at this position.

Bases for Q146E-SOD's low metal ion incorporation - kinetics of folding based on CD

We also tested the possibility of a kinetic contribution to Q146E-SOD's lower metal ion content. The unfolded state was populated by heating samples of WT-holoMnSOD, WT-*apo*MnSOD and Q146E-*apo*MnSOD each to a temperature at which 50 % of secondary structure had melted (70 °C, 60 °C and 85 °C, respectively, see right-hand end of blue traces in Figure 5). After incubating for 30 min the samples were cooled at a rate of 1 °C/min and θ_{222} was measured at 5 °C intervals (Figure 5, dark blue curves, right to left). Although almost full starting ellipticity was recovered upon cooling in the presence of 100 μM Mn^{2+} (solid blue triangles), only half the lost ellipticity was recovered in the absence of Mn^{2+} on the time scale of the experiment (open blue triangles). However when the same samples were re-examined after overnight incubation at 4 °C, all had recovered almost full ellipticity (red triangles) indicating that the failure to recover full ellipticity during the cooling time was a kinetic effect, rather than a thermodynamic one, and that the rate of recovery was greater in the presence of Mn^{2+} than in its absence.

Refolding in the absence of Mn^{2+} could lead to low Mn content because it would quench the dynamics needed for Mn assimilation.⁶¹ Considering that successive temperature points in the cooling curves also represent successive time points (right to left, in Figure 5), the slopes of the curves can be compared to assess relative apparent rates of refolding for the different SODs $\pm Mn^{2+}$. Because recovery of ellipticity only occurred after cooling to 60–70°C for

Q146E-SOD, apparent rates in the interval from 60 to 20 °C were compared. All three types of SOD displayed apparent rates of approximately 250 deg cm²dmol⁻¹ /min in the presence of Mn²⁺ (250, 270 and 250 for WT-holo, WT-apo and Q146E-apo, respectively). However in the absence of added Mn²⁺, Q146E-SOD displayed an apparent rate of 150 deg cm²dmol⁻¹ /min vs. apparent rates of 85 and 100 deg cm²dmol⁻¹ /min for holo-Mn and apo-Mn WT-SOD. Thus it appears that Q146E refolds a little faster than WT-SOD in the absence of Mn²⁺. Thus the ratio of the refolding rate with Mn present vs without was 2.8 for WT- but only 1.7 for Q146E-MnSOD. The effect is not large, but is consistent with the lower metallation of Q146E-SOD as more rapid folding and closure of the protein in apo form is consistent with the lower metallation observed, although considerably more detailed experiments are needed in order to draw quantitative conclusions.

We tested whether Mn's effect on refolding is associated with functional binding rather than non-specific binding, by assessing catalytic activity and mobility in non-denaturing electrophoresis gels.⁴⁷ Samples of WT-apoMnSOD and Q146E-apoMnSOD were incubated with 100 μM Mn²⁺ at temperatures sufficient to melt half the secondary structure for 30 min, returned to 4 °C and held overnight, then analyzed by non-denaturing electrophoresis and staining for activity.⁵³ Panel A shows that WT-apoMnSOD recovers considerable activity as a result of the treatment and that this is concentrated in the upper band (lanes 3) corresponding to protein containing two Mn ions (panel B), as is the case for the WT-holoMnSOD control (lanes 1). By contrast before incubation WT-apoMnSOD was predominantly in the mono-Mn and apo-Mn forms and displayed very little activity (lanes 2). This demonstrates that Mn was incorporated functionally into the active site in the course of the heating and then cooling regimen used,⁵⁷ which was not observed for samples simply incubated with Mn at 4 °C (not shown).

However incubation near its T_m in the presence of 100 μM Mn²⁺ had little effect on the activity or electrophoretic mobility of Q146E-SOD (Figure 6), which retained absence of detectable activity, and the same multiplicity of species and population distribution among them. It is intriguing that Q146E-SOD displays at least three bands indicating that these samples include more than one state of SOD. Nevertheless, panel B indicates undetectable activity for Q146E-SOD (.02 Mn per site, Table 1) at loading levels that readily permitted visualization of activity from WT-apoMnSOD containing .16 Mn per site (2800 u/mg protein, Table 1).

As a second probe of Mn binding to Q146E-SOD we used CD-detected melting curves, as these change markedly in response to Mn binding to WT-SOD (Figure 7). The middle panel of Figure 7 shows that the control sample of WT-apoMnSOD that had been incubated and cooled with added Mn acquired a strong high-temperature transition attributable to holoMnSOD (solid red triangles) with near elimination of the low-temperature transition attributable to apoMnSOD, whereas WT-apoMnSOD that had been incubated and cooled without Mn present retained substantial low-temperature unfolding (the presence of some high-temperature unfolding suggests that the protein was able to capture contaminating metal ion, open red triangles). WT-holoMnSOD samples displayed qualitatively similar behaviour: material incubated and cooled in the presence of added Mn melted at high temperature whereas the sample to which Mn was not added displayed a stronger low

temperature transition, consistent with failure to recapture some Mn released in the course of heating. These controls further support attribution of the transition at $T < 60\text{ }^{\circ}\text{C}$ to WT-apoMnSOD and the transition at $T > 60\text{ }^{\circ}\text{C}$ to WT-holoMnSOD.

Q146E-SOD that had been incubated and cooled without added Mn displayed a very high T_m upon re-melting (open red triangles) confirming recovery of the stable Q146E-apoMnSOD structure (reversibility, bottom panel, Figure 7). But after incubation and cooling with $100\text{ }\mu\text{M Mn}^{2+}$ Q146E-apoMnSOD displayed a smaller-amplitude high-temperature transition near $90\text{ }^{\circ}\text{C}$ and a new feature near $47\text{ }^{\circ}\text{C}$ resembling the $50\text{ }^{\circ}\text{C}$ feature in the melting curve of Mn-reconstituted Q146E-SOD (0.13 Mn per site Figure 4). The correlation between this low-temperature transition and the presence of Mn^{2+} during refolding suggests that Q146E-apoMnSOD was able to acquire some Mn^{2+} at the relatively high Mn^{2+} concentration used and strengthens attribution of the low-temperature transition to Q146E-holoMnSOD. Unfortunately the concentrations of these samples were too low to permit quantification of acquired Mn^{2+} and future experiments should also address the possibility of gradual loss of Mn overnight at $4\text{ }^{\circ}\text{C}$, to determine whether the samples examined represent kinetically trapped results of exposure to high Mn concentration.

Kinetics of folding based on fluorescence quenching

We sought to directly compare the rates of events involved in metal ion binding to Q146E-apoMnSOD with those applicable to WT-apoMnSOD. By monitoring quenching of Trp fluorescence associated with binding of Co^{2+} one can determine the fraction of the active sites in the ‘open’ state receptive to Co^{2+} binding, the rate of Co binding and the rate at which additional sites undergo conversion to the ‘open’ state.⁵⁷ WT-apoMnSOD is relatively indifferent to the identity of the metal ion, permitting incorporation of Fe, Mn, Co, Ni or Zn when these were provided at sufficient concentration to unfolded apo-protein,⁶² and Co has been shown to reconstitute WT-apoMnSOD with kinetics very similar to those of reconstitution with Mn.⁵⁷

The amplitude of the transient attributable to Co^{2+} binding (fast phase) was smaller for Q146E- than for WT-apoMnSOD. Thus although we measured $48 \pm 9\%$ population of the open state for WT-apoMnSOD in agreement with Whittaker (Figure 8 shows transients from one experiment), only $31 \pm 1\%$ of Q146E-apoMnSOD sites were open. This is consistent with the greater stability of folded Q146E-apoMnSOD, although the much lower starting fluorescence intensity is perplexing. The rate constants for Co binding to WT- and Q146E-apoMnSOD were $1.10 \pm .02\text{ min}^{-1}$ ($n=2$) and $0.669 \pm .002\text{ min}^{-1}$ ($n=2$), respectively, at $45\text{ }^{\circ}\text{C}$. Faster Co^{2+} binding to WT is consistent with higher Mn incorporation in this protein. A preliminary comparison of the temperature dependence reproduced the finding that $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ optimizes the fraction of the population in the open form for WT-apoMnSOD⁶¹ but indicated that the fraction of Q146E-apoMnSOD in the open state decreases above $40\text{ }^{\circ}\text{C}$. The opening transition need not involve loss of secondary structure so a feature at this temperature is not necessarily observable by CD.

Discussion

For Fe- and MnSODs, the evolutionarily-selectable activity requires incorporation of the correct metal ion in the active site, so Mn binding, in addition to catalytic turnover, is subject to selection. Thus the finding that mutation of a conserved residue lowers incorporation of Mn is not surprising, even though the residue in question is not a ligand to Mn. Indeed, Whittaker has shown that mutation of other second-sphere residues in the active site hydrogen bonding network, His30 and Tyr34, also affects Mn uptake.⁶¹ Moreover it is very interesting that the Q146E mutation destabilizes the holoMn state since the corresponding Q69E mutation in FeSOD lowers the stability of the Fe³⁺-bound state relative to the Fe²⁺-bound state. Thus this residue's identity strongly and differentially affects metal ion binding, and our studies add to prior work in indicating that Gln146 plays a role in determining SOD's metal ion content.⁴⁶ However the very large *increase* in the T_m of the apoMn state upon mutation was not expected.

Additionally, although our data on metal-containing Q146E-SOD are limited, they suggest that Q146E-holoMnSOD has a *lower* T_m than does Q146E-apoMnSOD (Figures 3 & 7). Both findings are consistent with the low incorporation of Mn or Fe upon reconstitution as they place the equilibrium between Q146E-apoMnSOD and Q146E-holoMnSOD in favor of the apoMn form under the conditions used for the initial melting curves. Presumably the 100 μ M Mn²⁺ used for the reconstitutions sufficed to produce some Q146E-holoMnSOD, which was kinetically trapped in the active site at our storage temperature of 4 °C. However increasing the temperature to 47 °C resulted in loss of secondary structure specific to Mn-bound Q146E-SOD, and presumably Mn release. We expect that the resulting locally unfolded apo-protein would spontaneously form the Q146E-apoMnSOD which is stable at that temperature and appears to retain a comparable amount of secondary structure, based on the CD spectrum of Q146E-apoMnSOD (supplemental Figure S2). The result would be little net loss of secondary structure associated with metal ion loss, for Q146E-holoMnSOD. This is indeed observed. When the data in Figure 3 are re-plotted as magnitudes of ellipticity vs. temperature rather than percent ellipticity, the amplitude of ellipticity loss near 88 °C is seen to be comparable in samples with different initial metal contents (supplemental Figure S5), consistent with the superimposable crystal structures of WT-holoMnSOD and WT-apoMnSOD at 1.9 Å resolution.>Whittaker, 2011 #1371}

The T_m s we measure for WT-holoMnSOD and WT-apoMnSOD are consistent with those reported based on calorimetry under similar conditions.^{55, 63} However the increase in T_m produced by replacing Gln146 with Glu is remarkably large. Numerous studies report mutations' effect on T_m , with most single-residue substitutions only changing T_m by 5 °C or less, whereas mutations increasing T_m by more than 10 °C are much rarer.^{64, 65} The striking effect of mutating Gln146 is in part a reflection of the critical position it occupies, H-bonding with residues in different domains and secondary structural elements of the protein (Figures 1 and S1). Silverman's group found that the effects of mutating the analogous Gln in human MnSOD ranged from 20 °C depression of T_m to 15 °C elevation, relative to the WT T_m (although there was no accounting for metal ion content).²⁷ The identity of the residue at that position is clearly crucial, as replacement of Gln with Asn or His produced

only a 2 °C change whereas mutation to Glu produced a T_m 15 °C higher than that of WT-holoMnSOD, consistent with our findings here.

Reasons for Glu146's strong stabilization of the apoMn state are not obvious, especially given that no crystal structure is available for Q146E-apoMnSOD. However the crystal structure of WT-apoMnSOD is essentially superimposable on that of WT-holoMnSOD, arguing that absence of the metal ion need not produce a different structure in Q146E-apoMnSOD.⁴⁷ For FeSOD the Gln to Glu change does not affect the structure either, as the fold and active site of Q69E-FeSOD are almost superimposable on those of WT-FeSOD.⁴²

However Glu146's effect is distinct from that of Glu69, as Glu69 of FeSOD stabilizes the Fe^{2+} state with coordinated H_2O ($Fe^{2+} \cdot H_2O$) relative to the $Fe^{3+} \cdot OH^-$ state and suggests that we and others should have isolated the $Mn^{2+} \cdot H_2O$ state of Q146E-MnSOD rather than the apo form.⁴² The difference could be related to the stronger hydrogen bonding of Gln146 with coordinated solvent in the MnSOD active site.⁶⁶ This would be expected to increase the stabilization produced by a favourable H-bond between ionized Glu^- and coordinated H_2O on Mn^{2+} . However computations found that the shorter distance between the side chain of the active site Gln and the metal ion in MnSOD vs. FeSOD disfavours protonation of coordinated solvent to H_2O .^{12, 16} The shorter distance could be less compatible with the larger $Mn^{2+} \cdot H_2O$ unit vs. the smaller $Mn^{3+} \cdot OH^-$ unit (the bond between the lower-charge Mn^{2+} ion and neutral H_2O is longer than the bond between Mn^{3+} and OH^- which benefits from the attraction between opposite charges as well as the Jahn-Teller effect). Thus the slightly larger size of $Mn^{2+} \cdot H_2O$ may be more difficult to accommodate in the scaffold of MnSOD where the substituent Glu residue derives from position 146 instead of 69.

In contrast, in the absence of bound metal ion, we speculate that each of Glu146 and the ligand Asp167 could enjoy salt bridges with protonated ligands His81 and/or 26 (His171 has a salt bridge with Glu170 of the other monomer in the native structure, Figure 1). Indeed, deprotonation of a residue with a pK_a of 7.7 at 37 °C is required for metal ion binding to WT-apoMnSOD.⁶¹ The presence of two such interactions in Q146E-SOD could double this barrier to inserting a metal ion and modify kinetic gating. However in order to know whether such interactions in fact occur in Q146E-apoMnSOD, a high-quality crystal structure or NMR vs. pH will be necessary.

The current study provides evidence that Mn can bind to Q146E-SOD when present at sufficient concentrations, but also demonstrates that the holoMn form of Q146E-SOD is not the thermodynamically stable one, and suggests that Q146E-SOD's lack of metal ion incorporation *in vivo* may reflect a thermodynamic component in addition to the kinetic component that causes the MnSODs of thermophiles to be expressed in apoMn form.^{67, 68} The large upshift in T_m of apoMnSOD upon mutation of Gln146 to Glu reveals the large sacrifice in protein stability made by WT-SOD for the sake of metal ion affinity. We do not presume that this phenomenon is unique to MnSOD, but the 35 °C magnitude of the increase in T_m is surprisingly large. We surmise that this reflects in part the high overall stability of WT-holoMnSOD, consistent with its role as a first line of defense against oxidative stress¹ and intense selection for Mn binding because catalytic activity depends on it. The current work also suggests that MnSOD's conserved Gln suppresses accumulation of the apoMn

form (destabilizing the apo, not just favoring the holoMn form), thereby facilitating recycling of useless apoMnSOD protein. WT-apoMnSOD's slightly slower refolding than Q146E-apoMnSOD in the absence of added Mn could additionally extend the time in which the 'open' form is populated and thereby have dual advantages for metal binding.

Current thinking is that protein stability should be such as to maximize catalytic activity at the temperature at which the enzyme's proprietor organism lives, which will require that the native structure be stable but by a margin small enough to not suppress dynamics needed for function.⁶⁹ We extend this idea to enzyme maturation, wherein MnSOD serves as a provocative illustration that the most stable protein variant is not necessarily the most desirable, and that function is also served by conserved residues that *destabilize* inactive forms of the protein, in this case causing the *apo*-protein to be only marginally stable at *E. coli*'s habitual growth temperature.

CONCLUDING REMARKS

Our results provide an explanation for the low metal ion content of *E. coli* Q146E-MnSOD in terms of dramatically increased stability of the apoMn form by 35 °C relative to WT-apoMnSOD, and apparent destabilization of Q146E-holoMnSOD relative to WT-holoMnSOD, in 0.8 M Gdm HCl. These changes shift the equilibrium between apoMn- and holoMnSOD in favor of the former, such that only Mn trapped in active sites remains bound. It therefore appears that Gln146, which has previously been considered primarily as an agent of proton delivery to the active site and redox tuning, may also be conserved for its ability to destabilize apoMnSOD and favor metal ion incorporation. Furthermore, replacement of Gln146 with Glu failed to support Fe-based activity in MnSOD, suggesting that its effect of significantly raising the metal ion E° in FeSOD may not be portable to MnSOD. This work has important implications for enzyme engineering, that functional properties may not be compatible with optimization of structural stability and that the desirable amino acid may in fact considerably destabilize the structure. Thus computational schemes that suggest mutations to be made on the basis of their ability to minimize energy may not always provide the best guidance and may need to be applied to states of the enzyme other than the as-isolated state. In the case of MnSOD it is clear that a metal ion must be bound for catalytic activity, but in other cases the catalytically active state of the enzyme may be more cryptic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

apoMnSOD	MnSOD lacking the Mn ions
CD	circular dichroism
EDTA	ethylene diamine tetraacetic acid
FeSOD	Fe-specific SOD
FesubMnSOD	Fe-substituted MnSOD with active sites containing Fe instead of Mn
GdmCl	guanidium hydrochloride
holoMnSOD	MnSOD with active sites replete with Mn
ICP-OES	inductively coupled plasma optical emission spectroscopy
IPTG	isopropyl thiogalactopyranoside
MnSOD	used to indicate the Mn-specific SOD as opposed to an Fe-specific SOD but does not specify apoMn vs. holoMn form
NMWL	Normal Molecular Weight Limit
NTA	nitritotriacetate
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
Q146E	with Gln146 replaced by Glu
SOD	superoxide dismutase
WT	wild-type (non mutated)

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Figure 1.

Depiction of the active site of *E. coli* MnSOD based on the crystal structure 1D5N.pdb³⁴ and generated using Chimera.³⁵ The redox-active Mn is depicted as a violet ball coordinated by the side chains of three His' (H26, H81, H171), one Asp (D167) and a solvent molecule (small red ball). Amino acid C atoms share the rainbow colouring of the ribbon that subtends them (supplementary figure S1) and N and O atoms are blue and red, respectively. A hydrogen bond network (aqua dashed lines) includes solvent molecules (small red balls) and the side chains of Gln146, Tyr34, His30, Asp167 and Trp128. An H-bond between His171 and Glu170.B links the active site shown to that of the other (B) monomer of the dimer. Also shown is the side chain of the Gln69 that would be present in FeSOD's active site, modeled in by superimposing the entire structure of FeSOD on that of MnSOD but showing only the Gln69 that corresponds to Gln146 of MnSOD. Numbering is that of *E. coli* MnSOD (and *E. coli* FeSOD).

Figure 2.

The Q146E mutation increases the stability of the MnSOD protein more than does binding Mn. The temperature-dependence of ellipticity at 222nm (θ_{222}) reveals loss of secondary structure content as the temperature increases in the presence of 0.8 M GdmCl at pH 7.4. 15 μ M of SOD (dimers) was equilibrated in the buffer (5 mM KH_2PO_4 , 0.8 M GdmCl pH 7.4) and the temperature was scanned from 20 to 100 $^{\circ}\text{C}$ at 1 $^{\circ}\text{C}$ /min. At each temperature point (5 $^{\circ}\text{C}$ intervals) four spectra were collected and the average value of θ_{222} was recorded. Samples were WT-apoMnSOD (prepared by Mn removal from WT-holoMnSOD as per the methods (0.01 Mn/site), WT-MnrecSOD (prepared by Mn removal followed by reconstitution with Mn, $0.95 \pm .05$ Mn/site) or Q146E-apoMnSOD as-isolated (0.02 Mn/site for this preparation).

Figure 3.

The percentage of θ_{222} loss associated with the low-temperature T_m ($T_m < 60^{\circ}\text{C}$) correlates with holo-Mn sites in Q146E-MnSOD. Temperature-induced unfolding was compared among samples of Q146E-MnSOD containing Mn in different fractions of the active sites. Samples were Q146E-apoMnSOD as-isolated (0.02 Mn/site, 0.03 Mn/site) and Q146E-apoMnSOD prepared by denaturation followed by reconstitution with Mn (0.13 ± 0.01 Mn/site). Samples contained 15 μM of Q146E-SOD (dimers) in 5 mM potassium phosphate buffer at pH 7.4 with 0.8 M GdmCl to prevent protein aggregation, and the temperature was increased from 20 to 100 $^{\circ}\text{C}$ at 1 $^{\circ}\text{C}/\text{min}$. For data collected every 5 degrees, four spectra were collected and averaged at each temperature. For data collected every one degrees, θ_{222} was measured with a time constant of 1 s. Inset: plots of the percentage of θ_{222} loss associated with the transition above 60 $^{\circ}\text{C}$ (red) or the percentage associated with the transition below 60 $^{\circ}\text{C}$ (blue) vs. Mn content of the sample. Error bars are provided using small squares of the appropriate colour. The correlation between the percent amplitude associated with the transition below 60 $^{\circ}\text{C}$ and Mn content with intercept of zero produced a slope of 1.13 with $R^2 = 0.88$, the straight line with unconstrained intercept (shown in blue) had a slope of 0.92 and intercept of 2.1 with $R^2 = 0.97$, and the correlation between percent amplitude associated with the transition above 60 $^{\circ}\text{C}$ and Mn content produced a slope of -2.0 and intercept of 98% with $R^2 = 0.97$ (shown in red). Thus the transition below 60 $^{\circ}\text{C}$ appears associated with sites with Mn bound, and the transition above 60 $^{\circ}\text{C}$ is assigned to the sites lacking Mn.

Figure 4.

The percentage of total ellipticity loss associated with the high-temperature T_m ($T_m > 60^\circ\text{C}$) correlates with metal-containing sites in WT-MnSOD. Temperature-induced unfolding was compared among samples of WT-MnSOD containing Mn or Fe in different fractions of the active sites. Samples were WT-apoMnSOD (prepared by Mn removal from WT-holoMnSOD as per the methods, 0.01 Mn/site , $0.20 \pm .1\text{ Mn/site}$), WT-FesubSOD (prepared by Mn removal followed by replacement with Fe, $0.45 \pm 0.01\text{ Fe/site}$) or WT-MnrecSOD (prepared by Mn removal followed by reconstitution with Mn, $0.95 \pm .05\text{ Mn/site}$). Loss of secondary structure upon heating was monitored via the ellipticity $[\theta]$ at 222nm every 1°C or 5°C . Samples contained $15\text{ }\mu\text{M}$ of SOD (dimers) in 5 mM potassium phosphate buffer at pH 7.4 with 0.8 M GdmCl to prevent protein aggregation, and the temperature was increased from 20 to 100°C at $1^\circ\text{C}/\text{min}$. For data collected at 5°C intervals, four spectra were collected at each temperature and averaged. For data collected every one degrees, θ_{222} was measured with a time constant of 1 s . Amplitudes of transitions and their attendant uncertainties were extracted from each data set using the utility provided in the JASCO software and using the simplifying approximation of a single transition above 60°C to avoid over-fitting data with insufficient digital resolution to justify more detailed fits. Standard errors were derived from repetitions of the Mn content measurements and from the amplitude of the noise level in the CD curves. Inset: linear regression of the percentage of ellipticity loss associated with the transition above 60°C vs. metal content of the sample. A least-squares fit to a straight line with intercept of zero produced a slope of 0.91 with $R^2 = 0.91$, an unconstrained straight line fit produced a slope of 0.93 and an intercept of 8.6 with $R^2 = .99$ for the % amplitude at $T > 60^\circ\text{C}$ vs % sites metallated (shown in red). An

unconstrained straight line fit produced a slope of -0.96 and an intercept of 85 with $R^2 = .95$ for the % amplitude at $T < 60$ vs % sites metallated (shown in blue).

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Figure 5.

Recovery of ellipticity upon cooling is improved by presence of 100 μM Mn. WT-holoMnSOD (top, A), WT-apoMnSOD (middle, B, 0.01 ± 0.01 Mn per site) or Q146E-apoMnSOD (bottom, C) samples were incubated at temperatures near their respective T_{ms} for 30 min (70 $^{\circ}\text{C}$, 60 $^{\circ}\text{C}$ or 85 $^{\circ}\text{C}$, respectively) and then cooled at 1 $^{\circ}\text{C}$ / min to 20 $^{\circ}\text{C}$ while monitoring θ_{222} . In each case a sample containing 100 μM MnCl_2 (solid blue triangles and lines) was compared with one lacking it (open blue triangles and lines). Successive data points in the cooling trajectory run from right to left in this presentation (blue arrows). For comparison, full melting curves are shown in each panel for the original sample type in question (left to right progression, '1st melt'). Comparison with the 20 $^{\circ}\text{C}$ starting point of these curves demonstrate almost full recovery of ellipticity when samples are cooled in the presence of Mn. They also demonstrate that in the absence of Mn, recovery is less complete on the time scale investigated. However red triangles demonstrate that overnight incubation at 4 $^{\circ}\text{C}$ resulted in essentially full ellipticity even for samples not supplemented with Mn (open red triangles) compared with the sample supplemented with Mn (filled red triangles). Each sample consisted of 15 μM SOD (dimers) in 5 mM KH_2PO_4 pH 7.4, 0.8 M GdmCl with or without 100 μM Mn (filled vs. open triangles).

Figure 6.

Cooling in the presence of 100 μM Mn results in functional binding of Mn. Non-denaturing gel electrophoresis of WT-holoMnSOD compared with WT-apoMnSOD and Q146E-apoMnSOD before vs. after incubation with 100 μM Mn^{2+} at the appropriate T_m . WT-holoMnSOD as isolated, was compared with WT-apoMnSOD purified from a culture grown without added Mn, the same WT-apoMnSOD after incubation with 100 μM Mn^{2+} at 60 $^{\circ}\text{C}$ and cooling, Q146E-apoMnSOD as isolated, and Q146E-apoMnSOD after incubation with 100 μM Mn^{2+} at 85 $^{\circ}\text{C}$ and cooling. Proteins were analyzed using non-denaturing electrophoresis through 4–12 % acrylamide gels. One of a pair of replicate gels was subjected to the NBT assay procedure to visualize SOD activity in the form of colorless zones (Panel A)⁵³ and the other was stained using Coomassie brilliant blue R-250 to visualize all protein (Panel B). Approximately 2.8 μg protein were loaded in each lane of gel A whereas 5.5 μg protein were loaded in each lane for gel B. The two gels were run in parallel at 100 volts for ~ 2 hours.

Figure 7.

CD-detected melting reveals increased population of holo-protein for SODs cooled in the presence of Mn^{2+} . As for Figure 5, the panels display results for SODs that had been WT-holoMnSOD (top, A), WT-apoMnSOD (middle, B), and Q146E-apoMnSOD (bottom, C) before heating and cooling in the presence or absence of 100 μM MnCl_2 (Figure 5). Here, the composition of SODs resulting from heating and cooling in the presence vs. absence of 100 μM Mn^{2+} (Figure 5) were assessed by heating again while measuring $[\theta]_{222}$ every 5 $^{\circ}\text{C}$ (left to right direction, red arrows). In each case, temperature-induced unfolding of SODs that had been cooled in the presence of 100 μM Mn is depicted by filled red triangles (Mn remains present), and unfolding of SODs cooled in the absence of added Mn is depicted by empty red triangles (Mn remains absent). For comparison, the end-points of the cooling curves are included in blue using filled and empty triangles to indicate presence vs. absence of Mn. The control first-melt curves from control samples as-prepared are also shown.

Figure 8.

Fluorescence quenching at 45 °C reveals greater population of the ‘open’ state competent to bind Co^{2+} in WT-apoMnSOD (blue) than in Q146E-apoMnSOD (red). The amplitudes of the fast phases were used to estimate the population in the ‘open’ form and the amplitudes of the slow phases were used to estimate the population in the ‘closed’ form,⁵⁷ to permit calculation of the % population open. Tryptophan fluorescence was measured at 355 nm based on excitation at 280 nm using 5 nm excitation and emission slit widths. Each sample of 50 $\mu\text{g/ml}$ protein ($\approx 1 \mu\text{M}$ dimers) was pre-equilibrated at 45 °C for 5 min in advance of Co^{2+} addition to 10 μM and subsequent monitoring of fluorescence emission every second for 30 minutes. WT data are in pale blue with a dark blue line displaying the best-fit curve of $Y = 497 + (61.2 \cdot \exp(-.018 \cdot t)) + (45.7 \cdot \exp(-.00113 \cdot t))$ where t is time and fitting errors for each of the parameters in the foregoing were 0.3, 0.4, 0.0002, 0.2, 2×10^{-5} , respectively. Q146E data are in pink with a red line displaying the best-fit curve of $Y = 486 + (6.6 \cdot \exp(-.011 \cdot t)) + (15.3 \cdot \exp(-.00102 \cdot t))$ where t is time and fitting errors for each of the parameters

in the foregoing were 0.4, 0.4, 0.001, 0.2, 7×10^{-5} , respectively. Black dots are WT-apoMnSOD vs. time in the absence of added Co^{2+} .

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Table 1Metal content and catalytic activity of WT-MnSOD and Q146E-apoMnSOD as-isolated.^a

Growth medium	Ratio (Mn/Dimer)	Metal ion occupancy (per site)	Specific Activity (unit/mg protein)	Activity/Mn (unit/mg protein)
MnSOD not supplemented	0.32 ±0.1	16%	2840 ±80	18000 ±6000
HoloMnSOD supplemented	2.0 ± 0.1	100%	20,000 ± 2000	20,000 ± 2000
Q146E-apoMnSOD not supplemented	0.03 ±0.01	2%	1	50

^aMn content was quantified by ICP-OES. The protein specific activity was determined by the xanthine/xanthine oxidase/cytochrome c assay at pH 7.4.⁵² Protein concentrations were determined using absorbance at 280 nm, with extinction coefficients of 86600 M⁻¹ cm⁻¹, 83200 M⁻¹ cm⁻¹ and 89500 M⁻¹ cm⁻¹ per dimer for WT-holoMnSOD, WT-apoMnSOD and Q146E-apoMnSOD respectively. Activity per Mn is Specific activity corrected for the Mn content.

Table 2

Metal content and catalytic activity of WT-apoMnSOD and Q146E-apoMnSOD after reconstitution with Mn or Fe.^a

	Metal ions /Dimer	Metal content / active site	Specific Activity (unit/mg protein)	Activity/Mn (unit/mg protein)
WT-MnrecSOD	1.9 ± 0.1	95 %	14400 ± 100	15200 ± 100
Q146E-MnrecSOD	0.13 ± 0.01	13 %	5 ± 3	40 ± 30
Q146E-FesubSOD	0.14 ± 0.01	14 %	16 ± 14	100 ± 100

^aMn and Fe content were quantified by ICP-OES. The protein specific activity was determined via the xanthine/xanthine oxidase/cytochrome c assay at pH 7.4.⁵² Protein concentrations were determined from the absorbance at 280 nm with extinction coefficients of 86600 M⁻¹ cm⁻¹ and 89500 M⁻¹ cm⁻¹ per dimer for WT-holoMnSOD and Q146E-apoMnSOD respectively.

Table 3

Comparison of melting temperatures of metallated and apo forms of WT- and Q146E-MnSOD.

	T_m °C	Uncertainty °C (# reps) ^a	Note
WT-holoMnSOD	69, 81	2 (3), 2 (2)	Mn ²⁺ SOD, Mn ³⁺ SOD
WT-apoMnSOD	52	1 (2)	
Q146E-holoMnSOD	49	2 (2)	Oxidation state unknown
Q146E-apoMnSOD	88	1 (3)	

^aStandard deviations and number of separate experiments producing the T_m values. Standard errors of individual fits averaged 0.6 °C, with a maximum of 1.6 °C.