Crystal Structure of Y34F Mutant Human Mitochondrial Manganese Superoxide Dismutase and the Functional Role of Tyrosine 34†,‡

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ABSTRACT: Tyrosine 34 is a prominent and conserved residue in the active site of the manganese superoxide dismutases in organisms from bacteria to man. We have prepared the mutant containing the replacement Tyr 34 → Phe (Y34F) in human manganese superoxide dismutase (hMnSOD) and crystallized it in two different crystal forms, orthorhombic and hexagonal. Crystal structures of hMnSOD Y34F have been solved to 1.9 Å resolution in a hexagonal crystal form, denoted as Y34Fhex, and to 2.2 Å resolution in an orthorhombic crystal form, denoted as Y34Forth. Both crystal forms give structures that are closely superimposable with that of wild-type hMnSOD, with the phenyl rings of Tyr 34 in the wild type and Phe 34 in the mutant very similar in orientation. Therefore, in Y34F, a hydrogen-bonded relay that links the metal-bound hydroxyl to ordered solvent (Mn–OH to Gln 143 to Tyr 34 to H2O to His 30) is broken. Surprisingly, the loss of the Tyr 34 hydrogen bonds resulted in large increases in stability (measured by Tm), suggesting that the Tyr 34 hydroxyl does not play a role in stabilizing active-site architecture. The functional role of the side hydroxyl of Tyr 34 can be evaluated by comparison of the Y34F mutant with the wild-type hMnSOD. Both wild-type and Y34F had kcat/Km near 10³ M⁻¹ s⁻¹, close to diffusion-controlled; however, Y34F showed kcat for maximal catalysis smaller by 10-fold than the wild type. In addition, the mutant Y34F was more susceptible to product inhibition by peroxide than the wild-type enzyme. This activity profile and the breaking of the hydrogen-bonding chain at the active site caused by the replacement Tyr 34 → Phe suggest that Tyr 34 is a proton donor for O2⁻ oxidation or is involved indirectly by orienting solvent or other residues for proton transfer. Up to 100 mM buffers in solution failed to enhance catalysis by either Y34F or the wild-type hMnSOD, suggesting that protonation from solution cannot enhance the release of the inhibiting bound peroxide ion, likely reflecting the enclosure of the active site by conserved residues as shown by the X-ray structures. The increased thermostability of the mutant Y34F and equal diffusion-controlled activity of Y34F and wild-type enzymes with normal superoxide levels suggest that evolutionary conservation of active-site residues in metalloenzymes reflects constraints from extreme rather than average cellular conditions. This new hypothesis that extreme rather than normal substrate concentrations are a powerful constraint on residue conservation may apply most strongly to enzyme defenses where the ability to meet extreme conditions directly affects cell survival.
Manganese Superoxide Dismutase

(hydroxyl anion) as the fifth ligand in a trigonal bipyramidal arrangement (2). The hMnSOD is a homotetramer (2, 8, 9) that is more compact than the structures of the bacterial MnSODs as indicated by the shorter distances between metals in the individual subunits (2). The tetrameric structure of hMnSOD forms a ring of positive electrostatic charge surrounding the active sites to enhance attraction of substrate (2) similar to that observed in Cu/ZnSOD and mutants (10).

The metal in Mn superoxide dismutases cycles between oxidized and reduced states:

\[ \text{Mn}^{3+} + O_2^{-} \xrightleftharpoons[k_{-1}]{k_1} \left[ \text{Mn}^{3+} - O_2^{-} \right] \xrightleftharpoons[k_2]{k_3} \text{Mn}^{2+} + O_2 \]  

\[ \text{Mn}^{2+} + O_2^{-} \xrightleftharpoons[k_{-3}]{k_3} \left[ \text{Mn}^{2+} - O_2^{-} \right] + 2H^+ \xrightleftharpoons[k_4]{k_4} \text{Mn}^{3+} + H_2O \]

Initial studies of the MnSOD from B. stearothermophilus determined that its catalysis is complicated by the presence of an inactive form of the enzyme that can interconvert to an active form, but these studies did not designate the nature of the inactive form (11, 12). Steady-state constants for catalysis by MnSOD from T. thermophilus were obtained by Bull et al. (13) from stopped-flow experiments and for the hMnSOD using both stopped-flow and pulse radiolysis (8). In both studies, the rapid emergence of the inactive form of the enzyme was demonstrated. Bull et al. (13) observed the inactive form spectrophotometrically during steady state, and they suggested that the inactive form results from oxidative addition of \( O_2^- \) to the Mn(II) form of the enzyme, resulting in a side-on peroxo complex of Mn(III).

Here we have examined the function of the active-site residue Tyr 34, which is close to the manganese but not a ligand of this metal. Tyr 34 is evidently critical structurally or functionally as it is conserved in all of the euarkyotic and bacterial MnSODs reported to date and in the bacterial FeSODs as well. Tyr 34 forms a hydrogen-bond network that includes Gln 143 and the metal—ligand cluster, and the properties of this tyrosine may be linked to catalysis at the metal ion. The amide group of Gln 143 (Ne2) is 4.6 Å from the manganese and 2.7 Å from the hydroxyl group of Tyr 34 to which it hydrogen-bonds (2). In the complex of azide with Mn(III)SOD from T. thermophilus, the azide is a Mn ligand and forms a hydrogen bond to the hydroxyl group of Tyr 34 (14), which suggests by analogy a potential binding mode for superoxide. Recent reports on catalytic activity of Escherichia coli Y34F FeSOD mutant suggest that Tyr 34 is multifunctional and essential for maximal activity of FeSOD (15, 16).

We have replaced Tyr 34 with Phe, determined two independent crystal structures including the highest resolution hMnSOD structure to date, defined the thermostability, and measured the catalytic properties of the resulting mutant Y34F hMnSOD. This replacement caused minimal alteration of the active-site structure compared with the wild type, increased thermostability, and allowed us to test the functional role of the side-chain hydroxyl of Tyr 34 in catalysis. We observed a decrease in the maximal catalytic rate of superoxide decay \( k_{cat} \) by an order of magnitude for the mutant Y34F compared with the wild type. However, the values of \( k_{cat}/K_m \) for both enzymes were similar at 10^9 M^-1 s^-1, near diffusion-controlled. The mutant Y34F exhibits rapid product inhibition qualitatively similar to the wild-type MnSOD; however, the magnitude of this inhibition was significantly greater in the mutant. These features provide new information on the hMnSOD catalytic mechanism, possible roles of Tyr 34 in proton-transfer pathways, and the evolutionary conservation of active-site residues in metalloenzymes.

**MATERIALS AND METHODS**

PCR-Based Site-Directed Mutagenesis. The oligonucleotides GCATATAGAACGACAGCTCC and GGAGATCT-CAGCATACGATC were used as primers for PCR to amplify the hMnSOD cDNA [cDNA sequence reported by Beck et al. (17)]. The plasmid phMnSOD4 (ATCC 59947), which encodes hMnSOD, was subcloned into the TA cloning vector, pcRII (Invitrogen Corp.). Four primers were designed and used for PCR-based site-directed mutagenesis to create the mutant Y34F hMnSOD. First we designed a pair of oligonucleotides, primers 1 (5’ GCAGTTACTGTAT-TCTGCGA 3’) and 2 (5’ CCTTTAAACACAGCTCCCG 3’), which through PCR recreate the entire MnSOD coding region flanking the mutation. In addition we prepared two oligonucleotides, designated as primers 3 (5’ CCACGGGCCTTTCCGGTTAACACCTGT 3’) and 4 (5’ CAGGTTGT-TAACGAGGGCCCGGTTGG 3’), whose sequences were complementary to each other and contained the mutation of interest. Two separate PCR reactions were used to amplify the 5’ portion (primers 1 and 4) and 3’ portion (primers 3 and 2) of the MnSOD cDNA. The PCR products from these two reactions were purified by electroelution and used as template DNA for the second round of PCR using primers 1 and 2. The Y34F hMnSOD PCR product was cloned into the TA cloning vector (pcRII) and subsequently subcloned into the expression vector pTrc 99A (Pharmacia Corp.). The subcloning was accomplished by using the restriction sites, DraI and PstI, incorporated into primers 1 and 2, respectively. The DraI site, which corresponds to the N-terminal portion of the protein, was annealed to the NcoI site in pTrc 99A, recreating an ATG codon, whereas the C-terminal end of the cDNA was annealed to the PstI site of the vector. The mutation, along with the remainder of the coding sequence, was verified by DNA sequencing. This construct expressed hMnSOD in the mutant SodA’/SodB’ E. coli (strain QC 774) as a mature protein tagged with an extra Met at the amino terminus. Culture conditions included additional supplementation by 5 mM MnCl2. Yields of MnSOD mutant protein were on average 50 mg of protein/50 g of bacterial pellet.

Purification of Human MnSOD. The mutant Y34F hMnSOD used in kinetic and spectroscopic studies was purified from E. coli using a combination of heat treatment (60 °C) and ion-exchange chromatography (DE52 and CM52) according to the procedures of Beck et al. (18). The purity of the resulting samples was determined on SDS–polyacrylamide gels, which showed one intense band. The purified enzyme was dialyzed extensively against EDTA and a portion of the resulting protein was digested with nitric acid for manganese analysis by atomic absorption spectrometry (Perkin-Elmer 5100PC). These measurements were used to determine the concentration of enzyme.
Crystallography. Mutagenesis, expression, and purification for crystallization was done as described previously (19). The orthorhombic crystals were grown from solutions consisting of 10 mg/mL protein buffered in 25 mM potassium phosphate at pH 7.8 and 20% poly(ethylene glycol) (PEG) 4000. These needle-shaped crystals grew after 7 days and belong to space group P2₁2₁2 with unit cell dimensions of a = 75.2 Å, b = 79.8 Å, and c = 68.8 Å. Initial data were collected on a Siemens Xentronics area detector and rotating-anode X-ray generator to 2.8 Å resolution. The initial structure refinement of the mutant model derived from the isomorphous wild-type enzyme coordinates (2) was performed using the simulated annealing (SA) method in X-PLOR (20). High-resolution 2.4 Å data were later collected at the Stanford Synchrotron Radiation Laboratory (SSRL). The data were processed using DENZO (21) and the Rsym was 7.5%. The data are 94.5% complete. After several cycles of refinement, the model was rebuilt to σA-weighted 2Fo - Fc and Fo - Fc omit maps (22) using XFIT (23). The refinement against these data was completed in X-PLOR using the bulk solvent correction (24) with the manganese ion completely unrestrained during the final round of refinement to remove any force field bias. The final model consists of two Y34F MnSOD subunits and 252 solvent waters with an R-factor of 18.6% and Rfree of 26.9%.

Large, hexagonal crystals of Y34F, nonisomorphous to those of wild-type hMnSOD, were grown from Na₂KPO₄ at pH 7.0. Hexagonal crystals belonged to space group P6₁2₁2, with unit cell dimensions of a = b = 80.9 Å and c = 242.5 Å. Initial data were collected on the MAR image plate area detector to 2.8 Å resolution. A 1.9 Å resolution data set was then collected on the translated MAR image plate at the Protein Structure Facility at the University of California, San Diego. A total of five crystals were used to collect the complete data set. The data were processed using DENZO and the Rsym was 7.7% after scaling the data from five different crystals with 88% completeness to 1.9 Å resolution. The structure of the hexagonal form was eventually solved with AMoRe (25) using one monomer of MnSOD as a probe. The SA refinement using the X-PLOR package converged to an R-factor of 23%. The model was refit to SA-omit (20) and σA-weighted Fo - Fc (22) maps. The solvent molecules were located by using the program PICK (26) and examined in σA-weighted Fo - Fc omit maps. The final R-factor converged to 18.2% (Rfree of 23.6%) with 358 water molecules (Table 1). The average temperature factor B value for the protein atoms is 25.4 Å². The final model includes two Y34F MnSOD subunits in the asymmetric unit with the active site well-defined by the 2Fo - Fc electron density map (see Figure 1). The Y34Fortho data were collected at room temperature and the Y34Fhex data were collected at 10 °C. Atomic coordinates have been deposited in the Brookhaven Protein Data Bank under accession codes 1AP5 for Y34Fortho and 1AP6 for Y34Fhex.

Differential Scanning Calorimetry. A Microcal-2 high-sensitivity differential scanning calorimeter was used to obtain all denaturation profiles. The human MnSOD and mutant protein at 2.4–3.2 mg/mL in 2 mM potassium phosphate buffer (pH 7.8) were deaerated under mild vacuum for 5 min and immediately scanned at a rate of temperature increase of 1 °C/min. The baseline and change in specific heat (ΔCp) upon denaturation were corrected as previously described (19). The peaks of the differential scanning calorimetry profile were deconvoluted assuming a reversible, non-two-state model (27) and using the software package ORIGIN (Microcal, Inc.). ΔH, ΔS, and Tm, defined as the temperature of half-completion, for each transition were obtained from the best fits.

Pulse Radiolysis. These experiments were carried out at the Center for Fast Kinetics Research at the University of Texas at Austin using a 4-Mev van der Graaff accelerator. A single high-dose electron pulse was used to generate superoxide radical anions from oxygen in aqueous solutions containing 10 mM sodium formate as hydroxyl radical scavenger (28) and 50 μM EDTA in addition to buffer and enzyme. All pulse radiolysis experiments were carried out at 20 °C. The dismutation of O₂⁻ was followed spectrophotometrically from an absorbance band centered at 250 nm [ε = 2000 M⁻¹ cm⁻¹ (29)] using a cell with a path length of 2.5 cm. Progress curves for each set of 6–9 experiments were averaged. Exposure to ultraviolet radiation was minimized by opening a mechanical shutter a fraction of a second before each pulse.

RESULTS AND DISCUSSION

Mutant Enzyme Structure and Active-Site Geometry. The Y34F structure and active-site geometry is well-defined by the 1.9 Å electron density maps (Figure 1). The three-dimensional structure of the Y34F human MnSOD mutant is similar to that of the wild-type hMnSOD; each is a homotetramer in which subunits A and C (A and B in Y34Fortho) form the asymmetric unit (Figure 2A). The subunit consists of two structural domains: an N-terminal helical hairpin domain consisting of two long antiparallel α helices separated by a tight turn and a C-terminal α/β domain, containing a central three-stranded antiparallel β sheet and five α helices (Figure 2B). The two domains are joined by the active-site manganese, which is liganded to three histidines, one aspartate, and one hydroxyl group (Figure 2C). The structures are well refined with almost all φ–ψ angles in the energetically preferred regions in a Ramachandran plot (30). Residues 142 and 170 are consistent outliers in MnSOD structures, including the wild-type structure, indicating an unusual type of tight turn. The 1.9 Å resolution data for Y34F provides the most accurate and highest resolution hMnSOD structure to date.

| Table 1: Diffraction Data and Refinement Statistics for Y34Fortho and Y34Fhex MnSODs |
| crystal form | P2₁2₁2 | P6₁22 |
| resolution (Å) | 2.4 | 1.9 |
| total observations | 51722 | 130874 |
| unique reflections | 15865 | 35261 |
| completeness of data (%) | 94.5 | 88.3 |
| (F) > 2σ (F) | 92.8 | 87.6 |
| Rsym (%) | 7.5 | 7.7 |
| <I/Io> | 20.0 | 13.7 |
| R (%) | 18.6 | 18.2 |
| Rfree (%) | 26.9 | 23.6 |
| no. of atoms | 3400 | 3507 |
| no. of water molecules | 252 | 358 |
| rmsd bonds (Å) | 0.009 | 0.008 |
| rmsd angles (deg) | 1.4 | 1.4 |

*Rsym is the unweighted R value on F between symmetry mates. *Rfree is the R factor of 5% randomly selected reflections never used in refinement.*
For each of the four active sites within the hMnSOD tetramer, a channel made up of amino acid residues primarily from two subunits provides the most probable access for the superoxide substrate to the catalytic Mn(III) ion. This deep channel is shaped like a lopsided funnel about 34 Å wide at the mouth between Lys 51A and Lys 178B and 8 Å wide at the base between Tyr 34A and Arg 173B. The channel funnel is lopsided by having sides ranging from 14 to 31 Å in depth. This funnel appears suitable for electrostatic guidance of superoxide anion as it is lined by eight lysines.

**Figure 1:** Diagram showing the active site of the Y34F mutant of MnSOD (hexagonal crystal form) in its 2Fo − Fc electron density (1σ blue contours). The structure has been refined to 1.9 Å resolution with F34 and Q143 in good density.

**Figure 2:** hMnSOD Y34F tetrameric structure, subunit fold, and active-site structure rendered as Ribbons diagrams (38). (A) Tetramer. Individual subunits are colored: A, yellow; B, green; C, magenta; and D, blue. Manganese ions are shown as pink spheres and the side chains of Phe34 are shown in white ball-and-stick representation. The A/B and C/D dimers (A/C and B/D in Y34Fhex) each form a crystallographic asymmetric unit. (B) Subunit fold. The Y34F MnSOD subunit is colored to emphasize secondary structure and domain organization. The N-terminal domain (bottom) is made up of the N-terminal loop (blue) and two long α helices (α1 and α2) (pink). The C-terminal α/β domain (top) is composed of five α helices (α3–α7) (blue) and three β strands (β1–β3) (orange). The pink-colored manganese lies between the two domains. (C) Active site geometry. The trigonal bipyramidal geometry of the five manganese ligands and Phe34 are drawn in a ball-and-stick representation. Amino acids from both domains contribute to the active site. (D) Active-site channel. The channel is formed for each subunit by residues from both subunits of the asymmetric unit. The superoxide substrate may pass through this channel to reach the Mn(III) ion. Residues that lie between the channel and Mn(III) ion are shown in ball-and-stick representation.
from three subunits: 29A, 44A, 51A, 106B, 108B, 110B, 178B, and 130C. Arg 173, His 30, Gly 120, and Tyr 34 form the base of the funnel. The side chains of Tyr 34, His 30, and Gln 143 gate the Mn-bound water ligand from the channel solvent (Figure 2D).

**Structural Comparison of the Y34F Mutant and the Wild Type.** Superposition of the wild-type (2) and Y34F subunit structures reveals close structural similarity, with a root-mean-square deviation for 196 Cα atoms of 0.29 and 0.26 Å for the orthorhombic and hexagonal crystal forms, respectively. In the vicinity of the manganese, the positions of the three histidine ligands (26, 74, and 163), Asp 159, Gln 143, and the OH−/H2O are only slightly shifted compared to the wild-type protein (Figure 3A). The imidazole ring of His 74 in Y34Fortho (the orthorhombic crystal form) subunit A is rotated about 20° (10° in subunit B) relative to that in the wild-type and Y34Fhex (the hexagonal crystal form). However, the superior 1.9 Å resolution and 23.6% Rfree for the Y34Fmutant (2.12 Å in Y34Fortho and 2.13 Å in Y34Fhex) are slightly longer than in the wild-type (2.09 Å). Consistent results are also observed for the Mn−Asp Oδ1 distances (2.09 Å in Y34Fortho and 1.97 Å in Y34Fhex and 1.94 Å in the wild type), representing slightly expanded coordination geometry. The OH−/H2O ligand in Y34Fhex is about the same distance from the Mn ion as seen in the wild-type enzyme, suggesting that both mutant and the wild-type enzymes have a similar hydroxyl ligand at this position. The ligand OH−/H2O Y34Fortho has very weak electron density and a high B-factor (47 Å² in comparison with 17 Å² in Y34Fhex and 22 Å² in native), so this OH−/H2O is less well defined in this orthorhombic crystal form. This is surprising as this structure was solved from crystals grown at a slightly higher pH (pH 7.8) than the wild-type crystals (pH 7.5) and Y34Fhex (pH 7.0). The decreased occupancy of the water site in the Y34Fortho crystal form does not affect the coordination geometry of the other ligands and is consistent with this being an exchangeable site suitable for superoxide binding. Superposition of the two different crystal forms of Y34F mutants reveals an rmsd of 0.3 Å for 196 Cα atoms. Apparently the crystal packing did not change the overall structure significantly. Each of the two subunits in the asymmetric unit of the Y34F mutant (two crystal forms) can be superimposed with another subunit in the wild-type structure with rmsd for 196 Cαs in the range of 0.19−0.40 Å, representing very similar three-dimensional structures. In the native enzyme, the hydroxyl group of Tyr 34 forms a hydrogen bond to the amide moiety of Gln 143 (2.7 Å in distance); this interaction is absent in the mutant structure due to the missing hydroxyl group (Figure 3B).

**Thermal Stability.** The thermal stability of human Y34F MnSOD was determined by differential scanning calorimetry (DSC). Five peaks were resolvable for Y34F MnSOD (Figure 4), three overlapping small ones (labeled A) with Tm~ 63, 71, and 78 °C, another small peak labeled B with Tm~ 86 °C, and a much larger one (labeled C) with Tm~ 96 °C. This notation for labeling the transitions was chosen to correspond to that used previously for native and I58T MnSOD (19). Transition C is the main unfolding transition
of the enzyme. Surprisingly, Y34F is more stable than native MnSOD, with increases of 16 °C in \(T_m\) (B) and 6.7 °C in \(T_m\) (C). The identity of transitions A and B are unknown; however, the \(T_m\) of transition B corresponds to the thermal inactivation temperature (19). Thus, this transition, which occurs with a relatively small absorption of heat, results in an inactive enzyme. Transition A, which is not observed in native MnSOD, appears to have multiple components in IT8T and Y34F MnSOD and is extremely weak and nearly undetectable in Q143N MnSOD (31). From the IT8T mutant this transition has no effect on activity (19).

The estimated stabilization of the mutant compared to native, given as \(\Delta G^\circ\), was calculated from the thermodynamic parameters \(H\), \(S\), and \(T_m\) (Table 2). A reversible analysis of an apparently irreversible process is possible if there is little accumulation of the irreversibly denatured species during that part of the DSC scan used for analysis (27). Irreversible species will accumulate near the high-temperature end of each transition; therefore, to minimize errors in determining \(\Delta H\) and \(\Delta S\), the high-temperature end was excluded in the reversible curve-fitting (32). As expected for an irreversible transition, the high-temperature end of the DSC profiles are not well fit with the reversible model (Figure 4) because of the sharp drop in the entropy of the species during that part of the DSC scan used for analysis (32). Therefore, to fit the high-temperature tail (beyond the peak half-height on the downslope) excluded (Figure 4). Due to irreversibility, an accurate value for \(\Delta C_p\) is unobtainable, so \(\Delta G^\circ\) was calculated by two procedures: (1) by assuming constant \(\Delta H\) and \(\Delta S\) and (2) by estimating the temperature dependence of \(\Delta H\) and \(\Delta S\) using a value of 0.12 cal/g for \(\Delta C_p\), which is an average value for several small, globular proteins (33).

These methods give a stabilization \(\Delta G^\circ\) of 13−16 kcal/mol for transition B and 3.1−3.5 kcal/mol for transition C (Table 2). The magnitude of \(\Delta G^\circ\) for transition B is greatly affected by the large value of \(\Delta H\) for transition B, which is more than twice that for transition B of native MnSOD.

The surprisingly great stabilization resulting from the Y34F mutation indicates that the Tyr 34 hydrogen-bonded network does not stabilize the active-site architecture. Instead, the increased stability of Y34F suggests that the conservation of tyrosine at residue 34 reflects a functional optimization. These results are consistent with detailed studies on the catalytic residues of T4 lysozyme, which have supported the general theory that enzyme residues involved in function are not optimized for stability (34). However, our results on the structure, stability, and thermostability of Y34F MnSOD differ from an analysis on the Y34F mutation on E. coli Fe superoxide dismutase where activity and thermal inactivation results were interpreted to suggest roles for Tyr 34 in both stability and catalysis (15).

**Table 2: Thermodynamic Parameters for Reversible Unfolding of MnSOD**

<table>
<thead>
<tr>
<th>enzyme</th>
<th>component</th>
<th>(T_m) (°C)</th>
<th>(\Delta H^\circ) (kcal/mol)</th>
<th>(\Delta G^\circ) (kcal/mol)</th>
<th>(\Delta G^\circ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>B</td>
<td>70</td>
<td>142</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>native</td>
<td>C</td>
<td>88.9</td>
<td>90.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Y34F</td>
<td>B</td>
<td>85.9</td>
<td>351</td>
<td>15.5</td>
<td>12.7</td>
</tr>
<tr>
<td>Y34F</td>
<td>C</td>
<td>95.6</td>
<td>197</td>
<td>3.5</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\(a\) \(\Delta H\) and \(\Delta G\) are given per mole of tetramer. \(b\) \(\Delta G^\circ\) is \(\Delta G\) of the mutant calculated at the appropriate \(T_m\) of the native, assuming constant \(\Delta H\) (\(\Delta C_p = 0\)). \(c\) \(\Delta G^\circ\) was calculated assuming \(\Delta C_p = 0.12\) cal/g.

**Figure 5:** Comparison of superoxide decay catalyzed by (top) wild-type human MnSOD and (bottom) Y34F human MnSOD as determined by pulse radiolysis. Data show the decrease in superoxide as determined from its absorbance at 250 nm (\(e = 2000\) M\(^{-1}\) cm\(^{-1}\)). The solutions contained 0.5 \(\mu\)M wild-type MnSOD or 0.5 \(\mu\)M Y34F MnSOD, 50 \(\mu\)M EDTA, 10 mM sodium formate, and 2.0 mM sodium pyrophosphate at pH 9.6 and 20 °C.
Figure 6: Absorbance at 480 nm as a function of pH for Y34F human manganese superoxide dismutase (○) and the wild-type human MnSOD (●) at 20 °C. The solid line for wild type is a fit to a single ionization with a pK\textsubscript{a} of 10.1 ± 0.1; for Y34F MnSOD the value is pK\textsubscript{a} = 11.1 ± 0.1.

Hence, this product inhibition cannot be overcome by donation of protons from these buffers in solution.

The visible absorption spectrum of Y34F hMnSOD displayed a maximum at 480 nm with a shoulder at 600 nm, similar to published spectra of the oxidized form of the wild-type enzyme (8, 13, 18). Accordingly, the Y34F mutant was purified predominantly in the oxidized form, although we recognize that are dealing with enzyme with mixed oxidation states of Mn\textsuperscript{2+} and Mn\textsuperscript{3+}. The pH dependence of this absorbance for Y34F hMnSOD can be described by a single ionization with pK\textsubscript{a} near 9 for the wild-type enzyme this pK\textsubscript{a} was closer to 10 (Figure 6). There is evidence of enzyme denaturation above pH 11. An ionization of pK\textsubscript{a} near 9 is observed for wild-type FeSOD, describing the pH dependence of K\textsuperscript{a} and the binding of azide, among other properties (35). This pK\textsubscript{a} is believed to be caused by the addition of an hydroxide ligand to the metal in the ferric form; the pK\textsubscript{a} near 9 in the ferrous form is believed to be the ionization of Tyr 34 (14). From the crystal structure results, the replacement Tyr 34 → Phe has destroyed the hydrogen-bonding network involving this residue, Gln 143 and the metal-bound water; this is consistent with a destabilization of metal-bound hydroxide and hence an increase in the pK\textsubscript{a} and lower activity for Y34F. This suggestion is also supported by examination of spectral properties and azide inhibition of MnSOD from E. coli in comparison with FeSOD and iron-substituted MnSOD (36).

Catalytic Mechanism of Y34F Human MnSOD. The kinetic mechanism of Scheme 1 used to describe the wild-type MnSOD (8, 13) can also describe catalysis by Y34F MnSOD (Figure 7), as shown by pulse radiolysis. The fit was obtained using KINSIM (37), and the rate constants for Y34F MnSOD obtained in this manner are given in Table 3, where they are compared with the constants found for the wild-type hMnSOD under comparable conditions. In this simulation, the breakdown of the enzyme–substrate complex for enzyme in the reduced state, represented by k\textsubscript{−3} in Scheme 1, is much slower for Y34F compared with the wild type, whereas the values of k\textsubscript{3} are the same for both enzymes (Table 3). This indicates a longer lifetime for this complex and hence a greater probability it will form the inhibited complex, which is confirmed by simulations of the catalysis using the data of Table 3 and KINSIM. The rate constants in Y34F for actual formation of the dead-end complex itself,
$k_s$ and $k_-$ are similar to those for the wild type. Hence, the mutation Tyr 34 $\rightarrow$ Phe may be altering the inherent catalysis of superoxide decay and not the steps by which the inhibited complex is formed.

The value of $k_{cat}$ for the mutant is about 10-fold less than for the wild type (Table 4), while the ratio $k_{cat}/K_m$ is nearly identical for the two forms of MnSOD, near $10^8$ M$^{-1}$ s$^{-1}$, a value close to the diffusion-controlled limit. These values were obtained using the data of Table 3 and eqs 5–9 of Bull et al. (13). The results of Table 3 may not represent unique solutions to the appropriate kinetic equations for hMnSOD and mutants; however, they qualitatively represent trends in the catalytic pathway between the wild type and mutants of hMnSOD. Thus, at low concentrations of superoxide compared with $K_m$ (50 and 4 $\mu$M for the wild type and Y34F, respectively) the catalysis by these two enzymes is nearly identical, but near substrate saturation the mutant Y34F MnSOD is slower by 10-fold. This is consistent with a role for Tyr 34 in proton transfer in analogy with FeSOD (35). Perhaps Tyr 34 is itself a proton donor or through its participation in the hydrogen-bonded network is involved indirectly in proton donation required to dissociate hydrogen peroxide or the hydroperoxide anion $\text{HO}_2^-$. The crystal structure of Figure 3B shows that the hydrogen-bond chain from Tyr 34 and extending to the manganese-bound water is broken in the Y34F mutant. In the absence of this hydroxyl in Y34F there may be less proton donation to product peroxyl and maximal velocity (i.e., $k_{cat}$) is slowed. Certainly Y34F breaks the hydrogen-bond relay Mn(III)$\rightarrow$hydroxyl$\rightarrow$Gln 143$\rightarrow$Tyr34$\rightarrow$water$\rightarrow$His 30. It is a reasonable assumption that proton-transfer steps are involved in $k_4$ (Scheme 1), and the data of Table 3 show a reduced value of $k_4$ in Y34F in comparison with the wild type.

**Conclusions.** Characterization of the structure, stability, and activity of Y34F hMnSOD provides insights into the functional role of the conserved Tyr 34 that impact our understanding of superoxide dismutase activity and metalloenzyme evolution. (1) The side-chain hydroxyl of the conserved residue Tyr 34 in MnSOD contributes to catalytic efficiency in MnSOD but is not essential at normal cellular superoxide concentrations. (2) The structure of the active site is unchanged by the replacement of Tyr 34 $\rightarrow$ Phe with the phenyl rings of Tyr 34 in the wild-type and Phe 34 in the mutant Y34F superimposable for two independent crystal forms of Y34F. (3) Y34F not only retains the wild-type active-site architecture but also shows a major increase in thermostability, arguing that Tyr 34 conservation is not due to a structural role. (4) The kinetic results suggest that the replacement Tyr 34 $\rightarrow$ Phe does not affect the diffusion-controlled steady-state constant $k_{cat}/K_m$, which has a value near $10^8$ M$^{-1}$ s$^{-1}$ for both the wild-type and Y34F human MnSOD. (5) The replacement of Tyr 34 $\rightarrow$ Phe appears to affect the rate of maximal catalysis $k_{cat}$, reducing by about 10-fold the steps that determine $k_{cat}$. This and the breaking of the hydrogen-bonding chain at the active site caused by the replacement of Tyr 34 $\rightarrow$ Phe suggests that Tyr 34 is a proton donor or is involved indirectly by its hydrogen-bonded network to other residues and solvent for proton transfer. (6) The mutant Y34F shows enhanced product inhibition when compared with the wild type. This may be related to decreased proton transfer capability in the active site of the Y34F mutant. (7) Catalysis in the substrate-inhibited region by both Y34F and the wild type was not susceptible to enhancement by even up to 100 mM of buffer in solution, indicating that protonation from certain buffers in solution is not capable of enhancing the release of the inhibiting bound peroxide ion, as expected from the structurally sequestered nature of the MnSOD active site. (8) The combination of structural conservation, increased thermostability, and diffusion-controlled rate for Y34F under normal cellular superoxide levels prompts us to propose the hypothesis that conservation of active-site residues can reflect functional constraints from extreme rather than normal cellular conditions.

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**References**


Table 4: Values of the Steady-State Parameters for the Decay of Superoxide Catalyzed by Human MnSOD and Two Mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (ms$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type MnSOD$^a$</td>
<td>40</td>
<td>800</td>
</tr>
<tr>
<td>Y34F MnSOD$^a$</td>
<td>3.3</td>
<td>870</td>
</tr>
<tr>
<td>Q143N MnSOD$^b$</td>
<td>0.3</td>
<td>0.82</td>
</tr>
</tbody>
</table>

$^a$ Data for wild type (8) measured at 20 °C and pH 9.4 by pulse radiolysis; data for Y34F (this work) measured at 20 °C and pH 9.6 by pulse radiolysis. $^b$ Data obtained by stopped-flow spectrophotometry at 5 °C and pH 9.4 (31).