Interrupting the Hydrogen Bond Network at the Active Site of Human Manganese Superoxide Dismutase*

(Received for publication, June 9, 1999, and in revised form, July 19, 1999)

Cecilia A. Ramiolo, Vincent Leveque, Yue Guan, James R. Lepock, John A. Tainer, Harry S. Nick, and David N. Silverman

From the Departments of Pharmacology and Therapeutics, Biochemistry and Molecular Biology, and Neuroscience, University of Florida, Gainesville, Florida 32610, the Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, and the Department of Physics, University of Waterloo, Waterloo, Ontario N2L3G1, Canada

Histidine 30 in human manganese superoxide dismutase (MnSOD) is located at a site partially exposed to solvent with its side chain participating in a hydrogen-bonded network that includes the active-site residues Tyr166 and Tyr34 and extends to the manganese-bound solvent molecule. We have replaced His30 with a series of amino acids and Tyr166 with Phe in human MnSOD. The crystal structure of the mutant of MnSOD containing Asn30 superimposed closely with the wild type, but the side chain of Asn30 did not participate in the hydrogen-bonded network in the active site. The catalytic activity of a number of mutants with replacements at position 30 and for the mutant containing Phe166 showed a 10–40-fold decrease in $k_{\text{cat}}$. This is the same magnitude of decrease in $k_{\text{cat}}$ obtained with the replacement of Tyr34 by Phe, suggesting that interrupting the hydrogen-bonded active-site network at any of the sites of these three participants (His30, Tyr34, and Tyr166) leads to an equivalent decrease in $k_{\text{cat}}$ and probably less efficient proton transfer to product peroxide. The specific geometry of His30 on the hydrogen bond network is essential for stability since the disparate mutations H30S, H30A, and H30Q reduce $T_m$ by similar amounts (10–16 °C) compared with wild type.

This paper is available online at http://www.jbc.org

---

Human manganese superoxide dismutase is a homotetramer (a dimer of dimers) of 22-kDa subunits that carries out catalysis through a redox process in which the metal cycles between a number of mutants with replacements at position 30 and for the mutant containing Phe166 showed a 10–40-fold decrease in $k_{\text{cat}}$. This is the same magnitude of decrease in $k_{\text{cat}}$ obtained with the replacement of Tyr34 by Phe, suggesting that interrupting the hydrogen-bonded active-site network at any of the sites of these three participants (His30, Tyr34, and Tyr166) leads to an equivalent decrease in $k_{\text{cat}}$ and probably less efficient proton transfer to product peroxide. The specific geometry of His30 on the hydrogen bond network is essential for stability since the disparate mutations H30S, H30A, and H30Q reduce $T_m$ by similar amounts (10–16 °C) compared with wild type.

$\text{Mn}^{3+} + \text{O}_2^\cdot + \text{H}_2\text{O} \rightarrow \text{Mn}^{2+} + \text{H}_2\text{O}_2$.

**REACTION 1**

$\text{Mn}^{2+} + \text{O}_2^\cdot + \text{H}_2^\cdot \rightarrow \text{Mn}^{2+} + \text{H}_2\text{O}_2$.

**REACTION 2**

This is a very efficient catalysis; steady-state constants for human MnSOD obtained using both stopped-flow spectroscopy and pulse radiolysis (2) show that $k_{\text{cat}}/K_m$ is near diffusion control and $k_{\text{cat}}$ is approximately $4 \times 10^9$ s$^{-1}$. Measurements on MnSOD from *Thermus thermophilus* showed similar results (3).

The maximal velocity of catalysis has rate-contributing proton transfer steps as determined by the solvent hydrogen isotope effect of 2.1 for the turnover number $k_{\text{cat}}$ for catalysis by *T. thermophilus* MnSOD (3), and the enhancement of maximal velocity by proton donors in solution observed for catalysis by the very similar *Escherichia coli* iron superoxide dismutase (4). This protonation promotes the dissociation of product from the enzyme and perhaps decreases the extent of product inhibition. Recent studies using site-specific mutagenesis have clarified the role of Tyr34 in the active site of MnSOD showing that replacement of this residue with Phe leaves a mutant with considerable catalytic activity (5–8). Tyr34 in human MnSOD has properties consistent with its participation in the proton transfer events reported by $k_{\text{cat}}$; the replacement of Tyr34 with Phe in human MnSOD resulted in the decrease in $k_{\text{cat}}$ by an order of magnitude with no change in $k_{\text{cat}}/K_m$. Moreover, the side chain of Tyr34 forms a hydrogen bond with metal-bound azide, a substrate analog, in MnSOD from *T. thermophilus* (9).

There is an extensive hydrogen-bonded network in MnSOD that extends throughout the active-site cavity involving side-chain residues, the aqueous ligand of manganese, and other water molecules, part of which is shown in Fig. 1. This network has been identified by crystallography (8, 10) and by the changes in proton NMR chemical shifts for many active-site residues in reduced *E. coli* iron superoxide dismutase caused by the replacement of Tyr34 with Phe (7). In this network the solvent ligand of manganese is hydrogen-bonded to the side chain of Gln143, which in turn forms a hydrogen bond with the phenolic hydroxyl of Tyr34. The side-chain imidazole of His30 forms two hydrogen bonds in human MnSOD, one with the side-chain hydroxyl of Tyr34 through an intervening water molecule and a second with the side-chain hydroxyl of Tyr166 from the adjacent subunit in the dimer. This rather extensive hydrogen-bonded array could be involved in the proton transfer necessary to form product hydrogen peroxide by supporting a proton relay, or possibly some of these residues may be a source of the proton itself. Of course, the ultimate source of proton donation to product is from solution, and His30 and Tyr34 in human MnSOD are partially exposed to solvent. Tyr166 is buried (data of Borgstahl et al. (10)). His30, Tyr34, and Tyr166 are conserved in all forms of MnSOD sequenced to date (11).

Initial studies of MnSOD from *Bacillus steaerotherophilus* determined that its catalysis is complicated by the presence of...
an inactive form of the enzyme that can interconvert to an active form (12). Bull et al. (3) observed the inactive form spectrophotometrically in catalysis by T. thermophilus and suggested that it results from product inhibition, perhaps as an oxidative addition of O₂ to the Mn(II) form of the enzyme resulting in a side-on peroxo complex of Mn(III). Such an inhibited phase was also observed for human MnSOD (2). The mutant of human MnSOD containing the replacement Tyr¹⁶⁶ → Phe showed enhanced inhibition (8).

To investigate further the structural and functional role of this hydrogen-bonded network in the active-site cavity of human MnSOD, we have prepared and measured catalysis by mutants at two additional sites in this network, His³⁰ and Tyr¹⁶⁶. The crystal structure for the mutant containing the replacement His³⁰ → Asn showed that the hydrogen-bonded network was interrupted, and calorimetry showed that the main unfolding transition was decreased by 12 °C. The catalytic activities of these mutants showed that neither His³⁰ nor Tyr¹⁶⁶ is essential for the catalysis, but their replacement each caused substantial decreases in both k₅ₐ₆T/k₉₆ₙ and k₉₆₉, generally at least 10-fold. Since k₅₉₆T appears to have rate-contributing proton transfer steps, the decrease in k₅₉₆T caused by the replacements suggests decreased proton transfer in the maximum velocity of catalysis. The replacements at positions 30 and 166 also caused a significant decrease in the extent of product inhibition with wild-type MnSOD.

**MATERIALS AND METHODS**

**PCR-based Site-directed Mutagenesis**—The oligonucleotides GCAT- ATGAAAGCAGCAGCTCC and GGAGATCTGAGCTGACGCGCTG-3′ were used as primers for PCR to amplify the human MnSOD cDNA (cDNA sequence reported by Beck et al. (13)). The plasmid pMNHSOD4 (ATCC no. 59947), which contains human MnSOD, was subcloned into the TA cloning vector, pCRII (Invitrogen Corp.). A series of primers were designed to create the mutants H30X in human MnSOD (X = A, E, K, N, Q, or S). First, we designed a pair of oligonucleotides, primer 1 (5′-CCGACATGATCTCATTCGAGAAGCAGACGGCCTG-3′) and 2 (5′-CTGAGAATACAGTAAGCTGC-3′), whose sequences are complementary to each other and contain the recognition sites for BspI and PstI, respectively. Upon denaturation were corrected as described previously (19). The peaks of the differential scanning calorimetry profile were deconvoluted assuming a reversible, non-two-state model (20) using the software package ORIGIN (Microcal, Inc.). ΔCₚ, ΔS, and T⁻¹ were defined as the temperature of half-completion, for each transition are obtained from the best fits. ΔDG was calculated from these thermodynamic parameters setting ΔCₚ = 0 since the value of ΔCₚ for MnSOD is unknown. Assuming ΔCₚ = 0.12 cal/K gives ΔDG values 10–40% lower (8, 19, 21).

**Stopped-flow Spectroscopy**—Experiments are based on the stabilization of KO₂ in aprotic solvent and the subsequent large dilution of this solution by an aqueous solution of enzyme in a stopped-flow apparatus, as described by McClune and Fee (22). KO₂ was dissolved in dimethyl sulfoxide with solubility of KO₂ enhanced with 18-crown-6 ether (23). The stopped-flow spectrophotometer (Kinetic Instruments, Ann Arbor, MI) was capable of efficient mixing of this solution with an aqueous solution of enzyme in a stopped-flow apparatus, as described by McClune and Fee (22). Enzyme solutions were clarified at 10,000g for 10 min at 4 °C before use.

**RESULTS**

**Structure of H30N MnSOD**—Unlike the native and other functional mutant structures of MnSOD, which crystallize in space group P2₁2₁2₁ and have two MnSOD subunits in the asymmetric unit, H30N crystallized in space group P2₁2₁2₁ and had four MnSOD subunits in the asymmetric unit. Like the wild-type human MnSOD which is tetrameric (2), the crystal structure of H30N is also tetrameric. The subunit fold and

**Manganese Superoxide Dismutase**
Asn30 does not show residues in the active-site. Features of wild-type human MnSOD and H30N MnSOD (multicolored) showing residues in the active-site. Asn30 does not form a hydrogen bond to the adjacent water molecule or to Tyr166, and thus a hydrogen-bonded array in the active site is less extensive in the H30N mutant than in the wild type.

The structural crystal of the mutant H30N MnSOD showed minimal changes in the orientation of residues in the active site cavity, with Asn30 having the same dihedral angle about the Co-Cβ bond as His30 in the wild-type enzyme (Fig. 1). The primary structural changes in the H30N mutant involve altered local hydrogen bonds to solvent and side chains. In wild-type MnSOD, there is a water molecule that acts as a hydrogen-bonded bridge between the side chains of His30 and Tyr34 (Fig. 1). Such a water molecule between Tyr34 and Asn30 also exists in the H30N mutant; however, it has lost its hydrogen bonding to the side chain at residue 30. Therefore, the Mn-superoxide decay catalyzed by H30N MnSOD can be roughly fit to a single ionization of pKa of 9.4 ± 0.1 for wild-type, 9.3 ± 0.1 for H30N, and >10.5 for H30Q.

The least-squares superposition of the crystal structures of wild-type human MnSOD (purple) and H30N MnSOD (multicolored) showing residues in the active-site. Asn30 does not form a hydrogen bond to the adjacent water molecule or to Tyr166, and thus a hydrogen-bonded array in the active site is less extensive in the H30N mutant than in the wild type.

The crystal structure of the mutant H30N MnSOD shows a slight compression from the dimer interface region, and H30N has more compact tetrameric association than that in the wild type.

The crystal structure of the mutant H30N MnSOD showed similar to the wild type with a root-mean-square deviation for Cs values of 0.56 Å. The superposition of the H30N tetramer and native tetramer shows a slight compression from the dimer interface region, and H30N has more compact tetrameric association than that in the wild type.

The crystal structure of the mutant H30N MnSOD showed minimal changes in the orientation of residues in the active site cavity, with Asn30 having the same dihedral angle about the Co-Cβ bond as His30 in the wild-type enzyme (Fig. 1). The primary structural changes in the H30N mutant involve altered local hydrogen bonds to solvent and side chains. In wild-type MnSOD, there is a water molecule that acts as a hydrogen-bonded bridge between the side chains of His30 and Tyr34 (Fig. 1). Such a water molecule between Tyr34 and Asn30 also exists in the H30N mutant; however, it has lost its hydrogen bonding to the side chain at residue 30. Therefore, the Mn-superoxide decay catalyzed by H30N MnSOD can be roughly fit to a single ionization of pKa of 9.4 ± 0.1 for wild-type, 9.3 ± 0.1 for H30N, and >10.5 for H30Q.

The least-squares superposition of the crystal structures of wild-type human MnSOD (purple) and H30N MnSOD (multicolored) showing residues in the active-site. Asn30 does not form a hydrogen bond to the adjacent water molecule or to Tyr166, and thus a hydrogen-bonded array in the active site is less extensive in the H30N mutant than in the wild type.

The crystal structure of the mutant H30N MnSOD showed minimal changes in the orientation of residues in the active site cavity, with Asn30 having the same dihedral angle about the Co-Cβ bond as His30 in the wild-type enzyme (Fig. 1). The primary structural changes in the H30N mutant involve altered local hydrogen bonds to solvent and side chains. In wild-type MnSOD, there is a water molecule that acts as a hydrogen-bonded bridge between the side chains of His30 and Tyr34 (Fig. 1). Such a water molecule between Tyr34 and Asn30 also exists in the H30N mutant; however, it has lost its hydrogen bonding to the side chain at residue 30. Therefore, the Mn-superoxide decay catalyzed by H30N MnSOD can be roughly fit to a single ionization of pKa of 9.4 ± 0.1 for wild-type, 9.3 ± 0.1 for H30N, and >10.5 for H30Q.

The least-squares superposition of the crystal structures of wild-type human MnSOD (purple) and H30N MnSOD (multicolored) showing residues in the active-site. Asn30 does not form a hydrogen bond to the adjacent water molecule or to Tyr166, and thus a hydrogen-bonded array in the active site is less extensive in the H30N mutant than in the wild type.

The crystal structure of the mutant H30N MnSOD showed minimal changes in the orientation of residues in the active site cavity, with Asn30 having the same dihedral angle about the Co-Cβ bond as His30 in the wild-type enzyme (Fig. 1). The primary structural changes in the H30N mutant involve altered local hydrogen bonds to solvent and side chains. In wild-type MnSOD, there is a water molecule that acts as a hydrogen-bonded bridge between the side chains of His30 and Tyr34 (Fig. 1). Such a water molecule between Tyr34 and Asn30 also exists in the H30N mutant; however, it has lost its hydrogen bonding to the side chain at residue 30. Therefore, the Mn-superoxide decay catalyzed by H30N MnSOD can be roughly fit to a single ionization of pKa of 9.4 ± 0.1 for wild-type, 9.3 ± 0.1 for H30N, and >10.5 for H30Q.

The crystal structure of the mutant H30N MnSOD showed minimal changes in the orientation of residues in the active site cavity, with Asn30 having the same dihedral angle about the Co-Cβ bond as His30 in the wild-type enzyme (Fig. 1). The primary structural changes in the H30N mutant involve altered local hydrogen bonds to solvent and side chains. In wild-type MnSOD, there is a water molecule that acts as a hydrogen-bonded bridge between the side chains of His30 and Tyr34 (Fig. 1). Such a water molecule between Tyr34 and Asn30 also exists in the H30N mutant; however, it has lost its hydrogen bonding to the side chain at residue 30. Therefore, the Mn-superoxide decay catalyzed by H30N MnSOD can be roughly fit to a single ionization of pKa of 9.4 ± 0.1 for wild-type, 9.3 ± 0.1 for H30N, and >10.5 for H30Q.

The crystal structure of the mutant H30N MnSOD showed minimal changes in the orientation of residues in the active site cavity, with Asn30 having the same dihedral angle about the Co-Cβ bond as His30 in the wild-type enzyme (Fig. 1). The primary structural changes in the H30N mutant involve altered local hydrogen bonds to solvent and side chains. In wild-type MnSOD, there is a water molecule that acts as a hydrogen-bonded bridge between the side chains of His30 and Tyr34 (Fig. 1). Such a water molecule between Tyr34 and Asn30 also exists in the H30N mutant; however, it has lost its hydrogen bonding to the side chain at residue 30. Therefore, the Mn-superoxide decay catalyzed by H30N MnSOD can be roughly fit to a single ionization of pKa of 9.4 ± 0.1 for wild-type, 9.3 ± 0.1 for H30N, and >10.5 for H30Q.
The initial velocities (µM s⁻¹) of the catalyzed decay of superoxide at 20 °C; the uncatalyzed rates have been subtracted. Solutions contained 10 mM Caps buffer at pH 10.0, and the concentration of H30N MnSOD was 0.25 mM. The solid line is a least squares fit of the data to the Michaelis-Menten equation with $k_{cat} = (5.3 \pm 0.7) \times 10^4$ s⁻¹ and $k_{cat}/K_m = (6.6 \pm 1.4) \times 10^4$ M⁻¹ s⁻¹.

Steady-state kinetic constants for the decay of superoxide catalyzed by human MnSOD and mutants at pH 9.4 or 9.6 and 20 °C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (µM s⁻¹)</th>
<th>$k_{cat}/K_m$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>40</td>
<td>800</td>
</tr>
<tr>
<td>Y34F°</td>
<td>3.3</td>
<td>870</td>
</tr>
<tr>
<td>Y166F</td>
<td>1.1</td>
<td>95</td>
</tr>
<tr>
<td>H30N</td>
<td>4.3</td>
<td>130</td>
</tr>
<tr>
<td>H30A</td>
<td>2.4</td>
<td>61</td>
</tr>
<tr>
<td>H30S</td>
<td>2.3</td>
<td>63</td>
</tr>
<tr>
<td>H30Q</td>
<td>1.4</td>
<td>52</td>
</tr>
<tr>
<td>H30K°</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>H30E</td>
<td>0.052</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The value of $k_{cat}$ for wild-type MnSOD was determined by numerical methods using data obtained by pulse radiolysis (2). From Guan et al. (8). These data at pH 9.4, the others at pH 9.6.

The logarithm of $k_{cat}/K_m$ (M⁻¹ s⁻¹) for the catalyzed decay of superoxide catalyzed by wild-type human MnSOD (●) and H30N MnSOD (○) measured by stopped-flow at 20 °C. For H30N, the following buffers (10 mM) and pH values were used: glycyl-glycine (pH 8.0); Taps (8.4, 8.8); Ches (9.2) glycine (9.6), Caps (10.0, 10.4). All solutions contained 50 mM KCl to the most active of the mutants at position 30 (Table I). The solvent hydrogen isotope effects on the steady-state constants were determined for catalysis by H30N MnSOD in solutions containing 10 mM glycyl-glycine buffer at pH 9.4 (uncorrected pH meter reading) and 20 °C. The ratio of $k_{cat}$ measured in H₂O with that in D₂O (0.98 atom fraction D) was $Dk_{cat}/k_{cat}$ = 2.1 ± 0.1. The corresponding solvent hydrogen isotope effect on $k_{cat}/K_m$ was $Dk_{cat}/K_m = 1.2 ± 0.4$. For Y166F MnSOD under these conditions $Dk_{cat}/K_m = 2.3 ± 0.1$ and $Dk_{cat}/K_m = 1.6 ± 0.4$.

Another change caused by the replacement of His30 by Ala, Asn, and Gln and of Tyr166 by Phe is that the resulting mutants show less product inhibition in their catalysis of superoxide dismutation than does wild type. Product inhibition in MnSOD is characterized by a region of zero-order decay of superoxide (3). For H30N the extent of product inhibition was about 4-fold less than with wild type. Product inhibition is prominent in both wild-type (2, 3) and Y34F MnSOD (8). It is not clear why product inhibition is less in the position 30 mutants, but probably part of this absence is due to the slower catalytic activity of the mutants, i.e. there is less product inhibition when the rate of product formation is lower. Other factors may enter, however, such as the effect of His30 on the stability of the enzyme-peroxide complex. Further work on this topic is in progress.

Thermal Stability—The thermal stability of the mutants with replacements at position 30 was determined by differential scanning calorimetry and was compared with the stability of the wild-type MnSOD. For wild-type human MnSOD two prominent transitions have been observed (19); transition C is the main unfolding transition at 89.9 °C, and transition B at 70 °C corresponds to the thermal inactivation temperature. Transition B must entail a minor conformational change in MnSOD since the calorimetric enthalpy associated with it is less than 5% of the calorimetric enthalpy of transition C. None of the mutants at position 30 have a transition that can be clearly associated with the inactivation transition. Only transition C, the main unfolding transition, is resolvable. This suggests that either the two transitions have been superimposed for the mutants or that the inactivation transition B has such a small calorimetric enthalpy for the mutants that it is not detectable. However, the main unfolding transition C, which can be determined unambiguously, is the determinant of conformational stability.

In 20 mM potassium phosphate, pH 7.8, the $T_m$ (transition C) for wild type MnSOD is 89.9 ± 1.1 °C, and the $T_m$ values for the mutants range from 74 to 80 °C (Table II). This indicates that the conformational stability of the mutants is considerably less...
than wild type. Using a value of 116 kcal/mol tetramer for $\Delta H$ of the wild type, these shifts in $T_m$ correspond to $\Delta \Delta G$ values of $-3.0$ to $-5.0$ kcal/mol (Table II).

**DISCUSSION**

In wild-type MnSOD, there is a hydrogen-bonded chain extending from the aqueous ligand of the manganese to the side chain of Tyr34 and then through a hydrogen-bonded water to the His30 side chain, which in turn is hydrogen-bonded to the phenolic hydroxyl of Tyr166. The side chain of His30 is partially exposed to bulk solvent, Tyr166 is buried. However, in H30N MnSOD the side chain of Asn30 does not appear hydrogen-bonded to the corresponding water molecule nor to Tyr166 (Fig. 1). Thus, a hydrogen-bonded array involving residues 30 and 166 that may be involved in the protonation of product peroxide is not as extensive in H30N and Y166F as it is in wild type.

The main unfolding transition measured by differential scanning calorimetry for the mutants at position 30 listed in Table II decreased by 10–16 °C compared with wild type. It is interesting that the result for the mutant of human MnSOD containing the replacement Tyr34 $\rightarrow$ Phe enhanced stability of this transition by nearly 7 °C (8). Both replacements His30 $\rightarrow$ Asn and Tyr34 $\rightarrow$ Phe caused apparent breaks in the hydrogen-bond network in the active site. Hence, the effects of these replacements are more complex than assigning them to the break in the hydrogen-bonding network. Of relevance is the mutation Q143N which stabilizes $T_m$ by 1.8 °C (21). Y34F and Q143N are in close proximity, and both mutations cause similar reductions in molecular volume equivalent to one O and one CH2, respectively. Thus, this region may be under some strain, which is reduced by the Y34F and Q143N substitutions resulting in increased protein stability. In contrast, all position 30 mutations of Table II reduce stability by 3.0–5.0 kcal/mol tetramer, suggesting that either the H2O-His30-Tyr166 hydrogen bonds or the geometry of His30 in the active site or both are essential for stability.

Both $k_{cat}$ and $k_{cat}/K_m$ for the catalysis of superoxide dismutation decreased by approximately an order of magnitude upon replacing His30 with Asn in human MnSOD (Table I). Thus, His30 is not essential for stability. This conclusion was also reached for H30A MnSOD from Saccharomyces cerevisiae by Borders et al. (28), although their results using a pyrogallol autoxidation assay determined that catalytic activity in this mutant was approximately unchanged compared with wild type.

It is interesting that the conservative mutations H30N, Y34F, and Y166F at each of three sites that interrupt the hydrogen-bonded array of side chains and water molecules in the active site cavity appear to have about the same effect on the maximal turnover number $k_{cat}$ (Table I). There was about a 10-fold decrease compared with wild type in $k_{cat}$ with several different side chains placed at residue 30 (Ala, Asn, Gln, Lys, Ser). Since proton transfer events appear to be rate-contributing for $k_{cat}$, we interpret changes in $k_{cat}$ upon these replacements in part as effects on intramolecular proton transfer, although there may be effects on other steps of the catalysis as well. The fact that conservative substitutions at residues 30, 34, and 166 reduce $k_{cat}$ for MnSOD by about an order of magnitude may signify that we have reduced the effectiveness of the proton delivery network of the wild type by each of these mutations. This is perhaps another manifestation of the hydrogen-bond network observed in the crystal structure and with the ionization of Tyr34 in Fe(II)SOD, which affects the NMR chemical shifts of many active site residues (7). It is interesting that this is approximately the magnitude by which $k_{cat}$ for CO2 hydration catalyzed by carbonic anhydrase II is reduced when the intramolecular proton shuttle His64 is replaced by Ala (29). Moreover, the observation that many mutations at position 30 result in about the same reduced value of $k_{cat}$ compared with wild type may signify that no residue we have used to replace His30 participates in a proton transfer network that is as effective in catalysis as the network in the wild type. The observation that the positively charged Lys30 gives $k_{cat}$ comparable to Ala or Asn demonstrates that residue 30 can be positively charged, as can His30, with retention of considerable activity. Glutamate at position 30 results in a decrease in $k_{cat}$ by about 3 orders of magnitude; this site cannot achieve effective catalysis with this negatively charged residue, a result perhaps due in part to repulsion of the superoxide radical anion.

For the wild type MnSOD, $k_{cat}/K_m$ at 8 $\times$ 108 M$^{-1}$ s$^{-1}$ is very near encounter controlled. The 6-fold lower value for the mutant H30N indicates a change in rate-limiting step, in the sense that diffusion is less limiting. The pH profile for $k_{cat}/K_m$ for H30N retains some pH dependence indicating a group with $pK_a$ near 9, as does the wild type (Fig. 4). This is possibly the pK_a for hydroxide binding as in E. coli Mn(III)SOD (6) or possibly Tyr34 as seen in E. coli Fe(II)SOD (7), although it does not affect $k_{cat}$.

The other mutants with replacements His30 $\rightarrow$ Ala, Gln, and Ser showed no apparent pH dependence in $k_{cat}/K_m$; this is the only significant kinetic difference we have observed among the mutants containing Ala, Gln, and Ser at position 30. Catalysis by the mutant Y34F MnSOD showed $k_{cat}$ decreased but $k_{cat}/K_m$ unchanged compared with wild type (8). This was interpreted as an effect of Tyr34 on the proton transfer processes that limit $k_{cat}$ but have no effect on $k_{cat}/K_m$. The effect on catalysis of mutation at residue 30 appears more complex than at position 34. The effect of the replacement of His30 by other residues is to substantially reduce both $k_{cat}$ and $k_{cat}/K_m$ (Table I).

**Conclusions—** The substitution His30 $\rightarrow$ Asn caused an interruption of an extended hydrogen-bonded array in the active site of human MnSOD. This substitution resulted in breaking the hydrogen bond from His30 to an adjacent water and to Tyr166. 2) His30 and Tyr166 are not essential for catalysis by MnSOD, although conservative replacements at these sites caused a decrease in catalysis by about 10-fold. 3) Since both His30 and Tyr166 are part of an extended hydrogen-bonded array, their replacement reflects on the role of this array in catalysis. The similar decreases in $k_{cat}$ caused by the many substitutions at position 30 and 166 demonstrate the approximately 10-fold decrease on $k_{cat}$ of interrupting this array. 4) The replacement His30 $\rightarrow$ Asn caused a 12 °C decrease in the main unfolding transition of MnSOD.

**Acknowledgments—** It is a pleasure to thank Kristi Totten and Harold Frey for their technical assistance. We are grateful to Dr. Carrie Vance for helpful discussions and to Amy Hearn for help with the absorption spectra. We thank Dr. Chingkuang Tu for his guidance and support and Dr. Peter Bratt for assistance with EPR measurements.

**REFERENCES**

Manganese Superoxide Dismutase
