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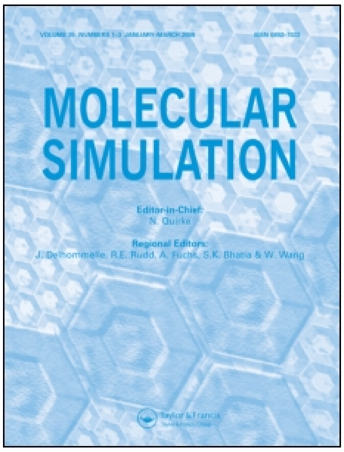

## Molecular Simulation

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# Molecular Dynamics Simulation of Substrate-Enzyme Interactions in the Active Site Channel of Superoxide Dismutase

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# MOLECULAR DYNAMICS SIMULATION OF SUBSTRATE-ENZYME INTERACTIONS IN THE ACTIVE SITE CHANNEL OF SUPEROXIDE DISMUTASE

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Molecular dynamics simulations of the diffusion of superoxide ion down the active site channel of the enzyme superoxide dismutase were performed with a parallelized version of GROMOS on the Intel iPSC/860. Our model consisted of a spherical assembly of 6968 atoms centered at a copper ion in the enzyme. Trajectory analysis revealed that the anion is directed toward the copper ion through the cooperative motions of several active site residues. Other mechanistic and structural motifs recurring through five full trajectories are examined. In addition to these qualitative results, an upper bound has been calculated for the rate constant for displacement by substrate of the water molecule that is coordinated to the copper. This required an analysis of the dynamics of crossing a free energy barrier that has been characterized in previous work. Strong frictional effects due to Coulombic interactions lead to a rather small rate constant; the transmission coefficient is less than 0.01. The mechanism of the enzyme therefore may involve diffusion of substrate up to the bound water followed by electron transfer mediated by this water, rather than displacement of the water by substrate with subsequent electron transfer.

KEY WORDS: Superoxide dismutase, active site channel, rate constant

## INTRODUCTION

Superoxide ion ($O_2^-$), the most common toxic side-product of aerobic respiration, inactivates enzymes such as catalases and peroxidases [1]. Its conjugate acid, OOH, readily initiates the auto-oxidation of lipids by abstracting the allylic hydrogen [2]. Superoxide dismutase (SOD), which catalyzes the disproportionation of superoxide ion to dioxygen and hydrogen peroxide with a rate that is close to the limit of diffusion control, is therefore one of the most important and efficient enzymes in aerobic life forms. It is also potentially useful in medical and industrial applications. Thus, research concerning this enzyme has been flourishing [3-8]. In this paper, we describe molecular dynamics simulation studies of the events that immediately follow the diffusional encounter of $O_2^-$ with the surface of SOD. The remainder of this section briefly reviews the background and motivation for this work.

Most of the research on SOD has focused on the bovine and human Cu/Zn enzymes. Hereafter, SOD refers to bovine superoxide dismutase, unless otherwise specified. The corresponding human SOD residue numbers can be obtained through incrementing the bovine residue numbers by two. When referring to bovine side chains, we put a hyphen in between the residue name and its sequence number; the hyphen is omitted for the corresponding residue in the human enzyme.

It is generally believed that SOD dismutes superoxide ion via two one-electron transfer reactions [2]:

$$\text{SOD-Cu}^{2+} + O_2^- \longrightarrow \text{SOD-Cu}^{+} + O_2$$

$$\text{SOD-Cu}^{+} + O_2^- + 2H^+ \longrightarrow \text{SOD-Cu}^{2+} + H_2O_2$$

The active site copper lies at the bottom of a 10 Å deep channel from the surface of the enzyme; the opening of the ion channel to the active site is about 10 Å wide at the surface of the enzyme. Competitive inhibition of SOD by monovalent anions indicates accessibility of the copper to the chloride ion (ionic radius 1.81 Å) and bromide ion (1.95 Å) but not to iodide ion (2.16 Å). The bottom of the ion channel should therefore be around 4 Å in diameter [9]. Hence the ion channel is usually represented schematically as in Figure 1. Four imidazole rings, each from a different histidine residue, are ligated to the copper ion, roughly in a square-planar arrangement. The fifth apical coordination site of copper is occupied by a water molecule. Whenever a water is bonded to the copper, it is referred to as the apical water.

Three active site residues, Asp-122, Thr-135 and Arg-141, are thought to be important in the dismutation of superoxide ion. The amino acid sequences of the SOD from 15 species have been aligned with the human sequence. Asp 124 (corresponding to bovine Asp-122) is conserved in all species; in other words, it is an invariant active site residue. Mutation of Asp 124 to glycine inactivates the enzyme [10, 11]. In the crystal structure of bovine SOD, the two carboxylate oxygens of Asp-122, OD1 and OD2, hydrogen-bond to HE of Cu ligand His-44 and HE of Zn ligand His-69, respectively, suggesting that this side chain is necessary to orient the Cu and Zn ligands correctly. Mutation of Thr 137 in human SOD to isoleucine decreases the reaction rate by about five times at high pH, implying that this residue may be involved in important hydrogen bonding [12]. SOD as a whole unit is negatively charged. Yet the enzymatic rate decreases with increasing ionic strength. Analysis of the electric field near the active site has suggested that while Lys-120, Glu-131 and Lys-134 direct the long range approach of the superoxide ion, Arg-141 has only a local orienting effect [13, 14]. In support of these conjectures, chemical modifications of the lysine residues near the active site (including the two mentioned above), inverts the dependence on ionic strength. No such inversion occurs for derivatization of Arg-141 [15]. However, replacement of Arg 143 in human SOD by lysine, isoleucine, or glutamate does cut the dismutation rate down to about 50, 10 and 1%, respectively [16, 17].

The rate of transport of superoxide ion to the active site channel can be increased by modifying certain side chains, such as Glu 132 and Glu 133 in human SOD [18–20]. If such re-engineering of the enzyme causes the initial encounters between the anion and the enzyme to become fast enough, the dismutation rate would be determined by the subsequent chemical reactions.

The present work is intended to provide a picture of the channel penetration process that immediately follows the initial, diffusional encounter. This work builds on a previous molecular dynamics simulation study of the free energy surface (or potential of mean force) for displacement of $O_2^-$ in the SOD channel [21]. In particular, we calculate trajectories of $O_2^-$ over the principal energy barrier on this

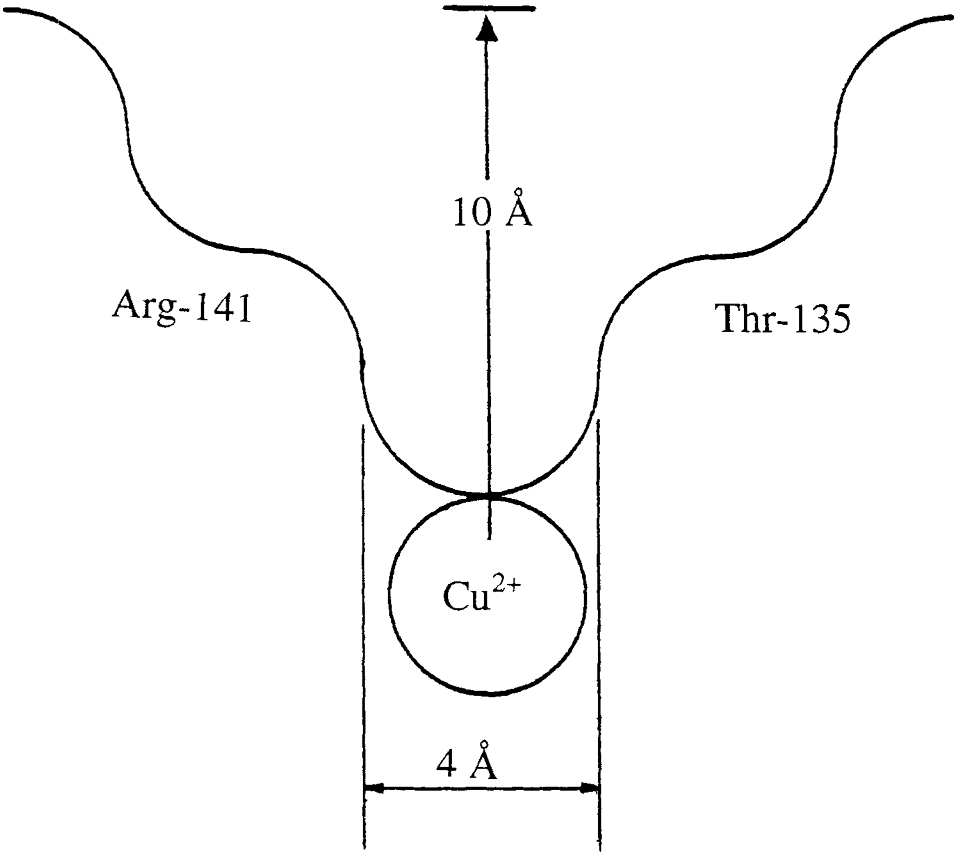


**Figure 1** Schematic representation of the active site channel in SOD.

surface. The trajectories are analyzed to provide both quantitative information (rate and transmission coefficient) and qualitative information(structural changes of mechanistic importance). Ultimately, we hope to develop a kinetic model that can combine the Brownian dynamics simulation results for the initial encounters with the molecular dynamics simulation results for the subsequent events to describe in detail the complete enzymatic mechanism, starting from the approach of superoxide ion to the SOD, and ending with the final electron and proton transfer.

## MODEL FOR SOD

The system in our simulation consists of a superoxide ion, 1629 atoms from SOD and 1779 water molecules, altogether forming a 26 Å radius sphere with a copper at the center. All protein atoms and the water oxygens in the outermost 6 Å shell are restrained by a harmonic potential, the former to the X-ray coordinates and the latter to the dynamically relaxed positions from a larger model of the hydrated protein [21]. The position restraints suppress the motion of the atoms that are more than 15 Å from the copper. Accordingly, our analysis will focus on atoms located less than 11 Å from the metal. All covalent bonds are constrained to their equilibrium distances by the SHAKE algorithm. The SPC/E model for water is employed. All simulations were performed using the GROMOS package, either in its original form [22], or in a parallelized form for the Intel iPSC/860 parallel supercomputer [23].

Previous molecular dynamics calculations have identified a local maximum in the potential of mean force when one of the atoms of the superoxide ion is about 4.5 Å from the copper [21]. This 2 to 3 kcal/mol barrier was taken as the activation barrier for the substitution of the substitution of the apical water by $O_2^-$. To generate a representative set of configurations near the transition state, we performed 800 picoseconds of umbrella sampling by applying a harmonic force to confine one of the oxygen atoms of the anion near the above free energy maximum. 400 configurations were then chosen such that consecutive configurations are separated from each other by about 2 picoseconds to ensure dynamic independence. The distances between the restrained oxygen and the copper were selected to be within 4.50 ± 0.01 Å to avoid artifacts due to removal of the harmonic force. Detailed procedures for equilibration and the computation of the potential of mean force (PMF) can be found in a previous paper [21]. A cutoff distance of 20 Å was used in these studies because of the charged nature of $O_2^-$. Each picosecond of simulation takes about 25 minutes of Cray-YMP CPU time. Many of the calculations were performed on an Intel iPSC/860, where one picosecond simulation takes about 84 minutes on an eight-processor system.

Half of the 400 configurations have the constrained oxygen moving toward the copper initially. Each trajectory is propagated in the absence of the harmonic force for up to two picoseconds. For the trajectories that initially move toward the copper, let $T_a$ represent the fraction for which the previously restrained oxygen does not return to the free energy maximum. Similarly, $T_b$ represents the fraction of trajectories that initially move away from the copper and do not return to the barrier. Then according to the absorbing barrier method of Berne and Straub [24], the transmission coefficient $\kappa$ can be obtained from the plateau region of the function:

$$\kappa = T_a T_b / (T_a + T_b - T_a T_b)$$

Previous studies of other systems suggested that the plateau region is usually attained in less than two picoseconds of simulation time [24–26], hence the choice of the above time scale.

To allow study of the mechanism of barrier penetration, the velocities of all atoms in some initial states generated by umbrella sampling were reversed. The new states were then allowed to propagate, again in the absence of the harmonic force. Time reversal of these "reverse" trajectories, followed by continuation with the original forward trajectories, yielded examples of longer trajectories, a few of which display movement of $O_2^-$ from the surface of SOD to the active-site copper [27]. In this way, we obtained five successful trajectories describing first the motion of the superoxide ion down the 10 Å channel, and then the replacement of the apical water. These trajectories are terminated when the center of mass of the anion is more than 10 Å from the copper or when the superoxide ion comes into direct contact with the copper for more than 10 picoseconds. The initial configurations of

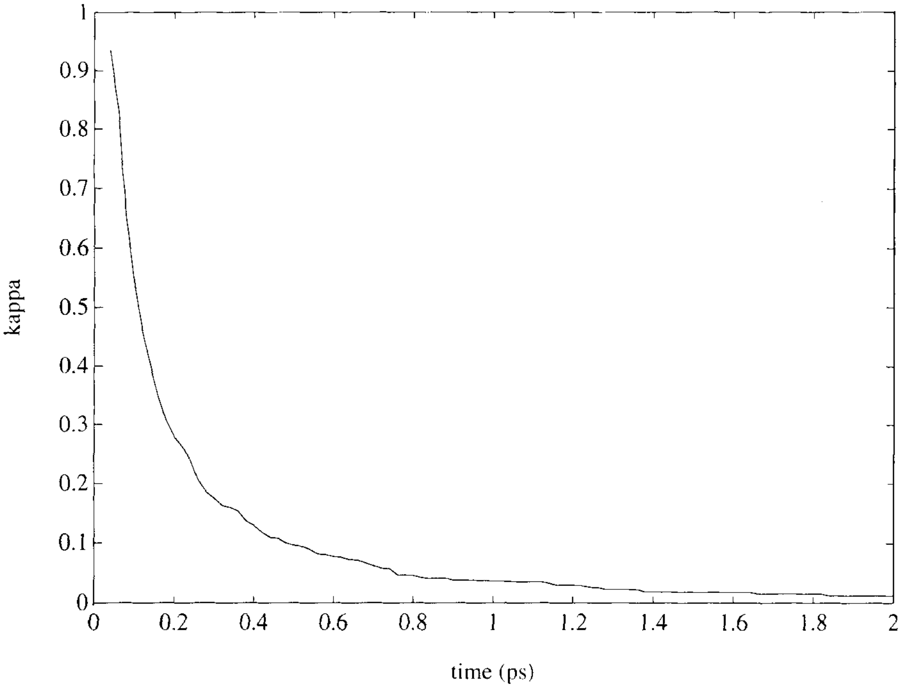


**Figure 2** Results of the absorbing barrier method to determine the transmission coefficient, $\kappa$.

two successful trajectories are separated by less than one picosecond of umbrella sampling. They display rather similar mechanistic features so we only describe one of them in this paper.

## RESULTS AND DISCUSSION

The rate constant $k$ is given by the following equation [27]:

$$k = 0.5\kappa\langle|\dot{\xi}|\rangle\left[\rho(\xi^{\neq})/\int\rho(\xi)\mathrm{d}\xi\right]$$

where $\xi$ is the reaction coordinate (in this particular case, it is the distance between the copper and one of the oxygen atoms of the anion), and $\dot{\xi}$ is the average speed of barrier-crossing. $[\rho(\xi^{\neq})/\int\rho(\xi)\mathrm{d}\xi]$ is the probability density of finding the superoxide ion at the top of the free energy barrier relative to the reactant well. It is an equilibrium quantity which can be calculated from the potential of mean force for $O_2^-$ displacement [21].

The results of the absorbing barrier calculations are shown in Figure 2. The transmission coefficient estimated from the current data appears to be less than 0.01. A more definite value would be available only if additional trajectories were run for longer times to verify that a plateau had been attained in $\kappa$. In any event, the transmission coefficient for this system is much smaller than that in the diffusion

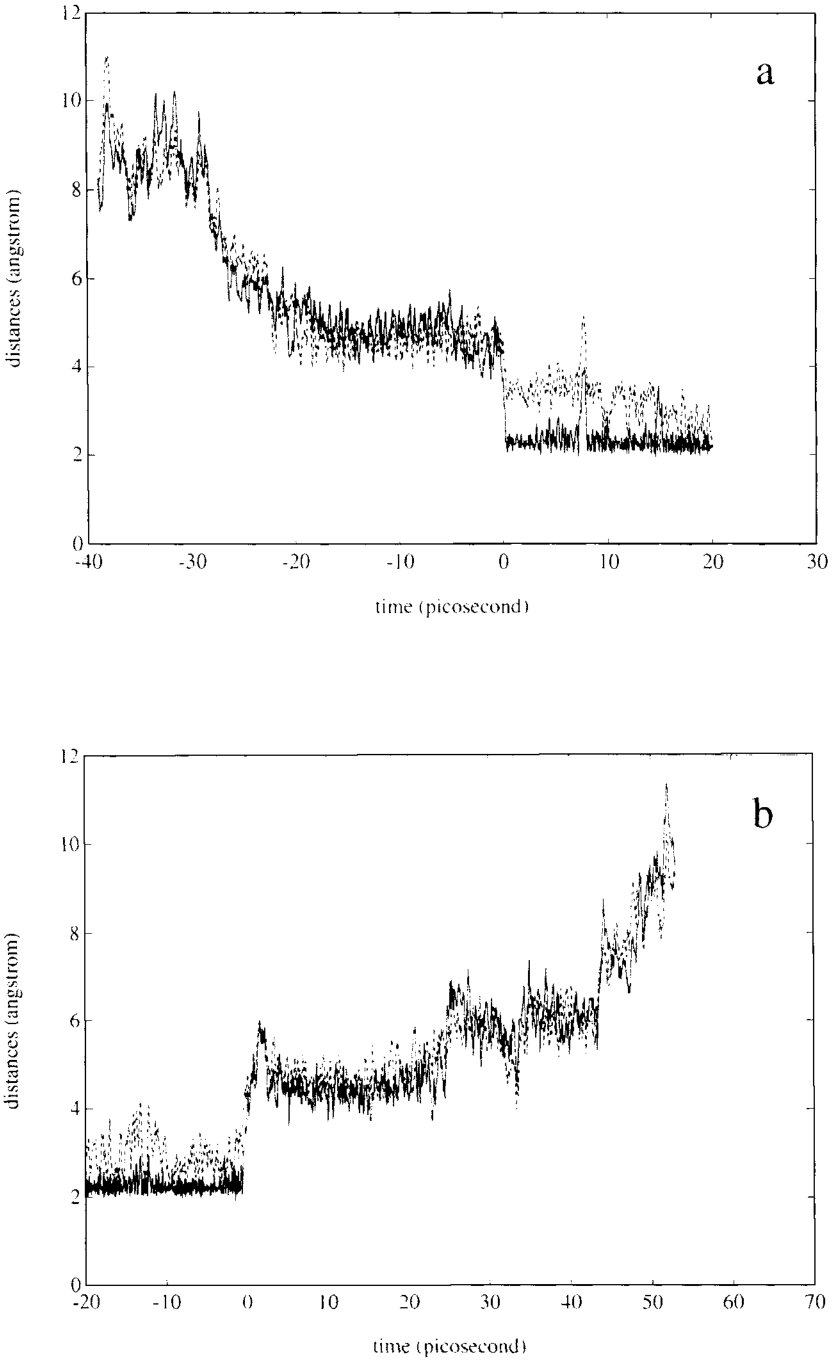
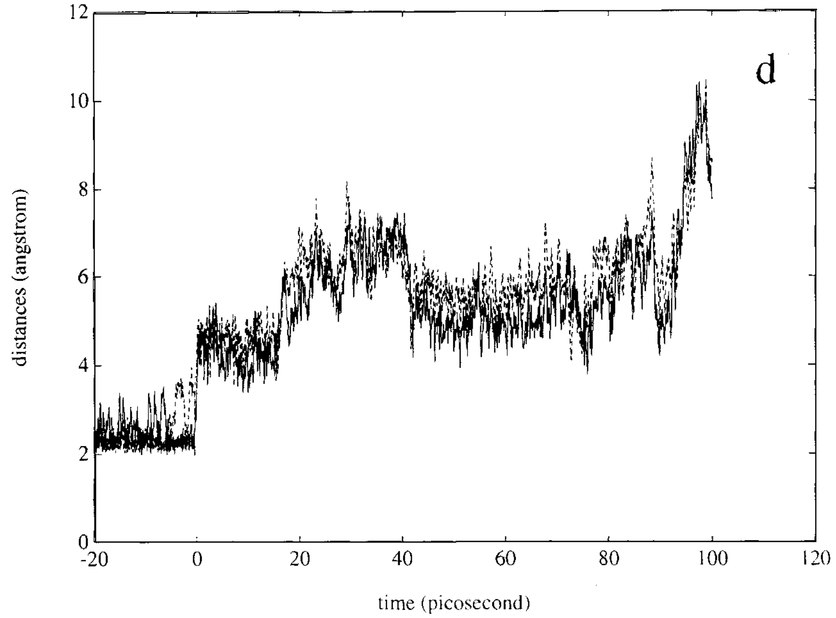


**Figure 3** Distances between the copper ion and the oxygen atoms of the superoxide ion as a function of time for the four successful trajectories.



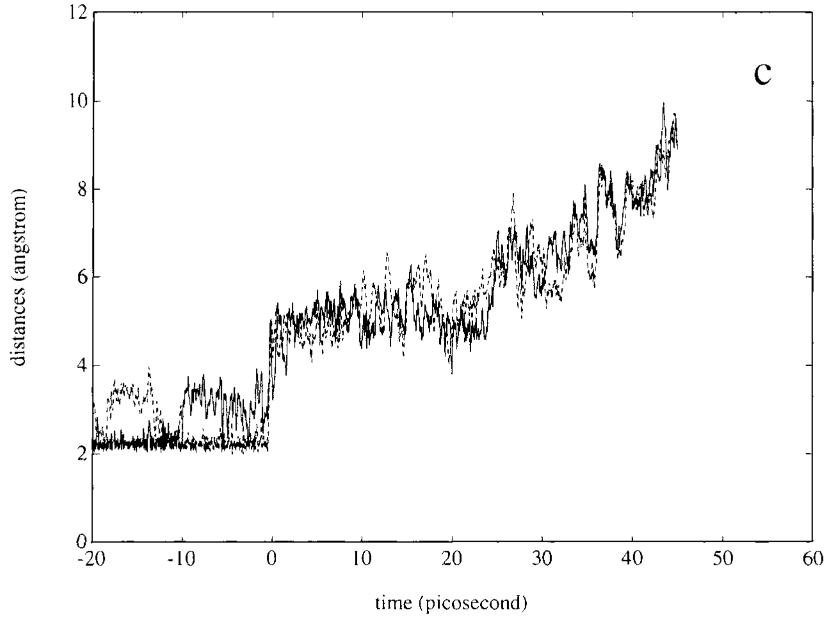


**Figure 3** *continued*

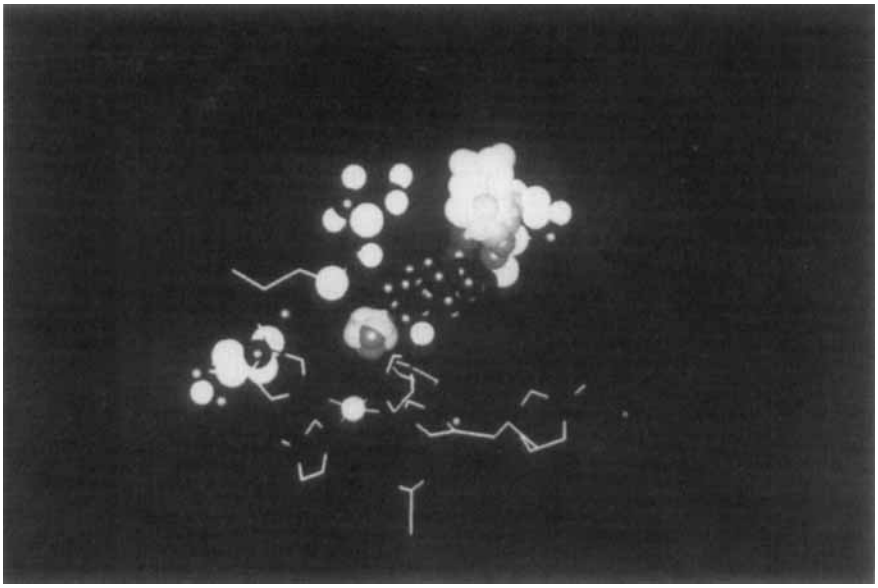


**Figure 4** Trajectory of one of the oxygen atoms of superoxide ion. As the anion diffuses down the active site channel, its colour changes from yellow → brown → red → purple → deep blue and finally pale blue. The positions are shown for the four residues His-46, Asp-122, Thr-135 and Arg-141. (See Colour Plates)

of sodium ion inside a periodic poly-(L,D)-alanine $\beta$-helix (0.11) [28], rotational isomerization of tyrosine rings in the bovine pancreatic trypsin inhibitor (0.22) [25] and for dioxygen escaping from myoglobin (1.0) [26]. This is not surprising, since the small size and net charge of the substrate are expected to lead to strong coupling to the charged groups in the protein and hence a more diffusive motion. To illustrate this point, we plot the distances between the copper and the two oxygen atoms of superoxide ion as a function of time for several successful trajectories in Figure 3. Displayed in Figure 4 are some of the structures that occur in the first trajectory.

Although the estimation of $\kappa$ is very rough, it should be a reliable upper bound. The average speed of barrier crossing for 400 initial configurations is $5.18 \times 10^{12}$ Å $s^{-1}$. The width of the reactant state is roughly 1 Å. By mapping out the potential of mean force, the activation barrier for the substitution of the apical water is between 2 to 3 kcal/mol [21]. The corresponding probability of finding the substrate at the transition state ridge can therefore be estimated to be 0.035 to 0.0065 Å$^{-1}$, respectively. Hence the rate constant at 300 K is estimated to be less than $0.17 \times 10^9$ to $0.9 \times 10^9 s^{-1}$.

Suppose that one cycle of the dismutation mechanism can be represented by the following scheme [21]:

$$\mathrm{SOD} + \mathrm{O}_2^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{SOD/O}_2^- \xrightarrow{k_2} \text{product}$$

where SOD/$O_2^-$ indicates the structure in which the superoxide ion is at the reactant well for the substitution of the apical water. Note that these rate constants are defined somewhat differently than in our earlier work [21]; in particular, $k_1$ here includes the movement of $O_2^-$ from the surface of SOD partway into the channel. The value of $k_1$ at low salt concentration has been estimated by Brownian dynamics simulation [20, 29] as $k_1 = k_D(b)\beta = 2.9 \times 10^9 M^{-1} s^{-1}$. Here $k_D(b) = 2.6 \times 10^{10} M^{-1} s^{-1}$ is an analytic result [20], and $\beta = 0.11$ is obtained from the simulation [29]. The value of $k_{-1}$ is estimated as $k_{-1} = k_1/K = 2.0 \times 10^9 s^{-1}$. Here the equilibrium constant K ($1.4 M^{-1}$) is approximated by the volume available to $O_2^-$ in the reactant well for the substitution of the apical water, multiplied by the potential of mean force for $O_2^-$ in the well relative to $O_2^-$ in solution [21]. Applying the steady-state approximation for SOD/$O_2^-$, the effective forward rate constant

$$k_f = \frac{k_1 k_2}{k_{-1} + k_2}$$

is less than $2.3 \times 10^8$ to $9.0 \times 10^8 M^{-1} s^{-1}$. The experimental dismutation rate is around $2 \times 10^9 M^{-1} s^{-1}$. The discrepancy between the calculated and experimental rates suggests that substitution of the apical water molecule by $O_2^-$ may not be required in the mechanism of SOD. Instead electron transfer may be mediated by the bound water molecule.

Now let us turn our attention to the mechanistic features observed in the full trajectories. As suggested-above, within the reliability of our model potential, only the movement of $O_2^-$ from the surface of SOD to contact with the apical water molecule may be of functional importance, but the subsequent substitution step may be a model for inhibition by univalent anions, for example. In contrast to the X-ray crystallographic data, our solution simulation suggests that when $O_2^-$ is in the active site channel, the His-44 to Asp-122 hydrogen bond is usually lost. Instead, there is a bridging water between HE of His-44 and the amide oxygen of Asp-122. Occasionally, HE of His-44 comes close to Asp-122, developing substantial electrostatic interaction and even hydrogen-bonding between the two. For the other hydrogen bond, the story is completely different. The amide oxygen of Asp-123 competes strongly with Asp-122 for hydrogen bonding with HE of His-69, freeing OD2 occasionally. Moreover, an amide hydrogen of Asn-137 sidechain is located near Asp-122. It hydrogen-bonds with OD1 for most of the time, and occasionally OD2 as well. Of course, rotation about carbon-carbon single bonds occurs readily. Over a long period of simulation, OD1 and OD2 may become equivalent.

When the superoxide ion is at the surface of SOD, it is usually either in direct contact with Arg-141 or Thr-135 or in between the two. On its journey down the active site channel, the anion was found to always come close (2 to 3 Å) to the polar hydrogen (HD1) of the imidazole ring of His-46. (A typical hydrogen bonding distance between hydrogen and oxygen is about 1.7 Å.)

After the superoxide ion becomes the second neighbor of the apical water, most of the time the bridging water between the former and the latter is pulled away by the guanidinium group of Arg-141. Occasionally, this task can be achieved by His-118 or Gly-136. In the first case, the anion will hydrogen-bond to the apical water even after the superoxide ion comes into direct contact with the copper. In the second case, the bonding between the two is not stable, that is, frequently other water molecules intervene.

The average distance between copper and the apical water, as determined by our simulation is 2.4 Å. The corresponding experimental values are 2.1 and 2.4 Å, respectively, as estimated from two separate experiments of proton nuclear magnetic resonance [30, 31].There can be considerable lengthening of this bond, up to about 4.2 Å even when the superoxide ion is more than 4 Å from the copper. The copper can remain four-coordinated for 1 to 2 picoseconds. As mentioned before, the bottom of the ion channel should be about 2 Å in radius. Simultaneous accommodation of the superoxide ion and a water molecule in the vicinity of copper would be too crowded. Steric hindrance may be partly responsible for this $S_N1$-like character. Long before any direct interaction between the anion and the metal, strong hydrogen-bonding between the superoxide ion and the apical water has been developed.

Less than one picosecond before the substitution of the apical water by the superoxide ion, a unique conformation is always attained: there are two bridging waters between Asp-122 and the superoxide ion; one hydrogen-bonded to OD1, the other to OD2. There is another water molecule bridging the anion and the guanidinium group of Arg-141. The same is true for the superoxide ion and the amide oxygen of Thr-135. Meanwhile the amide hydrogen of Asn-137 is hydrogen-bond to the carboxylate group of Asp-122. The polar hydrogen (HE) of the imidazole ring of His-69 can oscillate between the carboxylate oxygen, OD2 of Asp-122 and the amide oxygen of Asp-123. Prior to the formation of the above conformation, this hydrogen always stays away from Asp-122, that is, it is always in the vicinity of the amide oxygen of Asp-123. In this configuration, there can be substantial lengthening of the bond between the copper and apical water.

In summary, it involves the concerted effort of several active site invariant residues to attain this conformation. The determination of the relevance of this conformation and the solvent separated conformation for the subsequent electron transfer awaits future quantum mechanical calculations. It has been speculated that the superoxide ion can be in direct contact with Arg-141 and the copper simultaneously [15]. Such conjecture is not born out in our simulations. There are always some intervening water molecules.

Notice that, before binding of $O_2^-$ to the Cu, there is already a vacant coordination site. Hence the formation of the copper-superoxide ion bond is quite rapid (see also Figure 3). Usually it is accompanied by some rotation of the carboxylate group of Asp-122 and some motion of the amide hydrogen of Asn-137. After the substitution, the apical water always hydrogen-bonds to Asp-122.

To give the reader a feeling of the cooperative motions of the active site residues that assist the substrate's traversal down the ion channel, we give a detailed description of one trajectory in the Appendix. The selected trajectory, the first trajectory in Figure 3 and 4, is meant to illustrate most of the important features of our simulation.

## CONCLUSION

We have performed a molecular dynamics simulation to investigate the diffusion of the superoxide ion down the active site channel of SOD. The present work is meant to be a bridge between the previous Brownian dynamics simulations and future quantum mechanical calculations. It seems that His-46, Thr-135 and Arg-141 may guide the anion toward the copper either by direct electrostatic interaction or by manipulating the water molecules next to the superoxide ion. The rate constant for the substitution of apical water by $O_2^-$ is so small that this may not be a mechanistic step in the enzyme. The small value of that rate constant is due to strong electrostatic frictional effects, which lead to a remarkably small transmission coefficient. The functional mechanism instead may involve electron transfer through the apical water molecule.

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## APPENDIX

The following is a possible interpretation of our simulation results. At the beginning, the center of mass of the superoxide ion is more than 10 Å from the copper. The water molecules are named according to their orders in the input file. For example, w321 is the 321th solvent molecule. Asp-122, Thr-135, Gly-136, Asn-137 and Arg-141 are active site invariant residues. In our simulation, the carboxylic groups of Asp-122 and Asp-123 are deprotonated, while the guanidinyl group of Arg-141 is protonated.

### *Starting configuration*

There is a bridging water, w321, between Thr-135 and superoxide ion. The same is true between Arg-141 and the anion, w386 being the intervening water molecule. w363 is hydrogen-bonding to and directly under the anion. One of the hydrogen atoms of the apical water is oscillating between OD1 and OD2 of Asp-122. (In the other three trajectories, initially, there is a water molecule in between the apical water and Asp-122.) Moreover, OD2 of Asp-122 hydrogen-bonds to the amide hydrogen of Asn-137.

### *9 to 14 picoseconds*

There are two amino groups attached to the guanidinium carbon. The lower amino group of Arg-141 moves downward, carrying w285 toward HD1 of His-46 (which is a polar hydrogen of a imidazole ring). Subsequently, a strong electrostatic interaction develops between w285 and HD1.

Following this is the downward diffusion of w386 from the upper amino group of Arg-141 to the lower one, pushing w285 toward the center of the ion channel. w285 first hydrogen-bonds with w276 and then becomes the bridge between the superoxide ion and the apical water. The initial motion of w285 seems to be effected partly by His-46 as well.

Simultaneously, w363 is attracted toward the hydroxyl group of Thr-135, creating some space for the superoxide ion to go down. Another water molecule, w321 which is above the radical anion, moves down as well, until it hydrogen-bonds with

Gly-136. This water molecule then links the superoxide ion and the amide oxygen of Gly-136 for most of the rest of the trajectory, holding the anion near the apical water.

### *17 to 18 picoseconds*

The bridging water w285 is pulled to the rim of the ion channel by Arg-141. The superoxide ion now comes into direct contact with the apical water. During this period of time, the radical anion is in the vicinity of one of the hydrogen atoms (HD1) of the imidazole ring of His-46. In this particular trajectory, the closet distance is around 2.55 Å. For other trajectories, it can be even shorter, down to about 2.03 Å.

### *34 picosecond*

The amide hydrogen of Asn-137, which is hydrogen-bonding to OD2 of Asp-122, goes up, causing the aspartate group to rotate. The rotation in turn moves w347 and the apical water toward the superoxide ion, forming the unique conformation described above. The anion is connected to the amide oxygen of Thr-135 and the guanidinium group of Arg-141 by w321 and w285, respectively. Within the next 250 femtoseconds, the distance between copper and the oxygen atoms of the apical water increases from 2.31 to 3.44 Å.

### *39 picosecond*

The copper-superoxide ion and copper-apical water distances are 3.72 and 3.74 Å, respectively. Within 250 femtoseconds, the former shortens to 2.56 Å. The drastic shortening is not only the result of the motion of the anion, but also the movement of the cation and the surrounding ligands, that is, a change from a square-planar like conformation to a more square-pyramidal arrangement. After the substitution, the apical water always hydrogen-bonds to superoxide ion and Asp-122 simultaneously. The superoxide ion is in direct contact with the copper at least for the next 20 picoseconds.

All the molecular simulations in this paper are performed using the GROMOS package. The SHAKE algorithms has been employed to allow the use of a large step size, namely 2 femtoseconds. The cutoffs for the van der Waals interactions and the electrostatic interactions are 8 and 20 Å, respectively.

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