# Simulation of the Bimolecular Reaction between Superoxide and Superoxide Dismutase: Synthesis of the Encounter and Reaction Steps

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**Abstract:** Brownian dynamics simulations of the diffusional encounter of reactants can be combined with more detailed molecular dynamics simulations of subsequent events by use of Markov chain models. These methods are used here to show that fluctuations in the encounter complex of the enzyme superoxide dismutase and its substrate reduce the overall bimolecular reaction rate by 50% compared to simulations based on a rigid enzyme. The possible utility of these methods for other systems is also discussed.

## 1. Introduction

The enzyme Cu/Zn superoxide dismutase (SOD) is important in nature and potentially important in pharmaceutical and industrial applications as a scavenger of the superoxide radical $O_2^-$.[1-4] Wild-type SOD catalyzes the dismutation of the superoxide radical to molecular oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) at a rate that is approximately controlled by the initial diffusional encounter of the reactants.[5-7] The rates are, in fact, similar to those predicted by Brownian dynamics simulations in which the solvent is represented as a continuum and reactions are simply assumed to occur when the superoxide radical reaches a prescribed distance from the active site Cu.[8-12] Such simulations recently guided the synthesis of faster, mutagenized SOD's, in which the electrostatic field around the enzyme was modified to improve the steering of $O_2^-$ diffusion to the active site.[13,14] However, separate molecular dynamics simulations of the superoxide radical in the active site of SOD suggest that postencounter events influence the overall rate and will ultimately limit the activity of mutagenized forms of SOD.[15,16] These postencounter events include the passage of the superoxide radical through a narrow but hydrated channel of 10-Å length from the surface of the enzyme to the active site Cu and, finally, the transfer of electrons and protons. In this paper, we apply to SOD a new theoretical method that combines Brownian dynamics simulation results for the initial diffusional encounter and molecular dynamics simulation results for the subsequent events. This integrated method allows more complete and accurate analysis of binding and catalysis kinetics.

## 2. Theory

The bimolecular rate constant for substrate disappearance can be factored as[17,18]

$$k = k(b)\beta \tag{1}$$

where $k(b)$ is the rate at which the substrate initially reaches a spherical surface of radius $b$ centered on the enzyme. If $b$ is chosen large enough that the potential of mean force $U$ between the reactants is centrosymmetric for distances larger than $b$, then $k(b)$ can be found by solving the one-dimensional diffusion equation. The result is[19]

$$k(b)^{-1} = \int_b^\infty \frac{e^{U(r)/k_BT}}{4\pi D(r) r^2}\, dr \tag{2}$$

where $k_BT$ is Boltzmann's constant multiplied by temperature, and $D(r)$ is the relative diffusion coefficient. The quantity $\beta$ is the probability that a substrate started at a random location on the "$b$ surface" will reach the active site and react before escaping. Due to the irregular geometry of enzymatic systems, analytical expressions for $\beta$ are not available and numerical methods must be used to estimate this quantity.

In applying the numerical techniques, previous studies have assumed a continuum description of the solvent and the interior of the enzyme and a Brownian description of the dynamics of the substrate. Here, we use a new method to couple this type of description of the initial encounter of substrate and enzyme to a more detailed description of the subsequent events in the complex. In particular, when the substrate is closer to the active

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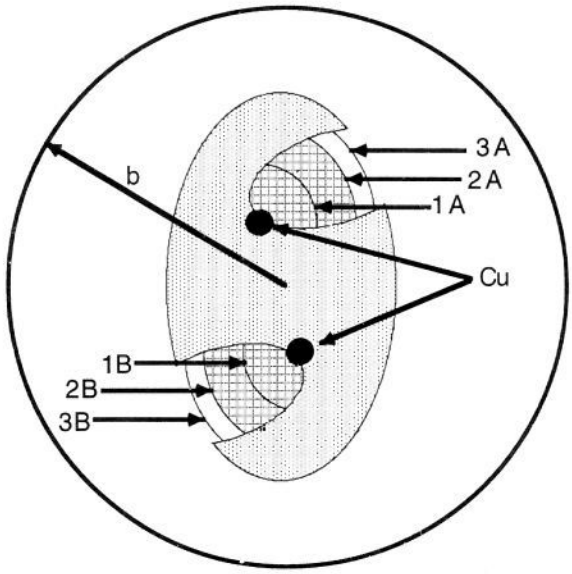


**Figure 1.** Schematic diagram of the SOD enzyme. Beyond the surface of radius *b*, the interaction between the enzyme (shaded region) and the substrate is centrosymmetric; an analytic diffusion model can be used to describe substrate motion in this region. Between the *b* surface and the surfaces labeled **2A** and **2B**, continuum electrostatics and Brownian dynamics simulations provide a reasonably accurate description of the substrate's motion. Between the surfaces labeled **2A** and **2B** and the reactive surfaces labeled **1A** and **1B** (the cross-hatched regions in the figure) we used molecular dynamics to simulate the motion of all atoms including explicit water.

site coppers than the surfaces **2A** or **2B** at the mouths of the active sites (the cross-hatched region in Figure 1), we use molecular dynamics to simulate the motion of all atoms, including explicit water, in the active site region. For simplicity, we assume that the reaction between the superoxide and the copper takes place instantaneously and irreversibly if the substrate reaches either surface **1A** or **1B**. More advanced simulation techniques, which use quantum mechanical models in this region, are under development.

This system can be abstracted to the state diagram shown in Figure 2A.[20] In Figure 2 each node represents a "state" of the substrate and the arrows denote all possible transitions from one state to a second state. For example, a substrate located on surface **2A** is in state 2A, and it has a probability of $T_{2A,3A}$ of reaching surface **3A** and a probability of $T_{2A,1A}$ of reaching surface **1A**. It cannot reach any other state in the diagram without passing through one of these two states and therefore $T_{2A,3A} + T_{2A,1A} = 1$. A substrate that escapes is in the state labeled 4.

Since the SOD enzyme is modeled here as a symmetric dimer, $T_{2A,3A} = T_{2B,3B}$ and $T_{2A,1A} = T_{2B,1B}$, and the state diagram simplifies as in Figure 2B. In Figure 2B, $T_{3,3}$ is the probability of a substrate diffusing from surface **3** in one active site to surface **3** in the other active site before reaching surface **2** in the initial active site or escaping.

The *T*'s can be arranged to form a transition probability matrix **T**:

$$\mathbf{T} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ T_{2,1} & 0 & T_{2,3} & 0 \\ 0 & T_{3,2} & T_{3,3} & T_{3,4} \\ 0 & 0 & 0 & 1 \end{bmatrix} \tag{3}$$

In the transition probability matrix, the rows represent the state the substrate is coming "from" and the columns represent the state the substrate is going "to". For instance, the entry in the second row and third column is the transition probability from state 2 to state 3, $T_{2,3}$. Note that once a substrate reaches state 1, where it is assumed to irreversibly react, or state 4 (escape), it will not leave that state (i.e. $T_{1,1} = 1$ and $T_{4,4} = 1$).

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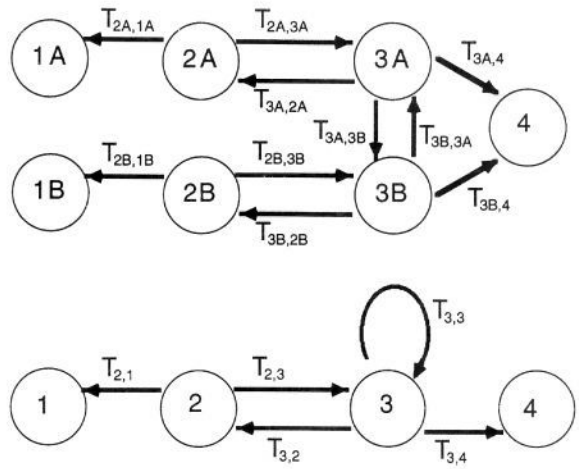


**Figure 2.** (A, top) State diagram for the Markov chain used to integrate the results for the different spatial domains shown in Figure 1. The states in the diagram correspond to a substrate at the respective surfaces in Figure 1. A substrate that escapes is in the state labeled 4. The arrows denote the possible transitions from one state to a second state. (B, bottom) The same as part A but assuming that the enzyme in solution will exist as a symmetric dimer (i.e. $T_{2A,3A} = T_{2B,3B}$, and, $T_{2A,1A} = T_{2B,1B}$).

The possible states of a substrate can be summarized by a state vector $\mathbf{P} \equiv [P_1, P_2, P_3, P_4]$. The components of the vector are the probabilities that a substrate molecule is on surface **1**, **2**, or **3** ($P_1$, $P_2$, or $P_3$, respectively) or has escaped ($P_4$) at a particular stage in the evolution of the Markov sequence of events. An initial state vector $\mathbf{P}^0$ can be determined by starting substrates on the *b* surface and determining the probability that they reach surface **2** ($P_2^0$) before escaping ($P_4^0$):

$$\mathbf{P}^0 = [0 \quad P_2^0 \quad 0 \quad P_4^0] \tag{4}$$

where $P_2^0 + P_4^0 = 1$. Using the initial state vector $\mathbf{P}^0$ and the transition probability matrix **T**, $\beta$ can be calculated:[20]

$$\mathbf{P}^\infty = \mathbf{P}^0\mathbf{T}^\infty \tag{5}$$

where

$$\mathbf{T}^\infty = \lim_{N\to\infty} \mathbf{T}^N \tag{6}$$

and

$$\mathbf{P}^\infty = [\beta \quad 0 \quad 0 \quad P_4^\infty] \tag{7}$$

where $\beta + P_4^\infty = 1$. $\mathbf{P}^\infty$ is the resulting state vector after an infinite number of steps or, equivalently, an infinite time (i.e. steady state). The calculation of $\mathbf{T}^\infty$ (eq 6) is easily accomplished by brute force, converging in less than 100 iterations. The resulting value of $\beta$ can be used in eq 1 to yield the desired rate constant.

Elaborations of the simple decompositions used in this study will sometimes be necessary. For example, only small patches of the surfaces **2** and **3** are accessible to the substrate in the present case, because of the narrowness of the active site channel of SOD. Where large regions of the surface are accessible, it may be necessary to decompose the surface into sectors and expand the state vector and transition matrix accordingly. Fortunately, the theory of Markov processes is well-developed and readily admits such generalizations. Furthermore, use of time-dependent Markov processes will allow us to generalize the above steady state analysis to the situation of non-steady state reaction conditions.

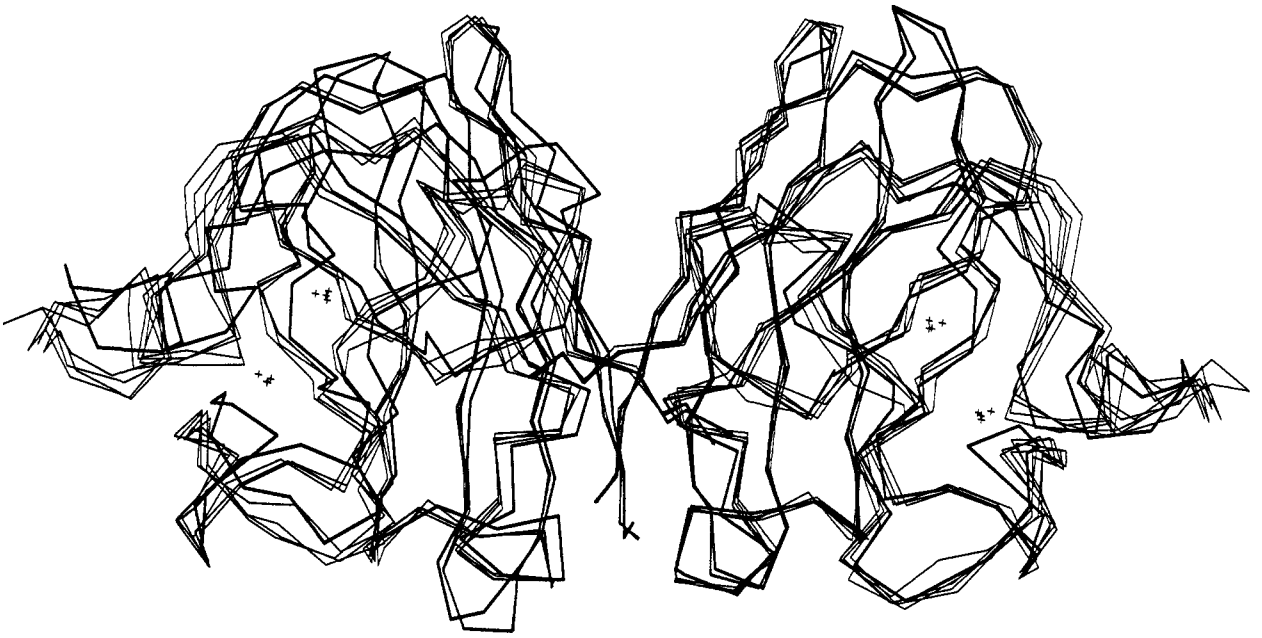

Figure 3. $\alpha$-Carbon traces of the X-ray crystal structure (in bold) and the five structures selected from the molecular dynamics simulation for analysis. The crosses represent the positions of the metal cofactors in the active sites.

## 3. Methods

**1. Molecular Dynamics Simulations.** The system in the molecular dynamics simulation consisted of the superoxide radical, 1629 atoms of SOD, and 1779 SPC/E water molecules, altogether forming a 26-Å sphere centered on the copper in one of the active sites. Protein atoms and water oxygens in the outermost 6-Å spherical shell were restrained by a harmonic potential, the former to the X-ray coordinates and the latter to dynamically relaxed positions from a larger model of the hydrated protein.[15] All covalent bonds were constrained to their equilibrium lengths using the SHAKE algorithm. A relatively large electrostatic cutoff distance of 20 Å was used in these studies because of the net charge on the superoxide radical. Details of the equilibration and simulation can be found in previous publications.[15,16] All molecular dynamics simulations were performed using the GROMOS package implemented in a parallelized form for the Intel iPSC/860 supercomputer.[21]

Surface **2** was chosen to be a spherical surface, of radius 9 Å, centered on the copper. This is approximately the location of a local minimum in the potential of mean force near the mouth of the channel.[15] To generate a representative set of configurations, we performed 150 ps of umbrella sampling by applying a harmonic force to confine one of the oxygen atoms of the superoxide near the 9-Å free energy minimum. From this simulation, 55 "starting configurations" were selected such that the copper–oxygen atom distance was 9.0 ± 0.3 Å. All selected starting configurations were separated by at least 2 ps to ensure dynamic independence.

Surfaces **1** and **3** were chosen to be spherical surfaces of radii 6 Å centered on the copper, where it has been suggested that the reaction takes place,[16] and 10 Å centered on the copper. The restraining potential was removed and molecular dynamic simulations were performed on each of the 55 configurations while the copper–oxygen distance was monitored. Of the 55 trajectories run, 8 times the superoxide reached surface **1**, $T_{2,1} = 0.146 \pm 0.048$, and the remaining 47 times it reached surface **3**, $T_{2,3} = 0.854 \pm 0.048$. The statistical error of $\pm\sigma$ for these calculations was estimated by using $\sigma = (P(1-P)/N)^{1/2}$, where $P$ is the estimate of the probability (e.g. $T_{2,1}$) and $N$ is the number of trajectories simulated.

**2. Brownian Dynamics Simulations.** In the Brownian dynamics simulations, the coordinates of the atoms in the enzyme were used to construct a detailed map of the excluded volume of the enzyme and to estimate the electrostatic fields surrounding the enzyme using the finite-difference Poisson equation.[19] The diffusional motion of the superoxide radical, modeled as a single sphere of hydrodynamic radius 2.05 Å and unit negative charge, was then simulated using the algorithm of Ermak and McCammon.[22] Details of Brownian dynamics simulations on the superoxide/superoxide dismutase system can be found in previous publications.[12,20] All Brownian dynamics simulations were performed using the UHBD program[23] with the GROMOS parameter set.

To calculate the components of the initial state vector (i.e. $P_2^0$ and $P_4^0$) the excluded volume and electrostatic fields of the X-ray crystal structure of SOD were constructed. A substrate particle was then placed at a random location on the $b$ surface of radius 41.5 Å and the trajectory was numerically simulated until the substrate reached surface **2** or the substrate reached a spherical surface of radius 60 Å, the "$m$ surface", centered on the enzyme (i.e. concentric with the $b$ surface). If the substrate reached the m surface, an analytical solution of the diffusion equation was used to determine if the substrate escaped or if the substrate returned to the $b$ surface, at which the numerical simulation was continued. Details of this procedure can be found in Luty et al.[18] Of the 2000 individual trajectories simulated, the probability of the superoxide radical reaching surface **2** was $P_2^0 = 0.156 \pm 0.008$.

To calculate the remaining components of the transition probability matrix $T_{3,2}$, $T_{3,3}$, and $T_{3,4}$, five enzyme structures, dynamically separated by at least 20 ps, were selected from the above 55 starting configurations of the molecular dynamics simulations. These structures should more accurately represent the conformation of the enzyme when the substrate is in the active site, relative to the X-ray crystal structure. To create symmetric SOD dimers, the atoms in the dynamical region of the molecular dynamics simulation were first duplicated. The original dynamical region and the duplicated dynamical region were then superimposed on the two subunits of the dimeric crystal structure of SOD by aligning the corresponding restrained atoms using QUANTA.[24] All atoms of the crystal structure which were present in the dynamical regions were then eliminated. $\alpha$-Carbon traces of the X-ray crystal structure and the five selected structures are presented in Figure 3.

Substrates were started on surface **3** by randomly drawing from a list of approximately 700 positions (for each structure) which were found accessible to the substrate during previous simulations. Trajectories were propagated until the substrate reached surface **2**, reached surface **3** in the other active site, or escaped. One thousand trajectories were run for each of the five enzyme structures. The average probability of a substrate started on surface **3** reaching surface **2** was $T_{3,2} = 0.736 \pm 0.006$, the probability of reaching surface **3** in the other active site was $T_{3,3} = 0.003 \pm 0.001$, and the probability of the substrate escaping was $T_{3,4} = 0.261 \pm 0.006$.

Finally, for comparison, we performed Brownian dynamics simulations using the X-ray crystal structure of SOD without taking into account the

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detailed dynamics in the active site. Substrate particles were started at a random location on the *b* surface of radius 41.5 Å and the trajectories were numerically simulated until the substrate reached surface **1**, where it was assumed to instantaneously and irreversible react, or the substrate reached the *m* surface. As before, an analytical solution of the diffusion equation was used to determine if a substrate that reached the *m* surface escaped, or returned to the *b* surface, at which numerical diffusion was restarted. For the 2000 trajectories performed, the probability of the superoxide radical reacting was $\beta = 0.119 \pm 0.007$.

## 4. Results and Conclusions

Using eq 5 with values of $T_{2,1}$ and $T_{2,3}$ from the molecular dynamics simulations and $P_2^0$, $T_{3,2}$, $T_{3,3}$, and $T_{3,4}$ from the Brownian dynamics simulations yields a value of $0.061 \pm 0.032$ for $\beta$. Using a solvent dielectric constant of 80, a temperature of 300 K, and charges of −1 e and −4 e on the substrate and enzyme, we estimate the rate of first encounter as $k(b) = 26.51 \times 10^9$ $M^{-1}$ $s^{-1}$ (with eq 2). From eq 1, the estimated rate constant for the hybrid Brownian dynamics–molecular dynamics simulation is $k = 1.62 \pm 0.86 \times 10^9$ $M^{-1}$ $s^{-1}$. This is only about one-half the rate constant obtained by the conventional, fully Brownian dynamics simulation, $k = 3.14 \pm 0.19 \times 10^9$ $M^{-1}$ $s^{-1}$. The experimental rate constant is not known at zero ionic strength, but it is likely to be in the range $k = 1 \times 10^9$ $M^{-1}$ $s^{-1}$ to $6 \times 10^9$ $M^{-1}$ $s^{-1}$.[6,7]

Examination of the structures of the enzyme from the molecular dynamics run shows that, in the presence of substrate, the active site channel closes up somewhat compared to the X-ray structure. For example, for surface **3** of the Markov analysis, the total area accessible to a substrate probe of 2.05 Å radius is 177 $Å^2$ for both active sites channels in the X-ray structure, compared to an average of 49 $Å^2$ for the five structures from the molecular dynamics simulation. The new method is therefore able to capture contributions to the kinetics that have not been included in previous Brownian dynamics simulations of diffusion-controlled reactions. In the present case, these new contributions arise primarily from the changes in the enzyme–substrate interactions due to structural fluctuations in the enzyme. They are not due to the differences between (inertial) molecular dynamics and (diffusive) Brownian dynamics *per se*, because Brownian calculations of $T_{2,1}$ (using the five enzyme structures selected from the molecular dynamics simulations) gave an average result very similar to that calculated by molecular dynamics.

There are, of course, many questions that remain to be addressed in computational studies of the activity of SOD. For the specific model considered here, the calculated rates are rigorously robust with respect to the locations of the *b* surface and the *m* surface.[17,18] Surface **1** was shown to be a reasonable choice for the location of electron transfer in a detailed molecular dynamics analysis of the kinetics of displacement of the water molecule that is directly coordinated to the active-site copper.[16] Some variation of the overall rate constant would be expected if the locations of surfaces **2** and **3** were changed, however. Ideally, these surfaces should be far enough from the reactive surface **1** that the system would be unperturbed by the initial presence of a reactant at surface **2**. This would eliminate the need for any approximations in the calculations of $P_2^0$ or the transition probabilities. To keep the molecular dynamics calculations within available resources for this study, surface **2** was located at a local minimum in the potential of mean force for superoxide at the mouth of the channel to the active site.[15] This seems a natural approximation for the calculations of transition probabilities, because superoxide will have relatively long residence times in this location. The side chains at the mouth of SOD relax somewhat around the superoxide, and this is reflected in the trajectories from which the transition probabilities are estimated. All of these issues, as well as other restrictions of this first study (e.g., the assumption of zero ionic strength), will be explored in the future.

The method used here, with further development, should be helpful in studies of a wide variety of systems. It has long been recognized, for example, that there is no channel wide enough to allow oxygen to move between the heme binding sites and the exterior of hemoglobin or myoglobin in the X-ray structures of these proteins.[25] Molecular dynamics simulations of myoglobin have provided estimates of the (unimolecular) rate constant for oxygen to move to the protein exterior.[26,27] The methods described here could be used to couple such calculations to simulations of ligand encounter to allow comparison with the experimental bimolecular rate constant. A more recent example is acetylcholinesterase; again, access of substrate to the active site is blocked in the X-ray structure, so that a Brownian dynamics study had to define a reactive zone at the surface of the enzyme.[28] The present methods can be used to couple such encounter simulations to simulations of the substrate capture by the fluctuating enzyme.

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