Computer Simulations of the Diffusion of a Substrate to an Active Site of an Enzyme

K. SHARP, R. FINE, B. HONIG

Computer simulations of the diffusion of a substrate to an enzyme active site were performed. They included the detailed shape of the protein and an accurate description of its electrostatic potential. Application of the method to the diffusion of the superoxide anion to the protein superoxide dismutase revealed that the electric field of the enzyme enhances the association rate of the anion by a factor of 30 or more. Calculated changes in the association rate as a function of ionic strength and amino acid modification paralleled the observed behavior. Design principles of superoxide dismutase are considered with respect to insights provided by the simulations. A possible means of enhancing the enzyme turnover rate through site-directed mutagenesis is proposed.

MANY ENZYMES FUNCTION AT rates that depend on the diffusion-limited association of their substrates with the active site (*1*). A well-studied example is the protein superoxide dismutase (SOD), which dismutes the negatively charged superoxide radical. Apparently, SOD uses electrostatic forces to increase the association rate constant of superoxide. We applied a new simulation technique to elucidate the mechanism of the electrostatically enhanced association of superoxide with SOD. Our results illustrate the interaction of a substrate with an enzyme and reveal how electrostatic potentials around macromolecules are involved in biological recognition.

SOD has an extremely high enzyme rate constant ($\sim 10^9\ M^{-1}\ sec^{-1}$) (*2*), while the naturally occurring concentrations of superoxide are very low (diffusion-dependent conditions). This rate constant is surprisingly high since the substrate must react with a copper ion in the active site, which takes up less than 0.1% of the total surface area of the protein. When the ionic strength is increased from 0 to 150 m*M* (physiological ionic strength), the rate decreases by 30%. The modification of charged residues can alter the rate and the dependence on ionic strength (*3*). These observations have led to the conclusion that electrostatics control the association rate (*4*). Paradoxically, the protein has a net charge of −4 at *p*H 7 (*5*). This would suggest that there should be a net repulsion between the substrate and enzyme that would reduce the association rate if the detailed shape of the electric fields were not considered. Furthermore, this repulsion would decrease with increasing ionic strength and thus increase the rate. This is contrary to what is observed experimentally.

The presence of positively charged copper and zinc atoms in the active site and the positions of other positively charged residues determined from the structure at a resolution of 2 Å (*6*) produce a region of strong positive potential which emanates from the active site and attracts the superoxide to the copper (*4*, *7*). This conclusion was based on calculations in which coulombic potentials were used and thus did not include the effects of the different polarizabilities of the solvent and the protein. Later calculations, which accounted for the dielectric discontinuity at the protein-solvent interface, showed that the region of positive potential that extended out from the active site channel was further enhanced by the focusing of field lines into the channel and away from the low dielectric of the protein interior (*8*). The calculations suggested that, despite its net charge, SOD produces an electrostatic potential that is capable of enhancing the association rate of the substrate and thus account for the observed behavior. However, in the absence of a direct calculation of the association constant this conclusion is tentative.

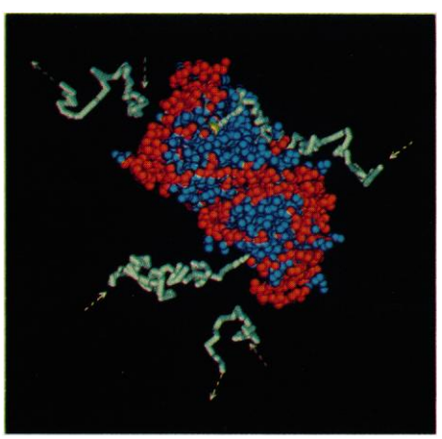

Fig. 1. Simulation of the association of the superoxide anion with the active site of superoxide dismutase (SOD) in the presence of the electrostatic field of the enzyme. Four typical Brownian dynamics trajectories that were generated with Eq. 1 are shown. The superoxide trajectories are shown in white and the active site copper atoms are shown in yellow. For clarity only those portions near the protein surface are shown. The entering and exiting portions of the trajectories are indicated by the broken arrows. The protein is color-coded according to the potential at its surface. Regions of positive and negative potential are colored blue and red, respectively. The longest dimension of the protein is about 60 Å. Two of the trajectories shown reach the active site while the other two exit the simulation space. The upper right trajectory undergoes two-dimensional diffusion across an attractive region of the protein surface before entering the active site. Actual simulations were run with much shorter steps than illustrated here.

We have used a method of Brownian dynamics adapted to irregular bodies (*9*) to study the factors that determine the association rate of the superoxide anion with SOD. Brownian dynamics simulates the diffusion process and is appropriate for the study of enzyme-substrate and protein-protein association (*10–13*). However, previous studies have been limited to a spherical representation of the protein and a simple coulombic form for its potential.

Our study combines the technique of storing complex force fields on a grid (*14*) with a detailed calculation of the electrostatic field of SOD, which includes the effects of ionic strength and the different polarizabilities of the solvent and protein (*8*). In addition, the grid is also used to store information about the accessibility of each region of space to the diffusing substrate (*9*). This enables us to represent in detail the shape of the enzyme to be used in the simulations. The association of superoxide with SOD can thus be studied with a realistic model of both the protein shape and electrostatic field.

Sharp *et al.* (*9*) have described the details of the Brownian dynamics simulations that were used to calculate the association rate, which we briefly summarize here. SOD was modeled by a collection of about 8000 1.5 Å cubes that were placed in the center of a 65 by 65 by 65 grid such that the longest dimension filled about two-thirds of the grid. The target location was defined by the collision surface of the two exposed copper atoms in the active sites. The protein shape was represented to an accuracy of half a grid unit, or about 0.7 Å. The protein was considered fixed, whereas the superoxide substrate, which was modeled as a 1.8 Å sphere, diffused in the region outside the protein. The electric field was calculated for every point of the grid by using a dielectric of 2 for the protein and 80 for the solvent and by incorporating ionic strength effects as described earlier (*8*).

K. Sharp and B. Honig, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032.
R. Fine, Department of Biological Sciences, Columbia University, New York, NY 10027, and Department of Structural Biology, Brookhaven National Laboratories, Upton, NY 11973.

The probability of the superoxide ion hitting the copper atom after starting at some initial distance R_s from the protein center was determined by running trajectory calculations that allowed the superoxide, represented as a point negative charge, to "diffuse" within the grid. Trajectories were stopped if they went beyond a distance R_e from the protein center or when they hit the target. Within the simulation space the motion was described by the equation

$$x_i = x_i^0 - (\partial U/\partial x_i)\,(D\tau/kT) + n_i(2D\tau)^{1/2} \quad \text{for } i = 1, 2, 3 \qquad (1)$$

where x_i is the new cartesian coordinate at a short time τ after beginning at position x_i^0, k is Boltzmann's constant, T is the absolute temperature, $U(x_i)$ is the potential function, D is the bimolecular diffusion constant, and the n_i are three sets of independent normally distributed random numbers such that $\langle n_i n_j \rangle = \delta_{ij}$. The equation $-\partial U(x_i)/-\partial x_i = f_i$, for $i = 1, 2, 3$, was used to calculate the force f_i on the superoxide particle. Some typical superoxide trajectories that were generated by the application of Eq. 1 are shown in Fig. 1.

After corrections were made for the truncation of trajectories that leave the simulation space (*12*), the association rate of the diffusing particle with the target molecule k_1 was calculated with the equation

$$k_1 = 4\pi D R_s P \qquad (2)$$

where P is the probability of a trajectory finding the target.

In these types of simulations errors can arise if the time step is not sufficiently small and if the starting and exit radii are not sufficiently large. By running simulations in which the time was systematically reduced and the radii were increased, we found conditions where the calculated association rate was independent of these simulation parameters. These values (τ = 0.1 psec, R_s = 60 Å, and R_e = 70 Å) were used in all of the simulations described here. Sufficient hits were collected to give an estimate of the rate to within a 5% coefficient of variation based on Poisson statistics unless indicated otherwise in the tables. The superoxide diffusion constant was taken as 100 Å^2 nsec^{-1} (*15*).

Table 2. Effect of the protein on the association rate of superoxide with SOD. The association rate was simulated at zero ionic strength. The coefficient of variation is 5% unless indicated otherwise.

Target	Net charge	Target area (Å^2)	Association rate ($10^{10}\,M^{-1}$ sec^{-1})	Relative rate*
Cu^{2+}	2	129	1.07†	1
SOD	0	12,000	2.36	2.21
Cu-SOD	0	10	0.041	0.038
Cu^{2+}-SOD	4	10	1.9	1.78
Cu-SOD with 0.0025 monopole field		−4	10	0.027 ± 0.003
All charges (full field)	4	10	1.37	1.28
Attractive field		10	1.53	1.43
Repulsive field		10	0.039 ± 0.010	0.036
All charges ($Glu^{119} \rightarrow$ Lys)	0	10	2.30	2.15
All charges ($Glu^{134} \rightarrow$ Lys)	0	10	2.40	2.24

*Rates are relative to the naked cupric ion. †Calculated with ionic radii of 1.4 and 1.8 for Cu^{2+} and O_2^-, respectively, and with a dielectric of 80 by using equations 2 and 5 from Sharp *et al.* (*9*).

One experimental observation that provides evidence for the effect of the electric field in guiding the substrate to the active site is the decrease in enzyme rate with increasing ionic strength (*3*). This effect occurs even at low salt concentrations from 0 to 150 mM. Previous work has shown that the correct dependence of rate on ionic strength was only obtained when the protein was represented as a low dielectric cavity in the high dielectric solution (*9*). Two further experimental observations that confirmed the importance of electrostatic effects on the enzyme rate were the unchanged ionic strength dependence upon neutralization of the catalytically important Arg^{141} and the reversed dependence on ionic strength upon neutralization of all the lysines (*3*). The two-dielectric model (*8*) was used to calculate the electrostatic fields around the native enzyme and these two amino acid–modified forms. The simulated rates as a function of ionic strength are shown in Table 1.

The association rate of the native enzyme decreased about 30% as the ionic strength was increased from zero to 144 mM. The simulated association rate of the Arg^{141}-modified protein increased slightly up to 50 mM and then decreased at 144 mM, but the rates were only about 60 to 75% of those for the native enzyme. This suggests that some of the decrease in enzyme rate determined experimentally upon neutralization of this group is attributable to a decrease in the electrostatic attraction as well to the disruption of the catalytic mechanism suggested previously (*3*). Another important result is that the reversal of ionic strength dependence in the lysine-modified enzyme is reproduced in the simulated rates (Table 1). These results confirm that the essential features of the association are being correctly modeled in these simulations. The increase in association rate is caused by a lowering of the negative potential barrier around the now predominantly negative protein which results from increased solvent screening, as was suggested from an analysis of the field maps alone (*8*).

The simulations in Table 1 were designed to simulate real conditions. In Table 2 we list the results of a number of hypothetical conditions in order to understand what features of SOD are important in enhancing the superoxide association rate. Comparisons are made to the isolated cupric ion, which is the simplest system capable of dismutation, and which has a rate constant of $8 \times 10^9\,M^{-1}$ sec^{-1} (*4*).

For the isolated cupric ion, the calculated association rate is only about 20% greater than the dismutation rate, which implies that most collisions result in a reaction. The association rate with the whole protein in the absence of a field is $2.36 \times 10^{10}\,M^{-1}$ sec^{-1}. The high collision rate is due to the large size of the protein compared with the copper ion (the protein is about equivalent to a sphere with a 30 Å radius). The maximum measured enzyme rate for SOD is 2×10^9 to $3 \times 10^9\,M^{-1}$ sec^{-1} (*2*). Compared to those for the whole neutral protein, about 10% of the collisions resulted in a reaction. This is very efficient if we consider that the two copper atoms form only 0.083% of the total surface area. This high efficiency is due to the electrostatic field, since for the neutral protein-copper complex (Cu-SOD) the association rate to the copper was only $4.1 \times 10^8\,M^{-1}$ sec^{-1}, which is a factor of 60 less

Table 1. The effect of amino acid modification on the relative association rate of superoxide with SOD. The association rate is relative to that for the whole neutral protein ($2.36 \times 10^{10}\,M^{-1}$ sec^{-1}). The coefficient of variation in calculated rates is 5% unless indicated otherwise.

Protein	Ionic strength (mM)			
	0	0.01	0.05	0.144
All charges	0.58	0.56	0.54	0.41
Neutral Arg^{141}	0.33	0.35	0.36	0.29
Neutral lysines	0.0009 ± 0.0001	0.0011 ± 0.0001	0.027	0.07

than for the whole protein and a factor of 5 less than the measured enzyme rate. If only the field due to the copper embedded in the protein (Cu^{2+}-SOD) was included, the rate increased by a factor of 46 to 1.9×10^{10} M^{-1} sec^{-1}, which is ten times faster than the enzyme rate. Since the protein contains two active sites, the effective association rate for each copper buried in the protein is about 1×10^{10} M^{-1} sec^{-1}, which is the same as for the naked cupric ion. Apparently the loss of target area that results from the partial burial of the copper is compensated by the enhanced electrostatic potential caused by the focusing of field lines in the channel of the active site.

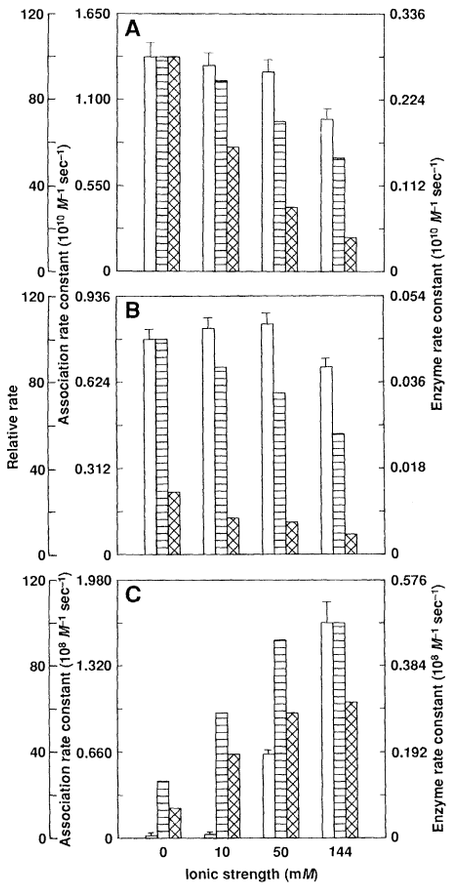

Fig. 2. Dependence of the simulated association rate and measured enzyme rate on ionic strength and the effect of amino acid modification for (**A**) native SOD, (**B**) SOD with neutral Arg^{141} enzyme, and (**C**) SOD with all lysines neutral. Enzyme rate data were taken from Cudd and Fridovich (*3*). Open bars represent simulated association rates. Error bars indicate the coefficient of variation in calculated rate based on Poisson statistics. Filled bars represent enzyme rates measured either by the pulse radiolysis method (crosshatched) or the riboflavin-sensitized photooxidation of dianisidine assay (shaded). Differences in values for the two assay methods probably result from the different superoxide concentrations required for the two assays (*20*). Both association rates and enzyme rates are scaled by the highest respective values obtained for each of the three protein forms to compare relative rates. The absolute scales for the association rate (on the left) are therefore different from those for the enzyme rate (on the right).

Since the field of the copper alone is large enough to account for the observed enzyme rate, we considered the role of the additional protein charges in determining the association rate. At zero ionic strength, the association rate we calculated for native SOD with all the charges included (1.37×10^{10} M^{-1} sec^{-1}) was comparable to the rate calculated for free cupric ions (2.2×10^{10} M^{-1} sec^{-1} per copper pair) and for the case of the charged copper in the neutral protein (Cu^{2+}-SOD, $k_1 = 1.9 \times 10^{10}$ M^{-1} sec^{-1}). The concentration of additional positive charges near the copper combine with the focusing effect of the active site cleft. Apparently the net effect of the protein charges (other than the copper atoms) is very small although the protein charges do reduce the association rate somewhat relative to the charged copper–neutral protein case.

It has been suggested (*7*) that the negative field of the protein, particularly the contribution of the conserved negatively charged glutamic residues Glu^{119} and Glu^{131} (*16*) near the mouth of the active site, plays a role in steering the ion to the active site. Two simulations were run to test this hypothesis, one with the positive portions of the potential map omitted and the other with the negative portions omitted (Table 2). The rate for the attractive part of the field was essentially the same as for the full field. In contrast, the repulsive part gave the same rate as for the neutral protein. The regions of negative potential apparently neither aided nor hindered the substrate in finding the copper. That is, they provided no net directionality or steering to diffusion away from or toward the active site. This may be because any directionality they provided near the active site was compensated by their role in repelling substrate molecules from the general region of the protein.

The specific roles of Glu^{131} and Glu^{119} were evaluated by calculating the rate constant for the hypothetical enzyme in which each of these residues was "mutated" into a lysine. The effect in either case was nearly a twofold increase in rate constant (Table 2). This shows that these residues actually decrease the association rate constant and suggests ways for designing a modified SOD which functions at higher rates than the native enzyme.

The simulated association rates from Table 1 and the measured enzyme rates are compared in Fig. 2. The experimental data are taken from Cudd and Fridovich (*3*) for two different types of assays, the riboflavin-sensitized photooxidation of dianisidine and the pulse radiolysis method. For each of the three forms of the protein the correct ionic strength dependence is reproduced. The decrease in association rate upon neutralization of the catalytically important Arg^{141} is about 30% at any ionic strength, compared with a decrease in enzyme rate of 85 to 95%. The greater decrease in the enzyme rate is caused by decreases in both the association and catalytic rates. For all conditions, the association rate is several times greater than the enzyme rate and ranges from a factor of about 3 for the lysine-modified protein to about 5 to 10 for the native enzyme.

Before we consider the implications of this large difference in association and enzyme rates we need to evaluate the uncertainty in calculating the association rate. A number of approximations and simplifications were made to perform these simulations. These included (i) the use of a grid to represent the protein shape and its field; (ii) the use of the linearized Poisson-Boltzmann equation in calculating the field; (iii) truncation of the field outside the grid; (iv) the use of a uniform diffusion constant for superoxide at the protein surface and within the active site; and (v) neglect of the rotational diffusion of the protein. The effects of these approximations have been considered previously (*9*) and are unlikely to cause more than a factor of 2 correction in the association rates. In any case, the trends reported in this work are independent of the exact value of the association constant.

Brownian dynamics simulations provide estimates of the association rate. This quantity must be distinguished from the enzyme rate determined experimentally by enzyme assays. This latter quantity is not directly determined in these type of simulations. The Michaelis-Menton model for enzyme kinetics (*1*) can be used to determine the relation between these two quantities.

$$k_e = k_2/K_m = k_1k_2/(k_1 + k_2) \qquad (3)$$

where k_e is the second-order enzyme rate constant (the turnover rate), k_1 is the second-order association rate constant, k_2 is the first-order catalytic rate constant, k_{-1} is the first-order substrate-enzyme dissociation constant, and K_m is the Michaelis-Menton constant. This expression is valid for substrate concentrations that are much less than K_m, which is the case for SOD (diffusion-dependent conditions).

It is evident from Eq. 3 that $k_e = k_1$ only if the dissociation rate is much less than the catalytic rate, otherwise k_e will be less than k_1 by the factor $(k_2/(k_{-1} + k_2)$. In either case the enzyme rate should be proportional to the association rate. Although never explicitly stated, it has generally been assumed that k_1 was approximately equal to k_e, that is,

about 2×10^9 to 3×10^9. (Larger values for k_1 were not considered since, as discussed above, even 2×10^9 was considered surprisingly large.) Our simulations suggest that k_1 is a factor of 5 to 7 greater than k_e ($\sim 1.4 \times 10^{10}$). Thus the problem is no longer to understand why k_e is so large but rather to understand why it is so small.

The answer lies in the factor $k_2/(k_{-1} + k_2)$, which is just the probability of reacting once the substrate has associated with the active site. This probability approaches 1 if ($k_2 >> k_{-1}$((diffusion-limited). Our calculations suggest that the reaction probability is about 0.2 and that k_{-1} is therefore comparable in magnitude to k_2. Turnover rates for SOD have been measured under saturation conditions at low temperature (*17*) and k_2 was found to be about 10^6 sec^{-1}. If we assume a reaction probability of 0.2, k_{-1} would be about 4×10^6 sec^{-1}.

A low reaction possibility could result from (i) the difficulty of accessing the copper because of bound water (*5, 6, 18*), competition from bound anions attracted by the positive potential (*19*), or the narrow channel at the bottom of the active site, or (ii) slow diffusion of protons to the active site or slow dissociation of the product OH^{-1} from the enzyme, both of which occur against the potential gradient.

Superoxide dismutase might be engineered to increase the enzyme rate (i) by increasing k_1 with the addition of positive charge near the active site channel, or (ii) by increasing k_2, which could increase the reaction probability by up to a factor of 5 to a value of unity. Increasing k_2 may be more difficult since the factors that influence it may be inherent properties of the active site channel. Perhaps the most striking conclusion from the results in Table 2 is that a complex protein such as SOD appears to mimic the effects of isolated cupric ions. The burial of the copper atom in the protein is offset by the focusing of the positive potential further out from the active site cleft into the solution due to the shape of the protein and its low dielectric constant. The charge distribution of the protein and its shape result in a quadropole-shaped electrostatic potential profile (*8*). The negative potential regions have no net effect on the association rate, whereas the positive regions, which are accessible to superoxide, provide a large collision surface and attract the ion efficiently to the copper atom.

The study of association of more complex substrates, protein-protein association (*13*) and protein–nucleic acid association, although computationally more demanding, is now within the realm of possibility by using extensions of the techniques described in this report.

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A New Method for Analyzing Powder Diffraction Patterns: Confirmation of a Predicted Phase of SF_6

LAWRENCE S. BARTELL, JACQUES C. CAILLAT, BRIAN M. POWELL

A recent computer simulation reproduced all of the solid-state transitions known for sulfur hexafluoride and predicted the unknown structure of its coldest phase. Subsequent neutron diffraction experiments that were performed to establish the structure of this phase could not be interpreted by conventional procedures. A method for analyzing Debye-Scherrer diffraction patterns was designed to avoid the many false minima that are encountered in standard analyses of low-symmetry phases. The structure found with this method confirmed the previous theoretical prediction.

CONSIDERABLE PROGRESS TOWARD understanding the structural and thermodynamic behavior of molecular solids was made several years ago in the molecular dynamics computer simulations of the sulfur hexafluoride (SF_6) system by Pawley and co-workers (*1, 2*). Although the octahedral SF_6 molecules are simple and quasi-spherical, the system is complex. Between its sublimation point (223 K) and 96 K, SF_6 exists in a body-centered cubic (bcc), plastic-crystalline phase (*3*). Under certain conditions it undergoes a transition to a trigonal structure when cooled below 96 K (*4*). At still lower temperatures it transforms into a phase whose symmetry is no higher than monoclinic (*4*). Molecular dynamics computations by Pawley and co-workers (*1, 2*) with a sample of 4096 molecules demonstrated the existence of all three phases. They reproduced the known lattice constants for the two higher temperature phases and the spontaneous transitions as the temperature was lowered.

For the coldest phase the computations predicted an uncommon packing arrangement, namely, a triclinic lattice (space group *P1*), with three molecules per cell. The prediction prompted an investigation of cold SF_6 in which neutron diffraction techniques were used. Because large single crystals could not be grown, measurements were made on polycrystalline samples in the low-temperature phase. Analysis of the crystal structure by conventional procedures proved intractable. Despite the examination of many thousands of sets of initial molecular configurations, no satisfactory unit cell was found in the subsequent refinements, so it was impossible to confirm or refute the predictions of Pawley and co-workers.

In concurrent research (*5*), submicroscopic crystals of SeF_6 and TeF_6 were obtained by homogeneous nucleation in their vapor phases in supersonic flow. To determine the lattice parameters of the microcrystals by electron diffraction, a procedure of analysis, documented in detail elsewhere (*6*), was devised. As a critical test the procedure was applied to the neutron data for SF_6, and an

L. S. Bartell and J. C. Caillat, Department of Chemistry, University of Michigan, Ann Arbor, MI 48109.
B. M. Powell, Neutron and Solid State Physics Branch, Atomic Energy of Canada, Ltd., Chalk River Nuclear Laboratories, Chalk River, Ontario K0J 1J0, Canada.