On the Mechanism of Action of Superoxide Dismutase: A Theoretical Study

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Abstract: Quantum chemical studies of the interaction of superoxide radical anion with a model of the active site of superoxide dismutase (SOD) suggest a new mechanism of action of the enzyme. The model for the active site of SOD was constructed from the crystal structure of the enzyme, and it contains the copper with its ligands, a model for the zinc ion, and an ammonium group as a model for Arg-141. The simulation of the enzymatic mechanism indicates that the presence of Arg-141 is responsible for a change in the redox chemistry of superoxide. In the absence of Arg-141, superoxide can reduce the copper and dissociate as oxygen. In its presence, however, the superoxide does not reduce the copper and forms a stable complex with the enzyme. This stable intermediate oxidizes another superoxide to oxygen with a concomitant reduction of the cupric to cuprous ion. In the resulting reduced form of the complex, superoxide has an increased proton affinity that induces proton transfer from Arg-141 to the superoxide. This form undergoes a charge redistribution that forms a complex between the oxidized form of the enzyme and a hydroperoxide anion. The enzymatic cycle is completed by the dissociation of hydrogen peroxide and the regeneration of the native enzyme. The stable complex between the enzyme and superoxide plays an important role in the activation of superoxide and can explain metal-mediated superoxide toxicity.

The Cu,Zn-superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of two superoxide radical anions to oxygen and hydrogen peroxide. It is a homodimer of molecular weight 32 000 that contains one copper and one zinc ion per monomer. The crystal structure of the enzyme was obtained to 2-Å resolution,[1,2] and it revealed the details of the active site. The copper, which is essential for enzymatic activity, is surrounded by His-44, His-46, His-61, and His-118 in a distorted square-planar geometry. The four ligands around the zinc are His-61, His-69, His-78, and Asp-81, and they are arranged in a tetrahedral structure. The distinctive structural property of the active site of SOD is the bridge between the copper and the zinc ions through the imidazolate group of His-61. The special properties of this structure have been discussed recently.[3] The details of the structure of the active site together with many physicochemical experiments (for a recent review see ref 4) led to a proposition for the mechanism of action of SOD.[3-11] This proposition is based on the following assumptions: (a) The enzyme is able to alternate between an oxidized and a reduced form and therefore the mechanism requires that the metal ion in the enzyme be both oxidizable and reducible by superoxide. (b) In the initial stage of this mechanism the reduction of the copper ion is associated with the uptake of a proton;[12-14] this was attributed to the protonation of the bridging imidazolate group. (c) In the reoxidation step the protonated bridging ligand is assumed to serve as a proton donor leading to the production of the hydroperoxide anion and the regeneration of the imidazolate bridge between the zinc ion and Cu(II).

The difficulties with this mechanism have been discussed[15,16] in relation to the mechanism of superoxide dismutation catalyzed by copper dihistidine complexes. An attempt to observe a complex between superoxide and copper dihistidine was unsuccessful, and thus the proposition that the dismutation of superoxide catalyzed by copper complexes involves such a complex[15] remained unsubstantiated. Notably, the role of several amino acid residues that are located near the active site and have a marked effect on the enzymatic dismutation is completely overlooked by this mechanism. Thus, the derivatization[17,18] of Arg-141, which is positioned only 6 Å away from the copper ion, diminishes the activity of the enzyme to approximately 10% of its original value. Likewise, the derivatization of 7 to 8 lysine residues diminishes the activity of the enzyme and inverts the dependence of its activity on ionic strength. Among the derivatized lysines are probably Lys-120 and Lys-134 that are positioned approximately 12 Å from the copper. Interestingly, the derivatization of the Arg-141 residue does not change the sensitivity of the enzymatic activity to ionic strength. Clearly, this recent work indicates that some amino acid residues near the active site have an important role in the mechanism of action of SOD. While it seems that the lysines play an important role in providing an attractive electrostatic field for the superoxide anion, the role of Arg-141 has to be of a different nature. Recently, the electrostatic potential and the electric field on the surface of the enzyme were calculated by an approximate method in which the charge distribution is represented by point charges on the atoms in the enzyme.[32] The analysis of the electric field near the active site indicated that the charged residues Lys-120, Glu-131, Lys-134, and Arg-141 play a role in directing the superoxide anion to the active site. This study also indicated that while Lys-120, Glu-131, and Lys-134 direct the long-range

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approach of superoxide, Arg-141 has only local orienting effects.

In this work we present a mechanism that differs from the one commonly accepted. An early account of this work, using a simplified model for the active site, was presented.[20] We present here results from quantum chemical calculations that provide evidence for the formation of a stable complex between superoxide and a copper complex that represents the active site of SOD. When Arg-141 is included in the model for the active site it plays an essential role in the formation of this complex by providing a hydrogen-bonding link that *changes the chemistry of the superoxide ion.* The consequence of this change is a different mechanism of action of SOD.

Computational Details

The model of the active site of SOD, shown in Figure 1, was constructed from the crystallographic coordinates taken from the Brookhaven Protein Data Bank.[21] The imidazoles of His-44, His-46, and His-118 were replaced by ammonia groups; the hydrogens were added with standard bond lengths and bond angles. The choice of ammonia to model an imidazole can be justified by the following observations. Inspection of the crystal structure of the active site of SOD indicates that the planes of the imidazole groups surrounding the copper ion do not lie in a single plane. This would suggest that the imidazoles do not exhibit any cooperative delocalizing effects. Also, ammonia groups were used in previous studies[34-39] to model the histidines that surround a zinc ion in carboxypeptidase and carbonic anhydrase. Of particular interest is the comparison of the interaction of a water molecule with two models of the active site of carboxypeptidase. In the first model[34] His-69 and His-196 were modelled by two ammonia groups, while in the second and more expanded model[39] they were modelled by two methylimidazole groups. The results of the interaction of a water molecule with the small model and the extended model are essentially the same, indicating that the ammonia groups in the small model are a good representation of the ligand field generated by the imidazoles. The imidazole ring of His-61 was taken in its entirety because earlier attempts to replace it by an NH_2^- group proved to be inadequate due to the tendency of the NH_2^- to reduce the cupric ion. The cationic effect of the zinc and its ligands, $[Zn(His)_3(Asp)]^+$, was modelled by a proton positioned in the location of the zinc ion. The relevance of this model, in which zinc is replaced by a proton, is supported by experiments that showed that at a slightly lowered pH, the enzyme that completely lacks zinc ions is as fully active as the holoprotein.[22,23] The terminal guanidinium group of Arg-141 was modelled by an ammonium group.

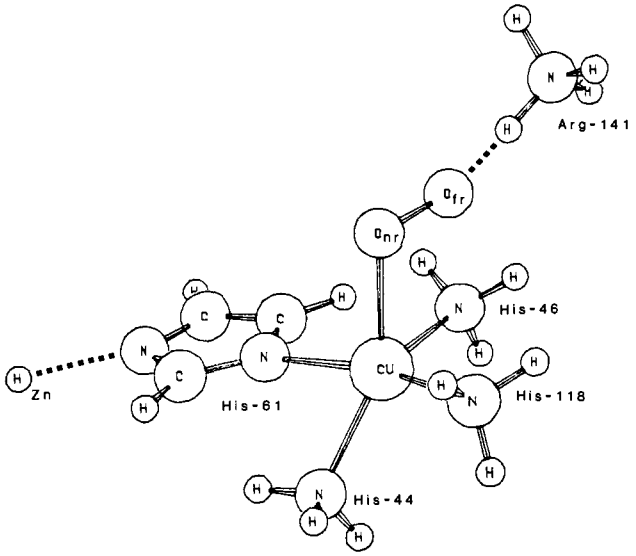

Figure 1. The model of the active site of SOD showing the surroundings of the copper, the model for Arg-141, and the mode of binding of superoxide.

Table I. Mulliken Charges of the Oxidized and Reduced Forms of a Model of the Active Site of SOD

group	oxidized[a]	reduced[b]
Cu	+0.77 (0.90)	+0.24
(NH_3) 44	+0.28 (0.04)	+0.12
(NH_3) 46	+0.32 (0.01)	+0.22
$(C_3N_2H_3)$ 61	−0.38 (0.03)	−0.43
H(Zn)	+0.67 (0.00)	+0.58
(NH_3) 118	+0.34 (0.02)	+0.23

[a] Doublet state; spin populations in parentheses. Total energy = −122.87523 hartrees. [b] Singlet state. Total energy = −123.19871 hartrees.

The relative position of the superoxide in the active site was obtained by initially positioning the superoxide 2.05 Å from the copper and orienting it to form a hydrogen bond with Arg-141. Subsequently, the geometry of the superoxide was optimized to minimize the close contacts with other groups in the active site. In the resulting geometry for an O–O distance of 1.4 Å, the Cu–O–O angle was 105° and the dihedral angle N44–Cu–O–O was −170°. In this geometry the distance between the distal oxygen of the superoxide (O-fr) and the nitrogen of Arg-141 was 2.787 Å and the angle N(Arg-141)–H–(O-fr) was 151.8°. The distance between the proximal oxygen (O-nr) and the copper ion was optimized for four different species: superoxide with O–O = 1.206 Å corresponding to an interatomic distance in the oxygen molecule, superoxide with O–O = 1.341 Å corresponding to an interatomic distance in the superoxide ion, hydroperoxyl radical with O–O = 1.330 Å, O–H = 0.976 Å, and O–O–H = 104.0°, and hydroperoxide anion with O–O = 1.498 Å, O–H = 0.961 Å, and O–O–H = 99.8°. The structural parameters for the hydroperoxide anion were taken from the theoretically optimized structure; experimental geometries were used for all other structures.[30] For the last two species the dihedral angle Cu–O–O–H was 110°, an angle that yields minimal close contacts in the complete active site of the enzyme. During the optimization of the Cu–(O-nr) distance all other parameters were kept fixed. The minimum-energy distance was calculated from fitting the data to a quadratic equation; the minimum energy at this distance was estimated from this equation.

All the calculations were performed by using a program that incorporates an extended version of Kahn's effective core potential (ECP) evaluation routine.[24] The 1s core electrons of oxygen and nitrogen were replaced by the ECP of Topiol et al.[25] and the 18 [Ar] core electrons of copper by a modified effective potential (MEP) recently developed by us.[26] This MEP was developed to correct for major valence atomic correlation energy errors and was shown not to cause systematic distortion of molecular binding energy curves of CuO. The basis set for copper and oxygen was the atom-optimized split-valence Gaussian basis used in ref 26 with the following modifications: the 4p and 4p′ functions on copper were replaced by a single Gaussian function with exponent 0.12;

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Table II. Charge and Spin Distribution upon Approach of Superoxide and Dissociation of Oxygen from the Active Model of SOD[a]

group	SOD(Ox) + O_2^- Cu–O = 6.15 Å	SOD(Re) + O_2 Cu–O = 2.05 Å	SOD(Re) + O_2 Cu–O = 6.15 Å
Cu	+0.84 (0.89)	+0.34 (0.01)	+0.29 (0.00)
(NH_3) 44	+0.27 (0.03)	+0.17 (0.00)	+0.17 (0.00)
(NH_3) 46	+0.31 (0.02)	+0.18 (0.01)	+0.20 (0.00)
$(C_3N_2H_3)$ 61	−0.37 (0.03)	−0.44 (0.00)	−0.44 (0.00)
H(Zn)	+0.65 (0.00)	+0.58 (0.00)	+0.58 (0.00)
(NH_3) 118	+0.31 (0.03)	+0.19 (0.01)	+0.20 (0.00)
O-nr	−0.39 (0.80)	+0.01 (0.97)	0.00 (1.00)
O-fr	−0.61 (0.20)	−0.02 (0.99)	0.00 (1.00)
energy, au	−154.43965	−154.57772	−154.60917

[a] The active site model of SOD does not include the ammonium group that models Arg-141. The energy of the fully dissociated SOD(Ox) and superoxide is −154.33337 au and that of the fully dissociated SOD(Re) and oxygen is −154.64255 au.

the 3d function on oxygen was eliminated. The basis set on the hydrogen-bonded proton of the model of Arg-141 was a [4/3,1] set. On all other atoms we used an atom-optimized minimal basis especially designed for ECP calculations (LP-3G).[27] This resulted in a total of 87 basis functions for the molecule shown in Figure 1. The various states reported here were calculated with the restricted open Hartree–Fock (ROHF) formalism.

Results and Discussion

The enzymatic mechanism of SOD was simulated in two stages. Initially the active site of the enzyme was limited to the copper ion and its surrounding ligands, excluding the model for Arg-141. A superoxide was brought toward the copper, and the resulting changes in charge distribution were analyzed to determine whether the enzyme can be reduced. In the second step of the simulation the importance of the model of Arg-141 was evaluated. The active site was extended to include the ammonium group that models the Arg-141 residue, and its effect on the properties of the superoxide anion as well as its ability to reduce the copper were evaluated. Finally, the formed complex was studied in relation to its tendency to become reduced and to undergo an intramolecular electron transfer which regenerates the oxidized form of the enzyme.

The Mulliken population analysis of the oxidized (doublet) and the reduced (singlet) states of the small model of the active site (i.e., excluding the model of Arg-141) are shown in Table I. The net charge of the oxidized complex is 2+ and it is distributed between the central metal ion and the ligands. The copper ion carries a charge of 0.77+ and most of the unpaired electron is localized on it. The ligands donate some of their charge density to the central atom and are consequently positively charged. The imidazolate of the bridging His-61 donates charge to both the copper ion and the proton H(Zn) that models the zinc ion with its ligands, but it still retains a net negative charge. The reduction of this model generates a closed-shell molecule that would have no detectable EPR signal. The additional electron added to the complex distributes between the copper and the ligands. More than 50% of it goes to the copper and the rest distributes itself among the ligands. This brings the net charge on the copper to 0.24+.

We now turn to the analysis of the approach of the superoxide anion to this model and the resulting changes in charge distribution. The charge distribution of the oxidized model of the active site changes very little upon the approach of superoxide to a distance of 6.15 Å. This is evident from the comparison of the first columns of Tables I and II. The stabilization of this complex seems to arise from the electrostatic interaction between the oppositely charged ions. The situation changes drastically upon the approach of the superoxide to a distance of 2.05 Å. Results in the second column of Table II indicate that the superoxide reduces the cupric to cuprous ion and consequently becomes a triplet oxygen bound to the cuprous complex. The dissociation of the triplet oxygen from the reduced complex, first to a distance of 6.15 Å and ultimately to the separated molecules, stabilizes the complex by 19.7 and 40.7 kcal/mol, respectively. Thus, our calculations indicate that the reduced form of the small model of the enzyme has no affinity for oxygen; in fact, the interaction of the reduced form of the model of the active site of SOD with oxygen is repulsive. One should keep in mind, however, that the reduced form of the enzyme is structurally different from the oxidized[28] form, a fact that was not modelled in our simulation. Also, the repulsive nature of the interaction between the reduced model and oxygen can be the result of the unoptimized O–O distance of 1.4 Å of the superoxide. Nevertheless, this stage of the simulation indicates that when the active site of SOD is modelled by copper and its immediate ligands, superoxide *can* reduce the enzyme. This is in agreement with the commonly accepted mechanism.

The situation changes significantly in the second stage of the simulation in which the model of the active site of SOD is increased to include an ammonium group that represents Arg-141, as shown in Figure 1. The energies associated with the various states of the separated molecules and the complexes in this stage of the simulation are summarized in Table III. The table is divided into three sections: In section A the energies of the dissociated molecules are presented. Four states of the enzyme are considered: the oxidized (ECu^{2+}) and reduced (ECu^{1+}) with Arg-141 protonated (i.e., NH_4^+) and the oxidized and reduced with Arg-141 deprotonated (i.e., NH_3). These four states of the enzyme can potentially interact with the superoxide and hydroperoxide anions or with oxygen and the hydroperoxyl radical giving rise to the six possible combinations shown in Table III. In section B of Table

Table III. Energies of the Separated Reactants and Complexes (in Hartrees)

	complex	geometry	energy	state
	A. Separated Molecules[a]			
1.	$ECu^{2+}\cdots H^+NH_3 + O_2^-$ (∞)	O–O = 1.341 Å	−165.85954	doublet; doublet
2.	$ECu^{2+}\cdots H^+NH_3 + O_2^-$ (∞)	O–O = 1.341 Å	−166.28231	singlet; doublet
3.	$ECu^{1+}\cdots H^+NH_3 + {}^3O_2$ (∞)	O–O = 1.206 Å	−166.27896	singlet; triplet
4.	$ECu^{2+}\cdots NH_3 + HO_2^-$ (∞)	ref 30	−166.25832	doublet; singlet
5.	$ECu^{2+}\cdots NH_3 + HO_2$ (∞)	ref 30	−166.25409	doublet; doublet
6.	$ECu^{1+}\cdots NH_3 + HO_2$ (∞)	ref 30	−166.56857	singlet; doublet
	B. Complexes with Superoxide (O–O = 1.341 Å)			
7.	$ECu^{2+}\cdots O_2^-\cdots H^+NH_3$	Cu–O = 2.010 Å	−166.31572	triplet
8.	$ECu^{2+}\cdots O_2^-\cdots H^+NH_3$	Cu–O = 2.016 Å	−166.31461	singlet open she
9.	$ECu^{1+}\cdots O_2^-\cdots H^+NH_3$	Cu–O = 2.197 Å	−166.55830	doublet
	C. Complexes with HO_2 or HO_2^- [30]			
10.	$ECu^{2+}\cdots O_2H\cdots NH_3$	Cu–O = 2.199 Å	−166.27178	triplet
11.	$ECu^{1+}\cdots O_2H\cdots NH_3$	Cu–O = 2.296 Å	−166.57175	doublet
12.	$ECu^{2+}\cdots {}^-O_2H\cdots NH_3$	Cu–O = 1.893 Å	−166.56874	doublet
12a.	$ECu^{2+}\cdots {}^-O_2H\cdots NH_3$	Cu–O = 1.893 Å	−166.60549	doublet

[a] Energy in hartrees calculated as the sum of the energies of the separated molecules: $E(ECu^{2+}\cdots H^+NH_3) = -134.40347$; $E(ECu^{1+}\cdots H^+NH_3) = -134.82624$; $E(ECu^{2+}\cdots NH_3) = -134.21835$; $E(ECu^{1+}\cdots NH_3) = -134.53283$; $E(O_2^-) = -31.45607$; $E({}^3O_2) = -31.45272$; $E(HO_2\ {}^2A'') = -32.03574$; $E(HO_2^-) = -32.03997$; $E_{corr}(HO_2^-) = -32.07672$ (see text for explanation [30]).

Table IV. Charge and Spin Distribution of the Complexes of SOD Augmented by Arg-141 with Superoxide

group	$ECu^{2+}\cdots O_2^{-}\cdots H^+NH_3$	$ECu^{1+}\cdots O_2^{-}\cdots H^+NH_3$
Cu	+0.87 (0.89)	+0.36 (0.00)
(NH_3) 44	+0.25 (0.03)	+0.15 (0.00)
(NH_3) 46	+0.23 (0.01)	+0.16 (0.00)
$(C_3N_2H_3)$ 61	−0.41 (0.03)	−0.45 (0.00)
H(Zn)	+0.64 (0.00)	+0.55 (0.00)
(NH_3) 118	+0.26 (0.03)	+0.17 (0.00)
O-nr	−0.24 (0.69)	−0.19 (0.82)
O-fr	−0.58 (0.32)	−0.72 (0.18)
(NH_3) 141	+0.41 (0.00)	+0.38 (0.00)
H-Arg	+0.57 (0.00)	+0.59 (0.00)
energy, au	−166.31572	−166.55830

III are shown the energies of the complexes with superoxide and in section C those of the complexes with HO_2 or HO_2^-. The last entry in the table (12a) was corrected for the atomic correlation energy of HO_2^-.[30]

The superoxide anion is attracted to the active site primarily by electrostatic forces. The total stabilization energy when superoxide interacts with the oxidized complex is 286.3 kcal/mol (lines 7 and 1 in Table III). The estimated electrostatic interaction from the charge distribution shown in Table IV is 222.1 kcal/mol, which is approximately 78% of the total stabilization energy. From the charge and spin distribution shown in Table IV for this complex it is clear that upon the formation of the complex between the extended active site and the superoxide, the superoxide *does not* reduce the cupric to the curpous ion. Moreover, even shortening the O–O bond to 1.206 Å, effectively forcing it into the geometry of an oxygen molecule, did not induce an electron transfer from the superoxide to the copper. In this geometry the optimized Cu–O distance is shortened to 1.992 Å but the energy is 6.6 kcal/mol higher than that of the complex with a regular superoxide ion (line 7 of Table III). Thus, the presence of Arg-141 changes the nature of the interaction between the superoxide and the copper in SOD. While in the absence of Arg-141 the approach of superoxide to copper causes *its* reduction, in the presence of the Arg-141 model the superoxide forms a complex with the active site without reducing the copper. The spatial disposition of Arg-141 relative to the copper is such that the incoming superoxide is within bonding distance from copper and simultaneously forms a hydrogen bond with Arg-141.

The charge and spin distribution shown in Table IV for the stable complex between SOD and superoxide is for the triplet state formed from the parallel coupling of the doublets of the superoxide and the copper complex. An antiparallel coupling of the spins of superoxide and the copper complex is also possible giving rise to an antiferromagnetic complex. The energy of this singlet open-shell molecule is shown in line 8 of Table III and is only 0.69 kcal/mol above the paramagnetic triplet state. At 37 °C this energy difference would result in a Boltzman distribution of 75% paramagnetic and 25% antiferromagnetic forms of the complex. The nearly equal probability of forming a paramagnetic triplet complex and an antiferromagnetic singlet open-shell complex results from the high localization of the spins on the copper and the superoxide as shown for the paramagnetic complex in Table IV.

The properties of the complex between SOD and superoxide are important for the subsequent steps in the enzymatic mechanism. This complex has a high electron affinity and therefore can act as the oxidant of another superoxide anion. This process is shown in Figure 2 and is marked "oxidation of superoxide". The energy of this process was evaluated by adding an electron to the complex between the oxidized form of SOD and superoxide and can be calculated from the difference between lines 9 and 7 in Table III. The charge and spin distribution of the reduced complex are shown in Table IV; they clearly indicate that the reduction takes place on the copper. Thus, regardless of whether the triplet or the singlet open-shell complex is reduced, the result is the same, i.e., a complex between the reduced form of the enzyme and

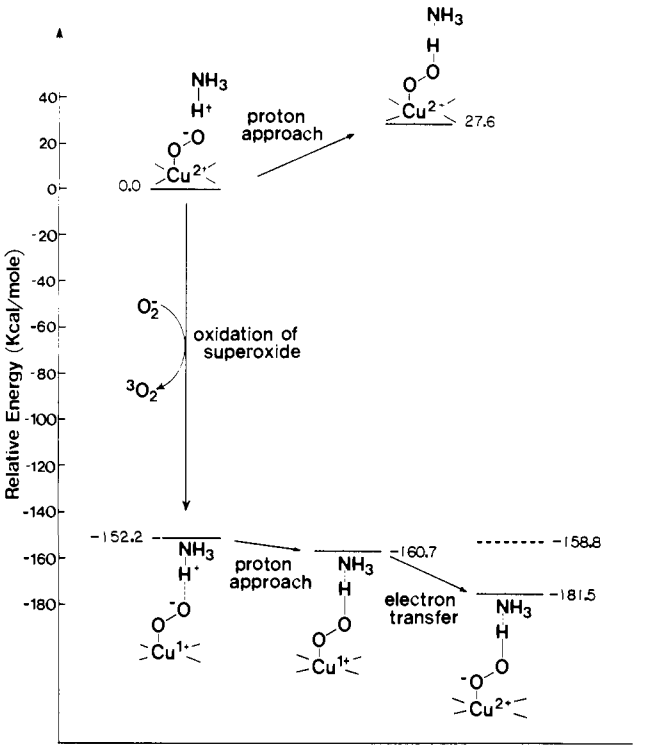
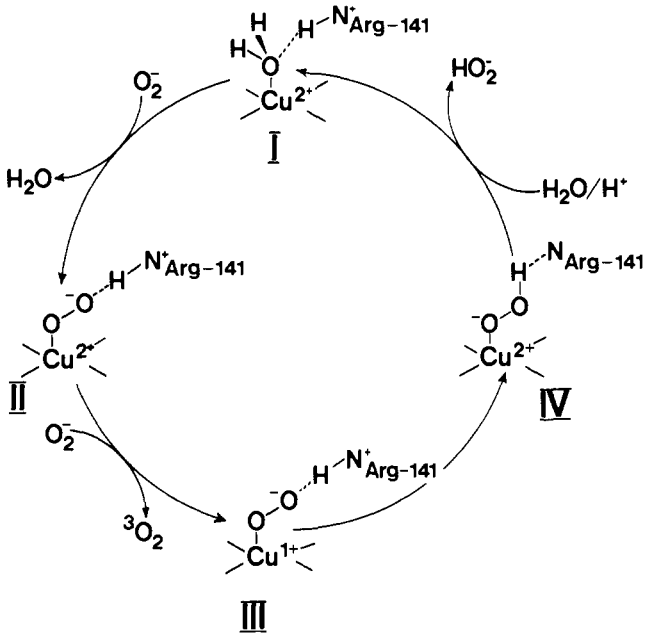


Figure 2. A diagramatic representation of the processes and energies involved in the mechanism of action of SOD.

superoxide. The immediate consequence of the reduction is a marked change in the proton affinity of O-fr (the distal oxygen) of the superoxide. In the oxidized complex the approach of the proton from the ammonium that models Arg-141 to O-fr raises the energy of the complex by 27.6 kcal/mol, as can be seen from the difference between line 10 and line 7 in Table III. However, upon reduction the energy of the complex is stabilized by 8.4 kcal/mol due to the approach of the proton (compare lines 11 and 9 in Table III). In this complex the charge and spin distribution indicate that the complex is between a neutral hydroperoxyl radical and a reduced copper complex. Taking into account the high electron affinity of the hydroperoxyl radical,[29] it was interesting to investigate whether an intramolecular electron transfer can be induced by the approach of the proton to O-fr. The calculation of the complex between the oxidized state of the model and a hydroperoxide anion shows that its energy is only 1.9 kcal/mol higher than that of the complex between the reduced model of SOD and a hydroperoxyl radical (lines 11 and 12 in Table III). This result, however, has to be corrected at least for the atomic correlation associated with the electron affinity of the hydroperoxyl radical.[30] In the construction of the MEP for copper[26] the atomic correlation due to the different electronic states of copper has been taken into account. The correction due to the error in electron affinity of the hydroperoxyl radical, which primarily comes from atomic correlation of the oxygen, is probably at least the 1.04 eV calculated with a larger basis set.[30] Thus, the corrected energy of the complex between the oxidized enzyme and hydroperoxide anion would be nearly 21 kcal/mole lower than the complex between the reduced enzyme and hydroperoxyl radical (compare lines 12a and 11 in Table III). These results are illustrated in the diagram in Figure 2 which shows that the reduction of the complex of SOD with superoxide triggers the protonation of superoxide by Arg-141, which in turn causes an intramolecular electron transfer from copper to the hydroperoxyl radical. The overall result of this process, upon dissociation of the hydroperoxide anion, is the regeneration of the oxidized form of the enzyme and the dismutation of two superoxide radical anions to an oxygen molecule and a hydroperoxide anion, which can easily be protonated to give hydrogen peroxide.

Scheme I summarizes the proposed mechanism that results from the computational simulation. Structure I is the native enzyme

Scheme I



in its oxidized form with a molecule of water bound to the copper. The crystal structure[1] shows a relatively long Cu–O bond of 2.85 Å, and the distance to one of the terminal nitrogens of Arg-141 is 3.30 Å. This structure is in agreement both with the proposition that the exchange of the water molecule by a superoxide anion is the rate-determining step[4] and with the finding that Arg-141 does not participate in the electrostatic facilitation of the reaction because it is hydrogen bonded to the water molecule in the active site.[19] Structure II is formed as a consequence of the exchange of the water in the active site by a superoxide ion. This structure has been shown by our calculation to be a stable complex with respect to the reactants in which the superoxide does not reduce the cupric ion. However, this complex is a strong enough oxidant to oxidize another superoxide to oxygen. In this outer-sphere redox reaction the cupric ion is reduced to cuprous ion as shown in structure III. This reduction triggers a sequence of events which ultimately produces the second product, i.e., hydrogen peroxide, and restores the oxidized form of the enzyme. First, the reduction triggers the proton approach from Arg-141 toward the superoxide; this process was impossible before the reduction. The proton transfer in turn induces an intramolecular electron transfer from the cuprous ion to the hydroperoxyl radical. This results in a complex between the oxidized enzyme and hydroperoxide anion shown in structure IV. Upon dissociation of the product from the enzyme its native form is restored and the catalytic cycle can take place again.

It is important to note that this mechanism does not preclude the possibility that the entire catalytic cycle can begin from the reduced state of the enzyme, as was shown by Fielden et al.[8] Even though the structural changes that take place in the active site of the enzyme upon reduction are not shown here, the superoxide can interact with the reduced form of the enzyme, form structure III, and continue the enzymatic catalysis as shown in Scheme I. The energy of the complex between superoxide and the reduced enzyme (line 9 in Table III) is lower than that of the separated molecules (line 2 in Table III). In fact, the entire process can be started by oxygen that shows an affinity for the reduced enzyme (line 7 and line 3 in Table III) and oxidizes the cuprous to cupric upon formation of structure II. It is interesting to note that the calculations predict that the hydroperoxide anion should show an affinity for the oxidized enzyme in its deprotonated form (line 12a and line 4 in Table III). This observation is in agreement with the reversal of the enzymatic reaction shown by Hodgson and Fridovich[31] to occur under the conditions of high pH and in the presence of an effective scavenger of superoxide. Recently, Bernofsky and Wanda[40] demonstrated that a large excess of SOD enhanced the release of protons in solutions of H_2O_2. They suggested that SOD catalyzes the formation of the complex, HO_4^-, from a superoxide anion and a hydroperoxyl radical. However, the reaction conditions described in their paper are favorable for the formation of a complex between SOD and superoxide, as described above, which will shift the reaction between H_2O_2 and oxygen to the right and thus enhance the release of protons.

Concluding Remarks

The computational simulation of the mechanism of action of SOD suggests that the enzyme does not undergo a sequential reduction and oxidation by superoxide. In this mechanism the enzyme forms a relatively stable complex with superoxide depicted in Scheme I as structure II. For the enzymatic mechanism this is the initial step that enables the sequence of events shown in Scheme I. This step also changes significantly the reactivity of superoxide. Superoxide is usually a weak oxidant because its reduction requires the formation of a doubly charged anion; it is also an inefficient hydrogen atom abstractor. Its activity as an oxidant is evident only in the presence of potential proton donors from which it abstracts a proton and then acts as an oxidant through the hydroperoxyl radical. When superoxide forms a stable complex with SOD, a process that was made possible primarily due to the presence of Arg-141, it effectively undergoes an activation. In its present form it can act as an oxidant, albeit in an indirect way, by enabling the outer-sphere reduction of the copper. In SOD the second step of the enzymatic reaction, i.e., the oxidation of another superoxide, retains its selectivity toward superoxide. This is probably made possible by the two lysine residues, positioned at the entrance to the reactive groove, that control the approach of anions to the active site by a positive electrostatic field. In other copper complexes the formation of the complex with superoxide may also be possible, but they may lack the super-structure of the protein that imposes the electrostatic selectivity toward anions, and in particular toward superoxide. When these complexes are formed they could act as activators of superoxide from a relatively benign reagent to a strong oxidant. Such complexes should have a longer lifetime than other powerful oxidants, e.g., hydroxyl radical, and could therefore reach vital sites in the cell to cause oxidative damage.

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(40) Bernofsky, C.; Wanda, S.-Y. C. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 231.