

respectively), but arenethiolato complexes  $[\text{Fe}_2\text{S}_2(\text{S-Ph}(p\text{-X}))_4]^{3-}$  exhibit rhombic spectra.<sup>26</sup> The reduced species of present peptide complexes **1**, **4A**, and **5** also exhibit rhombic spectra; see Figure 5.<sup>31</sup>

From a theoretical study of reduced 2Fe-2S ferredoxin, Gayda et al. reported that the difference between  $g_x$  and  $g_y$  values ( $g_x - g_y$ ) could be expressed by a linear function of the d orbital mixing.<sup>32</sup> Thus, the  $g_x - g_y$  value is important in the understanding of the electronic structure of the reduced 2Fe-2S complex. The  $g_x - g_y$  values of **1** and **5** are similar, but different from those of native plant-type ferredoxins. Large  $g_x - g_y$  values show weak  $d_{x^2-y^2}$  and  $d_{z^2}$  orbital mixing, and these small values indicate strong mixing. The orbital mixing is related to the distortion of the coordination geometry of Fe ions. For example,  $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-o-xyl})_2]^{2-}$ , which has a very small  $g_x - g_y$  value, has narrow S-Fe-S angles ( $106.4^\circ$ ) due to the  $\text{S}_2\text{-o-xyl}$  chelating ligands.<sup>33</sup> The  $g_x$

–  $g_y$  values of **1** and **4A** indicate that these peptide ligands exert a different distortion of the 2Fe-2S core. The difference in structure of the complexes **1** and **4A** is thus revealed.

In conclusion, a combination of spectroscopic data and electrochemical properties is necessary to infer the solution structures of 2Fe-2S peptide complexes. But among the data, the CD spectra are the most important in indication of chelation of the peptides. The splitting of the Cys  $\text{CH}_2$   $^1\text{H}$  NMR signals may also be utilized for diagnosis.

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**Registry No.** **1**, 99493-48-8; **2**, 99432-46-9; **3**, 99432-48-1; **4A**, 100815-65-4; **4B**, 100840-38-8; **5**, 100815-63-2;  $[\text{Et}_4\text{N}]_2[\text{Fe}_2\text{S}_2(\text{S-}i\text{-Bu})_4]$ , 100815-67-6;  $[\text{Et}_4\text{N}]_2[\text{Fe}_2\text{S}_2\text{Cl}_4]$ , 62682-81-9.

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## Ab Initio Calculations of the $\text{Cu}^{2+}\text{-O}_2^-$ Interaction as a Model for the Mechanism of Copper/Zinc Superoxide Dismutase

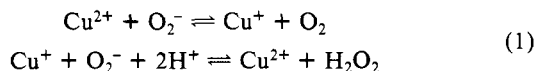
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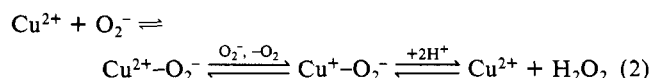
Ab initio calculations have been performed on the  $\text{Cu}^{2+}\text{-O}_2^-$  system by considering the effects of an ammonia ligand on copper and of an ammonium ion interacting with superoxide. This system is instructive as far as the active site of bovine erythrocyte superoxide dismutase is concerned. The calculations show that an intermediate  $\text{Cu}^{2+}\text{-O}_2^-$  is stable as long as superoxide is hydrogen bonded to the ammonium ion. As suggested by Osman and Basch, a second superoxide ion may reduce copper(II). Once copper(I) superoxide is obtained, calculations provide a pathway according to which a covalent bond is formed between superoxide and the ammonium proton, the Cu–O distance increases, and a second proton binds the proximal oxygen. This causes electron flow from copper(I) to superoxide, thus providing a copper(II)–hydrogen peroxide system. A further proton binding to the free ammonia molecule is required for the release of  $\text{H}_2\text{O}_2$ .

### Introduction

The mechanism of dismutation of superoxide by Cu/Zn superoxide dismutase was generally believed<sup>1</sup> to occur through a two-step mechanism of the type



Even though there is no firm evidence that electron transfer occurs through superoxide coordination, this is often assumed to be true. On the basis of this assumption, Osman and Basch have calculated the energetics of the copper(II)–superoxide interaction.<sup>2</sup> It is now being established that an active-site arginine residue (Arg-141) in its protonated form plays a role in the enzyme–superoxide interaction.<sup>3,4</sup> Osman and Basch have shown that a superoxide ion simultaneously interacting with copper(II) and Arg-141 does not oxidize to dioxygen, without breaking of the superoxide–arginine interaction.<sup>2</sup> This system can be reduced by a second superoxide ion, thus suggesting the possibility of a catalytic pathway completely different from that shown in eq 1, i.e.



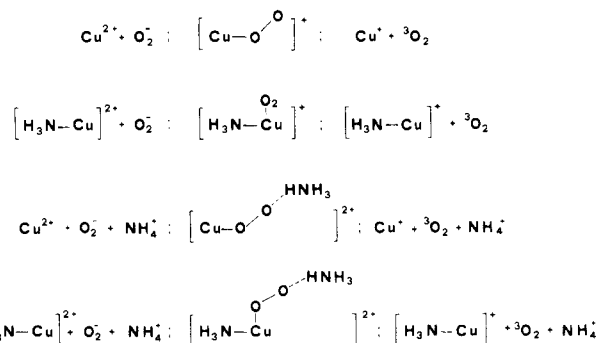
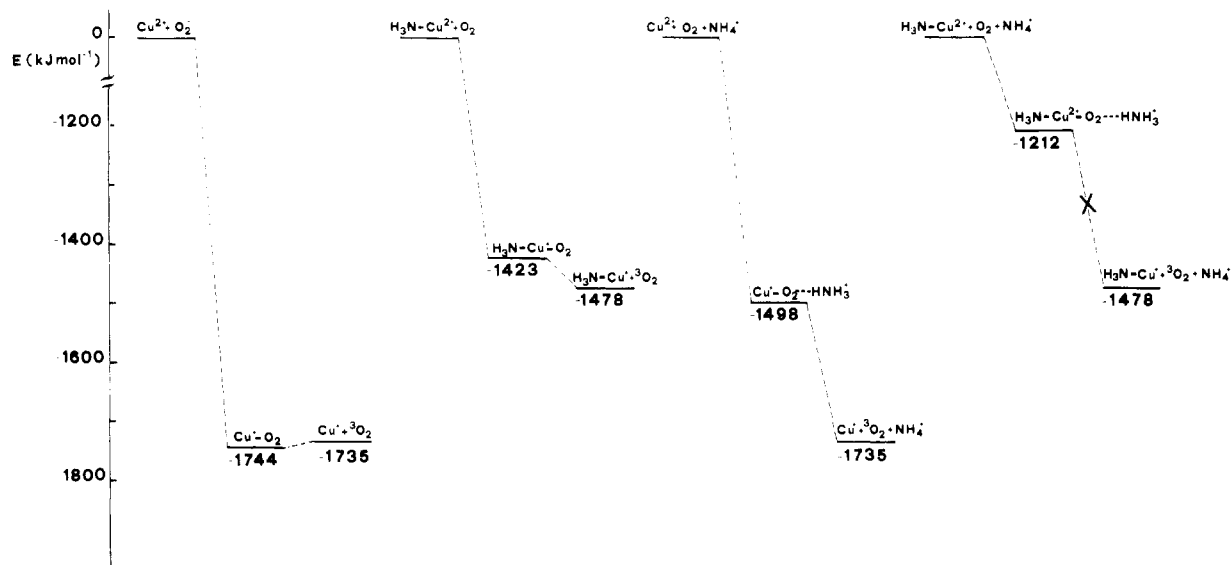
Their calculations are based on a molecular orbital approach including the core potential approximation with a split-valence

basis set: in this way they were able to treat a relatively large number of atoms, that is, three ammonia molecules and an imidazole ring to simulate the copper ligands from the protein and an ammonium ion to simulate Arg-141. Owing to the relevance of this problem, to its implications for enzyme-catalyzed superoxide dismutation in living systems, and, more generally, to the issue of binding of dioxygen and related species to metal ions in biological molecules, we have attempted to understand the factors that affect the energies of the copper(II)–superoxide system by evaluating the effect of an ammonia ligand on the  $\text{Cu}^{2+}\text{-O}_2^-$  moiety on one side and of the approach of an  $\text{NH}_4^+$  ion to the distal oxygen on the other. We have used ab initio Hartree–Fock calculations using a double- $\zeta$  valence plus polarization quality basis set: we found that the  $\text{H}_3\text{N-Cu}^{2+}\text{-O}_2^-\cdots\text{HNH}_3^+$  situation is the most stable among the various possible, thus supporting Osman and Basch's main conclusions. At this point, we turned to the evolution of this system toward its final products, with particular attention devoted to the possible steps in which the two protons

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Table II. Mulliken Populations of the Complexes

complex	Cu	O <sub>2</sub>	NH <sub>4</sub> <sup>+</sup> (Arg-141)	NH <sub>3</sub>
Cu <sup>+</sup> -O <sub>2</sub>	28.06	15.94		
Cu <sup>+</sup> -O <sub>2</sub> ...HNNH <sub>3</sub> <sup>+</sup>	28.03	15.96	10.01	
H <sub>3</sub> N-Cu <sup>+</sup> -O <sub>2</sub>	28.22	15.95		9.83
H <sub>3</sub> N-Cu <sup>2+</sup> -O <sub>2</sub> <sup>-</sup> ...HNNH <sub>3</sub> <sup>+</sup>	27.66	16.63	10.01	9.70
H <sub>3</sub> N-Cu <sup>+</sup> -O <sub>2</sub> <sup>-</sup> ...HNNH <sub>3</sub> <sup>+</sup>	28.36	16.75	10.03	9.86
H <sub>3</sub> N-Cu <sup>+</sup> -O <sub>2</sub> H...NH <sub>3</sub>	28.23	16.99 <sup>a</sup>	9.96 <sup>b</sup>	9.82
H <sub>3</sub> N-Cu <sup>2+</sup> -O <sub>2</sub> H <sub>2</sub> ...NH <sub>3</sub>	27.54	17.87 <sup>c</sup>	9.95 <sup>b</sup>	9.64
H <sub>3</sub> N-Cu <sup>2+</sup> -O <sub>2</sub> H <sub>2</sub> ...NH <sub>4</sub> <sup>+</sup>	27.53	17.83 <sup>c</sup>	10.02	9.62
H <sub>3</sub> N-Cu <sup>2+</sup>	27.41			9.59
H <sub>3</sub> N-Cu <sup>+</sup>	28.19			9.81

<sup>a</sup> Relative to O<sub>2</sub>H. <sup>b</sup> Relative to NH<sub>3</sub>. <sup>c</sup> Relative to O<sub>2</sub>H<sub>2</sub>.

The energies for all the systems investigated have been compared with those of the isolated H<sub>3</sub>N-Cu<sup>n+</sup> + NH<sub>4</sub><sup>+</sup> (NH<sub>3</sub>) + substrate moieties, with neglect of the residual interaction between the metal ion and the arginine-like group. This choice is based on the consideration that the inclusion of such an interaction would be a gross overestimate of the real effect, since in the absence of substrates one or more water molecules are likely to be present between the two groups, with a sizable shielding effect.

## Results

The energies for the systems shown in Chart I have been calculated (Table I and Figure 1) with the following fixed geometrical relationships between the groups: Cu-N = 2.07 Å; ∠N-Cu-O = 90°; Cu-O = 1.9 Å; ∠Cu-O-O = 136°; O-O = 1.3 Å (1.341 Å in free superoxide and 1.207 Å in triplet dioxygen); O...HNNH<sub>3</sub><sup>+</sup> = 2.5 Å; ∠O-O...H = 180°; ∠O...H-N = 180°. Table II shows that the reaction of superoxide ion with copper(II) leads to an adduct that is always described as copper(I)-dioxygen, unless an ammonia ligand is coordinated to the metal and an ammonium ion is hydrogen bonded to the coordinated superoxide. In the latter case the Mulliken population analysis clearly indicates that reduction of the metal ion does not take place. Since this is the situation that most closely mimics the enzyme active site, we have chosen it as the starting point for the catalytic cycle. It should be pointed out that, although the latter adduct is still energetically unfavored with respect to H<sub>3</sub>N-Cu<sup>+</sup> + O<sub>2</sub> + NH<sub>4</sub><sup>+</sup>, there is no pathway for such products to be reached, as long as the hydrogen bond between superoxide and NH<sub>4</sub><sup>+</sup> is present. At this point the breaking of the hydrogen bond would allow the system to evolve toward H<sub>3</sub>N-Cu<sup>+</sup>-O<sub>2</sub> + NH<sub>4</sub><sup>+</sup> and then to collapse to H<sub>3</sub>N-Cu<sup>+</sup> + O<sub>2</sub> + NH<sub>4</sub><sup>+</sup>. The addition of further nitrogen ligands, as extrapolated from the first two schemes of Figure 1, would increase the energy gap between coordinated and dissociated dioxygen; the possibility that this pathway is followed by the system cannot be ruled out. The present calculations confirm the important finding of the existence of the intermediate Cu<sup>2+</sup>-O<sub>2</sub><sup>-</sup>. As already proposed by Osman and Basch, such an intermediate can evolve through an alternative pathway; i.e., it can be reduced by a second superoxide ion, giving rise to a species described as Cu<sup>+</sup>-O<sub>2</sub><sup>-</sup>, as indicated by the Mulliken population (Table II). The energy of such a one-electron reduction product is by far lower than that of the starting system and is stable with respect to dissociation. Note that the formation of this species is predicted both by Osman and Basch and by the two-step mechanisms.

At this point we took into consideration the effect of allowing the hydrogen-bonded ammonium proton to approach the distal superoxide oxygen; the final O-H distance and the ∠O-O-H angle were taken equal to the experimental values in hydrogen peroxide (0.97 Å and 100°), without changing the O-N distance (3.5 Å). Since the energy change in transferring the proton from N to O is expected to be relatively small, it is possibly dominated by changes in the Cu-O distance and therefore the latter has been optimized in both cases. Figure 2 shows the total energy of these systems as a function of the Cu-O distance: it appears that the hydroperoxyl radical is slightly energetically favored. Interestingly, the optimized Cu-O distance (2.5 Å) turns out to be much larger than in coordinated superoxide (2.0 Å). On the other hand, the Mulliken population on copper is almost unaltered, indicating that

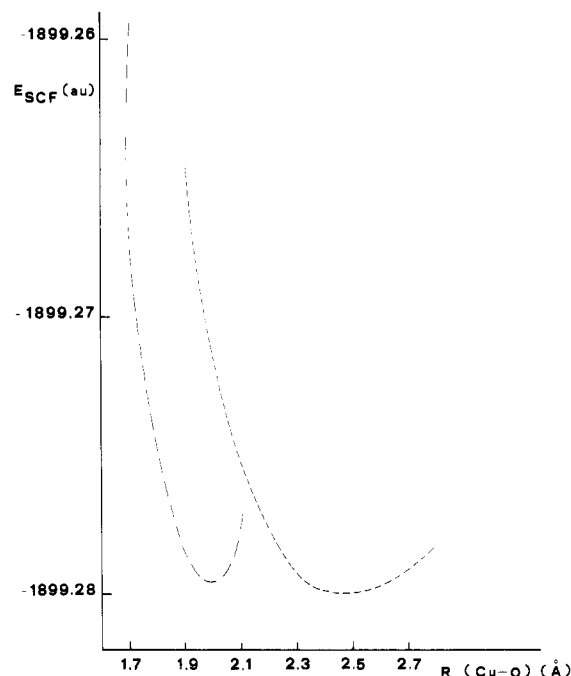


Figure 2. Copper-oxygen distance optimization in [H<sub>3</sub>N-CuO<sub>2</sub>...HNNH<sub>3</sub>]<sup>+</sup> (---) and in [H<sub>3</sub>N-CuO<sub>2</sub>H...NH<sub>3</sub>]<sup>+</sup> (-.-).

the proton transfer to superoxide is not enough to induce the formation of a hydroperoxide anion. It should be warned, however, that neglect of electron correlation energies tends to overstabilize Cu<sup>+</sup> by a few hundred kJ mol<sup>-1</sup><sup>15</sup> and slightly destabilize the hydroperoxide anion.<sup>16</sup>

Examination of the excited states of the H<sub>3</sub>N-Cu<sup>+</sup>-O<sub>2</sub>H...NH<sub>3</sub> system at the Hartree-Fock level shows that the first excited state that can be described as H<sub>3</sub>N-Cu<sup>2+</sup>-O<sub>2</sub>H...NH<sub>3</sub> lies at about 100 kJ mol<sup>-1</sup>. Therefore, there is the possibility that electron transfer from Cu<sup>+</sup> to HO<sub>2</sub> can occur already at this stage. Electron transfer occurs anyway if a proton is allowed to bind to the proximal oxygen of the hydroperoxyl radical (O-H = 0.97 Å, ∠Cu-O-H and ∠O-O-H = 90°): the Mulliken population on copper is now indicative of reoxidation to copper(II) (Table II). Therefore, the adduct can be described as hydrogen peroxide coordinated to copper(II). Of course the energy is also decreased with respect to that of the unprotonated species (Table I), although at the expense of a proton donor from the solvent. This derivative is stable with respect to the isolated H<sub>3</sub>N-Cu<sup>2+</sup> + H<sub>2</sub>O<sub>2</sub> + NH<sub>3</sub> species. If a proton is added to the NH<sub>3</sub> group in such a way as to form a hydrogen bond with the distal oxygen of the coordinated hydrogen peroxide, the system is unstable with respect to the dissociated species. Figure 3 summarizes the above results, showing that the entire sequence of events, although not unique, is consistent with a full catalytic cycle.

## Discussion

Our approach to the description of the copper-superoxide system differs from the Osman and Basch one in that a more limited number of copper ligands has been chosen, in order to perform the molecular orbital calculations without approximations, i.e. ab initio, and to analyze the effects of changes in the coordination number of the copper ion on the energetics of the copper-superoxide interaction. From the present results it can be safely established that the presence of a single ammonia ligand on copper is sufficient to prevent reduction to copper(I), provided that the superoxide ion can hydrogen bond to a nearby ammonium ion. A relatively large hydrogen-bonding distance (2.5 Å) is enough to stabilize a coordinated superoxide. From the observed trend in the energies as one passes from no coordinated ammonia

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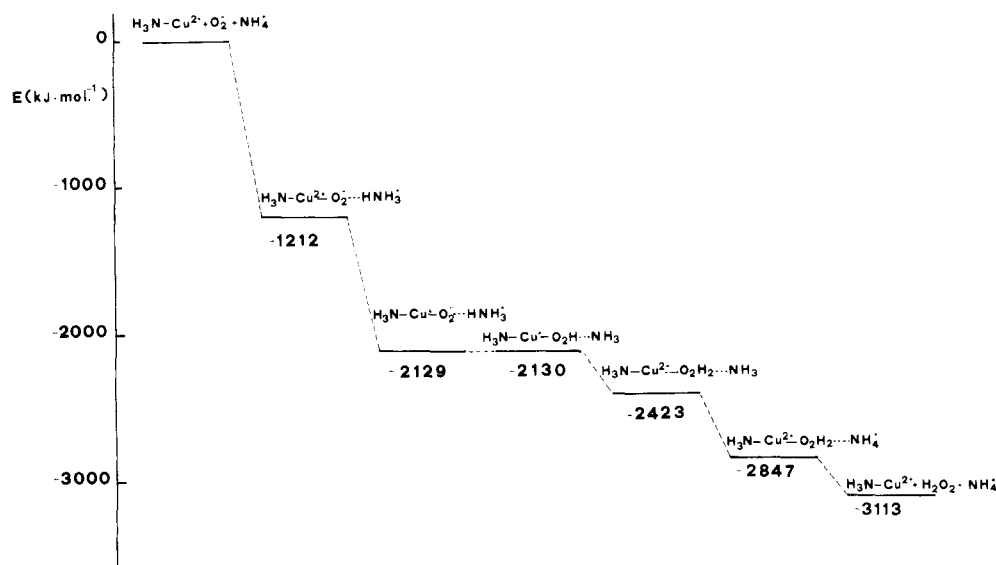


Figure 3. Possible catalytic pathway for the production of  $O_2$  and  $H_2O_2$  from 2 mol of superoxide ions and protons.

to one, it is clear that when the donor set in the equatorial plane is increased reduction of copper(II) becomes more and more difficult. This result is in complete agreement with Osman and Basch's data and gives more confidence in the idea that in superoxide dismutase the superoxide ion forms a stable adduct with the metal ion and that Arg-141 indeed plays a key role in determining the catalytic pathway.

Once it was established that our limited set of atoms is as good as a more extended set to try to understand the catalytic mechanism of  $O_2^-$  dismutation by  $Cu^{2+}$  complexes, we turned to the analysis of a possible detailed pathway for the entire catalytic cycle of SOD. In particular, we propose that proton transfer from the Arg-141 residue to the distal oxygen causes the copper-oxygen bond to lengthen considerably, making more likely the binding of a proton to the proximal oxygen; such a proton could be provided by the bridging histidine residue, which has been detached from copper upon reduction.<sup>3a,17</sup> Binding of this proton causes reoxidation of copper(I) in the copper(I)-superoxide adduct. This avoids the difficulties encountered by Osman and Basch in accounting for the electron-transfer step necessary to produce the hydroperoxide anion. The latter can only be released by addition of a second proton to the system. It is likely that the final product is neutral hydrogen peroxide and not a hydroperoxide anion; indeed, also Osman and Basch's calculations indicate that the hydroperoxide adduct is stable with respect to dissociation. Finally, it is proposed that reprotonation of Arg-141 occurs before and not after the release of hydrogen peroxide.

Our calculations show that the species depicted in Figure 3 represent a smooth sequence of possible intermediates that com-

pletely describes a catalytic pathway consistent with the existing kinetic data on the enzyme.<sup>1</sup>

### Conclusions

The importance of the presence of Arg-141 in the active site of superoxide dismutase has become increasingly evident from the dramatic decrease in catalytic activity observed when such a residue is chemically modified<sup>18,19</sup> and from its influence on anion binding<sup>20-22</sup> to the active site. Ab initio calculations provide increasing evidence that hydrogen bonding between Arg-141 and a coordinated superoxide anion prevents electron transfer to copper(II), thus drastically altering the commonly accepted picture for the superoxide dismutation mechanism. The purposely weak hydrogen-bonding interaction introduced in our model, much weaker than that used by Osman and Basch, strongly supports the idea that an intermediate  $Cu^{2+}-O_2^-$  adduct is formed in superoxide dismutase and that two different catalytic pathways are possible to reach the  $H_3N-Cu^+-O_2^- \cdots HNNH_3^+$  intermediate. The detailed scheme shown in Figure 3 suggests that reaction 2 should then be considered to be a realistic alternative description of the process.

**Registry No.** SOD, 9054-89-1; L-Arg, 74-79-3; Cu, 7440-50-8;  $O_2^-$ , 11062-77-4;  $NH_4^+$ , 14798-03-9;  $NH_3$ , 7664-41-7;  $H_2O_2$ , 7722-84-1;  $O_2$ , 7782-44-7.

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