Lyophilization Results in Cleavage of the Active-Site Histidine Bridge in the Four-Copper Form of Bovine Erythrocyte Superoxide Dismutase

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Bovine erythrocyte superoxide dismutase is typical of the copper- and zinc-containing superoxide dismutases found in the cytosol of eucaryotic cells.[1,2] A unique feature of the enzyme is the presence of a histidine imidazolate group bridging the copper and zinc ions in each active site of two identical subunits.[3] A catalytic mechanism for SOD has been proposed in which breaking and reforming of the copper–imidazolate bond plays an important role.[4] It is therefore of interest to determine the factors that govern the stability of the imidazolate bridge.

It has been shown by ESR spectroscopy that thiocyanate ion displaces the bridging imidazolate group from the copper ion in the copper sites of Cu_2Cu_2SOD.[5] ESR spectroscopy is a sensitive technique for determining whether the imidazolate bridge is intact in Cu_2Cu_2SOD because breaking of the bridge eliminates the antiferromagnetic exchange interaction between the copper ions and substantially modifies the ESR spectrum.[5,6] Using ESR spectroscopy, we have found that lyophilization results in cleavage of the imidazolate bridge between the copper ions in Cu_2Cu_2SOD and, most likely, also in Cu_2Zn_2SOD. Redissolving the lyophilized solid results in reformation of the bridge. Addition of sucrose to the protein solution before lyophilization prevents disruption of the bridge. The effect of lyophilization on the imidazolate bridge probably arises from a conformational change in the protein as a result of the loss of water of hydration.

ESR spectra of frozen solutions or lyophilized solids were obtained at 140 K by using a Varian E-line X-band spectrometer. Cu_2Zn_2SOD was isolated from bovine erythrocytes.[7,8] Cu_2Cu_2SOD was prepared as previously described.[6,9] Metal ion concentrations were determined by using a Varian AA-375 atomic absorption spectrometer. The protein concentration of Cu_2Zn_2SOD was determined from its absorbance at 258 nm,[7] while that of Cu_2Cu_2SOD was determined by using a microbiuret method.[10]

Figure 1 shows ESR spectra of Cu_2Zn_2SOD and Cu_2Cu_2SOD in solution and as lyophilized solids. ESR parameters[11] for SOD under various conditions are given in Table I. The solution spectrum of Cu_2Zn_2SOD (Figure 1b) exhibits features characteristic of considerable rhombic distortion, which are absent in the spectrum of the lyophilized enzyme (Figure 1a). The ESR parameters of the solid are similar to those of the thiocyanate complex of Cu_2Zn_2SOD in solution, where the imidazolate bridge is believed to be displaced by thiocyanate ion.[5,12] From these spectra it is not possible to determine with certainty whether the bridge has been broken upon lyophilization or whether a conformational change has occurred resulting in a more nearly square-planar geometry around the copper.

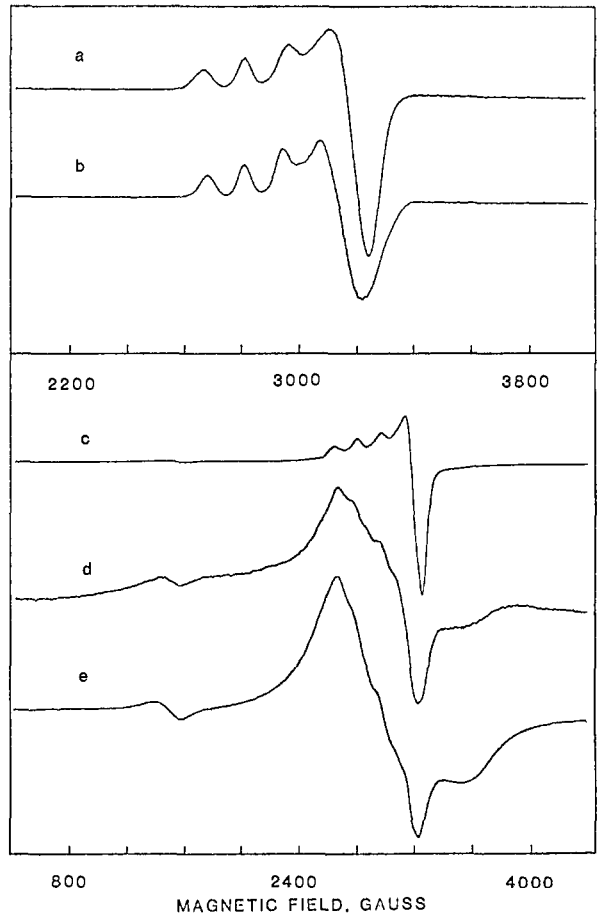

Figure 1. ESR spectra of (a) Cu_2Zn_2SOD as a lyophilized solid; (b) Cu_2Zn_2SOD in H_2O; (c) Cu_2Cu_2SOD as a lyophilized solid; (d) lyophilized Cu_2Cu_2SOD redissolved in H_2O; (e) freshly prepared Cu_2Cu_2SOD in H_2O. All spectra were recorded at 140 K with 100-kHz field modulation and at a modulation amplitude of 10 G. The microwave power was 50 mW and the gain was 200 for (a) and (b), 63 for (c), 800 for (d), and 320 for (e).

Table I. ESR Spectral Parameters of SOD under Various Conditions

sample	state	$g_\perp$ (or g_m)	$g_\parallel$	$A_\parallel$, cm^{-1}
(a) Cu_2Zn_2SOD	lyophilized from H_2O	2.062	2.256	0.0150
(b) Cu_2Zn_2SOD	aqueous solution of a	2.080	2.262	0.0134
(c) Cu_2Cu_2SOD	lyophilized from H_2O	2.067	2.272	0.0167
(d) Cu_2Zn_2SOD	in 10% aqueous sucrose	2.080	2.261	0.0133
(e) Cu_2Zn_2SOD	sample d lyophilized	2.069	2.256	0.0140
(f) Cu_2Zn_2SOD	sample e dissolved in H_2O	2.081	2.261	0.0132
(g) Cu_2Zn_2SOD	in 1% aqueous sucrose	2.082	2.262	0.0131
(h) Cu_2Zn_2SOD	sample g lyophilized	2.064	2.257	0.0142

The ESR spectrum of Cu_2Cu_2SOD in frozen solution is shown in Figure 1e. It is characteristic of antiferromagnetically coupled binuclear copper(II) complexes[13] and consists of broad absorption in the region 2000–4000 G and a weaker signal at 1200–1700 G. The latter signal arises from the $\Delta M_S = \pm 2$ transition. Lyophilization of Cu_2Cu_2SOD causes a drastic change in the ESR

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(1) Abbreviations: SOD, bovine erythrocyte superoxide dismutase; Cu_2Zn_2SOD, native superoxide dismutase having copper and zinc in their normal binding sites; Cu_2Cu_2SOD, SOD having copper in both the copper and zinc sites.

(2) (a) Fridovich, I. *Annu. Rev. Biochem.* **1975**, *44*, 147. (b) Valentine, J. S.; Pantoliano, M. W. In "Copper Proteins"; Spiro, T. G., Ed., in press.
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(9) Valentine, J. S.; Pantoliano, M. W.; McDonnell, P. J.; Burger, A. R.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4245. The Cu content of Cu_2Cu_2SOD was 1.98 copper ions per subunit.
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(11) ESR parameters were calculated as previously described (Malmström, B. G.; Vänngård, T. *J. Mol. Biol.* **1960**, *2*, 118). Mn(II), naturally present as an impurity in strontium oxide, was used to calibrate the magnetic field (Bolton, J. R.; Borg, D. C.; Swartz, H. M. In "Biological Applications of Electron Spin Resonance"; Swartz, H. M., Bolton, J. R., Borg, D. C., Eds.; Wiley-Interscience: New York, 1972; p 63).
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spectrum (Figure 1c). The characteristic features of the antiferromagnetically coupled pairs of copper ions are almost entirely absent. Instead, a spectrum typical of magnetically isolated copper ions in sites having axial symmetry is observed. This result demonstrates conclusively that the bridge has been broken. Redissolving the solid in water largely restores the spectrum of the bridged structure (Figure 1d).

The spectrum in Figure 1c consists of only a single signal, indicating that both copper ions in the active sites of lyophilized Cu_2Cu_2SOD must be in quite similar environments. This result is surprising since Cu_2Zn_2SOD and Ag_2Cu_2SOD, where the copper is in the native copper and zinc sites, respectively, have very different ESR spectra. Ag_2Cu_2SOD in particular has an ESR spectrum typical of copper in a tetrahedral site,[14] consistent with the known geometry of the zinc site in Cu_2Zn_2SOD.[3] Breaking of the imidazolate bridge in Cu_2Cu_2SOD appears to be accompanied by alteration of the geometry around the copper atom in the zinc site to form a more favorable square-planar structure. Since this phenomenon occurs not only on lyophilization but also in solution in the presence of thiocyanate ion,[15] the two effects are probably related.

Further evidence of this relationship is found in the ESR spectrum of zinc-free SOD,[15] which lacks the rhombic distortion evident in the spectrum of Cu_2Zn_2SOD. Maintenance of the distorted geometry of the copper site in native SOD depends upon the presence of zinc in the zinc site which, by coordinating to one of the histidine imidazoles already bound to copper, produces the bridging imidazolate structure. The geometry of the copper coordination sphere is also influenced by the tertiary structure of the protein, which is sensitive to the extent of hydration. This sensitivity is revealed by the present work where lyophilization not only breaks the imidazolate bridge in the case of Cu_2Cu_2SOD but also results in the appearance of a more axial spectrum for Cu_2Zn_2SOD, even though zinc is present. In turn, binding of zinc in the zinc site affects the tertiary structure of the protein, at least around the active site, as shown by changes in the NMR spectrum of the exchangable histidine N–H protons when zinc is added to the apoprotein.[16] The geometry of the copper site is thus determined by two factors which are themselves interdependent: the tertiary structure of the protein and the presence of zinc in the zinc site.

Addition of sucrose to Cu_2Cu_2SOD solutions prior to lyophilization stabilizes the imidazolate bridge in the solid. As little as 1% (w/v) sucrose, or approximately 30 sucrose molecules per molecule of protein, is effective in maintaining the antiferromagnetic interaction between the copper ions. The presence of sucrose has no effect on the ESR spectrum of Cu_2Cu_2SOD in frozen solution. Cu_2Zn_2SOD lyophilized from either 1% or 10% (w/v) sucrose solution has ESR parameters (Table I) different from the values for both the frozen solutions and the solid lyophilized in the absence of sucrose. In particular, the $A_\parallel$ value of the solid in the presence of sucrose is midway between the values for the solution and the sucrose-free solid. These results indicate that, although the presence of sucrose can provide substantial stabilization of the native structure of the protein upon lyophilization, the solid does not have a structure around the copper ion which is identical with the solution structure. Saccharides have been observed previously to stabilize metalloproteins during lyophilization,[17] although the manner by which they do so has not been elucidated.

Changes in the ESR spectrum of catalase upon lyophilization have been noted,[18] and Raman spectra of deoxyhemoglobin indicate a change in the vibrational characteristics of the iron–histidine bond when the solution is frozen.[19] These results, along with those presented here, indicate that significant chemical changes can occur at the metal centers of metalloproteins because of an alteration of the normal protein–water interaction.

Acknowledgment. This work was supported by research Grant GM-16449 from the National Institute of General Medical Sciences.

Registry No. SOD, 9054-89-1; Cu, 7440-50-8; Zn, 7440-66-6.

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(15) ESR spectra of Cu_2Cu_2SOD plus thiocyanate ion in frozen aqueous solution show a single axial copper signal with $g_\perp = 2.065$, $g_\parallel = 2.259$, and $A_\parallel = 0.0155$ cm^{-1}.[5] The ESR spectrum of zinc-free SOD in frozen solution has $g_\perp = 2.076$, $g_\parallel = 2.269$, and $A_\parallel = 0.0150$ cm^{-1}.[14]

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Chemistry of Exciplexes. 12. Chemistry of Heterodimers of Benzene and Anthracene[1]

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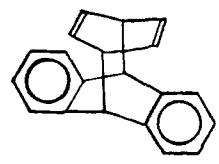
Cyclodimers of benzene with arenes are a group of energy-rich molecules which dissociate exoergically to their components.[3] There are three common types of such dimers: the $[4_{\pi_s} + 4_{\pi_s}]$, the $[4_{\pi_s} + 2_{\pi_s}]$, and the $[2_{\pi_s} + 2_{\pi_s}]$ dimers. This communication deals with the first synthesis of both meso heterodimers of anthracene with benzene in highly purified form (>99.8%), the $[4_{\pi_s} + 4_{\pi_s}]$ dimer **1** and the $[4_{\pi_s} + 2_{\pi_s}]$ dimer **2**, and their cycloreversions



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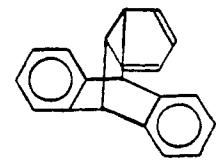

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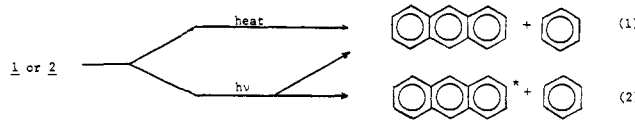
to anthracene and benzene. The cycloreversion of **1** is a chemical process involving $4n$ reacting electrons; that of **2** involves $4n + 2$ reacting electrons. The availability of both compounds has enabled us to study the role of conservation of orbital symmetry in cycloreversions in both the ground and the excited states.



Both **1** and **2** were synthesized by an application of photocycloaddition of substituted 1,3-cyclohexadienes (**3** and **4**, Scheme I) to anthracene[4] followed by conversion of substituents to an olefinic bond. The orientation of photocycloaddition was controlled by the choice of substrate and experimental conditions. The $[4_{\pi_s} + 4_{\pi_s}]$ adduct **5** was formed as the major adduct in the photocycloaddition of **3** to anthracene in acetonitrile, while the $[4_{\pi_s} + 2_{\pi_s}]$ adduct **7** was formed as the major adduct in the photocycloaddition of **4** to anthracene in dichloromethane. The for-

(1) Dedicated to Professor George S. Hammond on the occasion of his 60th birthday.

(2) Hedwig Loeb Undergraduate Scholar, University of Chicago, 1981.

(3) For a few known cyclodimers of benzene, see: (a) Schröder, C.; Martin, W.; Röttele, H. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 69–70. (b) Berson, J. A.; Davis, R. F. *J. Am. Chem. Soc.* **1972**, *94*, 3658–3659. (c) Grimme, W.; Köser, H. G. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 307–308; (d) Mak, K. T.; Srinivasachar, K.; Yang, N. C. *J. Chem. Soc., Chem. Commun.* **1979**, 1038–1039.

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