Role of the Bridging Histidyl Imidazolate Ligand in Yeast Copper–Zinc Superoxide Dismutase. Characterization of the His63Ala Mutant

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Received June 1, 1994

Copper–zinc superoxide dismutase (CuZnSOD or SOD) is composed of two identical subunits, each containing a Cu^{II} and a Zn^{II} binding site. One of the unique and interesting features of the SOD active site is the imidazolate ligand from His63 which bridges the catalytic copper and structural zinc ions.[1] Another striking feature of SOD is the pH independence of its spectroscopic properties and SOD activity over a wide range, *i.e.*, pH 5–9.5.[2,3] In order to investigate the role of the bridging imidazolate in this protein, we have prepared and characterized the H63A (His63 to Ala63) mutant of CuZnSOD.

The H63A mutant was prepared using oligonucleotide-directed mutagenesis on the cloned CuZnSOD gene from *Saccharomyces cerevisiae*.[4] The mutant gene was sequenced and expressed in *Escherichia coli* using the T7 RNA polymerase expression system,[5] and the protein was purified to homogeneity.[6] Apoprotein was prepared according to procedures reported previously.[7]

Addition of Cu^{2+} to apo-WTSOD and to apo-H63ASOD was followed by visible absorption spectroscopy (Figure 1). As has been reported previously,[8,9] addition of one Cu^{2+} per subunit in the copper site of the wild-type protein gives Cu_2E_2WTSOD[10] with a d–d band (λ_{max} = 670 nm) characteristic of Cu^{II} in the copper site (Figure 1Bc). Addition of a second Cu^{2+} per subunit produces Cu_2Cu_2WTSOD with a spectrum that corresponds to the superposition of the 670 nm band due to Cu^{II} in the distorted square pyramidal copper site and a 820 nm d–d band due to Cu^{II} in the distorted tetrahedral zinc site (Figure 1Bd).[8,9] Addition of Zn^{2+} prior to the second equivalent of Cu^{2+} blocks copper binding due to the higher affinity of the zinc site for Zn^{II} than for Cu^{II}.

Addition of one Cu^{2+} per subunit to apo-H63ASOD gave a d–d transition with λ_{max} = 670 nm (Figure 1Cc), remarkably similar to Cu^{II} in the copper site of Cu_2Zn_2WTSOD (Figure 1A)

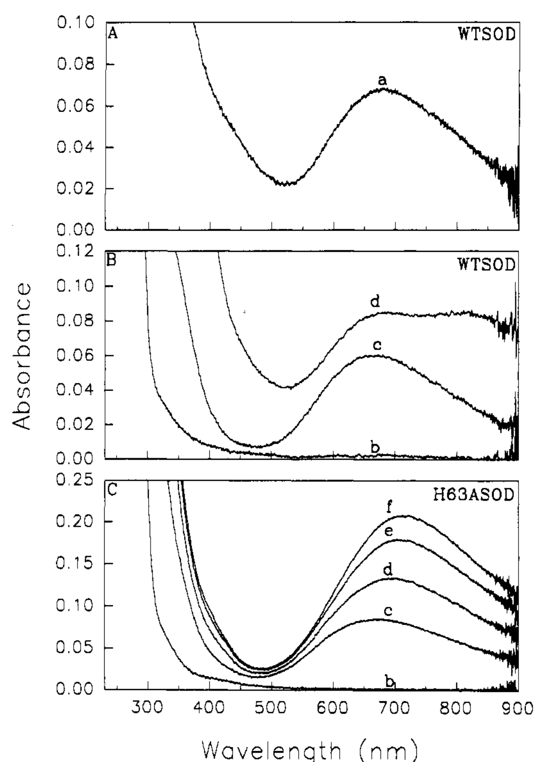

Figure 1. Electronic absorption spectra of (A) 0.3 mM native wild-type (WT) yeast CuZnSOD and Cu^{2+} addition to (B) 0.23 mM apo-WTSOD and (C) 0.39 mM apo-H63ASOD at room temperature in 100 mM sodium acetate buffer, pH 5.5, referenced against the same buffer. (a) Native yeast CuZnSOD; (b) apoprotein; (c) apoprotein plus 0.9 equiv of Cu^{2+} per subunit; (d) apoprotein plus 1.9 equiv of Cu^{2+} per subunit; (e) apoprotein plus 3.0 equiv of Cu^{2+} per subunit; (f) apoprotein plus 3.8 equiv of Cu^{2+} per subunit.

and Cu_2E_2WTSOD (Figure 1Bc). The addition of a second Cu^{2+} per subunit gave a spectrum corresponding to the superposition of the 670 nm transition and an additional d–d band with λ_{max} = 720 nm (Figure 1Cd). Binding of Cu^{2+} to the first site was stoichiometric, indicating that the binding constant is high. Binding of Cu^{2+} to the second site, presumably the zinc site, required an excess of Cu^{2+}, signifying that the binding was substantially weaker. Addition of Zn^{2+} prior to the addition of the second equivalent of Cu^{2+} did not block copper binding, indicating that the zinc site of the mutant, unlike that of the wild-type protein, does not prefer Zn^{II} over Cu^{II}. The large reduction of the metal-binding affinity of the zinc site and the higher energy of the d–d copper transition of Cu^{II} in the zinc site of the H63A mutant suggests that the distorted tetrahedral geometry of the zinc site of the wild-type protein is not maintained in the absence of His63.

In striking contrast to those of Cu_2Zn_2WTSOD, whose visible–UV and ESR spectral properties are invariant with pH over the range 5 < pH < 10.5,[3] the spectroscopic properties of Cu^{II} bound to H63ASOD are pH dependent (Figures 2 and 3). Cu_2E_2-H63ASOD[11] shows reversible pH-dependent behavior, drastically different from that of Cu_2Zn_2WTSOD. The pK_a of the pH-dependent transition was 9.3, as determined from changes in the visible absorption spectrum at 650 and 515 nm (Figure 2). At pH 5.6, the ESR spectrum was characteristic of Cu^{II} in an axially symmetric ligand environment, with $g_\parallel$ = 2.259, $g_\perp$ = 2.044, and $A_\parallel$ = 180 G (Figure 3B). Above pH 9.3, however, the spectrum was dramatically altered to one characteristic of Cu^{II} in the coordination environment of four tightly bonded ligands in a square planar geometry,[12–14] with $g_\parallel$ = 2.167, $g_\perp$ = 2.053, $A_\parallel$ = 225 G,

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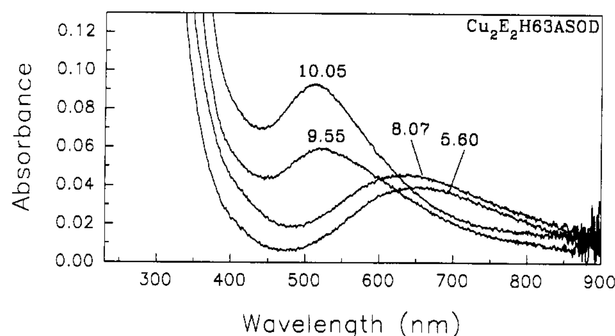

Figure 2. pH dependence of the electronic absorption spectra of 0.3 mM Cu_2E_2H63ASOD.[10] Cu_2E_2H63ASOD contains 0.9 equiv of Cu^{2+} per subunit at room temperature in 10 mM MES, 10 mM MOPS, 10 mM HEPES, and 10 mM sodium acetate buffer, initially at pH 5.6.

and the characteristic "fold-over" peak.[12] These changes were reversed when the pH was lowered to 5.6. By contrast, substantial changes in the spectral properties of Cu_2Zn_2WTSOD only occur at much higher pH (pH > 12) and are irreversible.[3] We conclude from these results that His63 plays a crucial role in maintaining the pH independence of the copper site.

Both bovine Cu_2E_2WTSOD and yeast Cu_2E_2WTSOD show a reversible pH-dependent metal migration of the Cu^{II} to the vacant zinc site of another subunit.[15] Nevertheless, we were able to monitor the ESR spectral properties of the copper remaining in the copper site as a function of pH under the conditions shown in Figure 3C. Interestingly, the pH dependence of Cu_2E_2-H63ASOD was quite similar to that found for yeast Cu_2E_2-WTSOD (Figure 3C). Thus, both the zinc and His63 are required to organize the tetrahedrally distorted zinc site and to prevent changes in the copper site geometry at high pH.

The pH-dependent changes in the ESR spectra of Cu_2E_2-H63ASOD and Cu_2E_2WTSOD are analogous to those observed for copper-substituted carbonic anhydrase and met-apo-hemocyanin.[12,16] The pK_a of the pH-dependent transition is similar to the pK_a for ionizing water bound to Cu^{II} complexes.[17] Thus, the Cu^{2+} ion in both Cu_2E_2H63ASOD and Cu_2E_2WTSOD is behaving in a fashion normal for small Cu^{II} complexes or copper-containing proteins. An abnormally high pK_a, such as that found for CuZnSOD, is strong evidence that the water ligand remains in an axial position on the Cu^{II} center even at relatively high pH since the interaction between the copper center and its axial ligands is much weaker than that with its equatorial ligands.[18] The changes observed at high pH strongly resemble those occurring

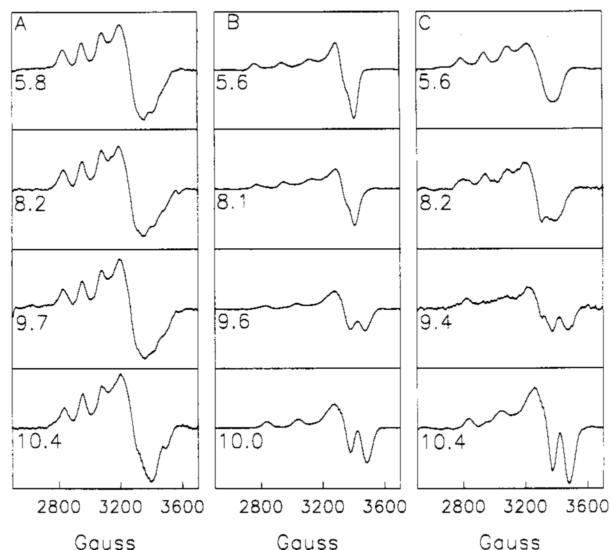

Figure 3. pH dependence of the 90 K X-band electron spin resonance (ESR) spectra of (A) 0.3 mM native yeast CuZnSOD, (B) 0.3 mM Cu_2E_2H63ASOD with 0.9 equiv of Cu^{2+} per subunit, and (C) 0.3 mM yeast Cu_2E_2WTSOD with 0.9 equiv of Cu^{2+} per subunit. The pH is shown in each panel. All protein samples were in 10 mM MES, 10 mM MOPS, 10 mM HEPES, and 10 mM sodium acetate buffer, initially at pH 5.6.

when cyanide binds to CuZnSOD at pH 8.8,[3] suggesting that the binding of OH^- and CN^- occur in a similar fashion. It is believed that CN^- binding to the Cu^{II} in CuZnSOD causes the axes of the copper to reorient, which places the CN^- in an equatorial position on the copper, creating a square planar geometry.[3,19]

The unusual bridging imidazolate His63 ligand has been postulated to play both structural and mechanistic roles in CuZnSOD.[2,19] Here we show that substitution of the alanine in place of the bridging histidine ligand in SOD dramatically perturbs the metal-binding properties of the protein. In the absence of the bridging imidazolate from His63, the zinc site geometry of SOD and the pH independence of the copper site are substantially altered. In addition, we find the SOD activity of H63ASOD to be pH dependent, giving an activity at physiological pH that is 250-fold less than that of the wild-type.[20] Future studies will address the mechanistic implications of these results in superoxide dismutase catalysis.

Acknowledgment. This work is supported by grants from the National Institutes of Health (GM2822 to J.S.V.) and the American Cancer Society (PF-3680 to L.M.E.).

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