Metal-Bound Histidine Modes in UV Resonance Raman Spectra of Cu, Zn Superoxide Dismutase

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Abstract: Ultraviolet resonance Raman [UVRR] spectra of Cu, Zn superoxide dismutase [SOD] contain bands arising from vibrations of metal-bound histidine ligands. Spectra in H_2O solution reveal several modes of the His61 side chain, which bridges the Cu^{2+} and Zn^{2+} ions as imidazolate. The disappearance of these bands signals disruption of the bridge when the pH is lowered to 3.0, or the Cu^{2+} is reduced to Cu^{+}. Binding of hydroxide [pH 12] or cyanide to the Cu^{2+} perturbs the imidazolate modes, reflecting geometry changes induced by these strong-field ligands. In D_2O solution several additional bands become enhanced which arise from histidine ligands that have undergone NH/D exchange. Some of these are attributed to Cu-bound ligands and others to Zn-bound ligands, on the basis of selective changes accompanying removal and replacement of the metals. Excitation profiles are similar for these bands, and for the bridging imidazolate bands; they are red-shifted relative to nonligating histidine. The detection of site-specific histidine ligand modes gives promise for wide applicability of UVRR spectroscopy in studying histidine ligation in metalloproteins. The single tyrosine residue of SOD, which is a target of active-site-catalyzed nitration by peroxynitrite, is found to have an elevated pK_a, 11.4, despite being exposed to solvent.

Introduction

The advent of reliable deep-ultraviolet lasers has made possible the systematic application of ultraviolet resonance Raman [UVRR] spectroscopy to proteins. Selective enhancement can be obtained for vibrational modes of aromatic residue side chains, and of the main chain amide bonds.[1,2] The UVRR spectra report on the local environment of the chromophores. It was early recognized that histidine would be an attractive target for UVRR studies, because of its importance as a catalytic residue in enzyme active sites, and as a ligand in metalloproteins. Unfortunately, the extent of resonance enhancement is low for the imidazole chromophore, and its Raman bands are usually undetectable in UVRR spectra of proteins.[1−3]

However, protonated histidine in D_2O does give a prominent UVRR band, at 1408 cm^{-1}, which is detectable in protein UVRR spectra, and can be used to measure the average protonation state of the histidine residues.[4] The combination of protonation and NH/D exchange elevates the Raman enhancement substantially, apparently because of mode composition changes together with a favorable match of the 1408 cm^{-1} mode eignevector with the excited-state distortion.[5] We have found that UVRR bands of metal-bound histidine also become detectable in protein spectra following NH/D exchange.[6,7]

The present work explores the nature of these metal-bound histidine UVRR bands in Cu, Zn superoxide dismutase [SOD]. This enzyme occurs in both eukaryotes and prokaryotes, protecting cells against oxidative damage by superoxide reaction products.[8] Interest in Cu, Zn SOD has been heightened by the finding that amyotrophic lateral sclerosis [ALS-Lou Gehrig's disease] can be linked to mutations in the Cu, Zn SOD gene.[9] Several of these mutations have been examined[10] in the light of the available crystal structures.[11] However, the lesion related to the disease is uncertain.[12]

The active site contains Cu^{2+} and Zn^{2+} in a histidine-rich environment [Figure 1].[12a,b] The Cu^{2+} is bound to four histidine ligands, in a tetragonal array, with an axial water molecule as a distant fifth ligand. The Zn^{2+} is bound tetrahedrally to three histidine and one aspartate ligand. Another aspartate plays an important secondary role by accepting H-bonds from the NH groups of both Cu^{2+}- and Zn^{2+}-bound histidines. One histidine ligand is common to both Cu^{2+} and Zn^{2+}, bridging the two metals via an imidazolate anion.

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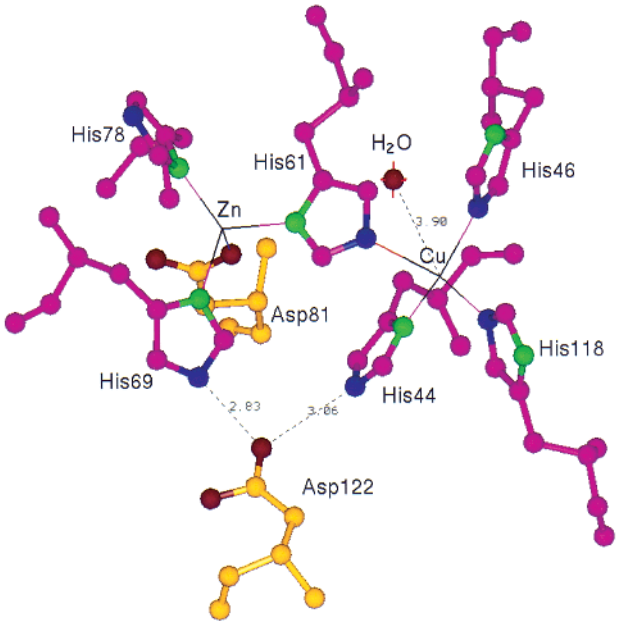

Figure 1. Active site structure of superoxide dismutase. N_δ and N_ϵ of His residues are shown in green and blue, respectively. Also shown are the (weak) bond between H_2O and Cu and the H-bonds between His44, His69, and Asp122 (length in Å) (PDB: 2SOD).[11]

The imidazolate bridge plays an important role in tuning the electronic properties of the copper ion, which is directly involved in catalysis. The mechanism involves two successive redox steps:[13]

$$O_2^- + [SOD]Cu^{2+} + H^+ = O_2 + [SOD][H^+]Cu^+ \quad (1)$$

$$O_2^- + [SOD][H^+]Cu^+ + H^+ = H_2O_2 + [SOD]Cu^{2+} \quad (2)$$

Thus the Cu^{2+}/Cu^+ potential must be poised between the O_2/O_2^- and O_2/H_2O_2 potentials, and this is accomplished by the coordination group, especially the bridging imidazolate. Although an X-ray crystal structure suggested that the bridge was retained in the reduced form of the enzyme,[14] a number of spectroscopic studies [including the present one],[15−20] as well as subsequent X-ray absorption (EXAFS) measurements,[21,22] established that the bridge is lost upon reduction. The Cu^+, which is three-coordinated,[21,22] is no longer bound to the N_ϵ atom of the bridging imidazolate, which bears a proton instead. This is the proton taken up by the enzyme in reaction 1, and delivered to the superoxide anion as it becomes reduced in reaction 2. Thus the bridging imidazolate modulates the ligand field of the $Cu^{2+/+}$ ion and facilitates proton transfer to the nascent peroxide product.

Because of its rich array of metal−histidine bonds, SOD is a useful system for evaluating the applicability of UVRR spectroscopy to issues of histidine ligation. Harada and coworkers reported a UVRR band arising from the bridging imidazolate,[23] and we have found several more imidazolate bands, as well as bands from other histidine ligands, once they undergo H/D exchange. Preliminary results of this work have been published elsewhere.[6] Hashimoto et al. have recently published an independent and complementary study of SOD UVRR spectra.[24] The present work contains a more comprehensive analysis of the ligating histidine UVRR bands.

Materials and Methods

Bovine SOD (approximately 98%) was purchased from Sigma without further purification. Apo SOD was prepared according to established procedures.[25,26] Briefly, native SOD was dissolved in a 50 mM sodium acetate/10 mM EDTA pH 3.8 buffer and centrifuged three times through centricon YM10 filters. The sample was then dissolved in 0.1 M $NaClO_4$/50 mM sodium phosphate pH 7.3 buffer. Further filtration with buffer (5×) was used to remove excess EDTA. Protein concentrations were determined spectropholometrically via the published absorption coefficients at 258 nm: 10.3 $mM^{-1}\cdot cm^{-1}$ for native SOD, and 2.92 $mM^{-1}\cdot cm^{-1}$ for apo SOD.[26]

Remetalated SOD (Cu2E2, Zn2E2, Cu2Cu2, and Zn2Zn2) were prepared following the standard procedures.[27] Cu2E2 and Zn2E2 were made by infusing $CuSO_4$ or $Zn(NO_3)_2$ into the apo SOD in 10 mM sodium acetate at pH 3.8 with a molar ratio of 2:1 (metal ions per protein dimer). Cu2Cu2 and Zn2Zn2 were prepared similarly but with a molar ratio of 4:1 in 10 mM NaAc at pH 5.5. Absorption spectra were used to verify the products. Cyanide-bound SOD was made by mixing SOD with a 4-fold excess of KCN in pH/pD 9.0, 50 mM Tris-HCl.[28] The reduced form of SOD was prepared by addition of a slight stoichiometric excess of sodium dithionite.

UVRR spectra were recorded with a Spex 1269 single monochromator equipped with an intensified photodiode array detector.[29] Samples were 0.3 mM in 50 mM sodium phosphate buffer. cw (229 nm) laser excitation was employed for most UVRR spectra. For the excitation profile experiments, native SOD was dissolved in pD 9.0 buffer with 0.2 M $NaClO_4$ as the internal intensity standard. The 229, 238, 244, and 257 nm laser lines were provided by an intra-cavity frequency doubled Ar^+ laser, while a newly implemented Q-switched Nd:YLF-pumped quadrupled Ti:sappire laser generated 225, 221, 217, and 213 laser lines.[30]

Raman cross sections were calculated based on peak heights of deconvoluted bands, which are a more reliable gauge of intensity than peak areas in complex spectra. The 934 cm^{-1} band from $NaClO_4$ was used to calculate the cross-section of each band from

$$\sigma = (I_{SOD}/I_{ClO_4^-})*(C_{ClO_4^-}/C_{SOD})*\sigma_{ClO_4^-}$$

The cross section values for $NaClO_4$ were taken from Dudik et al.,[31] using interpolation of the reported linear correlation with wavelength. The values used were (mbarn·molecule^{-1}·sr^{-1}) 0.90, 0.80,0.76, 0.63, 0.58, 0.47, and 0.41 for 213, 217, 221, 225, 229, 238, and 244 nm, respectively. Because the bands in the 1270 to 1380 cm^{-1} region are

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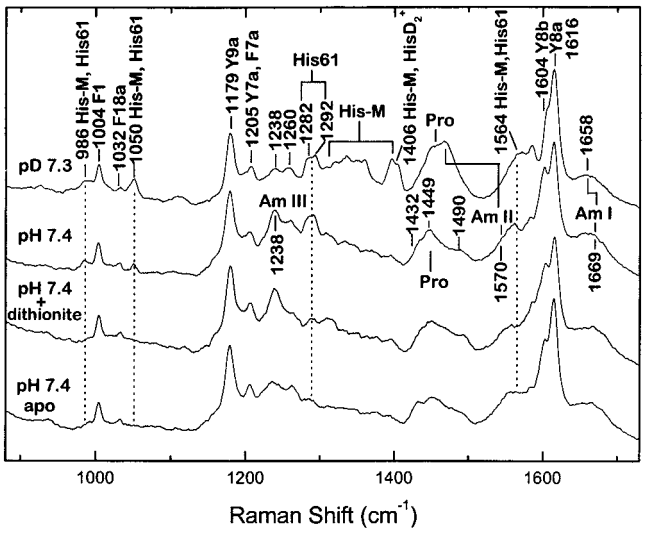

Figure 2. 229 nm-excited UVRR spectra of 0.3 mM SOD at pD 7.3 and pH 7.4 with and without dithionite. The spectrum of apo SOD is shown for comparison. Mode labeling: F = Phe, Y = Tyr, Pro = proline, Am = amide, His-M = metal-bound His, His61 = bridging His, and $HisD^{2+}$ = protonated His in D_2O.

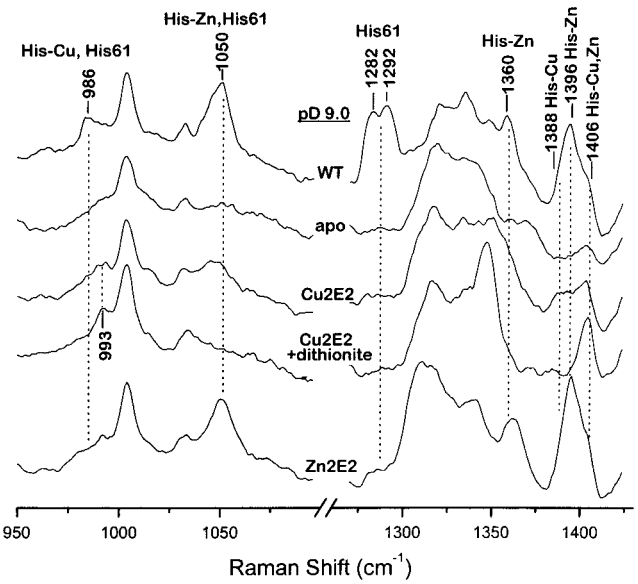

Figure 3. 229 nm-excited UVRR spectra of the indicated forms of SOD (0.3 mM) in D_2O at pD 9.0. Mode labeling: His-Cu = Cu-bound His, His-Zn = Zn-bound His, and His61 = bridging His.

not well separated, this region was deconvoluted into 8 bands (not shown) with fixed widths, to obtain peak heights.

Results

Aromatic and Amide Bands. When excited at 229 nm, the UVRR spectrum of SOD in neutral aqueous solution [Figure 2] contains the expected strong tyrosine bands Y9a [1179 cm^{-1}], Y7a [1205 cm^{-1}], and Y8a/b [1616/1604 cm^{-1}] and weaker bands from phenylalanine [F1, 1004 cm^{-1}; F18a, 1032 cm^{-1}]. There are also bands associated with amide I [1669 cm^{-1}], II [1570 cm^{-1}], and III [1238 cm^{-1}] and X-proline amide II [1449 cm^{-1}] modes of the backbone.[1,2] Amide modes are strongly enhanced at much shorter wavelengths, and are not usually detected at 229 nm, but SOD is mainly β-sheet in character, and the β peptide $\pi-\pi^*$ absorption is red-shifted, relative to α-helical peptides, thus accounting for the appearance of amide bands in the SOD UVRR spectrum.

Metal-Bound Histidine. Additional bands are seen at 986, 1050, 1282/1292, and 1564 cm^{-1}, and these are attributed to ring modes of the His61 imidazolate group, which bridges the Cu^{2+} and Zn^{2+} ions [Figure 1]. These bands have also been reported by Hashimoto et al.[24] The His61 bands all disappear when the metal ions are removed [apoprotein spectrum, Figure 2]. They also disappear when the Cu^{2+} is reduced with dithionite, consistent with other evidence that the imidazolate bridge is absent in reduced SOD.[11,17−22] The bond to the Cu^{+} ion is broken, and is replaced by a proton.

When SOD is incubated in D_2O, new bands appear in the UVRR spectrum [Figure 2, top], which are attributed to ring modes of imidazole ligands bound to the Zn^{2+} and Cu^{2+} ions. These modes become enhanced as a result of H/D exchange at the imidazole N atom opposite to the metal ion. Several new bands are discernible in the 1300−1400 cm^{-1} region, and in His61 the bands at 986, 1050, and 1564 cm^{-1} are augmented. [The 1564 cm^{-1} band shape is also affected by the shift of amide II from 1570 to 1480 cm^{-1}, as a result of amide NH/D exchange.[1,2]] The unique 1282/1292 cm^{-1} doublet band is unaltered in D_2O, confirming its assignment to the bridging His61 imidazolate, which has no exchangeable protons.

To investigate the nature of these metal-bound imidazole UVRR bands, we have examined SOD from which Zn or Cu has been removed selectively [Figure 3]. SOD can be prepared[27] with the zinc site empty [Cu2E2], or the copper site empty [Zn2E2], or with both sites filled with copper [Cu2Cu2] or zinc [Zn2Zn2] (the "2" reflects the fact that SOD is dimeric, and contains an active site on each monomer). When Cu is removed [Zn2E2 spectrum], the 1050, 1360, and 1396 cm^{-1} remain prominent, but these largely disappear when Zn is removed [Cu2E2 spectra], and are therefore assigned to modes of Zn-bound imidazole. On the other hand, bands at 986, 1388, and 1406 cm^{-1}, although relatively weak, are more prominent in the Cu2E2 than in the Zn2E2 spectra. Moreover, they are affected by dithionite addition to Cu2E2, and have altered frequencies and intensities for Cu^{+} relative to Cu^{2+}. Consequently these bands are assigned to modes of Cu-bound imidazole. The complex envelope between 1300 and 1350 cm^{-1} contains overlapping bands, which show significant changes upon metal removal or upon Cu^{2+} reduction. However, the apoprotein has significant remnant intensity in this region, possibly arising from the unligated histidine residues, making assignments uncertain.

Excitation Profile. Enhancement mechanisms were investigated by measuring excitation profiles of the metal-bound imidazole bands [Figure 4], using a tunable frequency-quadrupled Ti:sapphire laser and a frequency-doubled Ar^{+} laser. Intensities were determined by band deconvolution, keeping bandwidths fixed [see Materials and Methods section]. Because of band overlaps, the accuracy of the intensities is not better than 10%. However, the overall pattern is clear. All the coordinated histidine modes show broad excitation profiles, with weak maxima at ∼220 and 230 nm. These double-humped profiles are reminiscent of those found for histidine itself, but the histidine maxima are at ∼205 and ∼220 nm,[3] significantly blue-shifted with respect to the metal-bound histidine profiles. The UV absorption spectrum of SOD in the far-UV region is broad, and contains contributions from His, Tyr, and Phe as well as amide.

There is not much selectivity among modes associated with different metal centers. In particular there is no evidence for significant participation of charge-transfer transitions in the modes associated with Cu-bound histidine.[3] The resonances must

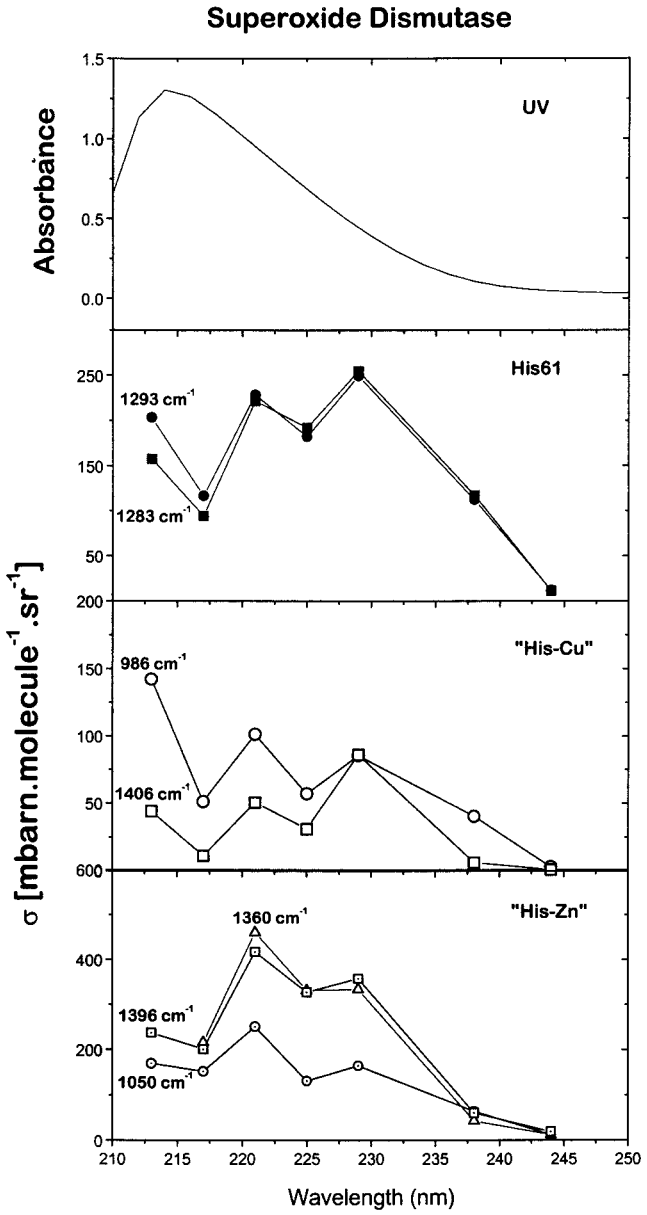

Figure 4. Excitation profiles of different histidine modes and the SOD absorption spectrum. Conditions: 0.3 mM SOD in D_2O at pD 9.0. 0.2 M $NaClO_4$ was used as the internal intensity standard.

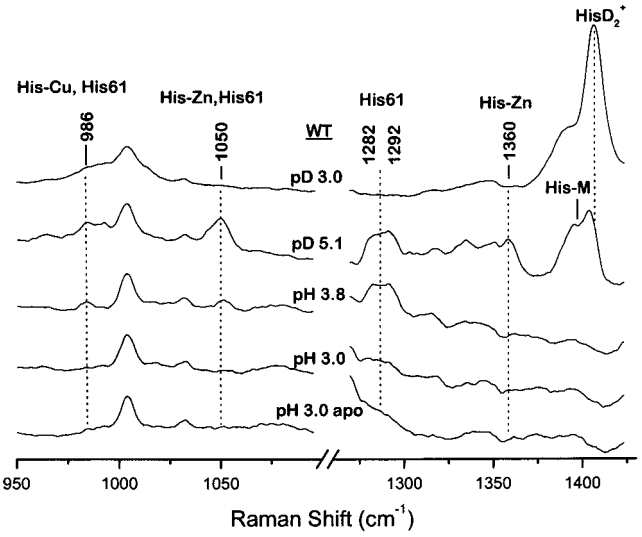

Figure 5. 229 nm-excited UVRR spectra of 0.3 mM wild-type SOD at pD 3.0, pD 5.1, pH 3.8, and pH3.0. Apo SOD at pH 3.0 is shown for comparison.

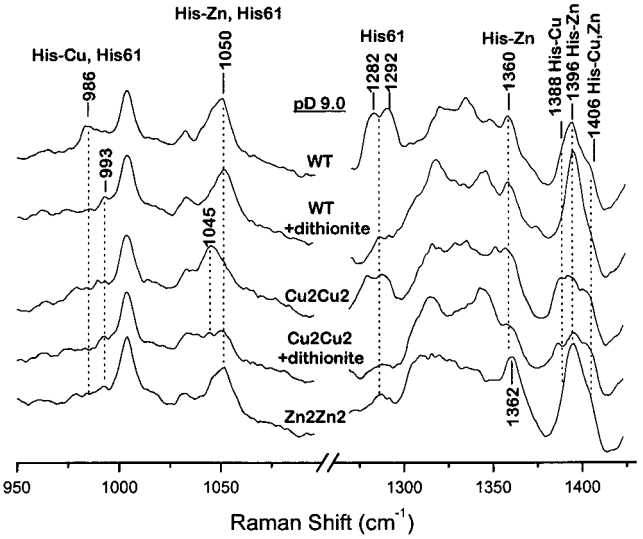

Figure 6. 229 nm-excited UVRR spectra of the indicated forms of SOD (0.3 mM) in D_2O at pD 9.0.

all derive from the imidazole $\pi-\pi^*$ transitions, which are somewhat red-shifted by binding of the imidazole to the metal ions.

Response to Low pH. The 1282/1292 cm^{-1} doublet band is a useful monitor of the status of the His61 bridging imidazolate ligand. As noted above, this band disappears upon Cu^{2+} reduction [Figure 2], and also upon removal of either Cu or Zn [Figure 3], signaling loss of imidazolate character. Likewise the bridge is broken when the pH is lowered to 3.0 [Figure 5]. In D_2O solution, lowering the pD to 3.0 greatly intensifies a band at 1409 cm^{-1}, which is due to protonated histidine residues, whose NH protons have been exchanged for deuterons.[4,32] The intensity of this band suggests that at pD 3.0, multiple histidine residues are protonated, as a result of metal depletion from the active site. Bovine SOD has two histidine residues which are not ligands to the metals. At pD 5.1, where the active site is intact [as evidenced by the 1282/1292 cm^{-1} bridging imidazolate band], these residues are partially protonated, and contribute modest intensity at 1409 cm^{-1}. The imidazolate bridge is still partially intact at pH 3.8 [Figure 5], close to the midpoint of the changes in absorption and EPR spectra that are associated with Zn release from the active site.[26,33]

Metal Substitution. Once Zn or Cu have been removed selectively, the empty site can be filled with the "wrong" metal, producing Cu2Cu2 or Zn2Zn2 SOD, whose UVRR spectra yield further information about the bound histidine ligands [Figure 6]. Inserting Cu in the Zn site partially restores the bridging imidazolate; the 1282/1292 cm^{-1} doublet is present in the Cu2Cu2 spectrum, at about half the intensity of the wild-type protein. This spectrum also shows an increase in the 1388 and 1406 cm^{-1} bands, at the expense of the 1396 cm^{-1} band, consistent with the inference from the Cu2E2 and Zn2E2 spectra [Figure 3] that the former arise from His-Cu and the latter from His-Zn modes. Interestingly, the 1050 cm^{-1} band, which is quite characteristic of histidine in the Zn site [see Figure 3], is distinctly shifted, to 1045 cm^{-1} in the Cu2Cu2 spectrum. The band becomes indistinct when dithionite is added, and loss of

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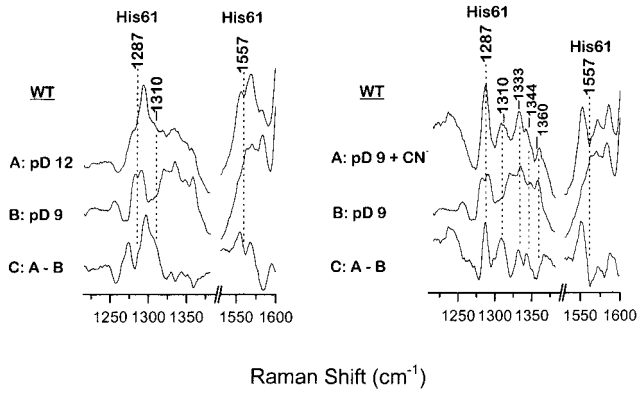

Figure 7. 229 nm-excited UVRR spectra of 0.3 mM wild-type SOD at pD 12 and pD 9, and of CN^--bound SOD at pD 9.0. Difference spectra are shown on the bottom.

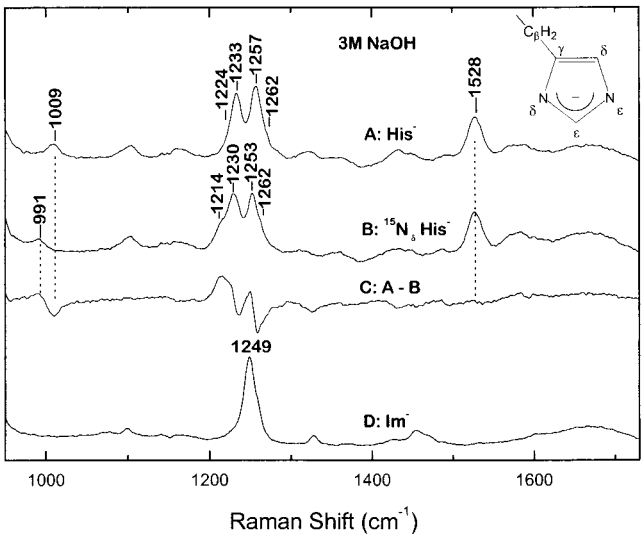

Figure 8. 229 nm-excited UVRR spectra of 5 mM histidine, $^{15}N_\delta$-histidine, and imidazole in 3 M NaOH. (Inset: The imidazolate form of the histidine side chain.)

the 1282/1292 cm^{-1} doublet indicates loss of the bridge when the Cu^{2+} ions are reduced in the Cu2Cu2 protein. The bridge is likewise absent in the Zn2Zn2 protein.

Cyanide and Hydroxide Binding. A significant perturbation to the bridge is detected [Figure 7] when cyanide is bound to SOD, as Hashimoto et al. have also reported.[24] The characteristic 1282/1292 cm^{-1} band is replaced by a single band at 1287 cm^{-1}, with intensity suggesting that the two components of the doublet have collapsed. In addition, there is a perturbation to the 1564 cm^{-1} band associated with His61. The difference spectrum contains a sigmoidal feature that indicates a downshift of this band upon cyanide binding. We found a similar effect at pD 12 [Figure 7], and attribute the changes to hydroxide binding. A downshift is again observed in the 1564 cm^{-1} band, and the 1282/1292 cm^{-1} collapses to a single band, as in the cyanide adduct.

Insight into the nature of this perturbation is gained from the spectrum of aqueous histidine in 3 M NaOH, which deprotonates the imidazole [Figure 8]. A double band is seen at 1233/1257 cm^{-1}, while imidazolate itself shows only a single band at 1249 cm^{-1}. The double band in histidinate must arise from coupling of the 1249 cm^{-1} imidazolate ring coordinate with a coordinate of the $C_\beta H_2$ substituent on the ring. Consistent with this explanation, both components shift down by 3−4 cm^{-1} when ^{15}N is substituted at the N_δ atom. Additional components can

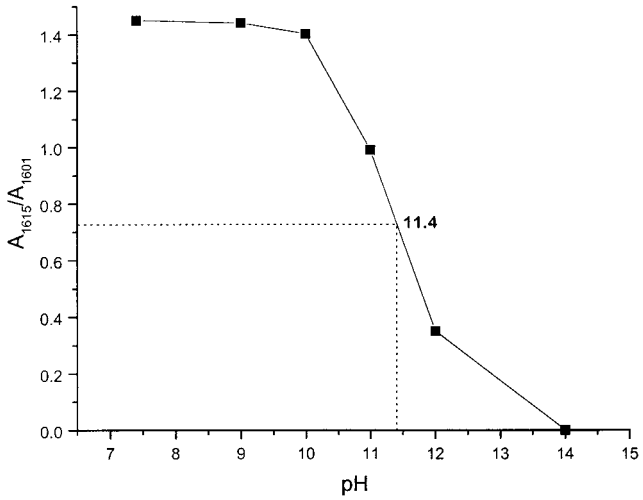

Figure 9. Titration curve of Tyr108 based on the ratio between the heights of 1615 (tyrosine) and 1601 cm^{-1} (tyrosinate) Y8a bands in the 229 nm-excited UVRR spectra. The pK_a is estimated to be 11.4.

be seen on the low- and high-frequency side of the doublet, which may reflect different orientations of the ring with respect to the ring−C_β bond, resulting in different extents of coupling. The double band of histidinate is some 40 cm^{-1} below the position of the 1282/1292 cm^{-1} doublet in SOD, and histidinate bands at 1009 and 1528 cm^{-1} are likewise about 40 cm^{-1} below the 1050 and 1564 cm^{-1} bands of SOD [Figure 2], which are assigned in part to His61. The higher frequencies in the protein are attributable to the polarizing effect of the bound Zn^{2+} and Cu^{2+} ions.

Tyrosine 108. Finally, we note that characteristic shifts in the Y8a/8b bands between tyrosine and tyrosinate[34,35] permit determination of the pK_a of the single tyrosine residue in SOD, Tyr108 [Figure 9]. This value is 11.4, significantly higher than the pK_a of aqueous tyrosine, 10.2. We note as well that the frequency of the strong Y8a band is slightly but systematically shifted by removal of metal from the active site. At pD 9.0, The frequency is 1613.5 ± 0.2 cm^{-1} in the native protein, or in the Cu2Cu2 and Zn2Zn2 forms, but is lowered to 1613.2 ± 0.1 cm^{-1} when either the Cu or the Zn site is empty or Cu^{2+} is reduced to Cu^+, and to 1612.2 cm^{-1} in the apoprotein.

Discussion

The present results, along with those of Hashimoto et al.,[24] establish several UVRR bands as arising from imidazolate modes of His61, because they are not present in the apoprotein, are insensitive to D_2O, and disappear when the active site is disrupted by lowering the pH to 3.0. The acid reaction has been studied by Valentine and co-workers, who established pH 3.8 as the approximate midpoint for the loss of zinc from the active site.[26,33] Retention of the active site to such a low pH reflects the importance of Asp122 in tying the site together by H-bonding with histidine ligands on both Zn^{2+} and Cu^{2+} (Figure 1). We infer that these H-bonds are lost when Asp122 is protonated at pH 3.0, resulting in loss of the Zn^{2+} and protonation of its ligands. Multiple histidine protonation is consistent with the large intensification of the 1408 cm^{-1} $HisD_2^+$ UVRR band at pD 3.0,[4] and also by the reported steepness of the low pH absorption and EPR spectral changes.[26,33] Significantly, mutagenesis of Asp124 in human SOD (corresponding

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to Asp122 in bovine SOD) to Gly or Asn abolishes the ability to bind Zn^{2+}.[36] At pH 3.0, most of the Cu^{2+} remains bound.[26,33]

As expected, the His61 bands disappear when either Zn^{2+} or Cu^{2+} are removed from the active site. When Cu^{2+} occupies both metal sites [Cu2Cu2] [Figure 6], the His61 1282/1292 cm^{-1} doublet is present at about half of the wild-type intensity, indicating partial formation of the imidazolate bridge. The bridge is not restored, however, when Zn^{2+} occupies both sites [Zn2Zn2].

The UVRR bands arising from His61 are also lost when SOD is reduced with dithionite [Figures 2 and 6]. This result was reported as well by Hashimoto et al.,[24] who also found a negative band in the difference spectrum at 1590 cm^{-1}, which they attributed to the newly generated neutral histidine ligand on Zn^{2+} when the imidazolate is protonated. Thus the UVRR evidence supports the view that the imidazolate$-Cu^{2+}$ bond is broken upon reduction to Cu^{+}, and is replaced with a proton. As discussed in the Introduction, delivery of this proton is a key element in the enzyme mechanism.[12b]

Cyanide binding has a marked effect on the His61 UVRR bands. The 1565 cm^{-1} band experiences a downshift, and the 1282/1292 cm^{-1} doublet collapses to a single band at 1287 cm^{-1}, as also reported by Hashimoto et al.[24] These authors suggested that the 1282/1292 cm^{-1} doublet might arise from a Fermi resonance, but the spectrum of histidinate ion [Figure 8] indicates that the doublet reflects coordinate mixing between a ring coordinate and a coordinate of the $C_\beta H_2$ substituent, since imidazolate has only a single UVRR band in this region. The 1233 and 1257 cm^{-1} bands of histidinate both shift 3−4 cm^{-1} upon $^{15}N_\delta$ substitution, consistent with a coordinate mixing mechanism. The ring coordinate is suggested to involve the $N_\delta-C_\epsilon$ bond, since Hashimoto et al.[24] reported a 37 cm^{-1} downshift upon C_ϵH/D exchange for the prominent 1278 cm^{-1} UVRR band of a zinc−imidazolate complex, which corresponds to the 1249 cm^{-1} UVRR band of imidazolate ion [Figure 8].

Coordinate mixing with a $C_\beta H_2$ substituent would be strongly influenced by changes in the dihedral angle about the ring$-C_\beta$ bond, through changes in mechanical coupling. The collapse of the 1282/1292 cm^{-1} His61 doublet upon cyanide or hydroxide binding [Figure 7] may reflect loss of coordinate mixing because of altered ring orientation. Cyanide binding rotates the tetragonal plane of the Cu^{2+} ligand field by interconverting the axial and equatorial ligand position.[28,37−41] Although cyanide replaces the weakly bound axial water molecule, it becomes part of the new equatorial plane. This change must be accompanied by geometrical rearrangement of the histidine ligands, and one of these changes may well be a reorientation of the His61 imidazolate ring.

When SOD is incubated with D_2O several new metal-bound histidine bands appear in the UVRR spectrum, because of the NH/D exchange. The mechanism for this enhancement has not been evaluated quantitatively, but it is probably similar to that of protonated histidine when the NH protons are exchanged for deuterons. In both cases, the NH/D exchange eliminates mixing of NH bending coordinates with ring coordinates, because of the much lower natural frequency of ND bending, thus concentrating the UVRR intensity into fewer modes.[5] His61 imidazolate enhancement is similarly explained, since there are no protons on the ring N atoms. In addition, the metal-bound histidine excitation profiles are red-shifted [Figure 4] relative to histidine itself, bringing the histidine ligand modes closer to resonance with the 229 nm excitation.

The multiplicity of metal-bound histidine modes may have several contributing factors. The orientation of the imidazole ring relative to the $C_\beta H_2$ substituent has already been mentioned as a factor controlling coordinate mixing. In addition, the N_δ and N_ϵ atoms are not equivalent, and the site of metal binding may influence ring mode frequencies.[24] For example, Miura et al. reported that in [nonresonance] Raman spectra of crystalline histidine−metal complexes the position of the ca. 1580 cm^{-1} band falls in nonoverlapping ranges for N_δ and N_ϵ binding.[41] In SOD the Zn^{2+} ion is bound through N_δ to all three of its imidazole ligands, while the Cu^{2+} ion is bound through one N_δ and three N_ϵ atoms [Figure 1]. Moreover, the strength of the metal−imidazole bond may have an effect. Miura et al. found a positive correlation between the frequency of the ca. 1580 cm^{-1} band and the length of the metal−ligand bond.[42] Finally, the ring modes may also be affected by H-bonds involving the distal NH protons, e.g. the H-bonds from the His69 and His44 N_ϵH atoms to the Asp122 carboxylate [Figure 1].

It is impossible to disentangle these factors on the basis of the data at hand, but some qualitative observations are possible from the metal removal and replacement experiments. For example, SOD in D_2O has a complex band centered at 1396 cm^{-1}, with shoulders at 1388 and 1406 cm^{-1}, which is not present in H_2O, and therefore is not contributed by His61. Removing the Cu^{2+} ion [Zn2E2] leaves mainly the 1396 cm^{-1} band, suggesting that it is contributed by the histidine ligands in the Zn^{2+} site, while removing the Zn^{2+} ion [Cu2E2] leaves mainly the 1388 and 1406 cm^{-1} bands, suggesting that they are contributed by histidine ligands in the Cu^{2+} site. Since the three bands are close in frequency, they probably arise from the same mode on [at least] three different histidine ligands, each having a distinctive frequency. Interestingly, when the Cu2E2 protein is reduced with dithionite, the 1388 cm^{-1} band disappears and the 1406 cm^{-1} band intensifies, suggesting a rearrangement of the Cu site as a result of the differing coordination propensities of Cu^{2+} and Cu^{+} .

The zinc site is responsible for the bands at 1050 and 1360 cm^{-1}, since both disappear when Zn^{2+} is removed, but are restored if either Zn^{2+} or Cu^{2+} is added back to the site [Figure 6]. His61 contributes part of the 1050 cm^{-1} intensity in the native protein, since the band is present in H_2O. However, the intensity is significantly higher in D_2O [Figure 2], and remains prominent in the Zn2E2 protein, indicating that another histidine in the Zn^{2+} site contributes to the intensity. We speculate that this ligand is His69, for which a rather strong H-bond is implicated by the 2.83 Å distance between the N_ϵ atom and an O atom of the Asp122 carboxylate [Figure 1]. A strong H-bond induces imidazolate character, and might provide resonance enhancement similar in some respects to His61 in the native protein. Interestingly the 1050 cm^{-1} band is shifted to 1045 cm^{-1} in the Cu2Cu2 protein, showing that the imidazole ring which gives rise to this band is sensitive to which metal is bound. On the other hand, the 1360 cm^{-1} band has no contribution from His61 [no enhancement in H_2O], and is not

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sensitive to which metal occupies the Zn^{2+} site [Figure 6]. Perhaps it arises from the remaining Zn site histidine, His78, which may have a different enhancement pattern from His69.

Finally, we comment on Tyr108, the sole tyrosine residue in bovine SOD. Interest in Tyr108 centers on its being a target for peroxynitrite induced nitration, which is catalyzed by the SOD active site.[43,44] Because of the large separation of Tyr108 from the active site (~18 Å), nitration occurs by encounter with a second SOD molecule, as indicated by the second-order kinetics.[44] Although the Tyr108 side chain is exposed to solvent, its pK_a 11.4 is a full unit higher than that of aqueous tyrosine. The elevation can be attributed to a nearby anion, which inhibits deprotonation. The residue next to Tyr108 is Glu107, and examination of the crystal structure[11] shows that the carboxylate side chain to be near the phenolic OH, the O···O distance being 3.48 Å. The pK_a elevation may modulate the reactivity of Tyr 108 toward peroxynitrite.

Additionally, the Y8a frequency is responsive to the metal content of the active site. This effect is attributable to the loosening of loop 4, 7, which contains Tyr108, when the metal are removed. This loop connects two β strands which contain three of the Cu-bound histidine residues, His44 and His46 (strand 4) and His118 (strand 7). Moreover strands 4 and 7 have extensive H-bond contacts with strand 5, which contains the bridging His61 ligand, as well as Zn-bound Asp81. The Try108 Y8A band monitors the progressive disorder of loop 4, 7 as the metal ions are removed.

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