a shift in the position of the stretching vibration observed in the infrared region that occurs at 2041 cm^{-1} for the monomer **3b** and that is found at 2068 cm^{-1} for the dinuclear complex **2b**. Variations in the observed LMCT bands (Table V) that are noteworthy are the "shift" of the position of the $N_3^- \rightarrow Cu(II)$ absorption from 370 nm in the **3b** to 405 nm in **2b** and the intensity increase for the $PhO^- \rightarrow Cu(II)$ band at 460–465 nm on going from the mononuclear azide complex to the dinuclear azide complex. This latter intensity increase is not observed for the corresponding chloride-containing compounds **3a** and **2a**. It also has been recently reported that the C–O(phenoxo) stretching vibrations in the resonance Raman spectra for phenoxo-bridged dinuclear Cu(II) complexes are observed at significantly higher frequencies than those of the corresponding mononuclear analogues.[34] Further studies on this and other systems will be necessary to evaluate the significance, if any, of chemical differences and spectroscopic variations observed that may be due to the dinuclear coordination of small molecules compared to that observed in mononuclear analogues.

Acknowledgment. We thank the National Institutes of Health (K.D.K., Grant No. GM 28962 and GM 34909) for support of this research.

Registry No. **1**, 86593-51-3; **2a**, 105582-79-4; **2b**, 86597-35-5; **3a**, 93532-00-4; **3b**, 105582-80-7; **4a**, 14870-25-8; **4b**, 105582-81-8; *L'*-OH, 97801-58-6; py2, 15496-36-3; Me_3SiN_3, 4648-54-8; 2-(bromomethyl)-phenyl acetate, 704-65-4; *o*-cresol, 95-48-7; *o*-tolyl acetate, 533-18-6.

Supplementary Material Available: Listings of bond lengths, bond angles, anisotropic temperature factors, and hydrogen coordinates and temperature factors for compounds **2b** (Tables VII–X) and **3b** (Tables XII–XV) (13 pages); listings of structure factors for **2b** (Table VI) and **3b** (Table XI) (23 pages). Ordering information is given on any current masthead page.

Contribution from the Department of Chemistry,
University of Florence, Florence, Italy

Characterization of the Cobalt(II)-Substituted Superoxide Dismutase–Phosphate Systems

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Received May 16, 1986

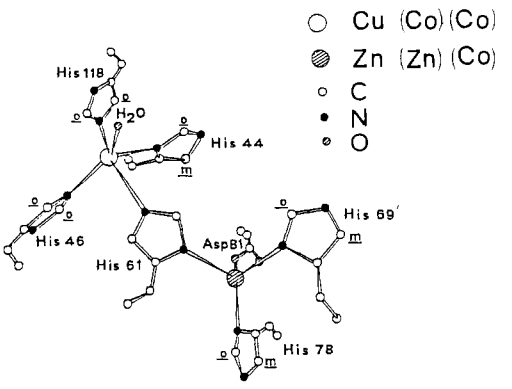
The systems containing Co_2Zn_2SOD or Co_2Co_2SOD and phosphate have been investigated by means of electronic and ^1H NMR spectroscopies. It is suggested that the cobalt ion at the copper site is bound to three histidines and one phosphate ion. Histidine-61, which bridges zinc and cobalt or the two cobalt ions, is detached upon addition of phosphate, which probably binds both Arg-141 and the cobalt ion. The shape of the electronic spectrum of the cobalt ion at the zinc site depends on the terminal or bridging mode of His-61.

Introduction

It has recently been shown that the demetalized erythrocyte copper–zinc superoxide dismutase (SOD) can bind cobalt(II).[1-3] The latter metal ion easily occupies the zinc site to give E_2Co_2SOD, where E stands for the empty copper site.[1] This is quite reasonable on account of the similar chemistry of cobalt(II) and zinc(II) and of the success obtained in substituting zinc(II) with cobalt(II) in zinc enzymes.[4]

The ^1H NMR spectra of E_2Co_2SOD indicate that the metal ion is bound to three histidines just as in the native enzyme;[5] this is confirmed by the ^1H NMR spectra of $Cu^I_2Co_2SOD$[6] and is consistent with the analysis of the ^1H NMR of Cu_2Co_2SOD.[5] A second pair of cobalt(II) ions is bound by the dimeric E_2Co_2SOD to form Co_2Co_2SOD.[2,3] This reaction is reported to be facilitated by the phosphate ion.[3] The interest in this kind of derivative resides in the characterization of apoSOD as metal chelator and in obtaining derivatives containing spectroscopic probes such as cobalt(II) capable of shedding light on the environment of the metal ion. We have shown that high-spin cobalt(II) is an excellent probe for ^1H NMR spectra in that it allows the detection of the proton signals of the metal ligands.[4,7] We have therefore attempted to understand further the nature of the interaction of cobalt(II) and the SOD ligands at the copper site. Furthermore, we have tried to understand the role of phosphate in the cobalt-binding process. Therefore, we have investigated the ^1H NMR spectra of Co_2Co_2SOD and Co_2Zn_2SOD in the presence of increasing amounts of phosphate at pH 7.4, and we have remeasured the electronic spectra of the two systems as well as ^{31}P NMR parameters.

Chart I



Experimental Section

Native SOD, purchased from Diagnostic Data Inc. (Mountain View, CA), was used without further purification. Demetalation was obtained as described elsewhere.[8] The Co_2Zn_2SOD derivative was obtained by adding 2 equiv of zinc(II) to the apoprotein at pH 5.9 and then raising the pH to 7.4 and adding 1.4 equiv of cobalt(II) in about 2 days. The Co_2Co_2SOD derivative was obtained by slow addition (2 days) of 3.6 equiv of cobalt(II) to the apoprotein at pH 7.4. Phosphate adducts of the metal-substituted proteins were obtained by titration with phosphate solutions at pH 7.4.

Electronic spectra were run on a Cary 17D spectrophotometer using microcuvettes of 1 cm light path.

Room-temperature 90-MHz ^1H NMR spectra were recorded on a Bruker CXP 90 spectrometer, using the modified DEFT sequence[9] described elsewhere[10] with a total recycle time of 0.2 s (8K data points).

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Spectra were typically obtained through block averaging of 5–20 spectra of 16 000 scans each. Chemical shifts were measured from the H_2O or HDO signals and reported from Me_4Si, assumed to be −4.8 ppm from the water peak.

Room-temperature ^{31}P T_1 NMR measurements were performed on a Varian FT-80 spectrometer, operating at 32.4 MHz. T_1's were measured through an inversion recovery 180°–τ–90°–AQ pulse sequence. The estimated error is ±5%.

Results

The 90-MHz ^1H NMR spectrum of Co_2Zn_2SOD in the absence of phosphate is reported in Figure 1A. As already discussed,[11] the three downfield signals, which are shaded in Figure 1A, are assigned to three NH signals of three coordinated histidines, which would correspond to His-44, His-46, and His-118. The scheme of the copper and zinc sites in the native enzyme is reported for convenience (Chart I).[12] The different intensities of the histidine NH signals are attributed to different exchange rates with solvent.[11] In D_2O as solvent, the three signals disappear. A fourth signal at 46 ppm is assigned to the meta-like proton of His-44, which, in terms of distance from copper, is similar to the NH's. Several other signals are observed between 20 and 40 ppm downfield from Me_4Si. They were assigned[11] to proton signals of residues close to cobalt(II) but not bound to it. The isotropic shifts (i.e. the shift from the diamagnetic region) would be due to through-space dipolar interactions arising when magnetic anisotropy is present. Among this group of signals the one at 34 ppm disappears when the spectrum is recorded in D_2O.

The ^1H NMR spectra indicate a quite complex behavior of the Co_2Zn_2SOD–phosphate system. At the beginning (spectra B and C of Figure 1), the three signals assigned as histidine NH become of comparable intensity without any major variation in the shifts (spectrum C). The variation in shift is also negligible for the signals at 46 and 36 ppm in the absence of phosphate. The signals between 20 and 40 ppm downfield broaden or disappear, indicating that some conformational changes in the protein are occurring without major involvement of the three bound histidines. The upfield signals in the −50 to −60 ppm region, assigned to non directly coordinated moieties, also dramatically broaden. The far-upfield and -downfield signals around +190 and −170 ppm are already washed out at phosphate concentrations corresponding to spectrum C, as well as the group of upfield signals below −20 ppm. Upon further addition of phosphate, two of the three NH signals disappear until they eventually reappear at high phosphate concentrations (spectra G and H of Figure 1). In the concentration range of spectra D–F it appears that these NH protons undergo a chemical exchange at a rate of the order of the difference in chemical shifts among the various sites. It is possible that the interconversion rate between Co_2Zn_2SOD–phosphate and Co_2Zn_2SOD is of the order of the difference in isotropic shift of the NH protons and this interconversion provides the different chemical sites for histidine NH.

The electronic spectra of Co_2Zn_2SOD in the presence of phosphate at pH 7.4 are also reported (Figure 2). Upon addition of phosphate the spectra increase in intensity as already reported.[3,13] From the variation of the molar absorbance at 600 nm with increasing amounts of phosphate (Figure 2A, inset) an affinity constant for a single phosphate interacting with cobalt ion of 260 M^{-1} is obtained. Such spectral dependence on phosphate concentration is also consistent with that previously reported.[13] From the variation of chemical shift of the signal at 46 ppm, an affinity constant consistent with the spectrophotometric value is obtained.

^{31}P NMR spectra of a 0.5 mM Co_2Zn_2SOD solution containing phosphate in the same concentration as that of spectrum G of Figure 1 show a T_1 value of 24.6 ms. This result is consistent with that of a previous report.[13]

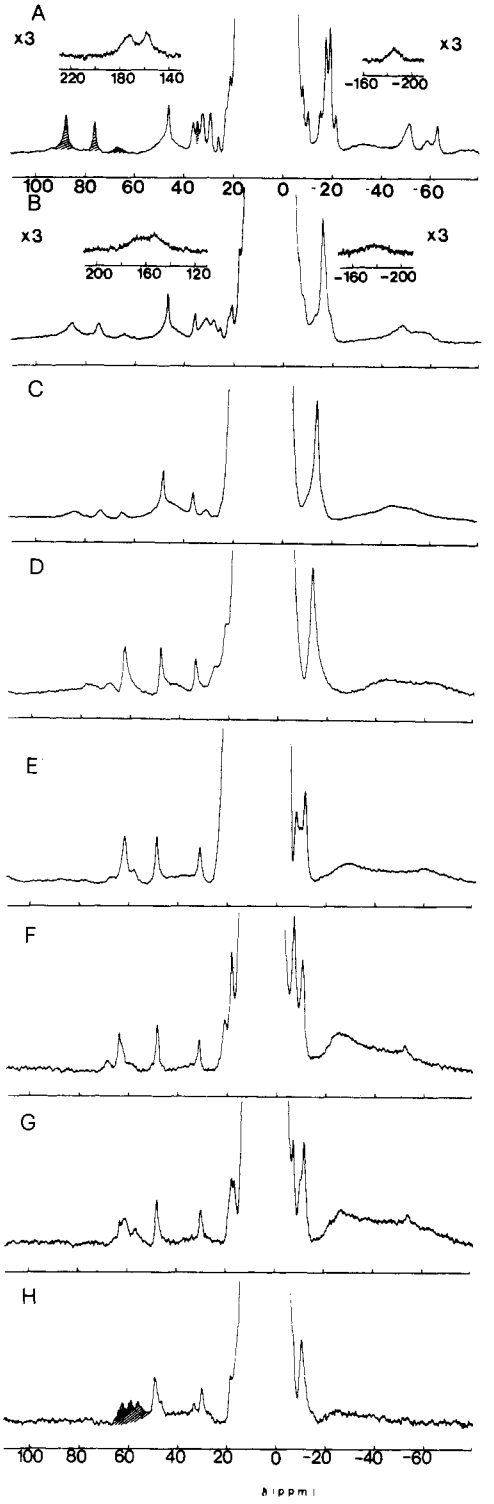

Figure 1. 90-MHz ^1H NMR spectra of Co_2Zn_2SOD in water at pH 7.4 (molar concentration of cobalt(II) in Co_2Zn_2SOD 2.2×10^{-3} M) (A) and in the presence of increasing amounts of phosphate. The molar concentrations (mM) are as follows for cobalt(II) as Co_2Zn_2SOD and phosphate, respectively; (B) 1.9, 1.1; (C) 1.7, 1.9; (D) 1.7, 3.8; (E) 1.6, 14; (F) 0.83, 26; (G) 0.80, 50; (H) 0.78, 200. The shaded signals disappear when the spectra are recorded in D_2O.

The ^1H NMR spectra of Co_2Co_2SOD essentially show the signals of Co_2Zn_2SOD plus those arising from the other cobalt(II) chromophore[11] (Figure 3A). The ^1H NMR spectra show a group of six signals, two of which are exchangeable protons, and two (at 48 and 36 ppm) that were already present in Co_2Zn_2SOD (at 46 and 36 ppm), as it appears from titration with phosphate (see below). Therefore, the two extra signals at 52 and 40 ppm are

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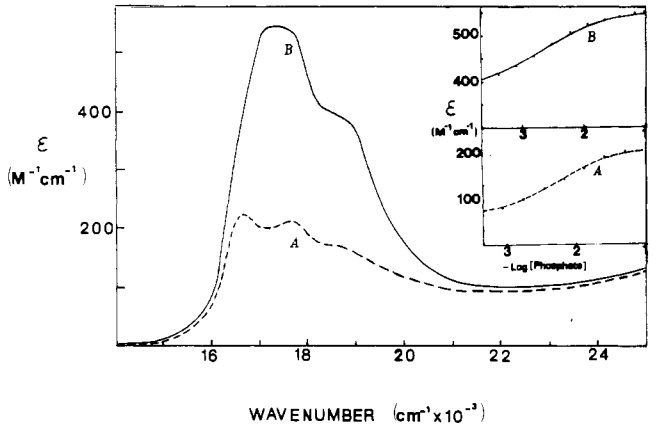

Figure 2. Limit electronic spectra of Co_2Zn_2SOD–phosphate (---) and Co_2Co_2SOD–phosphate (—) at pH 7.4. In the inset the relative variations of molar absorbance at 16.67 (A) and 17.20 × 10^3 cm^{-1} (B) are reported. The data are fit with affinity constants of 260 and 300 M^{-1}, respectively.

likely to belong to the meta-like protons of His-69 and His-78 coordinated to cobalt(II) in the zinc site. When phosphate is added, the behavior of the signals attributable to the histidines bound at the copper site closely parallel those of Co_2Zn_2SOD (Figure 3), although the three NH signals at 85, 72, and 64 ppm broaden at a lower phosphate concentration with respect to Co_2Zn_2SOD. The presence of the signals of histidines bound to cobalt in the zinc site makes the overall spectrum more complex. At a phosphate concentration of 2 × 10^{-2} M (Figure 3H), there are three signals at 61, 58, and 55 ppm, which disappear in D_2O. Their weak intensity is probably due to exchange with water. Their positions closely correspond to those of the NH signals in the Co_2Zn_2SOD–phosphate derivative. We assign only these 3 signals, as the overall spectrum is expected to contain at least 19 signals (of the protons of coordinated histidines) in a small chemical shift range. The small shift range observed is often encountered in tetrahedral cobalt(II) complexes,[7,14] and indeed it was observed also for Co_2Zn_2SOD in the presence of phosphate.

The electronic spectra of Co_2Co_2SOD with phosphate indicate the presence of two tetrahedral chromophores (Figure 2B). The affinity constant of phosphate for Co_2Co_2SOD is estimated from the electronic spectra to be 300 M^{-1}, i.e. close to that for Co_2Zn_2SOD. Such a value is consistent with the NMR titration.

Discussion

In contrast to results obtained with the native enzyme,[15] the experimental results provide evidence that phosphate interacts with the metalloprotein through direct binding to the metal ion in the case of cobalt occupying the site of copper. The electronic spectra indicate that upon phosphate binding the coordination around the cobalt ion in the copper site becomes tetrahedral,[4] since the intensity of the spectra in the visible region more than doubles; there is agreement on this within the scientific community.[3,13,16] Upon phosphate binding, the problem arises of which are the donor groups of cobalt at the copper site. On this question the ^1H NMR spectra provide independent and valuable information.

The ^1H NMR spectrum of Co_2Zn_2SOD in the presence of an excess of phosphate shows evidence of three exchangeable protons; it is reasonable to assign them to three NH protons of coordinated histidines. Indeed, the shifts of these protons are in the range typical of NH's of histidines coordinated to cobalt(II).[4,17] On the other hand, ^{31}P NMR data provide a $(fT_{1p})^{-1}$ value of 2.8 × 10^3 s^{-1}, where f is the molar fraction of the bound phosphate and

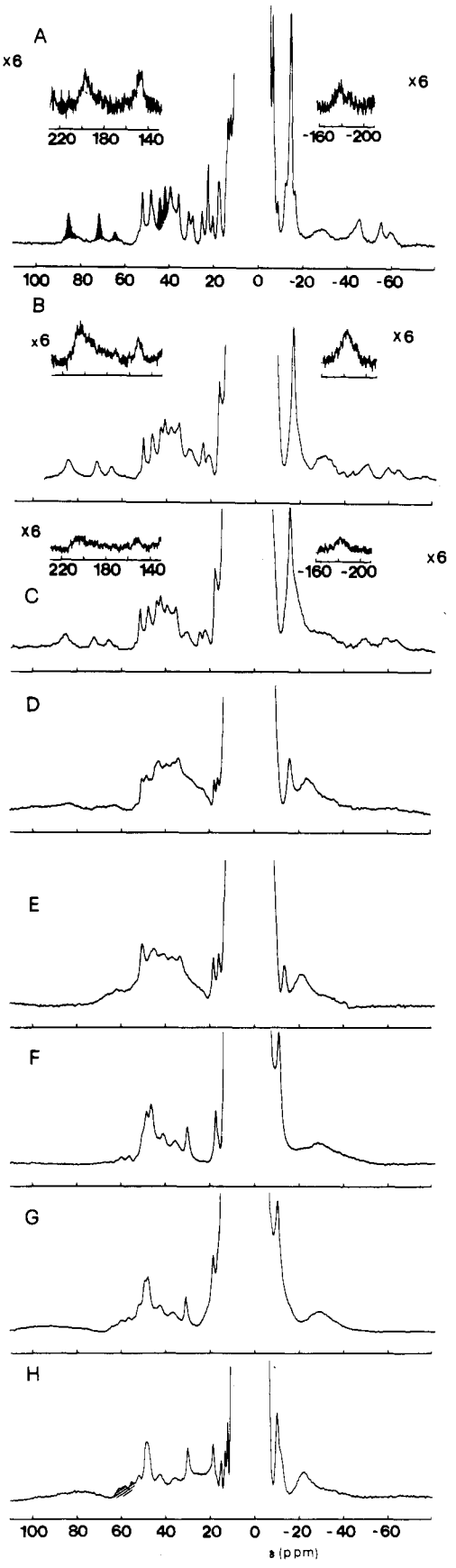

Figure 3. 90-MHz ^1H NMR spectra of Co_2Co_2SOD in water at pH 7.4 (molar concentration of cobalt(II) in the copper site 2.3 × 10^{-3} M) (A) and in the presence of increasing amounts of phosphate. The molar concentrations (mM) are as follows for cobalt (II) and phosphate, respectively; (B) 1.6, 0.37; (C) 1.6, 0.57; (D) 1.5, 1.4; (E) 1.3, 2.8; (F) 2.2, 5.0; (G) 2.1, 9.9; (H) 1.2, 19.

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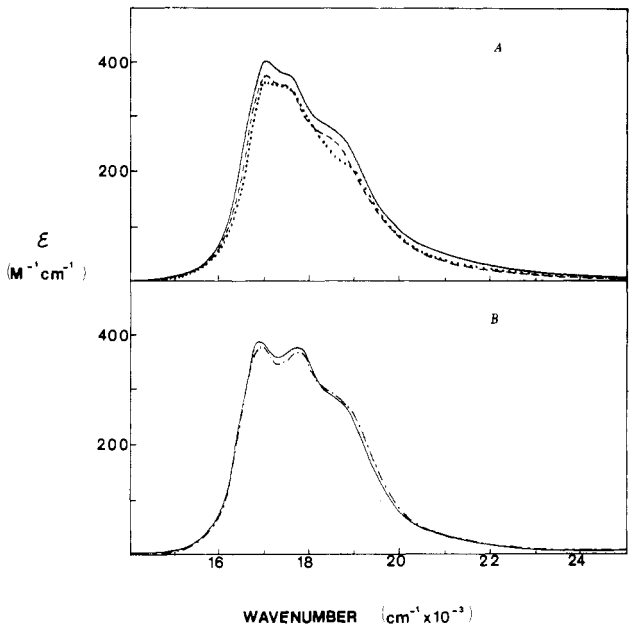

Figure 4. (A) Electronic spectra of E_2Co_2SOD (—) and $Cu^I_2Co_2SOD$ (---) and difference spectrum between Co_2Co_2SOD–phosphate and Co_2Zn_2SOD–phosphate (···). (B) Difference spectra between Cu_2Co_2-SOD and Cu_2Zn_2SOD (-·-) and between Co_2Co_2SOD and Co_2Zn_2SOD (—). Molar absorbances are per subunit.

T_{1p}^{-1} is the nuclear relaxation enhancement of the bulk phosphate nuclei in the presence of the paramagnetic metalloprotein. If we assume that $(fT_{1p})^{-1} = T_{1M}^{-1}$ is dipolar in origin, through the use of the Solomon equation[18] and with an electronic relaxation time of 1×10^{-11} s, a P–Co distance of 2.5 Å is found. The relaxation time is on the high-limit range; if it is taken shorter, an even shorter distance is calculated. The Solomon equation has been used in similar systems, providing analogous values.[19,20] A critical evaluation of the unpaired electron–nucleus coupling predicts that Solomon's equation in high-spin cobalt(II) systems is adequate to describe nuclear relaxation within a factor of 2 under any circumstance.[21] A distance of 2.5 Å is somewhat too short for a phosphate coordinated to a metal ion. However, if there is chemical bonding, there is delocalization of unpaired electrons from the metal ion to oxygen and phosphorus nuclei.[22] The dipolar coupling between unpaired spin density on phosphate and the ^{31}P nucleus gives a shorter metal-to-phosphorus distance.

The final conclusion is that phosphate is directly bound to the metal ion. Since the electronic spectra indicate tetracoordination and NMR data indicates three histidines plus one phosphate, the coordination sphere of cobalt(II) in the copper(II) site is univocally defined by His-44, His-46, His-118, and phosphate. It follows from the above discussion that His-61, which acts as the bridging histidine in the native enzyme, is not coordinated to the cobalt in the copper site in the phosphate adduct of Co_2Zn_2SOD.

In Co_2Co_2SOD the conclusions based on ^1H NMR spectra are less clear, although three exchangeable protons, corresponding to the three NH signals in Co_2Zn_2SOD, are observeable at 85, 72, and 64 ppm (see Figure 3A). It is therefore likely that the same conclusion holds also for Co_2Co_2SOD, i.e. that His-61 is no longer bridging cobalt(II) in the copper site but is terminally bound to the cobalt in the zinc site.

A further support to this hypothesis comes from the comparison of the electronic spectra of the cobalt ion in the zinc site among the various derivatives. Figure 4A shows three spectra of such a chromophore in the E_2Co_2SOD, $Cu^I_2Co_2SOD$, and Co_2Co_2SOD–phosphate derivatives. The first two are experimental spectra, the third is the difference between the electronic spectra of Co_2Co_2SOD–phosphate and Co_2Zn_2SOD–phosphate. Figure 4B shows the electronic spectra of the zinc-site cobalt(II) chromophore in Cu_2Co_2SOD and Co_2Co_2SOD. These spectra are the differences between Cu_2Co_2SOD and Cu_2Zn_2SOD on the one hand and between Co_2Co_2SOD and Co_2Zn_2SOD on the other. It is apparent that a modest but meaningful change in the positions of the bands around $17–18 \times 10^3$ cm^{-1} occurs between the spectra in Figure 4A,B. The Figure 4B derivatives share the common characteristic of having His-61 bridging between cobalt(II) in the zinc site and another metal ion in the copper site. In Figure 4A His-61 is surely not bridging so far as E_2Co_2SOD and Cu^I_2-Co_2SOD[6] are concerned. It had already been noted that the electronic spectrum of cobalt(II) in the zinc site depends on the presence or absence of metal binding to His-61.[23,24] Therefore, the electronic spectrum of Co_2Co_2SOD–phosphate is also indicative of His-61 not bridging the two metal ions.

Finally, the EPR spectra of Co_2Co_2SOD in the presence of phosphate deserve a comment. They have been previously reported as typical of $S = {}^3/_2$ ions.[3] Now, one would expect some magnetic coupling between the two cobalt ions if His-61 were bridging, since extensive magnetic coupling has been observed in the Cu_2Co_2-SOD[25] and in the Cu_2Cu_2SOD[26,27] derivatives. Under these circumstances no EPR spectrum of the $S = {}^3/_2$–$S = {}^3/_2$ pair would be expected, since the system would be better described by a singlet, a triplet, a quintuplet, and a septuplet.[28] Therefore, the EPR spectra of Co_2Co_2SOD in the presence of phosphate are, in our opinion, consistent with two magnetically uncoupled cobalt(II) ions and therefore not bridged by His-61. It is therefore concluded that the two metal ions are not bridged in the phosphate adduct.[29]

If we now consider the geometry and coordination number of cobalt in the copper site in the absence of phosphate, all the data are consistent with pentacoordination.[13,16] Such a coordination number may be reached through four histidine ligands and a water molecule. The electronic spectra of the cobalt in the zinc site are consistent with the presence of a histidinato bridge (Figure 4B).

The phosphate ion is known to inhibit native SOD;[30] it probably binds Arg-141 but not the copper ion.[30] When cobalt is at the copper site, phosphate may bind to both Arg-141 and the metal ion. The latter would be displaced from its original place, causing detachment from the bridging histidine and relaxing the metal-binding region from probable strains. The larger tendency of cobalt(II) to give rise to tetracoordination as compared to that of copper(II) may account for the overall different behavior with respect to the interaction with phosphate. The present structural characterization may also account for the fast uptake of cobalt(II) by apoSOD in the presence of phosphate.[2] It may also give further significance to the breaking of the bridge observed in Cu_2Cu_2SOD by NCS^- and phosphate.[31]

Acknowledgment. This work has been performed with the contribution of the Progetto Strategico Biotecnologie CNR.

Registry No. PO_4^{3-}, 14265-44-2; L-His, 71-00-1.

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