Formate as an NMR Probe of Anion Binding to Cu,Zn and Cu,Co Bovine Erythrocyte Superoxide Dismutases

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ABSTRACT: The binding of formate to bovine Cu,Zn superoxide dismutase has been studied by NMR spectroscopy. The distance between the copper ion and the proton covalently bound to formate has been evaluated from the broadening of the resonance of such proton. The effect on the copper-coordinated water molecule was evaluated from the bulk water relaxation effect by pulsed low-resolution NMR. The broadening of the resonance due to the formate carboxyl in the ^{13}C NMR spectrum gave further indications about the carbon–copper distance thus providing information about the orientation of the formate ion. Changes of isotropically shifted resonances of the Cu,Co enzyme, where cobalt substitutes the native zinc, indicate that rearrangements of imidazoles of the liganding histidines occur upon binding. Transient NOE experiments gave indication of the proximity of the formate proton to resonance H of the NMR spectrum assigned to the imidazole proton of the copper-liganding His 118 of the active site. 2D NMR NOESY experiments made clear that no important rearrangement of the liganding histidines occurred in the presence of a saturating amount of formate. The absence of relevant changes of the intensity of NOE cross-peaks which are sensitive to interatomic distances in the active site revealed that only slight changes have occurred. Molecular graphics representation on the basis of all the information obtained allowed us to locate the formate in the proximity of the active site. The formate binding occurs via hydrogen bonds through the carboxylate ion and the NH groups of the side chains of Arg 141 which is external to the copper coordination sphere and faces the active site of the enzyme.

Cu,Zn superoxide dismutase is the most efficient isoenzyme of the superoxide dismutase family in catalyzing the disproportionation of the superoxide radical into oxygen and hydrogen peroxide. This reaction constitutes a front-line defense against cytotoxicity arising from biological oxygen activation (Bannister et al., 1987).

The three-dimensional structure of the active site of Cu,Zn enzyme has been described by crystallographic analysis of the bovine erythrocyte enzyme (Tainer et al., 1982, 1983). The copper and zinc ions are coupled together by a bridging imidazole side chain which occupies a solvent-exposed loop of the rigid β barrel structure of each identical subunit (16 kDa) of the native dimeric enzyme.

An approach to the study of the structure of the active site of Cu,Zn superoxide dismutase in solution has been NMR spectroscopy of the Cu,Co derivative (Bertini et al., 1985) in which the magnetic coupling between Cu(II) and high-spin Co(II) allows the isotropically shifted ^1H NMR resonances of the histidines bound to the oxidized metal cluster to be detected.

In order to understand the mechanism of action of this enzyme, a series of studies has been carried out concerning the interaction of different anions with the bovine enzyme (Bertini et al., 1985). These molecules are of particular interest since they mimic the physiological substrate O_2^- (Rigo et al., 1977). It has been shown that, among the anionic inhibitors of the Cu,Zn enzyme, azide, thiocyanate, and cyanate produce similar variations in the NMR spectrum of the enzyme (Banci et al., 1987; Ming et al., 1988). Such variations indicate that the Cu-liganding His 46 is displaced from the copper coordination sphere (Banci et al., 1987, 1989).

On the other hand, CN^-, the strongest anionic inhibitor of the enzyme (Paci et al., 1988) and the most plausible analog of O_2^- as far as copper coordination is concerned (Tainer et al., 1983), has shown both similarities and some slight differences in the binding pattern (Paci et al., 1988; Banci et al., 1989).

Another class of inhibitory anions (acetate, borate, chloride, formate, perchlorate, and phosphate) does not coordinate to copper, as demonstrated by the invariance of the room temperature EPR spectrum of the enzyme. Therefore, the decrease of enzyme activity in the presence of such ions is due to a primary salt effect (ionic strength inhibition). NMR studies suggested that noncoordinating anions bind to the Arg 141 residue (Mota de Freitas et al., 1987; Desideri et al., 1988), the closest (5 Å) to copper outside the first coordination sphere (Tainer et al., 1983).

Also the formation of covalent bonds through chemical reactions either reversibly with butanedione or irreversibly with phenylglyoxal as to induce modification of the polar side chain of Arg 141 produces effects in the NMR spectrum of the Cu,Co derivative (Paci et al., 1991). This observation indicates that changes in the spatial arrangement of ligands occur around the copper ion which also are not detected by the EPR spectrum (Paci et al., 1991).

Among the above-mentioned anions, formate appears to be the most suitable once to be utilized for a more detailed NMR study. In fact, it bears a covalent proton, the NMR resonance of which might behave as a sensitive probe of paramagnetic effects if the anion is bound in proximity of the active site copper.

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The interaction between formate and the enzyme has been studied in the work described in the present paper along the following lines:

(a) the interaction of formate ion with the native paramagnetic Cu(II) enzyme has been studied following the broadening of the ^{1}H NMR resonance of the formate proton and that of the ^{13}C NMR resonance of the formate carboxylic group in order to estimate the copper–proton distance and the formate orientation; (b) bulk water relaxation time was measured with pulsed low-resolution NMR in order to ascertain whether formate displaces the copper-bound water molecule(s); (c) the Cu,Co enzyme was studied to follow changes induced by formate to the isotropically shifted resonances; (d) transient NOE[1] experiments were performed by selective excitation of the formate proton resonance in order to identify the protons that are in close proximity to the bound formate; (e) 2D NMR NOESY spectroscopy of the active site in the presence of saturating amount of formate was carried out to verify that the active site geometry of the cobalt derivative is substantially unchanged upon binding.

The results obtained allowed us to locate the formate ion in the active site by molecular graphics methods.

MATERIALS AND METHODS

All chemicals were of the highest purity commercially available and were used as received.

The enzyme was isolated from bovine erythrocytes (Mc Cord & Fridovich, 1969). The Cu,Co derivative was prepared and characterized as previously described (Paci et al., 1988).

^{1}H NMR spectra were recorded in 20 mM Tris–MOPS buffer at pH 7.00 with a Bruker AM400 spectrometer. The spectra of the formate covalent proton in the presence of different concentrations of native Cu,Zn enzyme were obtained in 10% deuterated water solution using a selective presaturation irradiation. The total recycle time was 3.2 s, enough to ensure the recovery of the longitudinal magnetization of the formate resonance. The line width of the formate covalent proton was measured directly in the magnified spectra, obtained without any digital manipulation. Data were fitted according to the methods proposed by Sykes (1969) for the case of fast exchange binding dynamics which allows the association constant as well as the line widths of both the free and bound form of the formate ion covalent proton to be obtained. An user-oriented best fit procedure allowed us to fit the experimental data taking into account as variables of the binding equilibrium the dissociation constant and the line width characteristic of the resonance of the formate proton in the bound form. The linewidth of the free form extrapolated from the plot was used to obtain a first set of values. The refinement procedure was performed using all these quantities as variables. Evaluation of the distance between this proton and copper has been estimated from the Solomon–Bloembergen equation which relates the line width to the distance from a paramagnetic center (Solomon, 1955; Bloembergen, 1957).

^{13}C NMR spectroscopy of the formate carboxylic carbon (0.125 M) in the presence and in the absence of paramagnetic enzyme (5 mM) was performed at 100.56 MHz with a single 60° pulse with a relaxation delay of 5 s in addition to the acquisition time. 1024 transients were accumulated over 8K of memory. Four zero fillings were applied in order to reduce the uncertainty due to the digital resolution. The line broadening observed was compared with the expected broadening of the carbon resonance calculated as a function of the carbon–copper distance. This estimate was performed using the binding constant obtained by ^{1}H NMR as above reported.

The measurements of the longitudinal relaxation time of the bulk water by pulsed low-resolution NMR were performed at different formate concentrations in the presence and in the absence of the native paramagnetic enzyme (2.0×10^{-4} M) with an inversion recovery pulse sequence on a Bruker P20 Minispec spectrometer operating at 20 MHz.

The spectra of the isotropically shifted resonances of the Cu,Co enzyme derivatives were obtained by using a modified pulse sequence (Hochmann & Kellerhals, 1980) to suppress water and signals due to protons of diamagnetic part of the enzyme. The total recycle time was 0.36 s. Typical spectra consisted of 10 000 scans of 4K data points over a bandwidth of 83 KHz. Chemical shifts were measured from bulk water resonance which was assumed to be 4.8 ppm from TMS. ^{1}H NMR resonances are labeled as previously reported (Bertini et al., 1985).

The nuclear Overhauser effect (NOE) of a given proton was observed, as usual, recording the modification of the spectrum intensity induced by the selective excitation at the resonance frequency of a selected proton. The spectrum thus obtained was subtracted from a reference spectrum obtained with the same excitation at a frequency far apart from the region where the resonances occur in order to ensure the same experimental conditions. The selective excitation can be obtained either by continuous irradiation (steady-state NOE) or by pulse excitation (transient NOE). The latter procedure consists of a selective pulse followed by a short delay (mixing time) which allows the magnetization transfer to build up (Wüthrich, 1986). Transient excitation instead of a steady-state experiment was used in the present work, but results are expected to be similar because of the very long relaxation time of the formate proton resonance with respect to that of the isotropically shifted resonances. The experiment was performed by insertion of the selective 180° pulse at the formate proton frequency via decoupler before the acquisition of the spectrum. Different delay times (from 1 to 7 ms) allowed the NOE buildup to be observed. The power of the selective excitation pulse was carefully adjusted to avoid spill-over effects and to give a rather narrow excitation bandwidth. Also, the selective pulse duration was chosen to be compatible with the length of the T_1 relaxation times of the resonances of the protons of the active site. During the experiment a cycling over the excitation frequencies as well as over the delay times was applied in order to minimize instrumental drifts or artifacts due to the long experimental time (up to 3 days). A presaturation irradiation of 1 s was applied in order to ensure an acceptable suppression of the water resonance and the recycle time was such as to allow the resonance of the formate proton to relax.

The 2D NMR NOESY spectrum of the fast relaxing isotropically shifted resonances of the Cu,Co derivative was obtained using the normal NOESY pulse sequence (Macura & Ernst, 1980); 512 experiments of a suitable number of transients were acquired in the phase-sensitive mode using the TPPI phase-cycling procedure (Marion & Wüthrich, 1983). Data were processed by TRITON 2D NMR software (Boelens & Vuister, 1990) running on a microVax Digital Equipment Corp. computer. A real absorption spectrum 1K × 1K was thus obtained. An accurate baseline correction was applied in both dimensions by a polynomial contained in the same program.

[1] Abbreviations: MOPS, 3-morpholinopropanesulfonic acid; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; TMS, tetramethylsilane.

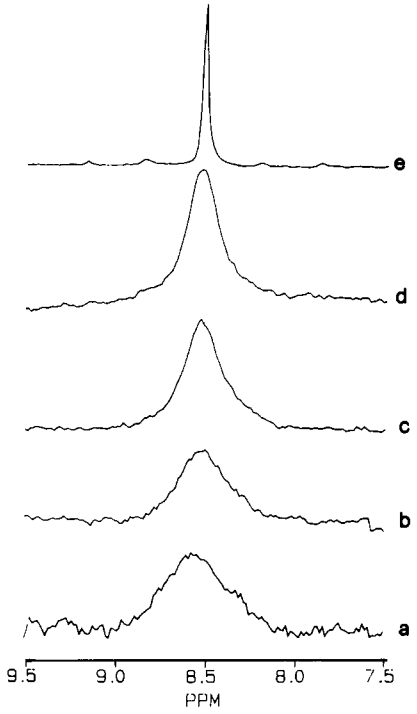


FIGURE 1: ^{1}H NMR of formate in the presence of increasing concentrations of Cu,Zn superoxide dismutase. Letters indicate solutions with the following different [formate]/[Cu,Zn enzyme] mole ratios: (a) 36.0; (b) 43.0; (c) 67.5; (d) 101.3. Spectrum e is that of the free formate at a concentration of 55 mM.

Molecular modeling of the structure of the formate ion and its molecular fitting with the enzyme active site was performed using an Evans & Sutherland PS390 graphics system linked to a microVax II using the last available version of the SYBYL program (5.41) from Tripos Associates. Stereoviews of the enzyme active site were generated from the coordinates obtained from EMBL Protein Structure Databank. Coordinates of the Cu,Co enzyme were obtained from the recently resolved crystal structure (Djinovich et al., 1992). The location of formate in the active site proximity was obtained by hand manipulation and using the X-ray atomic coordinates of both Cu,Zn and Cu,Co enzymes as a rigid scaffold to test the position of formate according to the experimentally collected data. No further refinement was applied in terms of energy. The final result was in agreement with van der Waals and NMR constraints.

RESULTS AND DISCUSSION

Binding of Formate to the Cu,Zn Enzyme

^{1}H NMR of Formate Proton. When small aliquots of a concentrated formate solution were added to a solution of Cu,Zn enzyme, a marked line broadening was observed as an effect of the fast exchange between the free and the paramagnetically bound form of the anion. Such a broadening strictly depends on the decrease of the T_2 relaxation time due both to the rather short electronic correlation time of the copper and to the coordination geometry, in particular on the inverse sixth power of the distance between the broadened proton and the metal ion.

In the presence of excess formate only a small fraction of the ligand added undergoes paramagnetic broadening, but the fast exchange dynamics still results in a broadening of the resonance of the free formate proton. Thus, following the dependence of the broadening of the formate proton resonance on the ligand concentration, it is possible to determine the line width of the bound form as well as the binding constant (see Materials and Methods).

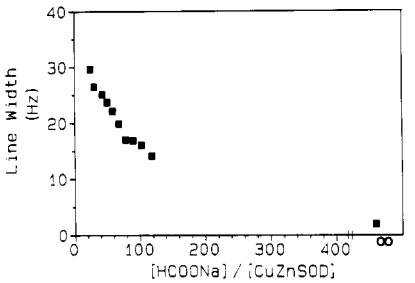


FIGURE 2: Plot of the line width of the NMR resonance of the formate proton as a function of the [formate]/[Cu,Zn enzyme] mole ratio.

Table I: Binding Parameters Obtained from the NMR Line-Width Measurements of the Formate Proton in the Presence of the Cu,Zn Enzyme and the Distance of the Formate Proton from the Copper Evaluated from the NMR Relaxation Data Obtained from the Data of Figure 2 as Reported in Materials and Methods

parameter	value
association constant (M^{-1})	4.2 ± 0.2
line width of the free formate (Hz)	2.0 ● 0.1
line width of the formate bound to the Cu,Zn enzyme (Hz)	7030 ± 20%
distance of the formate proton from copper (Å)	4.5 ÷ 4.7

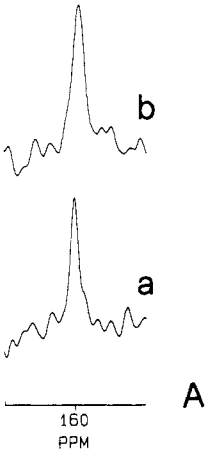
These effects are described in Figure 1. The addition of formate results in a narrowing of the resonance due to free formate until the line width eventually becomes identical to that of formate in the absence of the paramagnetic enzyme. The dependence of the half-height line width on the concentration of formate is shown in Figure 2. The values obtained for the binding constant and the line width of the bound form are reported in Table I. These values indicate that the negative charge of the formate oxygen binds close to the paramagnetic center. From calculation of the nuclear magnetic transverse relaxation time, as monitored by the line width of the resonance of the formate proton in the bound form, it is possible to state that the proton of formate is located at a distance of 4.5–4.7 Å from the paramagnetic center.

^{13}C NMR of Formate Carboxylate. The broadening of the carbon resonance of the formate carboxyl group in solutions containing saturating concentrations of formate was obtained by measuring the half-height line width in the presence of the paramagnetic enzyme as reported in Materials and Methods. The spectra obtained of the carboxylate carbon resonance in the absence as well as in the presence of Cu,Zn enzyme are reported in Figure 3A. As shown in the spectra, slight effects are induced by the enzyme copper–formate interaction on the line width of formate. The measure of the broadening observed was compared with the paramagnetic broadening expected for different distances between carboxyl carbon and the copper (Figure 3B). A value of approximately 5.3 Å was found for the carbon–copper distance.

This result indicates that formate places its hydrogen atom toward the copper and the carboxylate away from the metal. Molecular modeling indicates (see below) that the carboxylate is located in a position corresponding to the positively charged end of Arg 141.

Water NMR Relaxation

Pulsed NMR relaxation time measurements were performed in order to clarify whether, upon binding, formate displaces the copper-bound water molecules(s).



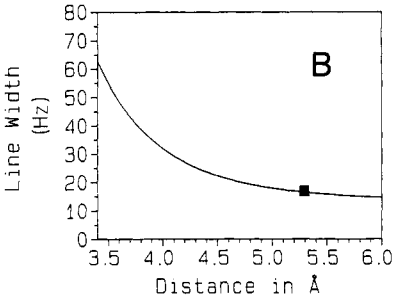


FIGURE 3: (A) ^{13}C NMR spectrum of formate in the absence (a) and in the presence (b) of Cu,Zn superoxide dismutase. The spectral region shown corresponds to that of the carboxyl carbon of formate. The spectra were obtained as reported in Materials and Methods. (B) The experimentally obtained line width of the carbon resonance (■) in the presence of enzyme is reported together with a solid curve showing the values expected for the line width calculated at different carbon–copper distances.

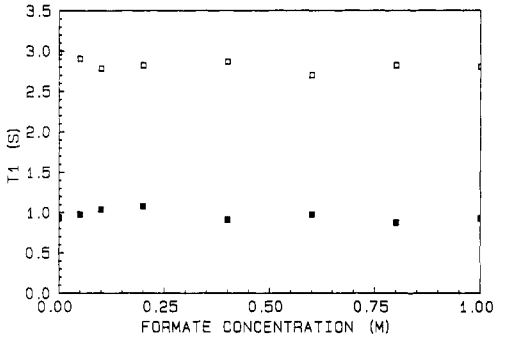


FIGURE 4: ^1H NMR T_1 relaxation time of protons of bulk water at increasing concentrations of formate in the presence (■) and in the absence (□) of paramagnetic Cu,Zn enzyme. The experiments were performed as reported in Materials and Methods in pulsed low-resolution NMR. The enzyme concentration was 0.2 mM.

The results obtained in the presence of increasing concentrations of formate in the presence as well in the absence of the paramagnetic Cu,Zn enzyme are reported in Figure 4 and indicate that binding of formate does not affect the coordination of water in the active site.

NMR of Hyperfine-Shifted Resonances of the Cu,Co Enzyme

Recent 2D NMR NOESY studies allowed definitive assignment of the isotropically shifted resonances of the copper-coordinated histidines of the Cu,Co derivative (Unpublished experiments; M. Sette, M. Paci, A. Desideri, and G. Rotilio, submitted for publication), on the basis of the interatomic distances obtained from the original X-ray crystal structure of this derivative (Djinovich et al., 1992). Figure 5 reports these assignments on a scheme of the structure. Capital letters in Figure 5 refer to hyperfine-shifted resonances in the high-

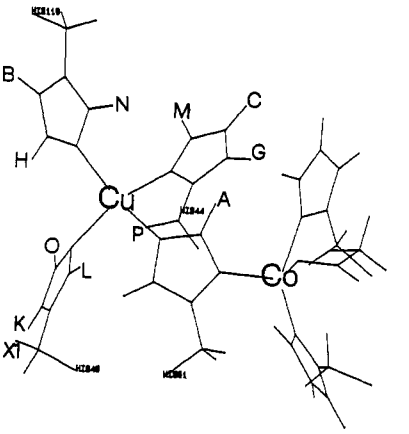


FIGURE 5: Scheme of the active site of Cu,Co enzyme with the related assignments.

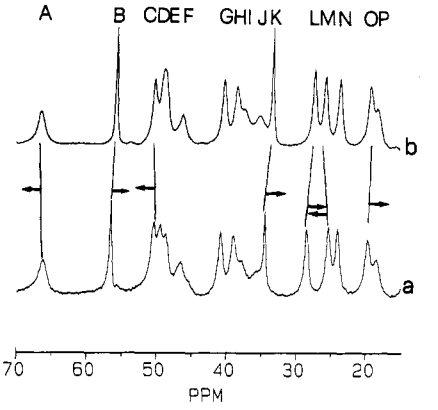


FIGURE 6: NMR spectrum of the isotropically shifted resonances due to the protons of the active site of the Cu,Co enzyme (a) and in the presence of a saturating concentration of formate (b). Letters label resonances as previously reported (Bertini et al., 1985).

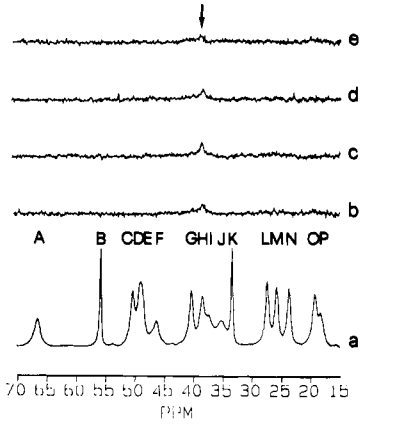


FIGURE 7: A 400-MHz transient NOE experiment. Spectrum a is a reference spectrum. Spectra b, c, d, and e report the difference spectrum between an irradiation on the frequency of the formate proton resonance and the reference spectrum after a variable delay time to allow the magnetization transfer to build up. Spectra were obtained as reported in Materials and Methods. Letters label resonances as in Figure 6. Delay times: (b) 1 ms, (c) 3 ms, (d) 5 ms, and (e) 7 ms. The magnetization transfer due to NOE of the formate proton with the band H is clearly visible.

resolution NMR spectrum as currently used for superoxide dismutase (Bertini et al., 1985).

Figure 6 reports the NMR spectra of the Cu,Co enzyme before and after the addition of a saturating concentration of formate. The magnitude of the changes of the isotropically shifted resonances is small, but the trend is similar to that observed in the presence of other anions. In particular, upon addition of formate (Figure 6b), the downfield shift of the A, C, and M bands and the highfield shift of the B, K, L, and O bands resemble the shifts observed in the case of azide binding to the Cu,Co derivative (Banci et al., 1987). This result indicates that these changes are not unique to copper-coordinating anions, in line with recent data (Paci et al., 1991) suggesting that the binding of anions to Arg 141, which is the nearest-neighbor residue outside the first copper coordination sphere (Tainer et al., 1983), induces a rearrangement of the spatial orientation of the coordinating imidazole rings.

Transient NOE between the Formate Proton and the Active Site Protons

In the presence of excess formate, NOEs between the formate proton and the active site protons were observed (Figure 7).

The NOE spectra reported in Figure 7 indicate that a dipolar interaction at short distance occurs between the formate proton and the Cε1 proton of His 118 to which resonance H is due (See Figure 5).

Different mixing times were used to observe the increase and subsequent decrease of the magnetization transfer. This behavior is due to the contemporary effect of the buildup of the magnetization transfer from the bound formate proton to the active site proton and the rather fast relaxation of the latter proton. The two processes influence the intensity of the transferred magnetization depending on the length of the delay time. In Figure 8 the time evolution of the intensity of the magnetization transfer is reported. This behavior indicates the proximity of the formate proton to a fast relaxing neighborhood due to the copper ion, thus confirming the binding pattern suggested above.

2D NMR NOESY of the Active Site Protons

In a series of 2D NMR NOESY experiments the NOEs between the active site protons of the Cu,Co derivative in the presence and in the absence of formate were recorded.

The very fast relaxation behavior of the isotropically shifted resonances of the active site protons (relaxation times range from 1 to 8 ms) and the consequent low intensity of the magnetization transfer make the NOEs very difficult to be observed. Nevertheless they are sensitive to small changes of interatomic distances. It is then important to establish whether the overall geometry of the active site is maintained in the presence of formate.

The NOESY spectrum reported in Figure 9 was obtained in the presence of a saturating amount of formate with a mixing time of 5.0 ms. Beside the vertical stripe of noise between 9 and −2 ppm produced by the residual resonance of water and of protons of the diamagnetic protein moiety, a number of cross-peaks are clearly distinguishable. Identical NOEs were observed in the same experimental conditions in the absence of formate (not shown). In particular the cross-peaks due to NOEs L/P and K/X1 show the same relative intensity in the presence or in the absence of formate. These two pairs of NOEs may be considered indicative that the global structure of the active site is unchanged. In fact, the first one occurs between two different histidines (His46Cδ2 and His44β,

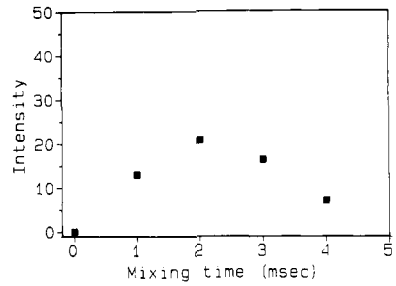


FIGURE 8: Plot of the intensity of the NOEs observed in Figure 7 as a function of the mixing time.

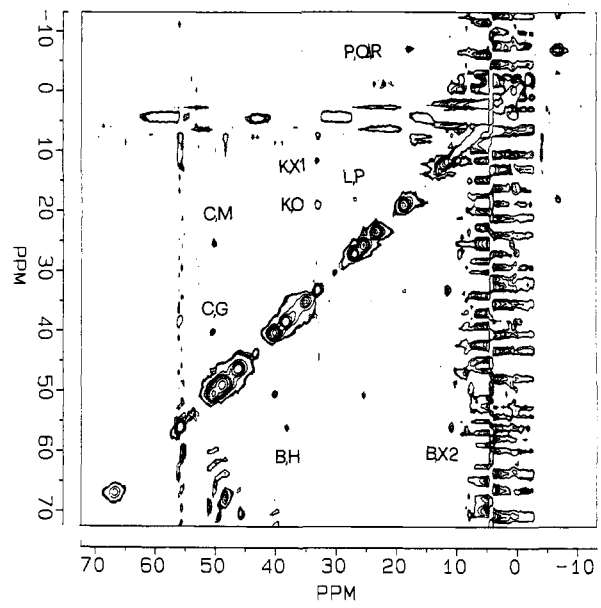


FIGURE 9: NOESY spectrum of the Cu,Co enzyme in the presence of saturating amounts of formate. The spectrum was obtained with a mixing time of 5 ms as reported in Materials and Methods.

respectively; see Figure 5). The latter NOE, on the other hand, occurs between an imidazole proton and the β proton belonging to the same residue (His46Nε1 and His46β, respectively; see Figure 5) and is therefore very sensitive to small conformational changes of this residue. The lack of effect by formate on the NOESY is therefore unequivocal evidence that the active site undergoes only small changes of the interatomic distances upon binding with formate.

Location of the Formate Proton in the Active Site by Molecular Graphics

Figure 10 shows a computer modeled structure of the active site geometry with the position of the formate ion as derived from the results reported above.

Since the active site conformation in solution upon the binding of formate as revealed from 2D NMR experiments is similar to that in the absence of formate the crystal coordinates of the unligated Cu,Co enzyme (Djinovich et al., 1992) were used for the modeling.

The formate proton was placed at a distance compatible with that established by NMR (4.65 Å) and with such an orientation as to locate the carboxylate carbon atom at a distance from the copper which is as close as possible to that measured from the broadening of the carbon resonance (5.3 Å, Figure 3). This operation was performed in such a way as not to affect the atomic radii of coordinated water. This location procedure produced a distance of 2.8 Å between the formate proton and proton Cε1 of His 118. This finding is

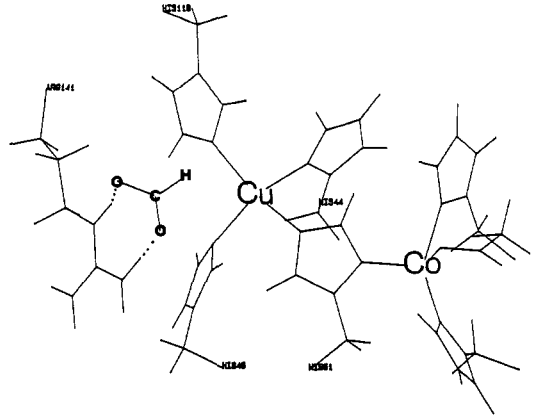


FIGURE 10: Location of formate in the active site of the Cu,Co enzyme obtained by molecular graphics as reported in Material and Methods.

in very good agreement with the above reported NOE found between the formate proton and resonance H. Moreover, no ambiguity arises from the other active site protons because they show interatomic distances longer than those for which NOEs are expected. In fact, 118 Cδ2 (N) and 118 Nδ1 (B) are at 4.3 and 4.6 Å, respectively, and protons 46 Nδ1 (K), 46 Cϵ1 (O), and 46 Cδ2 (L) are at 5.8, 3.6, and 7.3 Å respectively, while all the protons of the His 44 imidazole are at a distance higher than 6 Å.

The location of formate was found to satisfy both van der Waals and NMR constraints and appeared to involve binding of the formate negative charge with the charged surface of the Arg 141 side chain. In particular, two well-oriented hydrogen bonds (Figure 10, dotted lines) were found to stabilize the interaction between formate oxygens and the proximal and terminal nitrogens of the Arg 141 side chain (Nϵ and NH_2). The distance between the donor nitrogen atoms and the acceptor oxygen atoms was in both cases found to be 2.85 Å. Furthermore, the N–H–O angle was found to be 153° and 140° for the bonds involving the Nϵ and the NH_2 of Arg 141, respectively.

CONCLUSIONS

The results described above lead to the conclusion that no displacement of the copper-coordinated water molecule occurs upon binding of formate to the active site of Cu,Zn superoxide dismutase and that the anion binds to the polar termination of Arg 141 without entering the inner coordination sphere of the copper ion.

Small variations of the mutual orientations of the imidazole rings of the copper-coordinating histidines upon binding were monitored by the changes of the chemical shift of the isotropically shifted resonances. Such variations were found to follow the same trend observed for the interaction of the enzyme with other ions (Bertini et al., 1985) and when the side chain of Arg 141 of the enzyme is neutralized by chemical modification (Paci et al., 1991).

Nevertheless these changes do not significantly alter the interatomic distances of the active site as revealed by the 2D NMR NOESY experiments.

The mode of binding of formate to the active site of the enzyme is likely to mimic the interaction of the substrate O_2^- with the catalytically active copper ion.

However, it has been suggested that superoxide displaces a water molecule bridging the guanidinium side chain of Arg 141 and the copper-coordination sphere (Tainer et al., 1983). The formate binding can be then used as a model for the first step of the activation process of the substrate in the proximity of the catalytic center.

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