Fourier Transform Infrared Analysis of the Interaction of Azide with the Active Site of Oxidized and Reduced Bovine Cu,Zn Superoxide Dismutase†

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ABSTRACT: Binding of azide to the native and arginine-modified bovine Cu,Zn superoxide dismutase in the oxidized and reduced form and to the copper-free derivative has been investigated by Fourier transform infrared spectroscopy. The antisymmetric stretching band of the azide is shifted to higher energy upon coordination to the copper atom of the oxidized form of the native enzyme. Similar spectral changes occur upon interaction of the anion with the Cu-diethylenetriamine model compound. On the other hand, interaction of azide with the native reduced form of the enzyme results in a band shift toward lower energy with respect to the free anion band. The same shift is observed after reaction of the azide with free lysine or arginine but not when it is reacted with other amino acid residues. The antisymmetric band of the azide is not perturbed by addition of the reduced arginine-modified enzyme; it is likely shifted toward higher energy upon addition of oxidized arginine-modified enzyme while it is again shifted toward lower energy in the presence of the copper-free derivative of the unmodified enzyme. It is concluded that azide does not directly coordinate to the copper in the reduced form of Cu,Zn superoxide dismutase but it remains in the active-site pocket in electrostatic interaction with the guanidinium group of Arg141, which is an invariant residue in this class of enzymes.

Cu, ZnSOD[1] are a class of evolutionary conserved enzyme, which usually have two identical subunits each containing a metal cluster, the active site, constituted by a copper and a zinc atom bridged by a common ligand (His 61). The solvent-accessible copper ion in the oxidized state is coordinated to three more histidyl residues and to one water molecule, hydrogen-bonded to the guanidinium group of the invariant Arg 141 (*1*, *2*), while the buried, solvent-inaccessible zinc atom is coordinated to two more histidyl and one aspartic group. The reaction catalyzed by the enzyme is the disproportion of the superoxide anion into oxygen and hydrogen peroxide through a cyclic oxidation−reduction of the copper ion (*3*).

Cu,ZnSOD is competitively inhibited by singly charged anions such as N_3^- and CN^- (*4*). The inhibition has been attributed to a direct coordination to the copper when the metal is in the oxidized state as shown by the changes of the EPR spectrum observed upon titration with these anions (*5*, *6*). The direct coordination of N_3^- to the oxidized copper is also demonstrated by the presence of an intense LMCT band in the optical absorption spectrum (*7*). The fine details of such interaction has been described by the X-ray diffraction analysis of the N_3^- and CN^- derivatives, which has shown that, besides a direct interaction with the copper, the anions also form an ion pair with the guanidinium group of the fully conserved Arg 141 (*8*, *9*). Much less is known on the protein−anion interaction when the copper is in the reduced state. In solution, reduction of copper brings the detachment of the His 61 imidazole bridge from the copper site (*10*, *11*, *2*). This detachment has been claimed not to occur when the protein is in the crystal state (*12*, *13*); however, more recent works have demonstrated that breaking of the imidazolate bridge is occurring both in solution and in the crystal state, leaving the copper bound in a trigonal coordination with the remaining histidine residues (*14*−*16*). No crystal structure describing the interaction of the anions with the reduced copper protein is available. A systematic ^{35}Cl NMR study, monitoring the binding of the Cl^- to the enzyme, both native and selectively chemically modified at arginine or at lysine amino acid residues in the reduced state, provided convincing evidence that positively charged residues, located in the proximity of the native site, may serve as anion binding sites in the reduced proteins (*17*). However, up to now the anion's binding to oxidized and reduced Cu,ZnSOD using the same experimental approach, which allows a comparative investigation, has never been carried out.

Fourier transform infrared (FTIR) spectroscopy has been widely used to probe protein−ligand interaction (*18*−*21*) by

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[1] Abbreviations: SOD, Cu,Zn superoxide dismutase; DETA, diethylenetriamine; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; LMCT, ligand to metal charge transfer; NMR, nuclear magnetic resonance.

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monitoring the perturbation at the level of the ligand upon protein binding. In particular, reports on vibrational spectra of azide bound to metal sites in proteins indicate that this approach can provide important information on metal site structure and properties (*22*−*26*).

In this work we have investigated the perturbation of the antisymmetric stretching vibration of the azide anion in a copper model complex, in three different derivatives of Cu,ZnSOD, and in the presence of several free amino acids. In particular, infrared investigation of the azide in the presence of native and arginine chemically modified protein in the oxidized and reduced state, as well as in the presence of the copper-free Cu,ZnSOD, and comparison with the results obtained with the model system and in the presence of free amino acid residues enabled us to demonstrate that azide displays a different way of binding when the enzyme has the copper atom in the oxidized or reduced state. This finding is of functional relevance since, due to the analogy between azide and the superoxide substrate, it may be inferred that superoxide directly coordinates to copper when the metal is in the oxidized but not when it is in the reduced state.

MATERIALS AND METHODS

Samples Preparation. Diethylenetriamine and sodium azide were purchased from Fluka; arginine, lysine, alanine, glutamate, threonine, and phenylglyoxal from were Sigma. Cu-DETA was obtained by mixing equimolar amounts of DETA and cupric acetate (Fluka), following the procedure described by Morpurgo et al. (*7*). Bovine Cu,Zn superoxide dismutase was purified from bovine erythrocytes as previously described (*27*). Cu-depleted enzyme (E,ZnSOD) was made following the procedure of Calabrese et al. (*28*). The copper reduction was obtained by anaerobically adding small amounts of sodium dithionite to a deoxygenated Cu(II), Zn(II)SOD sample until the color of the protein was bleached, immediately before measuring the IR spectrum. Bovine Cu,ZnSOD was chemically modified at Arg141 with phenylglyoxal and purified by the method of Malinowski and Fridovich (*29*).

Cu,ZnSOD activity of the arginine-modified protein was less than 10% that of the native protein as measured by the pyrogallol method (*30*). Protein concentrations were determined by the Lowry method (*31*). Metal analysis of the copper-free SOD preparations by atomic absorption (Perkin-Elmer 3030) showed that the Zn concentration was the same as that the native enzyme, while less than 3% the native concentration of copper was present.

FTIR Measurements. FTIR spectra were measured at room temperature using a Bio-Rad FTS-40A FTIR spectrophotometer equipped with a PbS detector. Samples were placed in a demountable Specac cell with CaF_2 windows and a 0.05 mm spacer. For each experiment, the single beam spectrum in the 1800−2500 cm^{-1} wavenumber range was measured with 256 scans at 2 cm^{-1} resolution. The absorption spectra of the N_3^--containing samples and of the respective solvents were calculated with respect to the empty cell. The absorption spectra of the solvents were subtracted from sample spectra, after suitable normalization to obtain a zero absorption value at 2100 cm^{-1}. Protein and sodium azide concentrations used for FTIR measurements were 1.5 $\times$ 10^{-3} and 10^{-2} M, respectively. Care was taken to use an identical enzyme and N_3^- concentration for all the samples.

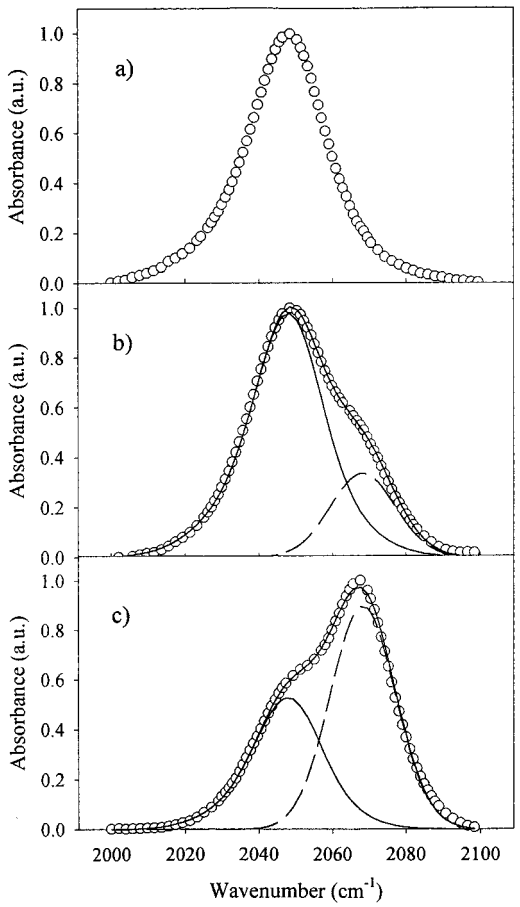

FIGURE 1: FTIR spectra of (a) 1 $\times$ 10^{-2} M azide free in water solution; the spectrum is not modified by the presence of different concentration of phosphate or Tris-HCl buffer, pH = 7 in the range 0−1 M; (b) 1 $\times$ 10^{-2} M azide in 0.1 M phosphate, pH 7, + 1 $\times$ 10^{-2} M Cu-DETA; (c) 1 $\times$ 10^{-2} M azide in 0.1 M phosphate, pH 7, + 5 $\times$ 10^{-2} M Cu-DETA. All spectra have been normalized to a maximum absorbance value of 1. (○) experimental points; (—): spectrum of free azide and overall synthesized band profile; (---) spectrum of bound azide. χ^2 values are 6 $\times$ 10^{-5} and 1.7 $\times$ 10^{-4} for panels b and c, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the FTIR spectrum of the azide anion free in solution (panel a) and in the presence of the Cu-DETA complex at two different [Cu]/[N_3^-] ratios (panels b and c). The free azide has a single band at 2047 cm^{-1}, while in the presence of Cu-DETA two bands were observed, the first at 2047 cm^{-1} and the second at 2068 cm^{-1}. The intensity of the high- and of the low-frequency bands increase and decrease, respectively, upon increasing the [Cu]/[N_3^-] ratio, indicating the occurrence of a single equilibrium and that the band of azide is shifted toward higher energy upon copper coordination. N_3^- is known to directly coordinate to the copper of the Cu-DETA complex, giving rise to an LMCT optical absorption band (*7*). This conclusion is confirmed by the spectral deconvolution (Figure 1b,c). The measured spectra were first normalized to unity maximum intensity; the normalized spectra of N_3^- were then deconvoluted as the sum of a fraction of the spectrum of free azide plus a

Table 1 [a]

sample	ν_1 (cm^{-1})	σ_1 (cm^{-1})	ν_2 (cm^{-1})	σ_2 (cm^{-1})	f_{bound} (meas)	f_{bound} (calc)
N_3^- + CuDETA 10^{-2} M	2068	9.0			0.25	0.22
N_3^- + CuDETA 5×10^{-2} M	2068	8.9			0.65	0.75
N_3^- + Cu(II)Zn(II)SOD	2058	7.2	2041	11.0	0.20	0.17
N_3^- + E,Zn(II)SOD			2040	11.0	0.17	
N_3^- + Cu(I)Zn(II)SOD			2043	11.7	0.16	
N_3^- + Arg			2041	11.1	0.23	
N_3^- + Lys			2042	9.8	0.24	
SOD Arg141(CuII)	2058	10.0			0.04	

[a] The experimental spectra (see Figures 1, 3, and 4), after normalization, are deconvoluted as the sum of a fraction of free azide (see Figure 1a) plus one or two Gaussian components. ν_1 and σ_1 denote the peak frequency and halfwidth of the Gaussian components used to fit the spectral contributions of bound azide. f_{bound} represents the total fraction of bound N_3^-. Spectra of N_3^- + DETA have been performed with $[N_3^-] = 2 \times 10^{-2}$ M; all other spectra with $[N_3^-] = 10^{-2}$ M. The estimated errors are ± 1 cm^{-1} for ν and ± 0.5 cm^{-1} for σ.

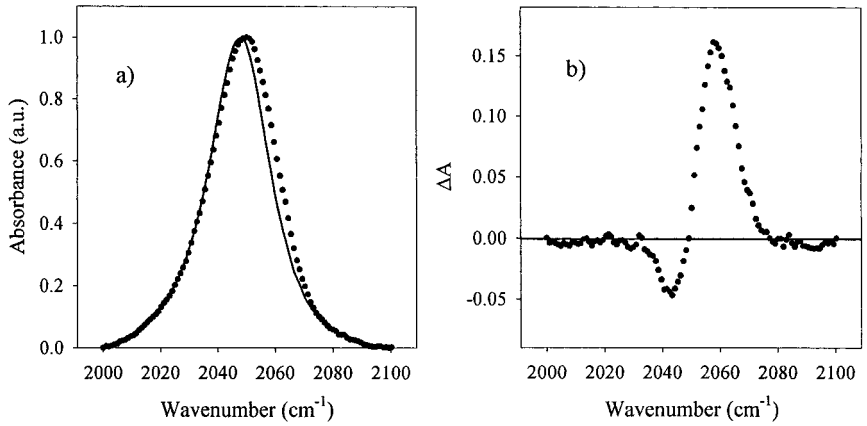

FIGURE 2: (a) FTIR spectrum of 1×10^{-2} M azide in the presence of 1.5×10^{-3} M Cu(II),Zn(II)SOD in 10 mM Tris-HCl, buffer pH 7 (●), as compared to that of free azide (—). (b) Difference spectrum. The measured spectrum has been normalized to unity maximum intensity.

Gaussian band (continuous and dashed lines in Figure 1b,c; see also Table 1). Under the assumption of identical molar extinction coefficients for free and Cu(II)-bound N_3^-, the percentage of bound azide was calculated from the areas of the low- and high-frequency bands and it turns out to be 0.25 and 0.65 for the experimental conditions of panels b and c, respectively, in Figure 1; these values compare fairly well with the values of 0.22 and 0.75 calculating assuming a Cu-DETA N_3^- association constant of 150 M^{-1}, as reported in the literature (*7*). Analogous results were obtained by deconvoluting the spectrum using a Voigtian line shape.

A behavior similar to that displayed by the Cu-DETA complex is observed in the FTIR spectrum upon addition of azide to the oxidized Cu(II),Zn(II)SOD enzyme (Figure 2a). In this case the perturbation of the azide band is more difficult to detect because the final enzyme concentration is lower with respect to that of the Cu-DETA complex and because the spectral shift is smaller (Table 1). However, the difference spectrum reported in Figure 2b clearly shows that also in this case binding of azide to copper brings a shift of the antisymmetric stretching band of the azide toward higher frequency, although less pronounced than that observed for the Cu-DETA− N_3^- complex; it should also be noted that the difference spectrum reported in Figure 2b has a nonsymmetric shape. The results are confirmed by spectral deconvolution (Figure 3 and Table 1): the experimental spectrum is obtained as the sum of a fraction of the spectrum of free azide (intense band at 2047 cm^{-1}) plus two less intense Gaussian bands, one at 2058 cm^{-1} corresponding to the Cu(II),Zn(II)SOD−N_3^- complex and the other at 2041 cm^{-1} corresponding to some other form of bound N_3^-. We have also tried to fit the experimental spectrum by assuming a single Gaussian for the bound azide, but in this case systematic misfits were obtained and the c_2 increased by a

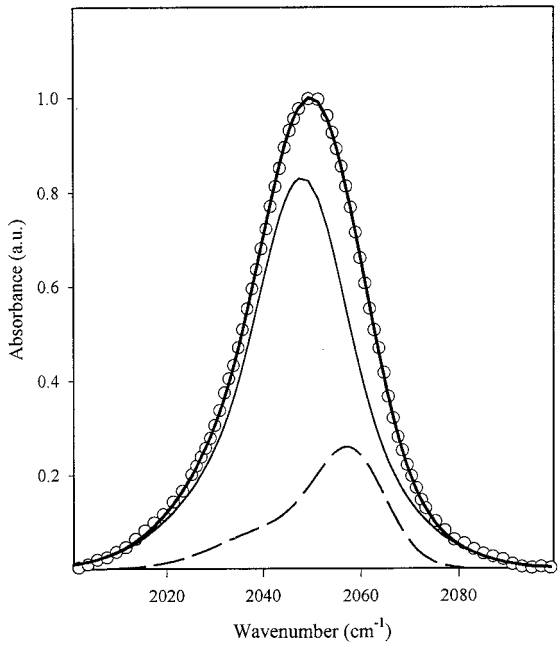

FIGURE 3: Deconvolution of the spectrum of Figure 2a corresponding to 1×10^{-2} M azide in 10 mM Tris-HCl pH 7.4, in the presence of 1.5×10^{-3} M Cu(II),Zn(II)SOD. Open circles are the experimental points; the continuous and dashed lines represent the spectra of free and bound azide, respectively.

factor of 3. The overall fraction of bound azide, calculated by the areas of the bands, amounts to 0.20, in good agreement with the expected value obtained from the Cu(II),Zn(II)-SOD$-N_3^-$ association constant (*5*, *6*).

In oxidized Cu(II),Zn(II)SOD, azide is known to directly coordinate to the copper and not to the solvent-inaccessible zinc (*5*, *6*, *8*) and to display an intense LMCT bound in the electronic spectrum (*7*, *32*). The fine details of the Cu(II), Zn(II)SOD$-N_3^-$ interaction have been described in a recent X-ray diffraction study, which has shown that azide coordinates to the copper atom, displacing the copper-coordinated water molecule and forming an almost square planar geometry with three histidines (His 44, 61, and 118) and with His 46 playing the role of the fifth axial ligand (*9*). Moreover, the linear anion forms an ion pair with the guanidinium group of the fully conserved Arg 141, which has been indicated to have a crucial role in determining the correct orientation of the superoxide substrate toward the catalytic copper ion (*33*, *34*). The concomitant presence of two positively charged groups (the Cu^{2+} ion and the guanidinium group) bridging the negative anion may give a suggestion for the interpretation of the FTIR spectra. The two bands (Figure 3) may originate from two populations of macromolecules having the N_3^- bound in the oxidized active site in two different ways: the first in which N_3^- is directly bound to the Cu(II) atom (higher frequency band) and the second interacting mainly with Arg141 (lower frequency band). Contribution to the lower frequency band can also come from the interactions of N_3^- with other positively charged residues located in the proximity of the active site, such as Lys 120 and Lys 134, which have been shown to play an important role in the attraction of the substrate toward the active site (*35*−*37*).

To verify the role of the positively charged residues in the interaction with N_3^-, the anion has been reacted with the E,ZnSOD derivative, in which the copper has been selectively removed (*28*). The spectrum reported in Figure 4a may be deconvoluted as the sum of the spectrum of free azide (intense band at 2047 cm^{-1}) plus one less intense Gaussian band at 2040 cm^{-1} (see also the data in Table 1). The lack of the copper atom in the E,ZnSOD enzyme confirms that the shift toward higher energy of the antisymmetric band of the azide is due to the interaction with the metal, while that toward lower energy is due to the interaction with some positively charged amino acid residues.

In agreement with this interpretation, an almost identical shift of the antisymmetric band of the azide is also observed upon reaction of the anion with an excess of free arginine or lysine residues ([aa]/[N_3^-] = 60), while the band is not shifted at all upon addition of other amino acids such as threonine, alanine, or glutamate, as shown in Figure 5a, where the interaction of N_3^- with the amino acid residues has been reported as difference spectra with respect to the free azide. The spectrum obtained in the presence of arginine (Figure 4b) or lysine may be deconvoluted as the sum of the spectrum of free azide (intense band at 2047 cm^{-1}) plus one less intense Gaussian band at 2041 cm^{-1} and at 2042 cm^{-1}, respectively (see the data in Table 1), indicating that the electrostatic interaction between the negatively charged anion and a positively charged group induces a shift of the azide band toward lower frequency.

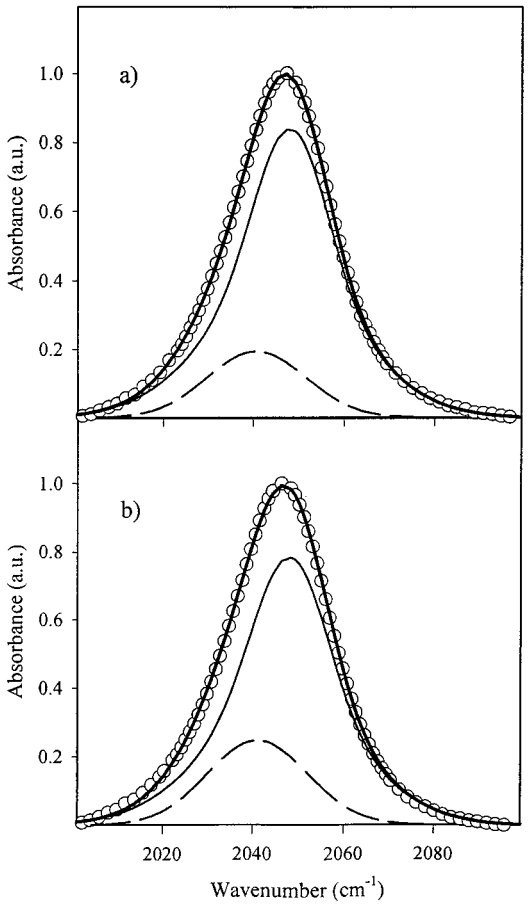

FIGURE 4: FTIR spectra of 1×10^{-2} M azide in 10 mM Tris-HCl pH 7.4, in the presence of (a) 1.5×10^{-3} M copper-depleted SOD [E,Zn(II)SOD] or (b) excess free arginine ([Arg]/[N_3^-] = 60). Open circles are the experimental points; the continuous and dashed lines represent the spectra of free and bound azide, respectively.

The perturbation of the N_3^- antisymmetric stretching band upon addition of Cu(I),Zn(II)SOD has also been investigated. For the sake of clarity, the results are reported in Figure 5b as difference spectra with respect to the free azide. The difference spectra obtained for the sample with the oxidized and with the copper-free enzyme are also reported for comparison. The difference spectra indicate that the interaction with the native oxidized enzyme mainly produces a shift of the antisymmetric stretching band of the azide toward higher energy, while in the other two cases the opposite effect (i.e., shift toward lower energy) is observed. This is confirmed by the spectral analysis, showing that the experimental spectrum measured in the presence of the reduced enzyme results from the sum of a fraction of the spectrum of free azide (intense band at 2047 cm^{-1}) plus one less intense Gaussian band at 2043 cm^{-1} (see Table 1). The opposite spectral shift observed in the oxidized and reduced forms of the enzyme, together with the similar effect observed for the reduced and copper-free enzyme and the free positively charged amino acid residues, strongly indicates that in the reduced enzyme there is not a direct copper−anion coordination but rather that the anion prefers to interact with positively charged groups, likely of amino acid residues, located in the proximity of the active site. The possibility that such a red shift is caused by the interaction of the anion with the Zn(II) can be excluded since the zinc ion is known

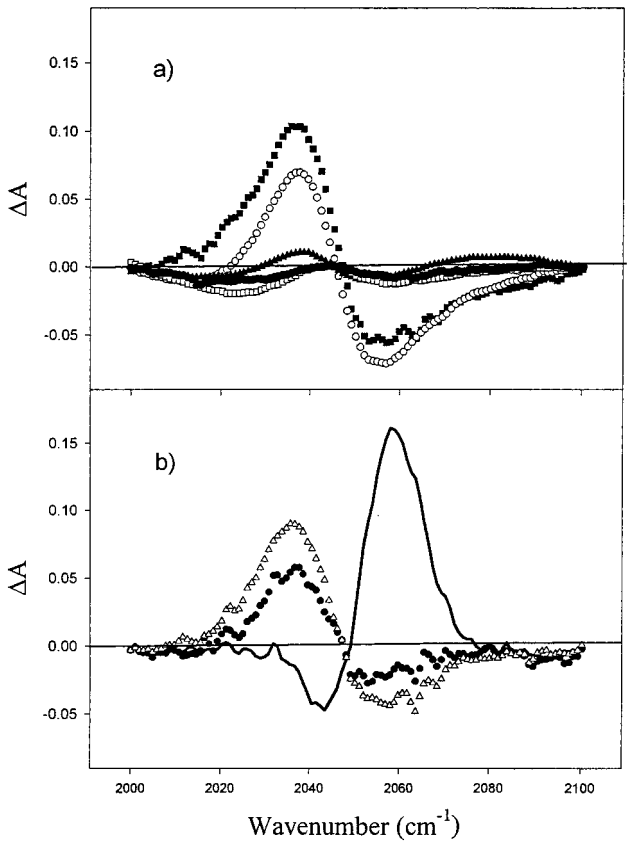

FIGURE 5: Difference spectra obtained with respect to free azide, after normalization. (a) N_3^- + excess free amino acids ([aa]/[N_3^-] = 60); (■) Arg; (○) Lys; (▲) Ala; (▽) Glu; (□) Thr. (b) (—), N_3^- + Cu(II),Zn(II)SOD; △, N_3^- + E,Zn(II)SOD; (●) N_3^- + Cu(I), Zn(II)SOD.

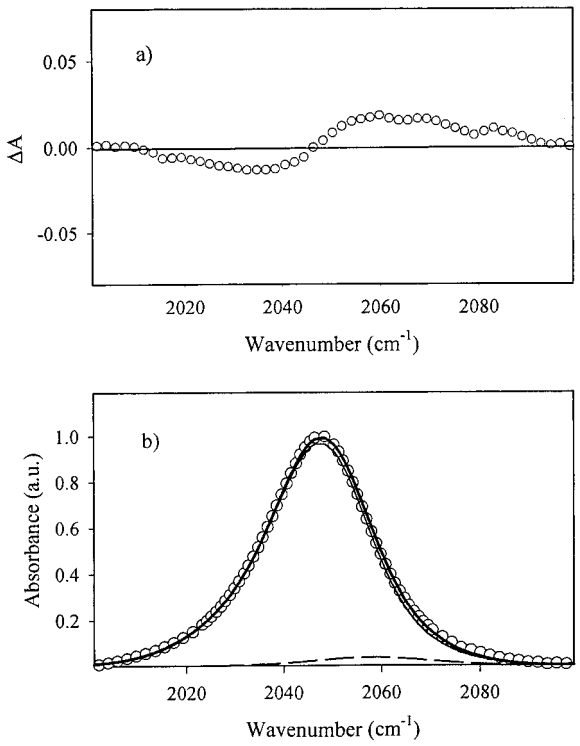

FIGURE 6: Effect of 1.5×10^{-3} M oxidized, Arg141 chemically modified SOD in 10 mM Tris-HCl pH 7.4, on a solution of 1×10^{-2} M azide. (a) Difference spectrum with respect to the free azide; (b) spectral deconvolution; symbols as in Figure 1. The χ^2 value is 1×10^{-4}.

to be buried and screened from the solvent (*9*, *15*) and it has never been reported to interact with anions (*38*, *3*). The small difference in the shift observed in the low-frequency band of the N_3^- interacting with free positively charged amino acid residues or with the copper-free or reduced enzyme likely reflects the different local environment felt by the anion.

The structural evidence coming from X-ray diffraction of the oxidized Cu(II),Zn(II)SOD, of a direct interaction between N_3^- and the fully conserved Arg 141, makes this residue the most likely target of the azide in the E,ZnSOD derivative and in the Cu(I),Zn(II)SOD. In line with this, a previous ^{35}Cl NMR study of a different derivative of Cu(I),Zn(II)SOD indicated Arg 141 as a possible interacting site for the anion (*17*). This hypothesis is supported by the conserved distribution of the electric field produced by the charge of the protein (*39*), which has the role of attracting the substrate toward the active site and which is specifically directed toward the guanidinium group of Arg 141 (*40*, *41*). Moreover, both simulations and active-site mutagenesis experiments have shown that, although positively charged residues such as Lys 120 and Lys 134 have a role in modulating the attraction of the substrate (*35*−*37*), only deletion of Arg 141 has a critical effect in the enzyme−substrate association and in the catalytic rate of enzyme (*34*), indicating that this residue has a specific role in the correct positioning of the substrate.

The perturbation of the N_3^- antisymmetric band upon addition of oxidized and reduced Cu,ZnSOD chemically modified at arginine 141, in which the guanidinium group has been selectively reacted with phenylglyoxal, has been investigated to clarify this point. The results relative to the oxidized, chemically modified enzyme are reported in Figure 6a as a difference spectrum with respect to the free anion and in Figure 6b as a normalized spectrum together with its spectral deconvolution. The perturbation introduced by the oxidized chemically modified enzyme is very small, almost within the limits of our spectral resolution; however, the presence of a small fraction (about 5%) of bound azide at 2058 cm^{-1} (see Table 1) is needed to exactly reproduce the spectrum, which displays a small blue shift. In the presence of the reduced, chemically modified enzyme the spectrum of the N_3^- is completely unaffected. In both cases, the presence of a band at 2041 cm^{-1} with a resulting overall red shift of the spectrum is excluded. The smallness of the spectral perturbation observed in the presence of the oxidized chemically modified enzyme is due to the fact that the affinity constant of the modified enzyme for anions is much lower than that of the native enzyme (*42*). The absence of any red shift of the N_3^- band upon addition of the reduced and oxidized, arginine-modified Cu,ZnSOD allows us to exclude the occurrence of an interaction with the positively charged lysine residues, leaving Arg 141 as the preferred target for the anion−enzyme interaction.

It is interesting to notice that the fraction of "bound" azide (Table 1) in the reduced form of the native enzyme (0.16) calculated from the band areas is similar to that observed when copper is in the oxidized state (0.20) or is absent (0.17), indicating that the enzyme provides, independently of the presence and of the oxidation state of the metal, an almost identical association constant for the azide and confirming

that Arg 141 has a crucial role in determining the enzyme−anion interaction (*34*).

CONCLUSIONS

The results presented in this paper indicate that the competitive inhibitor azide is able to directly coordinate to the oxidized but not to the reduced copper of Cu,ZnSOD; moreover, in the reduced enzyme an interaction between the N_3^- and the Arg 141 amino acid residue has been clearly demonstrated. Since N_3^- has been suggested to mimic the superoxide substrate, it may be envisaged that in the generally accepted mechanism of dismutation involving cyclic reduction and reoxidation of copper (*33*, *43*) the superoxide directly binds to the oxidized but not to the reduced metal. In light of this result the mechanism of superoxide dismutation proposed in two theoretical studies (*44*, *45*) must be reconsidered. Both these models of enzyme action suggest the interaction of substrate with Arg 141 in the reduced enzyme and together with our results provide a suitable explanation for the full conservation of Arg 141 in this class of enzymes (*46*).

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