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ARTICLE

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Solvent effects on the distribution of conformational substates in native and azide reacted Cu, Zn superoxide dismutase

An EPR study

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Abstract Native and azide reacted Cu, Zn superoxide dismutase in aqueous and mixed water-glycerol solution have been investigated by EPR spectroscopy at low temperature. An accurate computer simulation, based on a well established theoretical model which has been reformulated for rhombic symmetry, has shown that the EPR spectrum of the copper ion in the native protein shows a significant g and A strain in the parallel region. The strain arises from a distribution of the ligand field strengths onto the metal ion and this could be traced back to the existence of a multiplicity of conformational states in the protein molecule. The strain is reduced in the presence of azide which is known to bind directly to the copper atom and to give rise to a more relaxed configuration corresponding to a square pyramidal geometry in which the apical ligand occupies an elongated position. In both samples, addition of glycerol further reduces the strain, indicating that the solvent is directly coupled to the protein matrix, thereby modulating the structural heterogeneity displayed by the protein molecule.

Key words Superoxide dismutase · Conformational substates · Electronic paramagnetic resonance · Solvent effect

Introduction

It has recently been shown that electron paramagnetic resonance (EPR), aided by computer simulation, can be successfully applied to investigate the structural heterogeneity displayed by metallo-proteins (Hearshen et al. 1986; More et al. 1987; Yang and Gaffney 1987; Salerno 1985; Brill et al. 1986; Cannistraro 1990; Bacci and Cannistraro 1990; Bizzarri et al. 1991, 1992 and 1993). The g strain effect characterizing the low temperature EPR spectra of metallo-proteins can be interpreted by taking into account the presence of molecules frozen in many slightly different structures (Cannistraro 1990; Bacci and Cannistraro 1990; Bizzarri et al. 1991, 1992 and 1993). Different experimental and theoretical approaches show that a protein molecule can assume a very large number of different substates, called conformational substates (CS) (Frauenfelder et al. 1988; Goldanskii and Krupyanskii 1989), whose sampling is important for the biological function of the protein (Ansari et al. 1985). At physiological temperature, proteins fluctuate among CS; such behavior affecting the kinetic response of the molecules. By decreasing the temperature, the protein solution undergoes a glass-like transition and the fluctuations among CS are progressively suppressed (Frauenfelder 1987). Below the glass-temperature the molecules are frozen in many different CS whose distribution may be modulated by external agents such as pressure, pH, solvent composition (Hong et al. 1990; Frauenfelder et al. 1990; Di Iorio et al. 1991). However, the effect of the solvent on the dynamic coupling between the protein structure and the CS distribution is far from being fully elucidated.

The CS distribution and the dynamic properties of myoglobin, an almost completely α-helical protein, have been extensively studied by several experimental approaches, including X-ray and neutron diffraction analysis and Mössbauer, optical and EPR spectroscopy (Bizzarri et al. 1991, 1992 and 1993; Frauenfelder et al. 1979; Parak et al. 1982; Doster et al. 1989; Di Pace et al. 1992). EPR spectroscopy has shown that structural heterogeneity is reduced when the myoglobin samples are dissolved in the presence of glycerol or ethylene glycol (Bizzarri and Cannistraro 1993).

In an effort to understand how the dynamic properties depend on the secondary structure content of the protein, we recently started a systematic investigation of the dynamic properties of Cu, Zn superoxide dismutase (SOD),

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an almost entirely β-sheet protein (Tainer et al. 1982). Cu, Zn superoxide dismutase is an ideal model protein for dynamic investigations since its structural properties are now well established (Tainer et al. 1982; Djinovic-Carugo et al. 1992). The protein consists of eight anti parallel strands joined by three external loops which accomodate the copper and zinc metals constituting the active site. The average dynamics of Cu, Zn SOD have been worked out through a quasi-elastic neutron scattering investigation (Andreani et al. 1995) which indicated a mean square displacement of the non-exchangeable protons comparable to that observed in myoglobin (Doster et al. 1989). Investigation of the structural dynamics of the metal sites, undertaken by following the electronic absorption spectra of the metals as a function of temperature, indicated a higher rigidity of the solvent inaccessible zinc ion with respect to the solvent accessible copper atom (Cupane et al. 1994, 1995).

In this work, information concerning the dependence on the solvent composition of the local microheterogeneity of the copper has been obtained by investigating, through EPR spectroscopy, the native and the azide reacted form of the enzyme. The results indicate that the strain is reduced in the presence of azide and that in both the native and azide-reacted form, the presence of glycerol causes a decrease in the strain, as already observed in myoglobin (Bizzarri and Cannistraro 1993), suggesting a similar protein solvent coupling in these two structurally uncorrelated proteins.

Materials and experimental methods

Chemicals were purchased from commercial sources and were used without further purification. ^{63}Cu was obtained from Oak Ridge National Laboratory in the form of CuO; [^{63}Cu]$CuCl_2$ was prepared by dissolving CuO in HCl. Bovine Cu, Zn SOD was purified according to McCord and Fridovich (1969).

Copper-free protein was prepared as previously described (Rotilio et al. 1972) and was then reconstituted by stoichiometric addition of [^{63}Cu].

The protein used for the EPR spectra was 0.5 mM in 10 mM phosphate buffer pH 7.8. The pH remained unchanged after glycerol addition; moreover, it may be noted that the EPR spectrum of Cu, Zn SOD is pH independent in the 6–10 pH range (Rotilio et al. 1971).

The EPR spectra were recorded at 100 °K on a Bruker ESP 300 X-band spectrometer operating at 9.44 GHz microwave frequency, 100 KHz field modulation, 20 mW microwave power and 0.15 mT modulation amplitude.

Theoretical model for simulation of Cu-complex EPR spectra

The X-band EPR spectra of randomly oriented polycrystalline SOD samples, arising from the copper ion subjected to a ligand field of rhombic symmetry can be generally described by three partially superimposed patterns; each of them, centered at different g values (g_x, g_y, g_z), being characterized by four lines arising from the hyperfine coupling of the electron-spin (S = 1/2) to the copper nucleus (I = 3/2). The g_x and g_y patterns usually coalesce into a single broad line owing to the lower values of the hyperfine splitting and to the close g-values. The paramagnetic behaviour of the metal ion can be described (by neglecting the nuclear Zeeman and the quadrupole terms, which have been shown to be negligible in the study of other copper complexes (Giugliarelli and Cannistraro 1985), by the following spin hamiltonian

$$H_s = \beta(g_x S_x H_x + g_y S_y H_y + g_z S_z H_z) + A_x I_x S_x + A_y I_y S_y + A_z I_z S_z \quad (1)$$

where β is the Bohr magneton g_x, g_y, g_z and A_x, A_y, A_z are the components with respect to the principal axes, of the g-factor tensor and of the hyperfine constant tensor (coincidence of the g and A tensor axes is assumed).

If the spin hamiltonian, given in Eq. (1), is solved to the second-perturbative order, the following resonance frequencies can be determined for the allowed EPR transitions, (Bleaney 1960)

$$\nu_0 = \frac{1}{h}\left[g\beta H + K m_I + \frac{K'}{g\beta H} \right] \quad (2)$$

where m_I can assume the values $m_I = -3/2, -1/2, 1/2, 3/2$; g is given by the expression:

$$g^2 = [g'^2 + (g_z^2 - g'^2)\cos^2\theta] \quad (3)$$

where

$$g'^2 = g_y^2 + (g_x^2 - g_y^2)\cos^2\phi \quad (4)$$

ϕ being the angle between the projection of the magnetic field in the (x, y)-plane and the x-axis and θ being the angle between the magnetic field direction and the z-axis. K, which bears the same sign as A_z, can be expressed by:

$$K^2 g^2 = A_z^2 g_z^2 \cos^2\theta + B^2 g'^2 (1 - \cos^2\theta) \quad (5a)$$

where B is

$$B^2 g'^2 = g_y^2 A_y^2 + (g_x^2 A_x^2 - g_y^2 A_y^2)\cos^2\phi \quad (5b)$$

and finally, K′ is given by

$$K' = \left[\frac{A_x^2 A_y^2}{4B^2} \frac{A_z^2 B^2}{4K^2} + A_z^2 g_x^2 g_y^2 g_z^2 (A_y^2 - A_x^2)\cos^2\phi(1-\cos^2\phi)\cos^2\theta \right] \cdot [I(I+1) - m_I^2] - \left[\frac{1-\cos^2\theta}{2K^2 g^2} \left[\frac{g_z^2 g'^2}{g^2}(A_z^2 - B^2)\cos^2\theta + \frac{g_x^2 g_z^2}{g'^2} \right] \cdot (A_x^2 - A_y^2)\cos^2\phi(1-\cos^2\phi) \right] m_I^2 \quad (6)$$

For a powder like sample an integration over θ and ϕ should be performed; consequently, the EPR absorption spectrum $S(\nu, H)$ can be expressed by

$$S(\nu,H) = N\nu \int_0^{\pi} \int_0^{\pi/2} g_1^2 \, f[(\nu-\nu_0), \sigma_\nu^R] \sin\theta \, d\theta \, d\phi \tag{7}$$

where N takes into account all instrumental parameters; g_1^2 is the orientation dependent transition probability that, for the analyzed system, is given by

$$g_1^2 = \frac{1}{2}\left[\left(\frac{g_x g_y}{g}\right)^2 + g_y^2\right] \tag{8}$$

$f[(\nu-\nu_0)^2, \sigma_\nu^R]$ is the lineshape centered at the resonance frequency ν_0, and takes into account the electron spin magnetic relaxation phenomena. For Cu-complexes, f, whose residual linewidth σ_ν^R is mainly determined by the unresolved coupling of the electron spin to ligand nuclei (Froncisz and Hyde 1980), can be expressed by a Gaussian lineshape (Pilbrow 1990). An additional inhomogenous broading of the lines arises from a heterogeneity in the micro environment around the paramagnetic ions, which can be accounted for by a random distribution of some magnetic parameters. For copper-complexes, in particular, such a phenomenon, called the strain effect, generally consists of a progressive broadening together with a decrease of the intensity of the hyperfine lines in the $g_{||}$ region according to the value of the magnetic quantum number m_I (Brill et al. 1986; Cannistraro 1990; Froncisz and Hyde 1980; Pilbrow 1990; Hagen 1981, 1985, Fiamingo et al. 1989; Giugliarelli and Cannistraro 1985).

According to our previous work on other copper systems (Giugliarelli and Cannistraro 1985; Cannistraro and Giugliarelli 1986; Bizzarri et al. 1995), the inhomogenous broadening of the EPR lines was taken into account by introducing a spread on the g_z and A_z spin hamiltonian parameters. In addition, it has been hypothesized that a relatively small variability in the g_z and A_z parameters occurs. On such grounds, the following expression for the fluctuation of the resonance frequency ν_0 can be used

$$\Delta\nu_0 = \frac{\partial\nu_0}{\partial g_z}\Delta g_z + \frac{\partial\nu_0}{\partial A_z}\Delta A_z \tag{9}$$

It should be noticed that, since the presence of fluctuations in g_x, g_y, A_x and A_y does not significantly affect the EPR spectra, they have not been included in the simulations. If a Gaussian distribution for both g_z and A_z is assumed, from Eq. (9), the following expression for the variance of ν can be derived

$$(\sigma_\nu^S)^2 = \left(\frac{\partial\nu_0}{\partial g_z}\right)^2 \sigma_{g_z}^2 + \left(\frac{\partial\nu_0}{\partial A_z}\right)^2 \sigma_{A_z}^2 + 2\rho\left(\frac{\partial\nu_0}{\partial g_z}\right)\left(\frac{\partial\nu_0}{\partial A_z}\right)\sigma_{g_z}\sigma_{A_z} \tag{10}$$

In addition, if the fluctuations of g_1^2 due to g_z are negligible and the distributions of g_z and A_z are assumed to be orientation independent, Eq. (10) can be expressed as a power expansion of m_I and H as follows:

$$(\sigma_\nu^S)^2 = \left(\frac{g\beta}{h}\right)^2 (A m_I^2 + B m_I H + C H^2) \tag{11}$$

where A, B and C, are explicitly given by

$$\begin{aligned} A &= \frac{g_z^2}{\beta^2 K^2 g^6}[(A_z^2 - K^2)^2 \sigma g_z^2 + A_z^2 g_z^2 \sigma A_z^2 \\ &\quad + 2\rho(A_z^2 - K^2) A_z g_z \sigma g_z \sigma A_z]\cos^4\theta \\ B &= -\frac{2}{\beta K g}[(A_z^2 - K^2)\sigma g_z^2 + \rho A_z g_z \sigma g_z \sigma A_z]\frac{g_z^2}{g^4}\cos^4\theta \\ C &= \sigma g_z^2 \frac{g_z^2}{g^4}\cos^4\theta \end{aligned} \tag{12}$$

By including the g and A strain effects, the EPR absorption spectrum can be expressed by

$$S_{strain}(\nu,H) = \int S(\nu,H)\, F(g_z, A_z)\, dg_z\, dA_z \tag{13}$$

where $F(g_z, A_z)$ is a normal bivariate probability density function for the random variables g_z and A_z with a correlation coefficient ρ.

By integrating Eq. (13), the following expression for $S_{strain}(\nu, H)$ is obtained

$$S_{strain}(\nu,H) = N\nu \int_0^{\pi/2} g_1^2 \, f[(\nu-\nu_0)^2, \sigma_\nu^T] \sin\theta \, d\theta \tag{14}$$

where

$$(\sigma_\nu^T)^2 = (\sigma_\nu^R)^2 + (\sigma_\nu^S)^2 \tag{15}$$

From Eq. (15), it is seen that an additional linewidth parameters, σ_ν^S, due to the presence of the strain effect, must be introduced. Because of the dependence of σ_ν^S, upon the m_I value (see Eq. (11)), a different broadening of the hyperfine lines in the g_z region, according to the m_I value, may occur; such behavior finding a correspondence in the experimental EPR spectra of the Cu-complex (Hagen 1981; Cannistraro and Giugliarelli 1986).

In the framework of the frequency-swept formulation, the EPR signal can be obtained if the derivative with respect to the magnetic field of the absorption spectrum $S(\nu, H)$, taken at the fixed frequency, ν_C, is performed (Cannistraro and Giugliarelli 1986)

$$I(H) = \frac{dS(\nu_c, H)}{dH} \tag{16}$$

ν_C is the microwave frequency that for X-band is around 9 GHz. Finally, the following expression for the field-swept first derivative of the absorption $I_s(H)$, (mainly detected by an EPR spectrometer) can be derived according to the procedure described in Pilbrow (1984)

$$\begin{aligned} I_S(H) &= \frac{N\nu_c H)}{\beta}\int_0^{\pi/2} \frac{g_1^2}{g}\frac{1}{(\sigma_H^T)^3} e^{\left[-\frac{(H-H_0')^2}{2(\sigma_H^T)^2}\right]} \\ &\quad \cdot\left\{\left[1 - \frac{K'}{(g\beta H)^2}\right](H_0' - H)\right. \\ &\quad \left. + \left[\frac{(H-H_0')^2}{(2\sigma_H^T)^2} - \frac{1}{2}\right](Bm_I + 2CH)\right\}\sin\theta\, d\theta \end{aligned} \tag{17}$$

where

$$H'_0 = \frac{1}{g\beta}\left(h\nu_c - K m_I - \frac{K'}{g\beta H}\right) \quad (18)$$

and

$$\sigma_H^T = \frac{h\sigma_\nu^T}{g\beta} \quad (19)$$

Equation (17) was used within the framework of a best-fit program based on a Monte Carlo algorithm and spanning the parameter space (g_x, g_y, g_z, A_x, A_y, A_z, σ_x^R, σ_y^R, σ_z^R, σ_{g_z}, σ_{A_z}), to numerically reproduce, at a high level of confidence (normalized χ^2 less than 0.9), the experimental EPR spectra of Cu, Zn superoxide dismutase.

Results and discussion

The copper atom in Cu, Zn SOD is coordinated by four nitrogens and one oxygen of a fast exchanging water molecule, forming a distorted geometry which gives rise at 9 GHz to a rhombic EPR spectrum as shown in Fig. 1. The corresponding g tensors have been exactly known since the early seventies because of the resolution in the three directions of the 35 GHz EPR spectrum (Rotilio et al. 1972); however, up to now a complete simulation of this spectrum has never been reported. The reason is due to the fact that, in order to have a correct simulation, the introduction of a distribution for the spin hamiltonian parameters g and A (g and A strain) is required. In fact the experimental spectrum differs significantly from the simulated one in the absence of a distribution of these spin hamiltonian parameters and it is reproduced only by including a significant g and A strain (Fig. 1). In particular, only after the introduction of the strain has it been possible to reproduce the unequal spacing of the hyperfine lines in the low field magnetic region and their different line broadening. It must be remembered that in Cu, Zn SOD both ENDOR and ESEEM studies have clearly shown (Scholl and Huttermann 1992; Reinhard et al. 1994; Dikanov et al. 1994) that the nitrogen of the metal coordinating histidine may interact with the unpaired electron of the copper atom. In our simulation model the linewidth due to the unresolved coupling between the electron spin and the ligand nuclei is included in residual linewidth, σ_ν^R and the effect of strain is considered in the term σ_ν^S which is m_I dependent and accounts for the presence of a micro heterogeneity around the paramagnetic ion.

Addition of N_3^-, which is known to directly bind to the copper atom of Cu, Zn SOD and to give rise to a square pyramidal coordination around the metal, (Djinovic-Carugo et al. 1994) converts the spectrum from a rhombic to a more axial shape (Fig. 2). The occurrence of a more planar geometry gives rise to a decrease and an increase of the g_z and A_z value respectively, which produces a large overlap between the high field parallel hyperfine line with the perpendicular absorption line of the spectrum. The azide reacted enzyme has been recently reported to display a slight degree of rhombicity (Huttermann et al. 1995); however, the spectrum was fitted by assuming an axial shape, since any attempt to introduce a third component did not produce any significant improvement in the simulation. As in the native enzyme the four hyperfine lines in the g_z region are unequally spaced and show a different broadening with the low field one displaying the largest broadening. Such features may be reliably reproduced by simulating the spectrum (Fig. 2) with the parameters reported in Table 1. The values of σ_{g_z} and σ_{A_z}, indicating the extent of the parameter strain, introduced to obtain a good simulation of the EPR spectrum of the N_3^- reacted enzyme are lower than those used in the simulation of the native one indicating a decrease of the copper environment micro heterogeneity upon N_3^- binding.

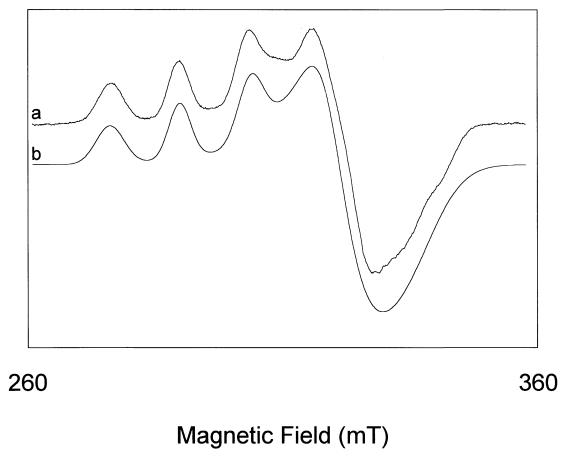

Fig. 1 Experimental (*a*) and simulated (*b*) EPR spectrum of [^{63}Cu] reconstituted native bovine Cu, Zn SOD in water. Settings: T = 100 K°, microwave power 20 mW; modulation frequency 100 KHz; microwave frequency 9.44 GHz; modulation amplitude 1 mT. The value of the parameters used in the simulation are reported in Table 1

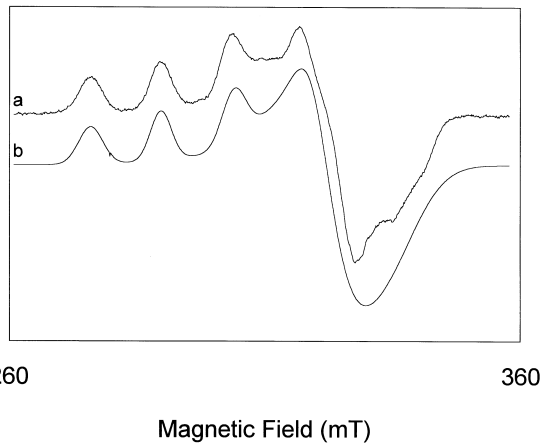

Fig. 2 Experimental (*a*) and simulated (*b*) EPR spectrum of azide reacted [^{63}Cu] reconstituted bovine Cu, Zn SOD in water. Settings as in Fig. 1

Table 1 Spin hamiltonian parameters used for the simulation of the EPR spectra (hyperfine coupling and linewidths are expressed in gauss)

Parameters	SOD	SOD + N_3^-	SOD 50% glycerol	SOD + N_3^- 50% glycerol
g_x	2.025	2.061	2.026	2.057
g_y	2.121	2.061	2.115	2.057
g_z	2.264	2.251	2.261	2.247
A_x	7.92	36.07	10.02	28.00
A_y	9.27	36.07	11.73	28.00
A_z	143.6	160.5	148.8	170.4
σ_x^R	67.20	39.07	64.40	34.00
σ_y^R	56.40	39.07	49.90	34.00
σ_z^R	20.00	23.25	19.00	22.30
σ_{g_z}	0.007	0.004	0.004	0.003
σ_{A_z}	15.0	5.2	12.7	3.0

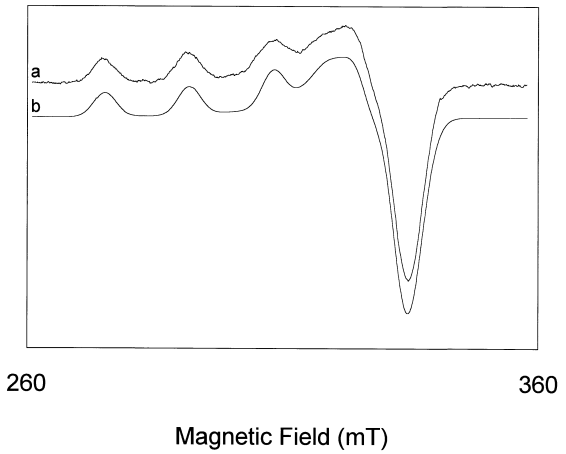

Fig. 3 Experimental (*a*) and simulated (*b*) EPR spectrum of [^{63}Cu] reconstituted native bovine Cu, Zn SOD in water-glycerol mixture (50%). Settings as in Fig. 1

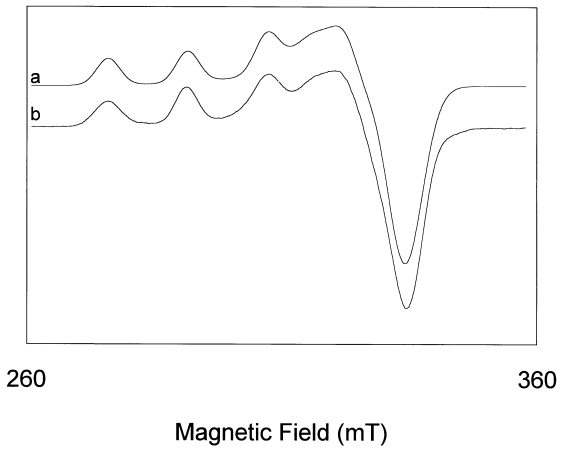

Fig. 4 Experimental (*a*) and simulated (*b*) EPR spectrum of azide reacted [^{63}Cu] reconstituted bovine Cu, Zn SOD in water-glycerol mixture (50%). Settings as in Fig. 1

In proteins the g and A strain effects have been connected to the existence of several biomolecular conformational substates, each one characterized by a slightly different arrangement of atoms or groups of atoms (Cannistraro 1990; Brill et al. 1986; Bizzarri et al. 1993); such heterogeneity being relevant to the biological functionality of the macromolecule (Frauenfelder et al. 1988). The strain reduction upon N_3^- binding suggests the occurrence of a more relaxed configuration and is in agreement with the result obtained through a low temperature optical spectroscopic investigation carried out in both the native and azide reacted enzyme (Cupane et al. 1994).

Since the low temperature optical spectra were recorded in the presence of glycerol, in order to make the samples transparent, the effect of glycerol on the EPR spectrum of native and N_3^- reacted Cu, Zn SOD has been investigated. The change of solvent composition gives rise to a slight variation of both the distribution and the mean values of the g and A tensor indicating a rearrangement of the copper region and possibly of the entire macromolecule, as induced by the solvent environment (Figs. 3 and 4). The simulation takes into account such variation since both the mean value and the distribution of the g and A tensors are free fitting parameters. The resulting simulations indicate that also in presence of glycerol the microheterogeneity around the copper atom is lower in the azide reacted than in the native enzyme. In the case of the native enzyme glycerol induces some perturbation in the high magnetic field region of the spectrum. The EPR spectrum of the azide reacted enzyme is well reproduced by simulation (Fig. 4) whilst, in the case of the native enzyme the simulation does not completely reproduce the experimental lineshape in the high magnetic field region (Fig. 3). It must be stressed however that the fit improves significantly only after introduction of the strain effect. In both samples the presence of glycerol reduces the value of the strain (Table 1). Such a reduction may be due to different mechanisms, but the similar perturbation of the spin hamiltonian parameters observed on the two samples suggests that the possible freezing-induced effects on the copper are mainly due to changes felt by the overall protein matrix instead of to a direct interaction with the metal ion. In accordance with this hypothesis glycerol is known not to directly interact with the copper of Cu, Zn SOD and its presence does not impair the biological activity of the enzyme which is only slightly reduced because of the decreased diffusion rate of the substrate toward the active site (Fielden et al. 1974).

A known effect of glycerol is to stabilize the globular state of a protein by inducing preferential hydration of the protein-water interface (Gekko and Tiamsheff 1981). As a result the system minimizes the free energy cost of segregation of solvent molecules, by adopting a compact conformation with the least surface area. Glycerol is also known to lower the dielectric constant of the solvent and this could result in stronger interactions between the charged aminoacid residues slightly modifying the protein

structure. Both these effects could cause the observed decrease of the protein structural heterogeneity and, in addition, they could be responsible for the small variations recorded in the g and A values.

It is interesting to notice that the heme microheterogeneity in myoglobin, a protein consisting largely of alpha-helix, is also reduced upon addition of glycerol (Bizzarri and Cannistraro 1993). The similar reduction now observed on the copper of Cu, Zn superoxide dismutase, a protein not containing any α helix and being mainly consituted by β strands suggests that glycerol acts with a similar mechanism on all globular proteins, independently of their structure. The ability of glycerol to determine a decrease of the H-bond rupturing capacity of an aqueous medium (Gerlsma et al. 1970) may be of relevance in explaining these results. In fact in the hypothesis that the presence of a multiplicity of water states is strictly coupled to the existence of the CS distribution of the protein (Doster et al. 1986), a slowing down of the solvent dynamics could be responsible for the reduced protein heterogeneity independently of its secondary and tertiary structure.

In conclusion we suggest that whatever the main effect of glycerol on the protein matrix the occurrence of a similar effect with two structurally uncorrelated proteins such as superoxide dismutase and myoglobin suggests that the coupling between the solvent and the protein dynamics properties may be identical for all globular proteins and that structural differences are not important.

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