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An EPR investigation on the structural heterogeneity in copper azurin and plastocyanin

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Abstract

The effects of cooling rate and of solvent properties on the active site heterogeneity of two copper proteins, azurin and plastocyanin, have been investigated at low temperature by electron paramagnetic resonance spectroscopy. The spectra of theses proteins have been analyzed, by an accurate computer simulation, in terms of a distribution of some relevant spin-Hamiltonian parameters. The results show that the structural heterogeneity of both proteins, quantified by the width of the distribution in the **g** and **A** tensors, is affected by both the freezing procedure and the solvent composition. In particular, the **g** distribution width is found to be reduced in the slow cooling regime; such a reduction appearing more significant when glycerol is added to the protein solutions. Despite of the similarity in the copper ion microenvironments of the two proteins, the effects are more pronounced in azurin. The results are discussed also in connection with the role played by the solvent and the rate of freezing in featuring the conformational substate landscape. © 1997 Elsevier Science B.V.

Keywords: Electron paramagnetic resonance; Azurin; Plastocyanin; Freezing rate; Glycerol

1. Introduction

The application of electron paramagnetic resonance (EPR) spectroscopy to the study of metalloproteins has given further insight on the structural heterogeneity of proteins at low temperature [1–11].

With the aid of computer simulations based on suitable theoretical models, the spectral features have been interpreted in terms of a distribution of ligand field strengths onto the paramagnetic metal ion, mainly situated in the active site [1–3,5,6,11].

Such a distribution has been postulated to arise from a characteristic structural disorder, which is reminiscent of that occurring in glasses [8,12–14]. In both systems, the intrinsic topological disorder, which determines a distribution of the relevant parameters used to describe the structural and dynamical properties of the systems, has been justified by admitting

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the existence of a huge amount of conformational substates (CS) [13–17]. In these CS, the protein molecules may slightly differ each other for structural arrangement of some groups of atoms and for the energy [16,18]. At room temperature, fluctuations among CS occur continuously and are crucial in determining the biological functionality of the protein [18]. In the activation of these fluctuations, the dynamical interaction of the protein–water system plays a very important role. In particular, the protein conformational motion has been considered as being driven by the fluctuations in the H-bond network of the hydration water [19]. This coupling suggests a close correlation between the solvent properties and the distribution of the CS. By lowering the temperature below 200 K, the transitions among CS can be suppressed and the protein system is frozen in local minima, giving rise to a static distribution of substates [16]. This distribution shows the imprint of the freezing pathway, which in turn depends on the solvent properties. In fact, different local minima can be explored, from the protein system, by changing the freezing condition as well as the chemico-physical properties, such as the viscosity and the dielectric constant, of the medium [17,20].

So far, the studies on the CS distribution have been mainly focused on heme proteins which, from a structural point of view, are formed by α-helices. The reason of this interest has probably to be ascribed to the fact that they are very well characterized systems by many experimental approaches [10,21,22].

However, recently the generality of these concepts has also been applied to azurin [23], a blue copper protein with a different secondary structure, essentially formed by β strands, and a different active site geometry.

In the EPR spectra of copper proteins the distribution in the site geometry entails a distortion, in the low field hyperfine pattern, centered at $g_{\|}$. Such a distortion consists in both different shapes and heights of the copper hyperfine lines and an unequal spacing between adjacent lines [8,9,24]. Recently, some of the authors [25] have revisited this problem and have shown that the hyperfine line shift is due to a superposition of Gaussian-like hyperfine lines to the background arising from the simultaneous presence of different paramagnetic ion orientations with respect to the magnetic field; such an effect being significantly enhanced in the presence of heterogeneity in the site geometry, otherwise called 'strain effect' [7,8,24].

Even if the protein heterogeneity at low temperature is supported by a wealth of evidence [9,10,16,23], it is still an open question whether the observed spread in the site geometry is mainly due to an intrinsic disorder of the macromolecules or whether the structural disorder induced by the freezing of the solvent around the protein prevails. The role of both contributions could be singled out by performing experiments under appropriate conditions apt to separately affect the two terms. In particular, information on the structural strain contribution can be obtained by changing the freezing conditions and the solvent properties. The freezing effects on biological molecules have been deeply investigated [26–28], whereas as it concerns the cooling rate effects, the results have always been presented at the margin of other studies [9,26–29]. On such a ground, we have systematically investigated how the freezing condition and the addition of glycerol to the protein solution, affect the copper site heterogeneity in azurin (AZ) and plastocyanin (PC). Glycerol has been chosen since it is extensively used to stabilize the protein structure and as glass making solvent in experiments needing optical transparency [16,18,30]. However, its effect on the modulation of CS distribution should be carefully clarified before the experimental results can confidently be connected to the protein CS. From this point of view, EPR spectroscopy is a useful tool because allows one to study the systems of interest with and without glycerol.

The parameters used in this work to quantify the protein heterogeneity are $\sigma g_{\|}$ and $\sigma A_{\|}$ which represent the width of the distributions of $g_{\|}$ and $A_{\|}$, respectively. The results show that the conformational heterogeneity of AZ is more pronounced than that of PC; moreover a reduction of the $\sigma g_{\|}$ values as due to a slow cooling procedure and to the addition of glycerol is registered in both proteins. It is suggested that the structural and dynamical modifications as induced by both the presence of glycerol and the freezing rate are involved in the reduction of the protein heterogeneity. The correlation between the solvent role and the conformational substate distribution is discussed.

2. Materials, experimental methods and simulation model for the analysis of the EPR spectra

AZ from *Pseudomonas Aeruginosa* was purchased from Sigma and used without further purification, PC was prepared from spinach leaves as described previously [31].

The proteins were dissolved, at a concentration of about 1 mM, in a phosphate buffer solution 10 mM, pH 7. Two criteria were applied to assess the purity of the proteins sample. Molecular weight homogeneity was established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10–15% gradient using the Pharmacia Phast System. In addition spectral ratios $A_{625}/A_{280} = 0.50$ and $A_{597}/A_{280} = 0.52$ was obtained for AZ and PC, respectively. Samples in mixed water–glycerol solvent were prepared by adding 40% v/v of glycerol to the phosphate buffer solution. The variation of pH in the presence of glycerol in solution was of 0.4 pH units. All samples have been analyzed in the rapid and slow cooling regime. Rapid cooling has been obtained by plunging the sample tube directly in the liquid nitrogen, whereas the slow cooling have been obtained by lowering the temperature from 300 to 170 K with a rate of 0.5°C min^{-1} using a Stelar temperature control. All the EPR spectra have been recorded at 77 K by an X-band ESP300 Bruker spectrometer. The experimental spectra of both proteins were independent on the protein batches used and on the samples preparation time. The microwave frequency has been measured with a Marconi 2440 counter. The analysis of the EPR spectra have been performed on a VAX 8350.

The low temperature EPR spectra of AZ and PC, show the spectral features of a powder-like Cu^{++} complexes with axial symmetry and can be described by the following spin-Hamiltonian

$$\mathcal{H} = \beta\left[g_{\parallel}H_zS_z + g_{\perp}(H_xS_x + H_yS_y)\right] + A_{\parallel}I_zS_z + A_{\perp}(I_xS_x + I_yS_y) \quad (1)$$

where β is the Bohr magneton, $g_{\parallel}$ and $A_{\parallel}$ are the g-value and the hyperfine component parallel to the molecular axis, respectively, $g_{\perp}$ and $A_{\perp}$ are the g-value and the hyperfine component perpendicular to the molecular axis, respectively. The other symbols have the usual meaning.

The resonance frequencies for the allowed EPR transitions can be obtained by solving, to the second perturbative order, this spin Hamiltonian (Eq. (1)) [32,33]:

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

where g, K and K' are given by:

$$g = \left(g_{\parallel}^2\cos^2\theta + g_{\perp}^2\cos^2\theta\right)^{\frac{1}{2}} \quad (3)$$

$$K = \left(g_{\parallel}^2A_{\parallel}^2\cos^2\theta + g_{\perp}^2A_{\perp}^2\cos^2\theta\right)^{\frac{1}{2}} \quad (4)$$

$$K' = \frac{A_{\perp}^2\left(A_{\parallel}^2 + K^2\right)}{4K^2}\left[I(I+1)\right] + \left[\frac{g_{\parallel}^2g_{\perp}^2}{2K^2}\left(A_{\parallel}^2 - A_{\perp}^2\right)\sin^2\theta\cos^2\theta - \frac{A_{\perp}^2\left(A_{\parallel}^2 + K^2\right)}{4K^2}\right]m_I^2 \quad (5)$$

and θ is the angle between the magnetic field direction and the molecular axis.

For a two level system ($S = 1/2$), the net absorption of the microwave radiation from the paramagnetic center as a function of the frequency, ν, and of the magnetic field H, can be expressed as [34]

$$S(\nu,H) \propto |V_{ij}|^2\,Vf\left[(\nu - \nu_0)^2,\ \sigma_{\nu}^R\right] \quad (6)$$

where $|V_{ij}|^2$ is the time-independent part of the transition matrix element, $f[(\nu - \nu_0)^2,\ \sigma_{\nu}^R]$ is the lineshape function centered at the resonance frequency ν_0 and σ_{ν}^R is the residual linewidth, which is mainly determined by the unresolved coupling of the electron spin to ligand nuclei. Actually, in powder-like or polycrystalline samples, the absorption arise from the different orientations of the paramagnetic centers with respect to the magnetic field, so that an orientation-dependent transition probability should be considered. This term, which is proportional to Vij, is given by [32]

$$g_I^2 = \frac{1}{2}g_{\perp}^2\left[\frac{g_{\parallel}^2}{g^2} + 1\right] \quad (7)$$

By integrating Eq. (6) over all the paramagnetic ion orientations, the total microwave absorption can be derived

$$S(\nu,H) = N\nu\int_0^{\pi/2} g_1^2 f\left[(\nu-\nu_0)^2,\sigma_\nu^R\right]\sin\theta\,d\theta \tag{8}$$

where N takes into account the instrumental parameters.

In the EPR lines of copper complexes and metalloproteins as well, the effects due to local strains onto the coordination of the paramagnetic center are taken into account by means of a gaussian distribution of the g and A values which ultimately takes into account for the distribution of the copper ligand bond lengths and angles [31,35–37]. With this assumption, the above expression becomes

$$S_{\text{strain}}(\nu,H) = \int S(\nu,H)F(g_\parallel,A_\parallel)\,dg_\parallel\,dA_\parallel \tag{9}$$

where $F(g_\parallel,A_\parallel)$ is a bivariate normal density function which describes the gaussian fluctuations of $g_\parallel$ and $A_\parallel$ in terms of the respective variances $(\sigma g_\parallel)^2$ and $(\sigma A_\parallel)^2$ and of the correlation coefficient ρ. By assuming small variation in the $g_\parallel$ and $A_\parallel$ values, the fluctuation in the resonance frequencies can be expressed as

$$\Delta\nu_0 = \frac{\partial\nu_0}{\partial g_\parallel}\Delta g_\parallel + \frac{\partial\nu_0}{\partial A_\parallel}\Delta A_\parallel \tag{10}$$

By integrating Eq. (9), the following results is derived

$$S_{\text{strain}}(\nu,H) = N\nu\int_0^{\pi/2} g_1^2 f\left[(\nu-\nu_0)^2,\sigma_\nu^T\right]\sin\theta\,d\theta \tag{11}$$

where

$$\left(\sigma_\nu^T\right) = \left[\left(\sigma_\nu^R\right)^2 + \left(\sigma_\nu^S\right)^2\right]^{\frac{1}{2}} \tag{12}$$

is now the total linewidth, and σ_ν^S is an additional term due to the strain effect. If the fluctuations of g_1^2 due to $g_\parallel$ are negligible and the distributions of $g_\parallel$ and $A_\parallel$ are assumed to be orientation independent, σ_ν^S can be expressed, in agreement with Refs. [25,36], as a power expansion of the nuclear quantum number, m_I, and of H as follows

$$\left(\sigma_\nu^S\right)^2 = \left(\frac{g\beta}{h}\right)^2\left(Am_I^2 + Bm_IH + CH^2\right) \tag{13}$$

where the coefficients A, B and C have been explicitly worked out [35].

Since in the EPR spectroscopy, the frequency is fixed and the spectrum is obtained by sweeping the magnetic field, the derivative field swept absorption spectrum as a function of the magnetic field H, can be obtained deriving Eq. (11) with respect to H

$$I_{\text{strain}}(\nu_c,H) = \frac{dS_{\text{strain}}(\nu_c,H)}{dH} \tag{14}$$

where ν_c is the microwave frequency.

Eq. (14) has been used to fit the low temperature experimental EPR spectra of AZ and PC. A best-fit program based on a Monte Carlo algorithm and spanning the parameter space allowed us to reproduce numerically, to a high level of confidence, the EPR experimental spectra.

3. Results and discussion

The EPR spectrum of AZ in aqueous solution recorded at 77 K under rapid cooling conditions, is shown in Fig. 1 (pattern a). The spectral features are typical of a type I copper complex with axial sym-

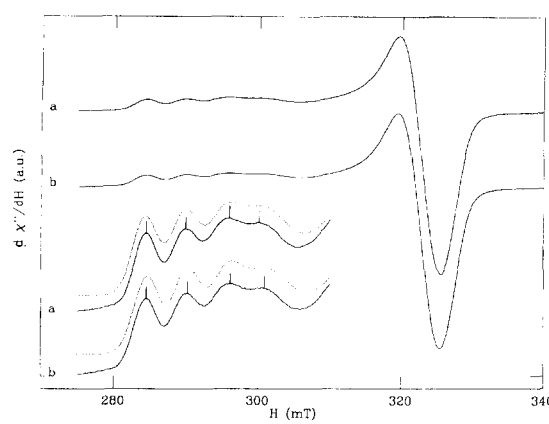

Fig. 1. Experimental EPR spectra of AZ recorded at 77 K in aqueous solution in (a) rapid and (b) slow cooling regime. Setting condition: $T = 77$ K, microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 5 G. The inset show the corresponding parallel pattern of the (solid line) experimental and (dashed line) computer simulated spectra at a higher gain. The spin-Hamiltonian parameters used in the simulations are listed in Table 1.

metry characterized by four hyperfine lines centered at $g_{\|}$ and separated by $A_{\|}$ and by a single, more intense, resonance line centered at $g_{\perp}$, at higher fields. The geometry of the copper site, Cu^{++}–N_2SS^*O, is known in detail from X-ray crystal structure and consists of a distorted trigonal bipyramidal symmetry [38].

The $g_{\|}$ region of the EPR spectrum, shown in the inset of Fig. 1 (pattern a, solid line) at an higher gain, is characterized by a distortion in the hyperfine pattern [1,7,8]. Owing to such a distortion associated to a partial overlap of the hyperfine lines with different m_I, a straightforward determination of the relevant spin-Hamiltonian parameters from the experimental spectra may be difficult and unreliable; an accurate computer simulation of the experimental spectra being then required. Realistic simulations of the experimental spectral features of metalloproteins have been obtained by using simulation methods in which some relevant parameters are distributed [1,7,8]. The rationale of this assumption is the presence, at low temperature, of an ensemble of molecules in different CS characterized by slightly different copper ligand bond lengths and/or angles which result into a distribution of ligand field strengths onto the paramagnetic metal ion. According to the simulation procedure discussed in the methods section, the experimental spectrum has been simulated. The agreement between the two spectra appears remarkable (pattern a, dashed lines) as evidenced by the χ^2 value. The overall parameters used for the simulation of the experimental spectra to which we will refer hereafter are listed in Table 1.

When the same sample of AZ is submitted to a slow cooling procedure, the corresponding EPR spectrum, obtained at 77 K, is shown in Fig. 1 (pattern b). The overall features of the spectrum remain unchanged, however, a magnification of the $g_{\|}$ region shows a distortion similar to that previously observed with an additional high field shift of the $m_I = 3/2$ hyperfine line. No appreciable modifications are observed in the $g_{\perp}$ perpendicular region. The corresponding simulated spectrum is also reported in the inset of Fig. 1 (pattern b, dashed line); again the agreement with the experimental spectrum is good. From data in Table 1, it can be inferred that the slow cooling procedure mainly results in a smaller value of $\sigma g_{\|}$ with respect to the rapid one.

Before discussing these data in detail, let us analyze the results obtained for the other copper protein, PC, submitted to the same freezing procedures.

The EPR spectrum of PC in aqueous solution, recorded at 77 K after rapid cooling is shown in Fig. 2 (pattern a). The general features and the spectral parameters (g and A values) are consistent with a distorted tetrahedral copper site geometry. Since the O atom in the AZ active site interacts weakly with the Cu^{++} ion, the geometries of the paramagnetic centers in the two proteins can be considered quite similar. The other ligand atoms are the same, but some differences do exist in the copper-ligands bond lengths. In particular, the smaller Cu–S(Met) bond length is believed to be one of the factors responsible of the highest redox potential of PC with respect to the other blue copper proteins [39].

The distortion of the $g_{\|}$ region shown in the inset

Table 1
Spin-Hamiltonian parameters used in the simulations of the EPR spectra

	% Glycerol	$g_{\|}$	$g_{\perp}$	$A_{\|}$	$A_{\perp}$	$\sigma g_{\|}$	$\sigma A_{\|}$	χ^2
Azurin								
Rapid cooling	0	2.2615	2.0441	54.0	4.1	0.0091	8.8	0.902
Slow cooling	0	2.2602	2.0439	54.0	3.8	0.0081	8.8	0.915
Rapid cooling	40	2.2590	2.0441	54.0	4.3	0.0078	8.8	0.906
Slow cooling	40	2.2578	2.0441	54.0	4.3	0.0063	8.8	0.913
Plastocyanin								
Rapid cooling	0	2.2442	2.0462	59.5	5.2	0.0048	6.0	0.901
Slow cooling	0	2.2420	2.0461	59.3	5.2	0.0045	6.0	0.911
Rapid cooling	40	2.2398	2.0439	59.5	5.2	0.0040	6.0	0.916
Slow cooling	40	2.2402	2.0438	59.5	5.2	0.0035	6.0	0.918

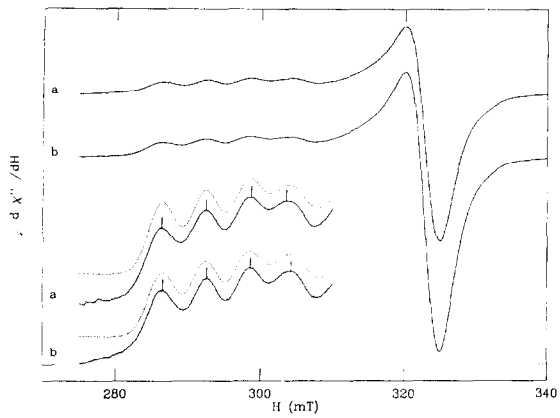

Fig. 2. Experimental EPR spectra of PC in aqueous solution in (a) rapid and (b) slow cooling regime. Setting condition as in Fig. 1. The inset shows an enlargement of the parallel pattern of the (solid line) experimental and (dashed line) computer simulated corresponding spectra. The spin-Hamiltonian parameters used in the simulations are listed in Table 1.

of Fig. 2 (pattern a, solid line) which has been already observed in AZ has been reproduced, to a good accuracy (pattern a, dashed line).

The slow cooling cycle on the same PC sample gives rise to the EPR spectrum shown in Fig. 2 (pattern b). An accurate comparison of the parallel regions at a higher gain (patterns a and b, solid lines) put into evidence a slightly shift of the $m_I = 3/2$ hyperfine line to higher field and a more regular pattern as a result of the slow cooling. On the other hand, the simulation of the experimental spectrum, also shown in the inset of Fig. 2 (pattern b, dashed line), gives a fitting parameter set characterized by a smaller value of $\sigma g_{\|}$ (see Table 1).

If we compare the simulation data listed in Table 1 for the rapid and slow cooling of both proteins, it can be noted that. A reduction of $\sigma g_{\|}$, which is more pronounced for AZ, is observed by lowering the freezing rate, whereas $\sigma A_{\|}$ does not depend on the freezing procedure.

In the explanation of the data obtained different aspects, in same way contrasting each other, should be taken into account. On one side, a slow cooling favors the growth of ice crystal of larger dimension [28,40] with a consequent increases of stress on the protein molecules leading to a higher heterogeneity. On the other side, it has been suggested that a lower freezing rate favors a local arrangement of the protein ligand to the metal ion involving a less strained structure reducing in this way the protein heterogeneity [28,29]. So that the overall reduction of the $\sigma g_{\|}$ observed in both proteins should then be ascribed to the prevailing of the latter effect. This should be not surprising, if we consider that Brill et al. have demonstrated, by EPR studies, that the structural inhomogeneities observed at low temperature in high-spin ferric myoglobins and hemoglobins, in which the paramagnetic center is shielded from the solvent, can be mainly assigned to the macromolecules, whereas the properties of the ice phase around them play a secondary role [28,41]. On the other hand, the copper site in AZ and PC is also located in an inner region at about 7 and 6 Å far from the protein surface, respectively [38,39], and the interaction with the solvent molecules is mediated by the protein matrix. In this connection, it should be also remarked that the g-strain present in the EPR spectrum of small copper complexes in amorphous phase can be completely released in the polycrystalline phase [42]. We may therefore suggest that the freezing rate effects observed on AZ and PC are essentially due to the intrinsic protein heterogeneity.

From simulation data in Table 1, it can be pointed out that: (i) the structural heterogeneity of AZ is greater than that of PC, (ii) the $\sigma g_{\|}$ value in AZ changes in a larger range with respect to PC. Both results could be related to a different conformational variability of the copper site in the two proteins, which in turn depends on the global structural differences. The structure of PC is, like that of AZ, a β barrel but it is more compact and lacks of the small α-helix portion, which forms the 'back flap' in AZ [39]. On the other hand a higher rigidity in the PC active site has also been suggested by looking at the temperature dependence of $g_{\|}$ in a large range of temperature (4–200 K) [29]. In addition, the smaller size of PC (99 residues against 128) should be also considered. In fact the more small is the size of a system, the more small is the number of accessible structural states [43]. This suggests a lower number of accessible structural states for PC with respect to AZ.

A more marked effect, with respect to the cooling rate, on the EPR spectra can be observed if glycerol

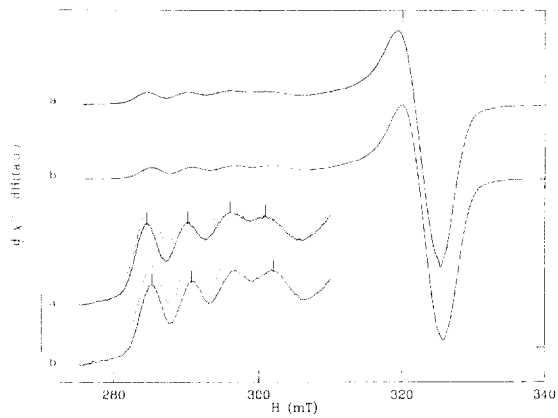

Fig. 3. Experimental EPR spectra of AZ in water/glycerol 40% v/v solution in (a) rapid and (b) slow cooling regime. Setting condition as in Fig. 1. In the inset an enlargement of the parallel pattern of the (solid line) experimental and (dashed line) computer simulated spectra is also shown. The value of the spin-Hamiltonian parameters used in the simulations are listed in Table 1.

is added to the protein solutions. In Fig. 3 (pattern a, solid line), the EPR spectrum of AZ in water–glycerol mixture recorded at 77 K after a rapid cooling and the corresponding amplification of the parallel pattern are shown. By comparing the low field hyperfine lines with and without glycerol in rapid freezing condition, an upfield shift of the $m_I = 3/2$ line is observed in the presence of glycerol in solution (Fig. 1 and Fig. 3, pattern a). The experimental EPR spectrum of AZ in the presence of glycerol has been simulated and the result is shown in the inset of Fig. 3 (pattern a, dashed line).

When the EPR spectrum on the same AZ sample in water–glycerol mixture is recorded after a slow cooling cycle the result shown in Fig. 3 (pattern b) is obtained. The amplification of the $g_{\|}$ region (Fig. 3 pattern b, solid line), shows that the distortion of the pattern, which is always present, appears less pronounced. The simulation of this spectrum (pattern b, dashed line), requires a smaller $\sigma g_{\|}$ with respect to the previous one.

The spectral behavior of PC samples in water–glycerol solution reflect the same trend as AZ, so that for the sake of simplicity we have reported only the simulation data (Table 1). From these data it can be noted that a reduction of $\sigma g_{\|}$, even if of less extent with respect to AZ, is obtained in the presence of glycerol.

The understanding of glycerol effects on the protein structure, needs some considerations. It is known that the presence of glycerol in solution determines an increase of the solvent viscosity as well as an increase of the chemical potential of the protein; the latter being due to the exclusion of glycerol molecules from the first hydration shell of the protein [44]. As a consequence, the protein will tend to reduce the contact area with the solvent favoring a mechanism of self-association. This packing makes the transition among those conformation which involve motions of large amplitude unfavorable, so that the protein heterogeneity is reduced. On the other hand, the presence of a third component in solution such as glycerol, which has the peculiarity to lower the freezing temperature, affects the crystal growth [40] as well as the dynamics of breaking and forming of the H-bond [45]. In the hypothesis that the presence of a multiplicity of water states is strongly coupled to the existence of the CS distribution [19], a slow down of the solvent dynamics, which is connected with a reduced heterogeneity in the water patches population, would be responsible for the reduced protein heterogeneity. Moreover, the reduction of the crystal size reflects in a lower induced strain on the protein matrix [2].

Spectral modification of the EPR spectrum of AZ in the presence of glycerol in solution has also been found by Groeneveld et al. [7]. However, the authors attribute the observed differences to the lower pH value induced by the glycerol in the starting borate buffer (pH 9.2), rather than to an alleviation of the g-strain. In our experiments, the pH of the solution (phosphate buffer, pH 7.0) is not altered, in a significative way, by the addition of the same percentage of glycerol used by Groeneveld et al., so that we might suggest that the modifications observed on the EPR spectrum can be due to a different protein heterogeneity.

A sharpening effect of glycerol has also been observed by Hagen [2] on the EPR spectra of PC and superoxide dismutase and has been assigned to the decreases of the number of protein conformations taking place in the presence of glycerol.

Evidence of spectral modifications, induced by glycerol, has also been found in myoglobin [11]. In particular, a narrowing of the crystal field parameters distribution and the decrease in the spread of the

iron–porphyrin displacement in the presence of glycerol and ethylene glycol have been observed and discussed in terms of a reduction in the heterogeneity of the conformational substates induced by addition of these cosolvents.

From the above observations, it appears quite clear that the dynamic properties of the solvent play a very important role in featuring the conformational substate landscape of the protein.

4. Conclusion

EPR spectroscopy reveals a suitable technique to investigate the AZ and PC structural heterogeneity observed at low temperature related to the presence of a frozen ensemble of molecules in different CS. By using a simulation method, in which the protein heterogeneity is explicitly considered through the width of the $g_{\|}$ and $A_{\|}$ distributions, the experimental spectra, have been simulated to a high level of confidence. The results show that the lowering of the freezing rate and the addition of glycerol to the aqueous solution reflect essentially a reduction of the $g_{\|}$ distribution width. The prevailing effect on the reduction of $\sigma g_{\|}$ is ascribed to the reduction of the intrinsic protein heterogeneity, involving a less strained structure and a lower number of conformational substates available for the protein molecules rather than to the properties of the ice phase around the macromolecules. In addition, although the similarity of the two proteins, AZ is characterized by higher $\sigma g_{\|}$ and $\sigma A_{\|}$ values indicating both a greater heterogeneity and conformational variability with respect to PC.

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