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ARTICLE

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Structural heterogeneity of blue copper proteins: an EPR study of amicyanin and of wild-type and Cys3Ala/Cys26Ala mutant azurin

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Abstract A comparative investigation of the effects of cooling rate and solvent physicochemical properties on the structural heterogeneity of wild-type and disulfide bond depleted azurin (Cys3Ala/Cys26Ala) and of amicyanin has been performed by EPR spectroscopy and computer simulation. By describing the spectral features of the EPR spectra in terms of Gaussian distributions of the components of the $\overleftrightarrow{\mathbf{g}}$ and $\overleftrightarrow{\mathbf{A}}$ tensors of the spin Hamiltonian, we have shown that either the cooling rate or the solvent composition affect the structural heterogeneity of the proteins. Such a heterogeneity has been quantified by the standard deviations $\sigma g_{||}$ and $\sigma A_{||}$ of the parallel components of the axially symmetric tensors. In particular, both parameters become smaller after the slow cooling cycle; such a reduction is more significant when glycerol is added as cosolvent to the protein solutions. The comparison of the $\sigma g_{||}$ and $\sigma A_{||}$ values found, for the copper proteins investigated, highlights that the reduction is more marked in the azurins compared to amicyanin and that the Cys3Ala/Cys26Ala azurin mutant has a structural heterogeneity lower than that shown by the wild-type protein. The remarkable similarity of the copper coordination sphere of the proteins suggests a more rigid structure of the azurin protein matrix in the absence of the disulfide bridge compared to wild-type azurin and of amicyanin with respect to both forms of azurin. The former result establishes an important role for the -SS- bond in modulating the flexibility of wild-type azurin.

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Introduction

To perform physiological functions, proteins fold naturally to their three-dimensional structure that is stabilized by the delicate equilibrium between the inter- and intramolecular interactions of the different residues and of these with the water molecules surrounding the proteins. The compactness of the structure is favored also by the presence of disulfide bridges. In fact, removal of one of these bridges entails a stability decrease of the native state in favor of the denatured one (Cooper et al. 1992; Guzzi et al. 1999). Along with the effect on the thermodynamic stability, the role of the disulfide bridge on the dynamic properties of the proteins is also an interesting aspect to be addressed.

Proteins are complex systems that allow a multiplicity of pathways for the folding process (Wang et al. 1996), which have been justified by admitting the existence of a great abundance of conformational substates (CS). Experimental evidence for the existence of the CS, for many years focused on heme proteins, has been recently provided also for the blue copper proteins, differing from the former mainly by the folding topology (essentially β-strands instead of α-helices) and by the active site geometry (Ehrenstein and Nienhaus 1992; Cannistraro et al. 1997; Guzzi et al. 1997). In these CS the protein molecules differ from each other by the structural arrangement of the amino acid side chains, by the position of the hydrogen bonds and by their energy (Frauenfelder 1987; Frauenfelder et al. 1988). The CS number depends on the temperature and on the physicochemical properties of the solvent in which the proteins are dissolved. At room temperature, fluctuations among CS occur continuously, whereas by decreasing the temperature below the glass transition temperature ($T_g \approx 180$ K) the protein molecules freeze in a particular conformation

and give rise to a static distribution of substates, which can be described as structural heterogeneity (Frauenfelder 1987, 1997). The application of EPR spectroscopy to the study of metallo-proteins has greatly helped in understanding the structural heterogeneity of proteins at low temperature (Brill 1977; Hagen 1981; Brill et al. 1986; Groeneveld et al. 1987; Cannistraro 1990; Aqualino et al. 1991; Guzzi et al. 1997). To each conformation or substate is associated a certain ligand field at the paramagnetic center. As a consequence, some relevant spin Hamiltonian parameters are distributed (Hagen 1981; Brill et al. 1986; Hagen 1989; Bizzarri and Cannistraro 1991).

Such effects are generally called *g*- and *A*-strain. In particular, in the $g_{||}$ region of the EPR spectra of blue copper proteins these effects are shown as a progressive line broadening, depending on the nuclear spin quantum number, m_I, value together with a distortion of the hyperfine lines; this distortion consists of an unequal spacing between adjacent hyperfine lines (Cannistraro 1990; Aqualino et al. 1991; Bizzarri and Cannistraro 1995; Cannistraro et al. 1997; Guzzi et al. 1997).

In principle, the observed spread in the active site geometry can be considered as due to the sum of two contributions. The first is due to intrinsic disorder of the protein matrix surrounding the copper site; the second one is due to structural disorder induced by cooling of the solvent around the protein. In fact, when it freezes, the solvent exerts a significant stress on the protein. This stress is transferred to the paramagnetic center, as an additional strain, through the protein matrix (Hagen 1989). To obtain information about the mode of action of these two contributions, both cooling rate and solvent composition investigations are performed. The effects of freezing on biological systems have been extensively studied (Ondrias and Rousseau 1981; Yang and Brill 1991, 1996), whereas the cooling rate effects have been presented as minor effects for a long time (Ondrias and Rousseau 1981; Aqualino et al. 1991; Yang and Brill 1996), and only recently have they been discussed with the same consideration (Guzzi et al. 1997).

With this background, we have investigated how the cooling procedure and the solvent composition affect the active site heterogeneity in azurin (Az), both wild type (wt) and double mutant (dm), and in amicyanin (Ami). Az and Ami, which function as electron transfer, belong to the same protein family, the cupredoxins. They have a very similar metal-binding site, Cu-N_2SS*, with an additional O-ligand for wt Az. From a structural point of view, the main difference between the two proteins is the presence in Az of a disulfide bridge connecting Cys3 to Cys26 and located at the southern end of the protein at about 26 Å from the metal ion. To obtain more insight into the role of the protein matrix, Az has been modified at a strategic site by replacing the two cysteine residues involved in the disulfide bridge with two alanines, by using the site-directed mutagenesis technique. The solvent composition has been altered by adding glycerol to the protein solution. Glycerol was chosen as co-solvent because it is widely used to achieve transparent protein samples, indispensable for the optical spectroscopy investigations on CS at low temperature (Di Pace et al. 1992; Bizzarri and Cannistraro 1993). Nevertheless, its use imposes us to ensure that it is an inert compound which does not alter the protein structure and dynamics.

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 the $g_{||}$ and $A_{||}$ values, respectively. The results obtained show that a reduction of the $\sigma g_{||}$ and $\sigma A_{||}$ values, due to a slow cooling procedure and to the addition of glycerol, takes place in all the proteins. The conformational heterogeneity of azurins is more pronunced than that of Ami. On the other hand, between the two azurin forms, the Cys3Ala/Cys26Ala mutant, i.e., that without the -SS- bond, has a structural heterogeneity lower with respect to the wt protein. It is suggested that the structural and dynamic modifications of the protein matrix, as induced either by the absence of the disulfide bridge or by the presence of glycerol and the cooling rate variation, are involved in the reduction of the protein heterogeneity.

Materials and methods

Az, wt from *Pseudomonas aeruginosa* and dm realized by site-directed mutagenesis, were produced in *Escherichia coli* and were purified as described elsewhere (Van de Kamp et al. 1990; Guzzi et al. 1999). Ami, from *Thiobacillus versutus*, was obtained as reported by Kalverda et al. (1994). The purity of the proteins was judged on a standard IEF gel electrophoresis.

The proteins were dissolved at a concentration of about 1 mM in 10 mM phosphate buffer solution, pH 7. Samples in the water-glycerol mixture were prepared by adding 50% v/v glycerol to the protein solution. The presence of glycerol in solution reduced the pH by 0.4.

All protein samples were analyzed in the slow and rapid cooling regimes. Rapid cooling was obtained by plunging the quartz tube containing the sample directly into liquid nitrogen, whereas in the slow cooling regime the temperature was lowered from 300 to 170 K, in steps of 5 K each over 10 min, using the ER 4111 VT Bruker temperature control unit.

All EPR spectra have been recorded at 77 K by a Bruker ER 200D-SRC X-band spectrometer, equipped with the ESP 1600 data acquisition system. The experimental conditions were as follows: 100 kHz magnetic field modulation, 10 mW microwave power, and 5 G modulation amplitude.

Theory and simulation model for analysis of the EPR spectra

The 77 K EPR spectra of Az, wt and dm, and of Ami show the spectral features of Cu^{2+} metal complexes ($S=1/2$, $I=3/2$) with axial symmetry and can be described by a spin Hamiltonian containing both Zeeman and hyperfine interactions:

$$H = \beta_e\left[g_{\|}B_zS_z + g_{\perp}\left(B_xS_x + B_yS_y\right)\right] + A_{\|}I_zS_z + A_{\perp}\left(I_xS_x + I_yS_y\right) \quad (1)$$

where β_e is the electron Bohr magneton, and $\sigma g_{||}$ and $\sigma A_{||}$ and $\sigma g_{\perp}$ and $\sigma A_{\perp}$ are the parallel and perpendicular components of the $\overleftrightarrow{\mathbf{g}}$ and $\overleftrightarrow{\mathbf{A}}$ tensors, respectively.

The resonance frequencies for the four allowed m_I-dependent EPR transitions can be obtained by solving to second-order perturbation theory the spin Hamiltonian of Eq. (1) (Bleaney 1960):

$$v_0(m_I) = \frac{1}{h}\left[\gamma\beta_e B + Km_I + \frac{K'}{g\beta_e B}\right] \quad (2)$$

where g, K, and K' are given respectively by:

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

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

and:

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

with θ the angle between the direction of the external magnetic field B and the molecular axis of the Cu^{2+} copper complex.

For a two-level system ($S=1/2$), the microwave radiation absorption from paramagnetic species is proportional to (Abragam and Bleaney 1970; Pilbrow 1984):

$$S(v, B) \propto \left\{\left|V_{\alpha\beta}\right|^2 f\left([v - v_0]^2, \sigma_v^R\right)\right\} \quad (6)$$

where $\left|V_{\alpha\beta}\right|^2 = |\langle\alpha|V(t)|\beta\rangle|^2$ is the transition probability between the two levels, orientation independent; v is the applied microwave frequency and v_0 is the resonance frequency; $f\left([v - v_0]^2, \sigma_v^R\right)$ is the lineshape function, centered at v_0, with residual linewidth σ_v^R, which is mainly determined by unresolved coupling of Cu^{2+} electronic spin to ligand nuclear spin.

In a polycrystalline sample, as well as complexes in a frozen solution, all orientations of the symmetry axis of a microcrystal with respect to the magnetic field are equally probable. So the microwave radiation absorption depends on the orientation-dependent transition probability. This term, which is proportional to $\left|V_{\alpha\beta}\right|^2$, is given by (Pilbrow 1990):

$$g_1^2 = \frac{1}{2}g_\perp\left[\frac{g_\parallel^2}{g^2} + 1\right] \quad (7)$$

Under this condition the net absorption can be expressed by:

$$S(v, B) = Cv\int_0^{\pi/2} g_1^2 f\left([v - v_0]^2, \sigma_v^R\right)\sin\theta\, d\theta \quad (8)$$

Here C is a constant taking into account all instrumental parameters in Eq. (6).

In metallo-proteins, strain effects have been taken into account by means of a Gaussian distribution of ligand-copper bond lengths and angles, which in turn affect the $\overleftrightarrow{\mathbf{g}}$ and $\overleftrightarrow{\mathbf{A}}$ values (Froncisz and Hyde 1980; Cannistraro 1990).

However, the presence of g- and A-strain does not allow us to obtain the spin Hamiltonian parameters from a direct inspection of the experimental EPR spectra, because the peaks of the hyperfine lines could be meaningfully shifted with respect to the position in the absence of strain (Bizzarri and Cannistraro 1995). To fully characterize the paramagnetic properties of the systems, computer simulation is required.

The approach used by us to simulate the experimental EPR spectra has been that suggested by Bizzarri and Cannistraro (Cannistraro 1990; Bizzarri and Cannistraro 1995). In this model the strain effect is introduced in terms of a correlated distribution of $g_{||}$ and $A_{||}$ values. With this assumption, an analytical expression for the EPR absorption of the various systems in the presence of strain can be extracted. In particular, under the hypotheses that small changes in $g_{||}$ and $A_{||}$ values occur and that changes in $g_\perp$ and $A_\perp$ values are negligible, the fluctuations in the resonance frequencies can be expressed as:

$$\Delta v_0 = \frac{\partial v_0}{\partial g_\parallel}\Delta g_\parallel + \frac{\partial v_0}{\partial A_\parallel}\Delta A_\parallel \quad (9)$$

In this way, the EPR absorption, $S(v, B)$, can be obtained by integrating Eq. (8) on the $g_{||}$ and $A_{||}$ values:

$$S_{strain}(v, B) = \iint S(v, B)F\left(g_\parallel, A_\parallel\right)dg_\parallel\, dA_\parallel \quad (10)$$

where $F(g_{||}, A_{||})$ is a bivariate normal probability density function, which describes the Gaussian fluctuations of $g_{||}$ and $A_{||}$ in terms of the respective variances $(\sigma g_{||})^2$ and $(\sigma A_{||})^2$ and of the correlation coefficient ρ. Under these circumstances, $S_{strain}(v, B)$ can be expressed as follows:

$$S_{strain}(v, B) = Cv\int_0^{\pi/2} g_1^2 f\left([v - v_0]^2, \sigma_v^T\right)\sin\theta\, d\theta \quad (11)$$

in which:

$$\left(\sigma_v^T\right)^2 = \left(\sigma_v^R\right)^2 + \left(\sigma_v^S\right)^2 \quad (12)$$

is the total linewidth. The additional term $\left(\sigma_v^S\right)$ is due to strain-induced broadening and is given by:

$$\left(\sigma_v^S\right)^2 = \left(\frac{\partial v_0}{\partial g_\parallel}\right)^2\left(\sigma_{g_\parallel}\right)^2 + \left(\frac{\partial v_0}{\partial A_\parallel}\right)^2\left(\sigma_{A_\parallel}\right)^2 + 2\rho\left(\frac{\partial v_0}{\partial g_\parallel}\right)\left(\frac{\partial v_0}{\partial A_\parallel}\right)\left(\sigma_{g_\parallel}\right)\left(\sigma_{A_\parallel}\right) \quad (13)$$

By solving the partial derivatives, $\left(\sigma_v^S\right)^2$ can be expressed as a power expansion of m_I and B as follows:

$$\left(\sigma_v^S\right)^2 = \left(\frac{g\beta_e}{h}\right)^2\left(\mathscr{A}m_I^2 + \mathscr{B}m_I B + \mathscr{C}B^2\right) \quad (14)$$

where $\mathscr{A}$, $\mathscr{B}$ and $\mathscr{C}$ depend on the magnetic parameters as explicitly reported by Cannistraro et al. (Cannistraro and Giugliarelli 1986; Bizzarri and Cannistraro 1995).

The recorded EPR spectrum intensity is the first derivative of the absorption spectrum and can be obtained by deriving Eq. (11) with respect to B:

$$I_{strain}(B) = \frac{dS_{strain}(v_c, B)}{dB} \quad (15)$$

Equation (15) has been used to fit the 77 K experimental EPR spectra of Az and Ami. To evaluate the goodness of the fit, a minimization procedure with the χ^2 test, making use of the following expression, has been used:

$$\chi^2 = \sum_{i=1}^{N}\left[\frac{I_{exp}(B_i) - I_{strain}(B_i, p)}{\sigma_i}\right]^2 \quad (16)$$

where $I_{exp}(B_i)$ is the intensity of the experimental EPR spectrum of the sample at discrete points in which the magnetic field sweep is divided, $I_{strain}(B_i, p)$ is the simulated spectrum that depends on the magnetic parameter set p, and σ_i is the standard deviation calculated for the i-th experimental point of the EPR spectrum.

Results and discussion

The results obtained in the present study for different blue copper proteins have been analyzed and discussed in close connection with the role played by the solvent and cooling rate as well as by protein-matrix modification in characterizing the CS energy landscape.

Figure 1 (solid line) shows the EPR spectrum of wt Az in aqueous solution, recorded at 77 K after rapid cooling.

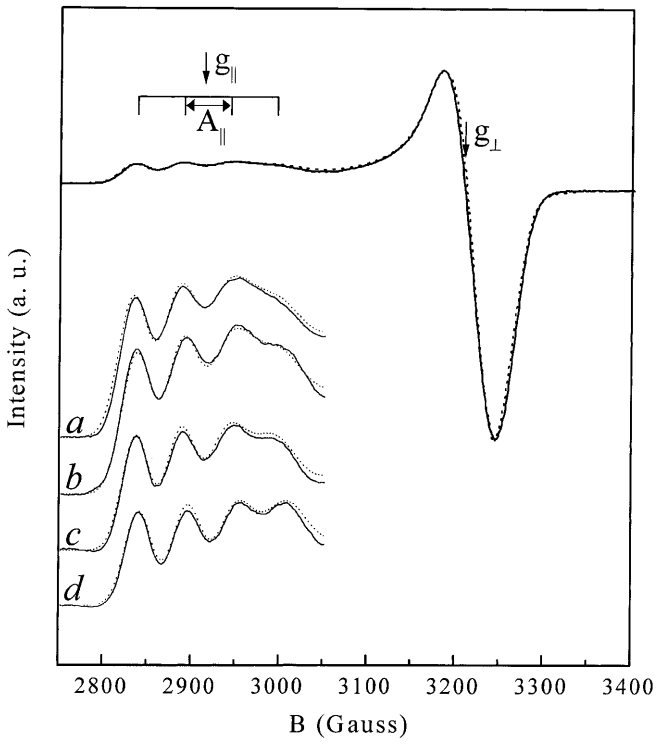

Fig. 1 Experimental (*solid line*) and simulated (*dotted line*) EPR spectra of wild-type azurin recorded at 77 K after rapid cooling. In the *inset* is shown the parallel region of the experimental (*solid line*) and simulated (*dotted line*) spectra of wt Az after (*a*) rapid and (*b*) slow cooling in aqueous solution and after (*c*) rapid and (*d*) slow cooling in a water-glycerol mixture (50% v/v). The corresponding spin Hamiltonian parameters used in the computer simulation of the experimental spectra are given in Table 1

After this cooling procedure the sample is in an amorphous phase. The spectrum is characterized by four hyperfine lines at low magnetic field centered at $g_{||}$ and separated by $A_{||}$, and by another more intense line at high field centered at $g_{\perp}$, resulting from the superposition of the four hyperfine perpendicular components due to a low $A_{\perp}$ value. Moreover, from $g_{||} > g_{\perp} > g_e$ it can be concluded that the copper site has a very distorted tetrahedral geometry. These spectral features are typical of a type-1 copper complex with axial symmetry (Brill 1977).

From the inset in Fig. 1 (pattern a, solid line), showing the $g_{||}$ region of the spectrum recorded at a higher gain, a distortion of the hyperfine lines is observed. Such a distortion is ascribed to (1) dependence of the line broadening on m_I and on applied microwave frequency and (2) partial overlap of the resonance lines with different m_I values. The latter effect is more evident in the $m_I = 1/2$ and $m_I = 3/2$ resonance lines, which form almost a single broad band. The presence of such a distortion, which is mainly related to a strain effect, makes it difficult to obtain the magnetic spin Hamiltonian parameters from the experimental spectrum. Other factors, such as different hyperfine splitting of the ^{65}Cu (30%) and ^{63}Cu (70%) isotopes or non-coinciding principal axes of the $\overleftrightarrow{\mathbf{g}}$ and $\overleftrightarrow{\mathbf{A}}$ tensors as possible sources of these line broadening, do not explain the behavior of the low-field hyperfine line of azurin (Aqualino et al. 1991).

To fully characterize the paramagnetic properties of the analyzed biosystems, a computer simulation procedure has been used. The simulated spectrum, obtained following the procedure described in the previous section, is shown in Fig. 1 (pattern a, dotted line). Both the visual inspection and the low χ^2 value suggest a good agreement between the experimental spectrum and the simulated one. The parameters used to simulate all the experimental spectra are listed in Table 1.

The EPR spectrum at 77 K of the same Az sample, but subject to a slow cooling process, shows the same spectral features of Az rapidly cooled. After such a cooling procedure, a polycrystalline phase is obtained. The parallel region of the spectrum is shown in the inset of Fig. 1 (pattern b, solid line).

By comparing the two $g_{||}$ regions, a reduced linewidth and a higher intensity of the hyperfine lines are observed. Moreover, the $m_I = 1/2$ and $m_I = 3/2$ resonance lines are better resolved and, in addition, there is a high-field shift of the whole spectrum, especially of the $m_I = 3/2$ line.

Table 1 Spin Hamiltonian parameters used to simulate the EPR spectra of blue copper proteins

	Glycerol (% v/v)	$g_{\|\|}$	$g_{\perp}$	$A_{\|\|}$ (G)	$A_{\perp}$ (G)	$\sigma g_{\|\|}$	$\sigma A_{\|\|}$ (G)	χ^2
Azurin wt								
Rapid cooling	0	2.2867	2.0628	51.90	4.1	0.0106	9.5158	0.932
Slow cooling	0	2.2837	2.0633	51.70	4.2	0.0093	9.2158	0.915
Rapid cooling	50	2.2851	2.0640	50.90	4.1	0.0085	8.0158	0.905
Slow cooling	50	2.2777	2.0635	55.00	4.2	0.0075	6.9158	0.897
Amicyanin								
Rapid cooling	0	2.2367	2.0425	54.00	5.5	0.0042	7.2158	0.929
Slow cooling	0	2.2510	2.0570	54.80	5.7	0.0040	7.0158	0.901
Rapid cooling	50	2.2507	2.0550	54.30	5.5	0.0037	5.6158	0.911
Slow cooling	50	2.2507	2.0550	54.00	5.5	0.0035	5.6158	0.905
Azurin dm								
Rapid cooling	0	2.2867	2.0628	51.50	4.1	0.0082	7.6158	0.912
Slow cooling	0	2.2867	2.0628	51.20	4.1	0.0078	7.2158	0.927
Rapid cooling	50	2.2851	2.0633	52.20	4.1	0.0076	7.0158	0.917
Slow cooling	50	2.2794	2.0648	53.70	4.1	0.0068	6.6158	0.921

The latter effect has been explained as being due to the overlap of each of the m_I resonance lines on the background of the adjacent ones (Bizzarri and Cannistraro 1995). No significant variations in the $g_\perp$ region are observed. The corresponding simulated spectrum is shown in the inset of Fig. 1 (pattern b, dotted line): there is a good agreement between the two spectra also in this case. It is noteworthy that in the slow cooling regime the $\sigma g_{||}$ and $\sigma A_{||}$ values are smaller than in the rapid one.

When amicyanin in aqueous solution undergoes rapid cooling, the EPR spectrum at 77 K (Fig. 2, solid line) also shows distortion of the $g_{||}$ region. Such a distortion is more reduced with respect to the one observed in wt Az, as evidenced by the higher resolution of the hyperfine lines, and it is well reproduced by the simulation model (see inset, pattern a, dotted line).

The slow cooling cycle on the Ami sample gives an EPR spectrum similar to that obtained after rapid freezing. However, by comparing the parallel regions at higher gain (patterns a and b, solid lines), a small linewidth and a higher intensity of the hyperfine lines, a slight shift to higher field of the spectrum, in particular of the $m_I = 3/2$ hyperfine line, and a little more regular pattern are observed as a result of the slow cooling procedure. The simulated spectrum (dotted line) is characterized by a fitting parameter set which shows smaller $\sigma g_{||}$ and $\sigma A_{||}$ values (Table 1).

Accurate comparison of the simulation data in Table 1 for the rapid and slow cooling of both proteins indicates a reduction of $\sigma g_{||}$ and $\sigma A_{||}$, by lowering the cooling rate. Such a reduction is more pronounced for wt Az.

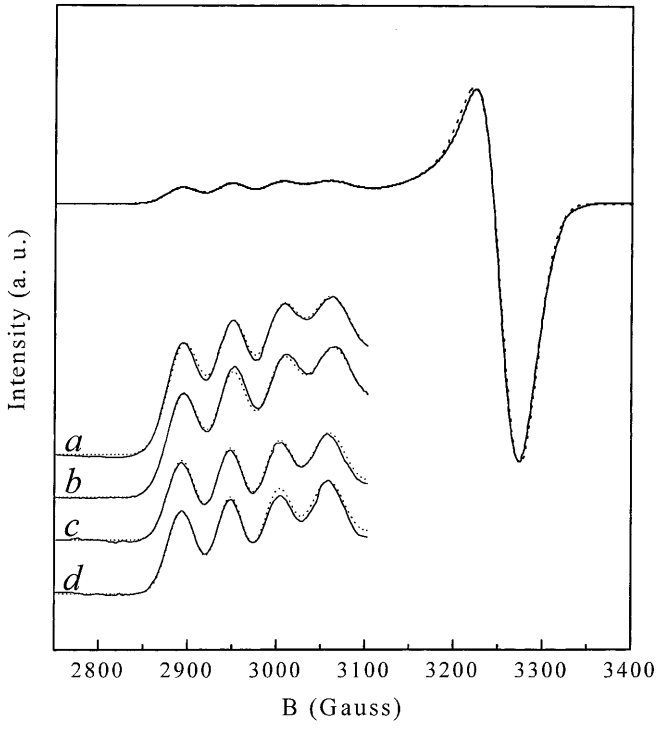

Fig. 2 As in Fig. 1 but for amicyanin

In explaining the obtained data, different aspects should be taken into account. The total protein heterogeneity can be considered as the sum of two contributions: one intrinsic, coming from the presence of the CS, and the other extrinsic, related to the external solvent. By lowering the cooling rate, the two contributions experience opposite variations. In fact, on the one hand the slow cooling favors a regular and continuous growth of ice crystals, which achieve larger dimensions with respect to the rapid cooling procedure (Ross 1965; Hagen 1989; Yang and Brill 1991). Thus the stress on the protein molecules increases and there is a rise of the solvent-induced heterogeneity, leading to a higher structural disorder in the protein. On the other hand, the slow cooling process allows the atoms constituting a protein molecule, especially the metal ion protein ligands, to reach an equilibrium position (Bacci and Cannistraro 1990; Yang and Brill 1991). The local arrangement of the protein ligands to a more stable position involves a less strained structure; the result is a reduction of the intrinsic protein heterogeneity. We observe in both proteins a reduction of $\sigma g_{||}$ and $\sigma A_{||}$ values on going from the rapid to slow cooling, meaning that the latter effect prevails.

Such a result is supported by other experimental studies. For example, low-temperature EPR spectra analyses of myoglobin and hemoglobin, ferric proteins in which the paramagnetic center is situated in a hydrophobic pocket, have demonstrated that the inhomogeneities of the hyperfine lines are mainly ascribable to structural disorder of the macromolecules, whereas the water freezing effects play a secondary role (Fiamingo et al. 1989; Yang and Brill 1991). Like these proteins, also in azurin and amicyanin the active site is inside the protein, located about 7.5 and 6 Å away from the protein surface, respectively (Adman 1991; Romero et al. 1993). Thus the interaction between the paramagnetic ion and the solvent is mediated by the protein matrix. The relevant role played by the protein matrix in *g*- and *A*-strain is also demonstrated by the presence of these effects in the EPR spectrum of small copper complexes in the amorphous phase, and their absence in the polycrystalline phase (Cannistraro et al. 1988). Our samples also show the strain effects after the slow cooling cycle. Accordingly, the disorder and size of the protein have to be taken into account. In other words, the cooling rate effects observed in the experimental spectra of Az and Ami are due to intrinsic protein heterogeneity. From the simulation data in Table 1, it can be pointed out that: (1) the structural heterogeneity of Az is greater than that of Ami; (2) $\Delta\sigma g_{||} = \sigma g_{||(\mathrm{rc})} - \sigma g_{||(\mathrm{sc})}$ and $\Delta\sigma A_{||} = \sigma A_{||(\mathrm{rc})} - \sigma A_{||(\mathrm{sc})}$, where the subscript indices "rc" and "sc" refer to rapid and slow cooling procedures, respectively, and are higher in Az than in Ami.

The less structural heterogeneity of Ami should be attributable to its smaller size with respect to that of Az (106 residues versus 128). In fact, the smaller is the protein size, the lower is the number of possible conformations (Gafert et al. 1995), assuming for the protein

at least 2^N conformations, where N is the number of amino acid residues. This suggests a lower CS number for Ami. The different heterogeneity could also be ascribable to the diverse composition of the protein matrix, i.e., a different primary structure and, consequently, to a different conformational landscape in the two proteins. Ami, like Az, has a β-type secondary structure, but a greater number of β-strands (9 versus 8) and the lack of an entire α-helix award it a more compact structure, determining a more narrow distribution of the ligand field at the copper site with respect to that observed in Az.

The strain reduction, as regards the cooling procedure, becomes more evident if 50% v/v glycerol is added as co-solvent to the protein solution. The EPR spectrum at 77 K of wt Az in the water-glycerol mixture after rapid freezing always shows axial symmetry. By comparing the $g_{||}$ regions of the spectra in the two solvents (inset of Fig. 1, patterns a and c), there can be observed, in the presence of glycerol, a higher resolution of the EPR spectrum, particularly for the $m_I = 3/2$ hyperfine line. This effect is coupled to a downfield shift of the $m_I = -1/2$ and $m_I = 1/2$ hyperfine lines. The $g_{||}$ region of the simulated spectrum is shown in the inset of Fig. 1 (pattern c, dotted line) and the corresponding parameters are given in Table 1.

The parallel region of the EPR spectrum of Az in the presence of glycerol, under slow cooling, is also shown in the inset of Fig. 1 (pattern d, solid line). This region is less distorted, as is confirmed by the lower $\sigma g_{||}$ and $\sigma A_{||}$ values used to simulate the EPR spectrum (pattern d, dotted line), with respect to rapid freezing of the same sample.

The EPR spectra of Ami in the water-glycerol mixture follow the same trend as Az, for the same stress cooling (Fig. 2, patterns c and d). From the simulation data in Table 1 can be noted a reduction of $\sigma g_{||}$ and $\sigma A_{||}$ in the presence of glycerol even if it is not as evident as in Az. Similar results have already been found for plastocyanin (Guzzi et al. 1997), a cupredoxin with a similar copper active site and folding topology.

To understand the glycerol effects on the protein structure, some considerations should be made. The addition of glycerol to the aqueous solution produces an increase of the chemical potential of the protein owing to the "preferential exclusion" of glycerol from the first hydration shell of the protein (Gekko and Timasheff 1981). To improve this thermodynamically unfavorable situation, the protein reduces contact with the solvent, favoring a rearrangement of the protein molecules to reach a minimum distance. This packing makes unfavorable the transitions among the CS which involve motions of large amplitude, whereas small amplitude motions continue occurring; this suggests a decrease of the intrinsic protein heterogeneity.

Such a behavior is also confirmed by the glycerol action concerning the inside of the macromolecule: the addition of glycerol to the protein solution makes the "core" of the protein denser and less compressible, owing to the partial collapse of the cavities in the protein formed by removal of the so-called "lubricating water" (Chirgadze and Ovsepyan 1972; Zak and Klibanov 1988) from the glycerol (Priev et al. 1996). This implies an internal stiffening of the biomolecule, with a consequent reduction of the amplitude of the motions induced by thermal excitation and microenvironmental fluctuations.

On the other hand, the presence of glycerol in the solution lowers the freezing temperature of the solvent, slowing the growth rate of the ice crystals, with a consequent reduction of their dimensions with respect to the case where no cryoprotective agents are present, for the same temperature (Ross 1965). The resulting effect is a reduced strain induced on the protein matrix and, accordingly, a weaker extrinsic heterogeneity in the presence of glycerol. Another possible mechanism, which could be responsible for the spectral modifications, is represented by the glycerol effect on the dynamics of formation and rupturing of the H-bonds. In fact, glycerol, by raising the solution viscosity, alters the protein-solvent coupling. Since the fluctuations among CS are strongly correlated to the fluctuations in the H-bond network in the hydration water shell (Doster et al. 1986), a reduction of the solvent dynamics should be responsible for the reduced structural heterogeneity.

To obtain more insight into the effects of the protein matrix on the structural heterogeneity, we have also investigated in detail the behavior of the Az mutant. Before analyzing the results of the cooling effects on the Az mutant, it should be pointed out that the double mutation does not affect the global protein folding, as has been shown by optical absorption, fluorescence emission, and circular dichroism experiments at room temperature (Guzzi et al. 1999), as well as by X-ray diffraction data (Bonander et al. 2000).

Figure 3 shows the EPR spectrum of dm Az, recorded at 77 K, after rapid cooling and in the inset are shown the $g_{||}$ regions of the spectra after the rapid and slow cooling cycles, without (patterns a and b) and with (patterns c and d) 50% v/v glycerol. The spectral features are typical of a type-1 copper complex with axial symmetry and the spectral behavior of the dm Az samples reflects, on the whole, the same trend as the previously investigated proteins, i.e., by lowering the cooling rate and by adding glycerol to the aqueous solution the structural protein heterogeneity is reduced. Nevertheless, the EPR spectra are more regular compared to those of wt Az after the same cooling procedures, as is evident by the higher resolution of the parallel hyperfine lines, in particular of the $m_I = 3/2$ resonance. The parameters used to simulate the experimental EPR spectra (see Table 1), for the same solvent and cooling rate, suggest that for dm Az: (1) the structural heterogeneity is reduced with respect to wt Az; (2) the $\Delta\sigma g_{||}$ and $\Delta\sigma A_{||}$ values are smaller than those found for wt Az.

Since the two forms of Az have an equal number of amino acids, and thus of conformations, the reduced

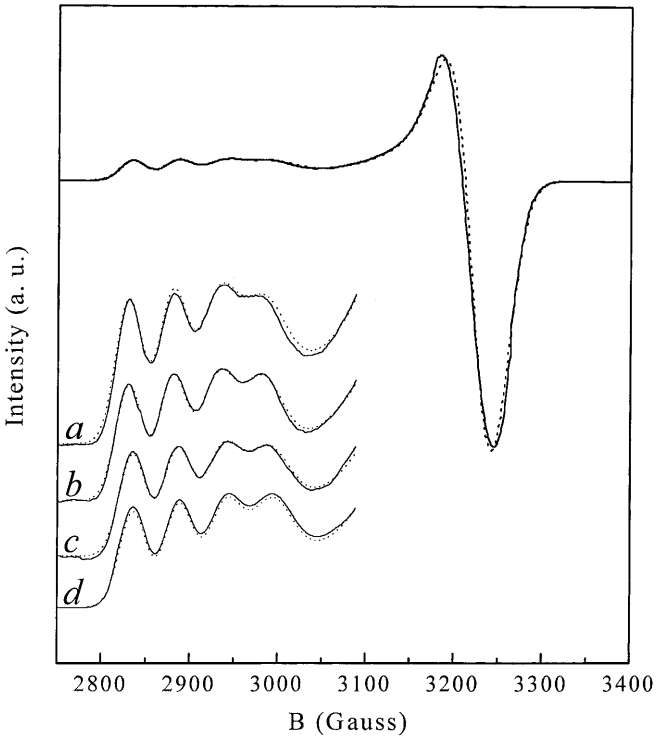

Fig. 3 As in Fig. 1 but for the Cys3Ala/Cys26Ala azurin mutant

structural heterogeneity of dm Az should be ascribed to a lower number of accessible states for the mutated protein. Our data suggest that the removal of the disulfide bridge in wt Az affects the dynamic behavior, making the protein matrix more rigid. By assuming that alanines are non-polar residues, the disulfide bridge is probably substituted by hydrophobic interactions that reduce the CS motions amplitude, inducing a compactness in the protein. This result is also supported by molecular dynamics simulation data, which show that the dynamics of dm Az is significantly depressed with respect to that of the wt protein (manuscript in preparation). The lower structural heterogeneity of dm Az explains why the $\sigma g_{||}$ and $\sigma A_{||}$ values are smaller and change over a shorter range. By analogy with Ami, it can be hypothesized that if the protein matrix is already more rigid in the native form, then the reduction of the cooling rate and the addition of glycerol to the protein solution are less effective in reducing the strain effects.

The results presented in this study allow us to highlight that: (1) the protein heterogeneity is strongly dependent on the protein matrix: (2) the disulfide bridge is a structural element that plays an important role in determining the flexibility of the protein matrix, which is significantly reduced after its removal. The latter result could also affect the biological functionality of Az. In fact, since the active site, where the redox reaction takes place, is deeply buried within the protein, the flexibility extent could be of some importance. Accordingly, it could be fundamental to warrant to the whole system the correct flexibility and in particular to allow the electron to shuttle between the biological partners of Az. This hypothesis, however, needs more experimental investigation.

Conclusions

EPR spectroscopy has revealed itself to be a suitable technique to enquire into the structural heterogeneity of Az, wt and dm, and Ami, observed at low temperature and strictly connected with the presence of a frozen ensemble of protein molecules in different CS. By using a simulation numerical method, in which the protein heterogeneity is taken into account through the width of the magnetic parameters' $g_{||}$ and $A_{||}$ distributions, the EPR experimental spectra have been simulated to a high level of confidence. The results show that lowering of the cooling rate and the addition of glycerol to the aqueous solution are reflected in a reduction of the $g_{||}$ and $A_{||}$ distributions width. The decrease of the $\sigma g_{||}$ and $\sigma A_{||}$ values is ascribable to a reduction of the intrinsic protein heterogeneity; this involves a less strained structure and a lower number of CS accessible to the proteins. A small contribution to the strain, instead, comes from the properties of the ice phase around the macromolecules.

It is interesting to note that dm Az has $\sigma g_{||}$ and $\sigma A_{||}$ values lower than those of the native protein; consequently, the disulfide bridge removal decreases the heterogeneity of the Az protein matrix. In addition, despite the similarity in the active site structure of Az and Ami, the latter is characterized by lower $\sigma g_{||}$ and $\sigma A_{||}$ values, for the same solvent composition and cooling rate, indicating both a smaller heterogeneity and conformational variability of Ami with respect to Az.

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