EPR and Magnetic Susceptibility Studies of Cobalt(II)- and Nickel(II)-Substituted Azurins from *Pseudomonas aeruginosa*. Electronic Structure of the Active Sites

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The electronic properties of cobalt(II)- and nickel(II)-substituted azurins from *Pseudomonas aeruginosa* have been investigated. EPR data for the cobalt derivative and paramagnetic susceptibility data for the nickel derivative are reported. The EPR spectrum of Co(II)−azurin shows the typical pattern of a Kramers' doublet ($\pm^1/_2$) associated with an $S = {}^3/_2$ ground state in a distorted axial symmetry environment. The temperature dependence of the EPR intensities shows that this Kramers' doublet is the excited doublet and, therefore, that the corresponding zero-field splitting parameter D is negative (~ -3.5 cm^{-1}). The mean g value is equal to 2.3. Nickel(II) azurin exhibits an effective magnetic moment $\mu_{eff} = 2.8\ \mu_B$ (Bohr magnetons), constant in the temperature range 120−30 K. The magnetic moment decreases and reaches the value of 1.80 μ_B at 5 K. From the temperature dependence of the susceptibility, the fitting of the data to the theoretical $S = 1$ susceptibility equation leads to a zero-field splitting parameter D of around 17.7 cm^{-1}. The spin Hamiltonian parameters that have been determined for the two metallosubstituted proteins are consistent with a highly distorted tetrahedral structure derived from an axially elongated trigonal bipyramid.

Introduction

Azurins are cupredoxins which participate in electron transfer chains of several denitrifying bacteria.[1,2] They are small blue copper proteins and consist of a single type 1 copper site bound to a single polypeptide chain of 125−130 amino acid residues and around 14 000 Da molecular weight. While the Cu(II)−azurin has been extensively characterized by UV-vis and EPR spectroscopies,[2] it is less adequate for NMR studies, due to the relatively slow electron relaxation of Cu(II).[3] The crystal structure of Cu(II)−azurin from *Pseudomonas aeruginosa* (*Pae*) has been recently determined to 1.93 Å resolution at different pH values.[4] The copper ion is strongly bound to the S_γ of Cys112 and to the N_δ of both His46 and His117 and weakly ligated by the S_δ of Met121 and the carbonyl oxygen of Gly45, resulting in a distorted trigonal-bipyramidal geometry (see Figure 1a).

The native Cu(II) ion has been substituted by Ni(II) and Co(II) in some blue copper proteins,[5−16] and the resulting paramagnetic molecules have been studied by UV-vis[5−8] and ^{1}H NMR spectroscopy.[10−17] It has been suggested that the copper substitution would have minimal effects on the metal site geometry because of the special rigidity of this part of the structure.[18] Thus, the metal binding site experiences minimal changes upon reduction, or even extraction, of the copper.[19−21] This has also been confirmed by recent X-ray crystallographic studies on Zn(II),−[22] Cd(II)−[23] and Ni(II)−azurins.[24] However, the arrangement of metal ligands in the Zn(II)− and Ni(II)−azurins is more tetrahedral than that in the Cu(II)−azurin, being described as distorted tetrahedral.[22,24] In these metalloderivatives, the carbonyl oxygen of Gly45 is more strongly coordinated and the metal−S_δ Met121 distance enlarges up to 3.3−3.4 Å.[22,24]

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(1) Sykes, A. G. In *Advances in Inorganic Chemistry*; Sykes, A. G., Ed.; Academic Press Inc: New York, 1991; Vol. 36, pp 377−408.
(2) Chapman, S. K. In *Perspectives on Bioinorganic Chemistry*; Hay, R. W., Dilworth, J. R., Nolan, K. B., Eds.; Jai Press Ltd: London, 1991; Vol 1, pp 95−140 and references therein.
(3) Bertini, I.; Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems*; Benjamin Cummings: Boston, 1986.
(4) Nar, H.; Messerchmidt, A.; Huber, R.; van de Kamp, M.; Canters, G. W. *J. Mol. Biol.* **1991**, *221*, 765−772.
(5) Tennent, D. L.; McMillin, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 2307−2311.
(6) Germanas, J. P.; Di Bilio, A. J.; Gray, H. B.; Richards, J. H. *Biochemistry* **1993**, *32*, 7698−7702.
(7) Strong, C.; Harrison, S. L.; Zeger, W. *Inorg. Chem.* **1994**, *33*, 606−608.
(8) Di Bilio, A. J.; Chang, T. K.; Malmström, B. G.; Gray, H. B.; Karlsson, B. G.; Nordling, M.; Pascher, T.; Lundberg, L. G. *Inorg. Chim. Acta* **1992**, *198*−*200*, 145−148.
(9) Ferris, N. S.; Woodruff, W. H.; Tennet, D. L.; McMillin, D. R. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 288−296.
(10) Hill, H. A. O.; Smith, B. E.; Storm, B. C.; Ambler, R. P. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 783−790.
(11) Blaszak, J. A.; Ulrich, E. L.; Markley, J. L.; McMillin, D. R. *Biochemistry* **1982**, *21*, 6253−6258.
(12) Moratal, J. M.; Salgado, J.; Donaire, A.; Jiménez, H. R.; Castells, J. *J. Chem. Soc., Chem. Commun.* **1993**, 110−112.
(13) Vila, A. J. *FEBS Lett.* **1994**, *355*, 15−18.
(14) Moratal, J. M.; Salgado, J.; Donaire, A.; Jiménez, H. R.; Castells, J.; Martínez-Ferrer, M[a]. *J. Magn. Reson. Chem.* **1993**, *31*, 541−546.
(15) Salgado, J.; Jiménez, H. R.; Donaire, A.; Moratal, J. M. *Eur. J. Biochem.* **1995**, *231*, 358−369.
(16) Piccioli, M.; Luchinat, C.; Mizoguchi, T. J.; Ramirez, B. E.; Gray, H. B.; Richards, J. H. *Inorg. Chem.* **1995**, *34*, 737−742.
(17) Bertini, I.; Turano, P.; Vila, A. *Chem. Rev.* **1993**, *93*, 2833−2932.
(18) Baker, E. N. *J. Mol. Biol.* **1988**, *203*, 1071−1095.
(19) Shepard, W. E. B.; Anderson, B. F.; Lewandoski, D. A.; Norris, G. E.; Baker, E.N. *J. Am. Chem. Soc.* **1990**, *112*, 7817−7819.
(20) Shepard, W. E. B.; Kingston, R. L.; Anderson, B. F.; Baker, E. N. *Acta Crystallogr.* **1993**, *D49*, 331−343.
(21) Nar, H.; Messerschmidt, A.; Huber, R.; van de Kamp, M.; Canters, G. W. *FEBS Lett.* **1992**, *306*, 119−124.
(22) Nar, H.; Huber, R.; Messerchmidt, A.; Filippou, A. C.; Barth, M.; Jaquinod, M.; van de Kamp, M.; Canters, G. W. *Eur. J. Biochem.* **1992**, *205*, 1123−1129.
(23) Blackwell, K. A.; Anderson, B. F.; Baker, E. N. *Acta Crystallogr.* **1994**, *D50*, 263−270.
(24) Moratal, J. M.; Romero, A.; Salgado, J.; Perales-Alarcón, A.; Jiménez, H. R. *Eur. J. Biochem.* **1995**, *228*, 653−657.

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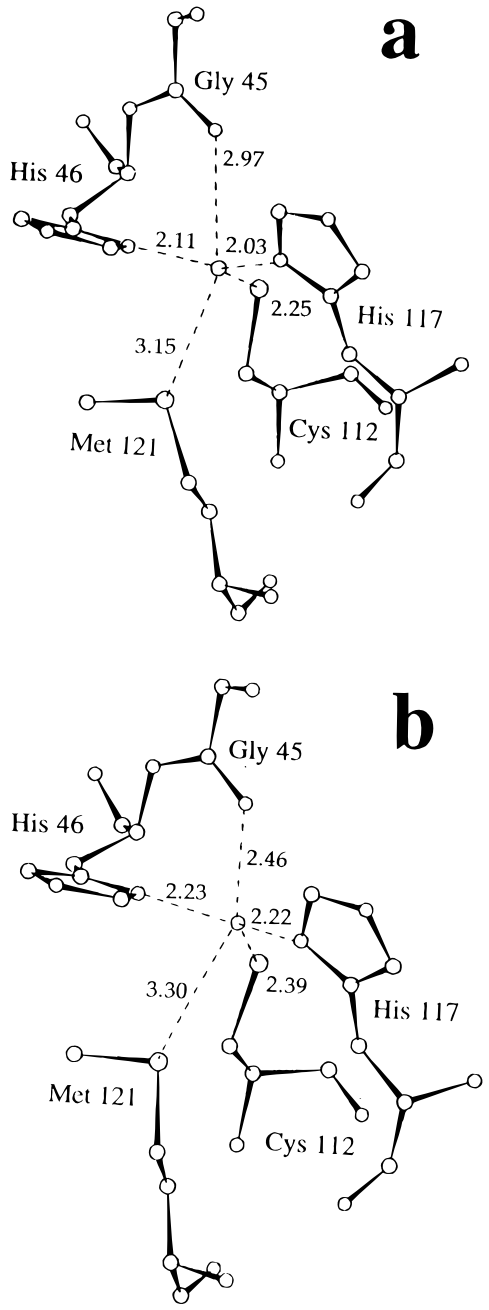


Figure 1. Schematic drawing of azurin metal centers as determined by X-ray crystallography. (a) Copper(II) azurin from ref 4 and (b) nickel(II) azurin from ref 24.

In contrast, the ^{1}H paramagnetic-NMR studies of Ni(II)−azurin in solution indicate that there is still some contact interaction between Met121 and this metal ion.[14] Moreover, ^{1}H NMR studies on Co(II)−azurin suggest a very close structural similarity between both the cobalt- and the nickel-substituted azurins.[15]

Axial ligand interactions in blue copper proteins have called the attention of researchers because they seem to be related with the tuning of the redox properties of these metal sites.[25−27] To understand and evaluate the possibility of metal−axial ligand interactions in the azurin derivatives, we have considered the active site electronic properties derived from EPR and magnetic susceptibility studies, which in general provide valuable information regarding the coordination geometry and electronic structure in biological systems.[28] Thus, the ground state zero-field splitting, which can be evaluated from low-temperature experiments, is directly related to the ligand field properties at the metal center. Various symmetries and rhombicities are present in the case of high-spin cobalt(II) metalloproteins.[29] EPR studies have been performed in some cobalt(II)-substituted proteins such as horse liver alcohol dehydrogenase,[30,31] (Cu−Zn) superoxide dismutase,[32] glyoxalase I,[33] and stellacyanin.[34] Additionally, a previous EPR study of Co(II)−azurin has been reported, in which a high-spin Co(II) center from g values at 4, 28, and 42 K has been proposed.[35] Magnetic properties have also been reported in the case of metallosubstituted proteins such as Co(II)-substituted (Cu−Zn) superoxide dismutase[36] and Co−stellacyanin.[37] Studies of the electronic structure of Ni(II)-containing sites are more difficult, since in general they are EPR silent because of their large zero-field splitting compared to incident microwave energy. Low-temperature magnetic measurements are one of the most powerful techniques to evaluate this zero-field splitting and, therefore, to possibly get information on the coordination symmetry of the metal ion. Thus, the magnetic properties of the Ni(II) enzymes urease[38] and of some Ni(II)-substituted proteins, such as carboxypeptidase A and carbonic anhydrase,[38] have been reported in the last few years. In the case of Ni(II)−azurin, Blaszak et al. have proposed a pseudotetrahedral environment from the value of the effective magnetic moment ($\mu_{eff} = 3.2\ \mu_B$), measured at room temperature by means of the Evans method.[11]

Experimental Section

Materials. All chemicals used were of analytical grade and purchased from Merck and Sigma. Water was bidistilled before use. DEAE Sephacel and CM Sepharose (Pharmacia) were used for ion exchange chromatography, and Sephadex G-100 (Pharmacia) was used for size-exclusion chromatography. The dialyses were carried out using cellulose dialysis tubing Sigma (MW cutoff: 3000), which had been previously boiled in distilled water for 15 min. Each dialysis was carried out for 6 h against a 30-fold excess of stirred buffer solution. All the measurements were carried out with 0.02 M CH_3COONH_4 as buffer.

Bacterial Growth, Protein Purification, and Preparation of Azurin Metalloderivatives. *Pseudomonas aeruginosa* bacteria (CECT110) obtained from the Spanish Type Culture Collection were grown in the medium described by Parr et al.[39] at 34 °C under nitrogen

(25) Gray, H. B.; Malmström, B. G. *Comments Inorg. Chem.* **1983**, *2*, 203−209.
(26) Lowery, M. D.; Solomon, E. I. *Inorg. Chim. Acta* **1992**, *198−200*, 233−243.
(27) Guckert, J. A.; Lowery, M. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1995**, *117*, 2817−2844.
(28) Palmer, G. In *Advances in Inorganic Biochemistry*; Darnall, D. W., Wilkins, R. G., Eds.; Elsevier: North-Holland, 1980; Vol. 2, p 153.
(29) Bertini, I.; Luchinat, C. In *Advances in Inorganic Biochemistry*; Eichorn, G. L., Marzilli, L. G., Eds.; Elsevier: New York, 1984; Vol. 6, pp 78−80.
(30) Makinen, M. W.; Yim, M. B. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6221−6225.
(31) Werth, M. T.; Tang, S. F.; Formicka, G.; Zeppezauer, M.; Johnson, M. K. *Inorg. Chem.* **1995**, *34*, 218−228.
(32) Rotilo, G.; Calabrese, L.; Mondovì, B.; Blumberg, W. E. *J. Biol. Chem.* **1974**, *249*, 3157−3160.
(33) Sellin, S.; Goran Eriksson, L. E.; Aronsson, A. C.; Mannervik, B. *J. Biol. Chem.* **1983**, *258*, 2091.
(34) Dahlin, S.; Reinhammar, B.; Ångström, J. *Biochemistry* **1989**, *28*, 7224−7233.
(35) McMillin, D. R.; Tennent, D. L. In *ESR and NMR of Paramagnetic Species in Biological and Related Systems*; Bertini, I., Drago, R. S., Eds.; Reidel Publishing Co.: Dordrecht, Holland, 1979; pp 369−379.
(36) Morgenstern-Badarau, I.; Cocco, D.; Desideri, A.; Rotilio, G.; Jordanov, J.; Dupre, N. *J. Am. Chem. Soc.* **1986**, *108*, 300−302.
(37) Solomon, E. I.; Wang, R. H.; McMillin, D. R.; Gray, H. B. *Biochem. Biophys. Res. Commun.* **1976**, *69*, 1039−1042.
(38) Clark, P. A.; Wilcox, D. E. *Inorg. Chem.* **1989**, *28*, 1326−1333.
(39) Parr, S. R.; Barber, D.; Greenwood, C.; Phillips, B. W.; Melling, J. *Biochem. J.* **1976**, *157*, 423−430.

atmosphere, using an automatic 14 l fermentor (New Brunswick Scientific Co. NB). The native azurin was isolated from the cell paste and purified in the oxidized state as described earlier.[39−41] The purity of azurin was checked by PAGE and UV-vis spectroscopy. Purified Cu(II)-azurin had an A_{625}/A_{280} ratio of 0.54−0.55. Copper was removed from the oxidized holoprotein by dialyzing a $\sim 5 \times 10^{-5}$ M azurin sample at 20 °C against 0.5 M KCN in 0.1 M phosphate buffer of pH 8.5.[42] The nickel(II) and cobalt(II) metalloderivatives were prepared at pH 8.5 by the addition of 2−3 molar equivalents of the metal ion to a $\sim 5 \times 10^{-5}$ M apoprotein solution at room temperature.[15] The concentrations of the azurin metalloderivatives were measured spectrophotometrically, using the extinction coefficients $\epsilon_{440} = 3.3 \times 10^3$ M^{-1} cm^{-1} for Ni(II)−azurin and $\epsilon_{330} = 4.0 \times 10^3$ M^{-1} cm^{-1} for Co(II)−azurin.[5]

Physical Techniques. Optical spectra in the UV and visible regions were recorded with a Varian Cary 1 or a Perkin-Elmer Lambda 9 spectrophotometer using 1 cm path length cuvettes. EPR spectra were recorded with a Bruker ER-200D spectrometer operating at the X-band frequency of 9.43 GHz and provided with a helium continuous-flow cryostat (Oxford Instruments). Microwave power was 10 dB and modulation amplitude 4 G. Water glass samples were mounted in standard 4 mm quartz EPR tubes. Variable temperature magnetization measurements were made on a SQUID magnetometer, at a magnetic field of 0.1 T, within the temperature range 120−5 K. Concentrations of the studied solutions (3.6 mM) were precisely determined as described here above. Experimental magnetization values were converted to molar susceptibilities. Glycerol was added to the samples (10%) as a cryoprotectant. All the solutions were carefully degassed using the freeze−pump−thaw procedure to remove dissolved molecular oxygen, that is especially important when the expected spin state of the protein sample is $S = 1$.[43] They were transferred to quartz holders, which we have especially designed for SQUID measurements, and kept under argon for storage prior to measurement or use. Separate measurements under identical conditions were made on the buffer solution containing the apoprotein at the same conditions, to subtract the large diamagnetic correction from the overall signal. Proton buffer was used since it has been checked, in experiments made with nickel urease, that the possible magnetic noise due to the slowly relaxing $I = {}^1/_2$ nuclei of water is within experimental error.[38]

Results and Discussion

EPR Spectroscopy of Co(II)−Azurin. The EPR spectra of Co(II)−azurin at 5 K (Figure 2) display a typical distorted axial pattern for high-spin Co(II) with three resonances, the perpendicular signal being split into two components due to rhombic distortion. The effective g values at two different pH conditions are 5.91 (g_y), 3.77 (g_x), and 2.01 (g_z), at pH=7.5 and 5.20 (g_y), 3.85 (g_x) and 2.0 (g_z) at pH = 4.5. In a previously published EPR study at 4.2 K, two g values ($g_1 = 5.2$ and $g_2 = 3.8$) were detected for the Co(II)−azurin.[35] The differences in the two components of the perpendicular signal can be related with a pH-induced conformational change originated by the titration of the His35 residue in the native protein,[4,44,45] which has also been reported for the Ni(II) and Co(II) metalloderivatives.[12,15] The comparison of the g values suggests that the rhombicity at pH = 7.5 is slightly higher than that at pH = 4.5, indicating that the conformational change implies some structural rearrangements in the metal binding site. However, the X-ray structure of Cu(II)−azurin at pH 5 and pH 9,[4] as well as NMR studies at various pH values on Co(II)−azurin,[15] revealed that the conformational transition affects mainly the surroundings

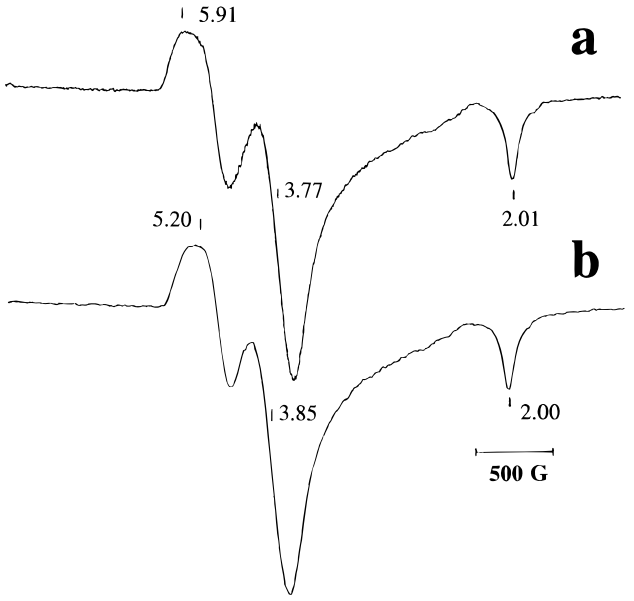


Figure 2. Electron paramagnetic resonance spectra (X-band) of Co(II)−azurin in H_2O solvent at 5 K (2 mM protein, 20 mM CH_3COONH_4): (a) pH 7.5 and (b) pH 4.5.

of His35, while the structural changes in the metal coordination site are probably not significant in view of the experimental error (≤0.1 Å).[4] It seems that these changes, although small, are large enough to produce significant variations in some spectroscopic properties of the metal site, as it can be observed by EPR[45] and NMR.[10−12,15,44]

On the other hand, in the EPR spectra (Figure 2) there is no evidence of resolved hyperfine structure from the ^{59}Co nuclear spin momentum, except at lower field, where the absorption-shaped component indicates possible ill-defined hyperfine transitions. These spectra are consistent with those expected for $S = {}^3/_2$ molecules subject to zero-field splitting where only the ($\pm{}^1/_2$) Kramers' doublet, identified from the effective g values, is resonant. A mean g value of ∼2.3 is calculated from the effective g factors, in agreement with second order spin−orbit coupling and an orbitally nondegenerate ground state. When the temperature is increased from 5 to 20 K, a maximum of intensity is found at 10 K. The same intensity variation is observed for the three resonances, confirming that they arise from rhombic anisotropy of the single doublet ($\pm{}^1/_2$). The intensity maximum shows that this doublet is the excited one and therefore, the predominantly axial zero field splitting D, is negative. At temperatures higher than 10 K, the intensities decrease according to Curie law behavior. When the temperature further increases, the signals vanish. The resonances are so extremely fast relaxing that they are only clearly visible below 20 K.

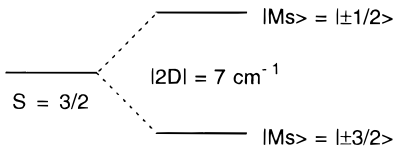
The value of the zero-field splitting parameter D can be evaluated from the intensity dependence of the transitions, according to the Boltzmann distribution of the two Kramers' levels, ($\pm{}^1/_2$) and ($\pm{}^3/_2$) associated with the ground spin state $S = {}^3/_2$. They follow the approximate expression[46]

$$F = \frac{1}{T(1 + e^{2D/kT})} \qquad (1)$$

where $2D$ is the energy gap between the two Kramers' levels and the rhombic parameter E is supposed to be $E = 0$. From this treatment, it is found that $D = -3.5$ cm^{-1}, as it is shown in Scheme 1.

(40) Ambler, R. P.; Wynn, M. *Biochem. J.* **1973**, *131*, 485−498.
(41) Mitra, S.; Bersohn, R. *Biochemistry* **1980**, *19*, 3200−3203.
(42) Hutnik, C. M.; Szabo, A. G. *Biochemistry* **1989**, *28*, 3923−3934.
(43) Day, E. P. *Methods Enzymol.* **1993**, *227*, 437−463.
(44) Adman, E. T.; Canters, G. W.; Hill, H. A. O.; Kitchen, N. A. *FEBS Lett.* **1982**, *143*, 287−292.
(45) Groeneveld, C. M.; Aasa, R.; Reinhammar, B.; Canters, G. W. *J. Inorg. Biochem.* **1987**, *32*, 143−154.
(46) Weil, J. A.; Bolton, J. R.; Wertz, J. E. *Electron Paramagneticc Resonance*; John Wiley: New York, 1994.

Scheme 1



In structurally defined Co(II) complexes, ranges of values of the splitting between the two doublets have been established from -36 to $+13$ cm^{-1} for tetracoordinate sites, from $+20$ to $+50$ cm^{-1} in pentacoordinate sites, and ≥ 50 cm^{-1} in hexacoordinate sites.[47] Although these ranges are probably not appropriate for highly distorted tetracoordinate Co(II) species,[31] it seems that a negative D value is only possible for tetracoordinate Co(II). On the other hand, possible distortions from trigonal-bipyramidal coordination that preserve the trigonal symmetry are described as the so-called tetrahedral distortion.[48] Thus, we interpret our results of the cobalt(II)−azurin active site on this line, i.e., the electronic structure probes a highly distorted tetrahedral geometry that preserves a trigonal coordination, with one of the axial ligands moving away from the cobalt center. The g values presented above, which indicate the existence of a rhombic field and a low-symmetry distortion, would be in agreement with this conclusion. It is interesting therefore to remark that Solomon et al. have proposed a distorted tetrahedral coordination geometry for Co(II)−azurin from infrared and visible circular dichroism and magnetic circular dichroism studies.[49]

Magnetic Susceptibility of Ni(II)−Azurin. Figure 3 displays the temperature dependence of the molar paramagnetic susceptibility χ_M and the temperature dependence of the effective magnetic moment μ_{eff} (inset). Curie law behavior is observed in the temperature range 120−30 K, leading to a constant μ_{eff} value of around 2.8 μ_B, in agreement with $S = 1$ as the ground state. Below 30 K, the deviation from Curie law behavior indicates that the zero-field splitting of the triplet state is operative. The effective magnetic moment decreases and reaches the value of 1.8 μ_B at 5 K.

The following spin hamiltonian is considered:

$$H = D_S\left(\mathbf{S}_Z^2 - \frac{1}{3}\mathbf{S}^2\right) + \beta g_S \sum_{i=x,y,z} (H_i \mathbf{S}_i) \qquad (2)$$

with D_S and g_S being the axial zero-field splitting parameter and the isotropic g factor, respectively, associated with the spin state S. In the case of a triplet $S = 1$ ground state, the molar susceptibility is derived according to the equation:

$$\chi_M = \left(\frac{2N\beta^2}{3kT}\right) g_S{}^2 \frac{e^{-D_S/kT} + \frac{kT}{D_S}(1 - e^{-D_S/kT})}{1 + 2e^{-D_S/kT}} \qquad (3)$$

The best fitting of the data to this theoretical curve gives the following values for the parameters defined above:

$$g_S = 1.98(0.1)$$

$$D_S = 17.7(1) \text{ cm}^{-1}$$

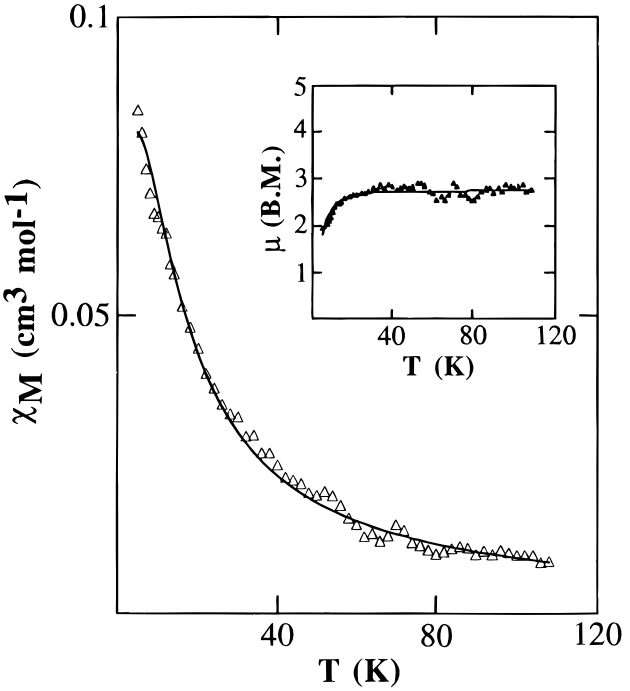


Figure 3. Temperature dependence of the magnetic susceptibility for Ni(II)−azurin in H_2O solvent between 120 and 5 K (3.6 mM protein, 20 mM CH_3COONH_4, pH 7.5). The inset shows the plot of magnetic moment data versus temperature. The solid lines represent the best fitting of the data to the theoretical curve (eq 3).

(The sign of D_S cannot be deduced from these magnetic measurements).

We have restricted the set of parameters to be fitted since magnetization or susceptibility measurements generally do not have enough resolution to provide g anisotropy and to determine reliably both the axial D and rhombic E zero-field splitting parameters. Additionally, the metal content of the solution, known from independent analysis, has not been considered as a parameter, not to scale the data to the expected spin state $S = 1$ in the large temperature range in which the Curie law is obeyed. Consequently, the metal concentration is only correlated with the g factor, and therefore, this g factor is the only variable parameter that we have considered in this region. On the other hand, at high temperatures, the obtained susceptibility values are of the order of magnitude of experimental errors, owing to the low metal content of protein solutions and to substantial scatter of the data associated with the decrease of paramagnetic signals with increasing temperature.

The magnitude of the zero-field splitting of distorted tetrahedral complexes depends strongly on the energy gap between the two levels of the $^3T_1$ parent ground state, which is connected with the spin−orbit coupling. It has been shown that D can vary between 30 and 50 cm^{-1} in a series of pseudotetrahedral nickel complexes of general formula NiN_2S_2, proposed as models for N-S coordinated nickel enzymes and nickel-substituted proteins.[50] Similar values of D have been reported from Ni(II)-substituted rubredoxin proteins with distorted tetrahedral centers.[51] In the case of distorted octahedral Ni(II) complexes, D is much smaller (for example, $D = 5$ cm^{-1} in trans $Ni(py)_4Cl_2$). In our study, it is evident that the observed

(47) Makinen, M. W.; Kuo, L. C.; Yim, M. B.; Wells, G. B.; Fukuyama, J. M.; Kim, J.E. *J. Am. Chem. Soc.* **1985**, *107*, 5245−5255.
(48) Banci, L.; Bencini, A.; Benelli, C.; Gattesci, D.; Zanchini, C. *Struct. Bonding* **1982**, *52*, 37−86.
(49) Solomon, E. I.; Rawlings, J.; McMillin, D. R.; Stephens, P. J.; Gray, H. B. *J. Am. Chem. Soc.* **1976**, *98*, 8046−8048.
(50) Frydendahl, H.; Toftlund, H.; Becher, J.; Dutton, J. C.; Murray, K. S.; Taylor, L. F.; Anderson, O. P.; Tieking, E. R. T. *Inorg. Chem.* **1995**, *34*, 4467−4476 and references therein.
(51) Kowal, A. T.; Zambrano, I. C.; Moura, I.; Moura, J. J. G.; LeGall, J.; Johnson, M. K. *Inorg. Chem.* **1988**, *27*, 1162−1166.

magnetic behavior corresponds to the temperature dependence expected for an orbitally nondegenerate ground state that implies low electronic symmetry. The *D* value that we propose lies between those corresponding to distorted tetrahedral and octahedral symmetry. These results suggest that five coordination is possibly achieved in frozen Ni(II)−azurin solutions, with a fifth ligand at longer bond length. In an earlier investigation of Ni(II)−azurin at room temperature, a higher value for the effective magnetic moment was found. On this basis was proposed a distorted tetrahedral coordination.[11] However, the magnitude of the effective magnetic moment at room temperature does not always provide a reliable method for distinguishing between tetracoordinate and pentacoordinate Ni(II) geometries. The large isotropic shifts presented by some of the Met121 ^{1}H NMR signals of Ni(II)−azurin suggest that this axial ligand is, at least, semicoordinated.[14] Since the coordination of the Gly45 axial ligand in this metalloderivative is clear from the X-ray structure and NMR data,[14,24] this metal site could be defined as pentacoordinated with a very weak fifth ligand (Met121), in line with the present results. Although it is worthwhile to emphasize that a reduced magnetic moment or zero-field splitting (due to second order spin-orbit coupling), compared to the expected ones in tetrahedral symmetry, can be also the result of ligand effects that could give a lower spin−orbit coupling constant.[52]

Concluding Remarks

Distorted tetrahedral coordination environments, leading to geometries intermediate between tetra- and penta coordination, are difficult to evaluate and give rise to apparent contradictions. The azurin metal site provides one of these examples. The existence of two possible axial ligands and a set of three closely interacting equatorial ligands condition the coordination of the metal, which adopts distorted coordination geometries whose main structural feature is the equatorial trigonal plane. Thus, the metal can approach the Met121 axial ligand more, as in the case of the native Cu(II),[4] or the Gly45 axial ligand, as in the Zn(II), Ni(II),[22,24] and probably Co(II)[15] derivatives, but it always stays close to the equatorial plane. In all these situations there is doubt about the existence of interaction with the fifth ligand (Gly45 in the case of Cu(II)−azurin or Met121 in the other metalloderivatives). Thus, for the Ni(II)−azurin, although the crystallographic distance corresponding to the supposed fifth ligand is large, the spectroscopic data indicate the existence of interaction with the metal. From the study of the electronic structures of both cobalt- and nickel-substituted azurins by EPR and low-temperature magnetic measurements, quite similar conclusions can be drawn. Distorted tetrahedral environments of the active sites, such as distorted five-coordinate trigonal geometry, with one axial ligand, probably Met121, moving away, may reconcile apparent contradictory results. However, in several similar studies the question of the reliability of zero-field splitting for distinguishing between tetracoordinate and pentacoordinate geometries in low-symmetry environments is raised.[31] Ligand field calculations are needed to assess this important point.

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(52) Gerloch, M. *Magnetism and ligand field analysis*; Cambridge University Press: Cambridge, 1983.