A W-Band Electron Paramagnetic Resonance Study of a Single Crystal of Azurin

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Abstract: Field-swept electron spin echo spectroscopy at 95 GHz on a frozen solution and a single crystal of azurin from *Pseudomonas aeruginosa* has enabled the determination of the complete *g*-tensor. Highly accurate principal values have been obtained ($g_x = 2.0393 \pm 0.0004$, $g_y = 2.0568 \pm 0.0007$, $g_z = 2.273 \pm 0.004$), and the orientation of the principal axes of the *g*-tensor with respect to the copper site has been established. One of the principal axes makes an angle of 15° with the Cu–Sδ(Met121) bond. The other axes lie almost in the NNS plane and are rotated by 24° with respect to the Cu–Sγ(Cys112) bond. The *g*-tensor does not corroborate descriptions of the copper site in terms of effective C_{3v}, C_{2v}, or C_s symmetry. The observed orientation of the principal axes presents a sensitive point of reference for future quantum-chemical considerations.

I. Introduction

Blue copper proteins like azurin function in redox-reaction chains of bacteria.[1] They are characterized by a single copper atom whose interconversion between the valence states I and II is a key element in the electron transport. Understanding of this process on a molecular scale requires detailed knowledge of the geometric and electronic structure of such proteins and in particular of this copper site.[2] Two developments have given momentum to the research in this area during the last years. First, genetic engineering techniques provide for the possibility of well-defined mutations in the primary structure of the proteins.[3] Secondly, the structures of several copper proteins have become available from high-resolution single-crystal X-ray studies.[4-8] These developments have paved the road for a more effective application of various spectroscopic methods. In this context we have started investigations of azurin and some of its mutants by electron spin echo (ESE) detected electron paramagnetic resonance (EPR) spectroscopy.[9]

Azurin is a low molecular weight protein containing 128–129 amino acids. The structures of azurins from different origins (*Alicaligenes denitrificans*[10] and *Pseudomonas aeruginosa*[6,11]) have been determined by X-ray diffraction studies. Five ligands surround the copper atom forming a site whose geometry can be best described as a trigonal bipyramid. The coordinating atoms of three strongly bound ligands, the Nδ atoms of histidines-46 and -117 and the Sγ atom of cysteine-112, form a distorted trigonal NNS plane from which the copper ion is displaced only very slightly. The other two coordinating atoms, the Sδ of methionine-121 and the carbonyl O of glycine-45, occupy the axial positions and interact weakly with the copper. Until recently, EPR spectroscopy was widely used in the distinction of various types of copper sites.[12] The EPR spectrum of blue copper proteins reveals an almost axially symmetric *g*-tensor and a remarkably small value of the copper hyperfine coupling in the $g_\parallel$ region. The application of higher microwave frequencies for azurin[13] and plastocyanin,[14] Q-band (35 GHz) instead of X-band (9 GHz), improved the resolution thereby clearly showing the splitting of the EPR lines owing to the difference in g_x- and g_y-values. A complete *g*-tensor is not available as yet for any blue copper protein. The orientation of the principal axes of the *g*-tensor with respect to the copper site is unknown which limits the value of EPR as a reference point for quantum-chemical descriptions of the copper site. To the best of our knowledge, there exists only one EPR study of a single crystal of a blue copper protein.[15] This investigation concerns plastocyanin, but, unfortunately, the orientation of only one of the principal axes of the *g*-tensor could be established.

Here we report the results of a high-frequency ESE-detected EPR study at W-band (95 GHz) on both a frozen solution and a single crystal of azurin from *Pseudomonas aeruginosa*. Owing to the superior sensitivity and resolution of EPR at this high frequency, a crystal of submillimeter size could be investigated in detail. Both the principal values and the directions of the principal axes of the *g*-tensor for azurin have been determined with great accuracy. When looked upon from the copper atom, one of the principal axes of the *g*-tensor makes an angle of 15°

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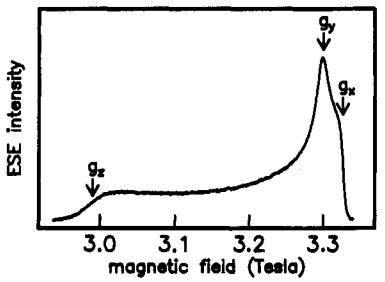

Figure 1. W-band ESE-detected EPR spectrum of a 2 mM solution of azurin at 1.2 K. The magnetic fields corresponding to the principal g-values are indicated by arrows.

with the Cu–Sδ(Met121) bond, while the other two are about in the NNS plane and rotated by 24° with respect to the Cu–Sγ-(Cys112) bond.

II. Experimental Section

Azurin from *Pseudomonas aeruginosa* was purified as described elsewhere.[16] The ESE experiments were performed both on a solution and on a single crystal of azurin. The concentration of the oxidized protein in the solution was 2 mM. This solution was buffered to pH 5.0 with a 20 mM acetate–buffer and contained 40% glycerol. The crystal of oxidized azurin (0.5 × 0.3 × 0.1 mm) was grown by vapor diffusion as reported previously[6] and kept in mother liquor (4.0 M ammonium sulfate, 0.7 M lithium nitrate, 0.1 M acetate, pH = 5.5). The space group of the crystal is $P2_12_12_1$, corresponding to orthorhombic symmetry. The unit cell of dimensions a = 57.8, b = 81.0, c = 110.0 Å contains 16 molecules with four molecules per asymmetric unit.

All ESE experiments were done at 1.2 K. A description of the W-band spectrometer, operating at 94.9 GHz, is given elsewhere.[17] Both the frozen solution and the crystal were contained in a quartz tube and mounted in a cylindrical cavity. Two-pulse echo experiments were performed using microwave pulses separated by 500 ns at a repetition rate of 10 Hz. The EPR spectra were obtained by monitoring the echo height while scanning the magnetic field in the region of 2.9–3.4 T. In order to obtain the principal values of the g-tensor from the EPR spectrum of the frozen solution, Argonne premium coal sample no. 4 was used as a reference.[18] The accuracy of the g-values is restricted by the line width of the canonical components in the EPR spectrum of this coal sample.

From X-ray diffraction the direction of the crystallographic axes for the crystal under study was known and consequently the approximate orientation of the crystal in the EPR spectrometer. The precise orientation (±1°) of the crystallographic a-, b-, and c-axes in a laboratory-fixed axes system was determined from the EPR experiments (vide infra). The spectrometer allows rotation of the external magnetic field (B_0) in a plane that contains the sample tube and rotation of the sample tube around its axis. In this way experiments at all possible orientations of B_0 with respect to the crystal can be performed without remounting the crystal.

III. Results

A. Field-Swept ESE Experiments. The ESE-detected EPR spectrum of the frozen solution of azurin is represented in Figure 1. Copper hyperfine splitting, clearly visible at low-field in EPR spectra at X-band,[13] is not resolved at W-band. This we attribute to g-strain effects, which were found to overshadow the hyperfine structure for blue copper proteins already in Q-band EPR spectra.[13] The shape of the spectrum reveals the near-axial anisotropy of the g-tensor. The principal values of the g-tensor are represented in Table 1.

For the azurin crystal, ESE-detected EPR spectra have been studied as a function of the orientation of the magnetic field with respect to the crystal. For each azurin molecule in the unit cell a local axes system is defined in which the g-tensor of that molecule is diagonal, the so-called principal axes system (x, y, z). Spectra observed for the magnetic field parallel to principal x- and z-axes are given in Figures 2 and 3. For example, the resonance field corresponding to the high-field transition in the top spectrum of Figure 2 is stationary with respect to small variations in the orientation of the magnetic field. The direction of the magnetic field for which this spectrum has been obtained therefore

Table 1. Average Angles between the Principal Axes of the g-Tensor and the Copper–Ligand Bonds with Their Standard Deviations[a]

	x	y	z
Cu-Sγ(Cys112)	112.8° ± 1.6°	24.1° ± 1.7°	97.1° ± 1.5°
Cu-Sδ (Met121)	79.0° ± 1.7°	99.2° ± 1.2°	14.5° ± 1.2°
Cu-Nδ (His46)	19.3° ± 2.5°	109.1° ± 2.5°	90.1° ± 2.7°
Cu-Nδ (His117)	124.5° ± 2.1°	145.3° ± 2.1°	88.6° ± 3.1°
Cu-O (Gly45)	77.2° ± 1.4°	101.9° ± 1.7°	162.3° ± 1.8°

[a] Principal values of the g-tensor: g_x = 2.0393 ± 0.0004, g_y = 2.0568 ± 0.0007, g_z = 2.273 ± 0.004.

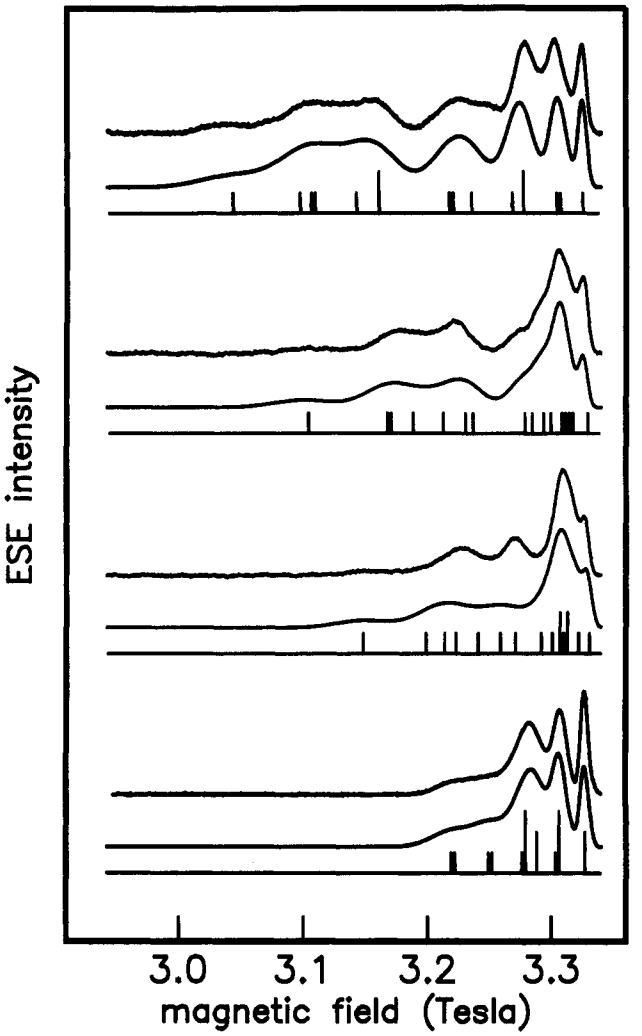

Figure 2. W-band ESE-detected EPR spectra of a single crystal of azurin at 1.2 K for four different orientations of the magnetic field with respect to the crystal. In each case the magnetic field is directed along the principal x-axis of the g-tensor of one of the four molecules in the asymmetric unit. For each orientation the top spectrum represents the experimental spectrum, the bottom one the calculated stick diagram, while the middle one, the simulated spectrum, has been obtained by attributing a certain line width to each stick (see text). The stick spectra clearly reveal that the EPR spectra derive from 16 paramagnetic centers in the unit cell; sticks have been drawn on top of each other when resonances were calculated within 3 mT.

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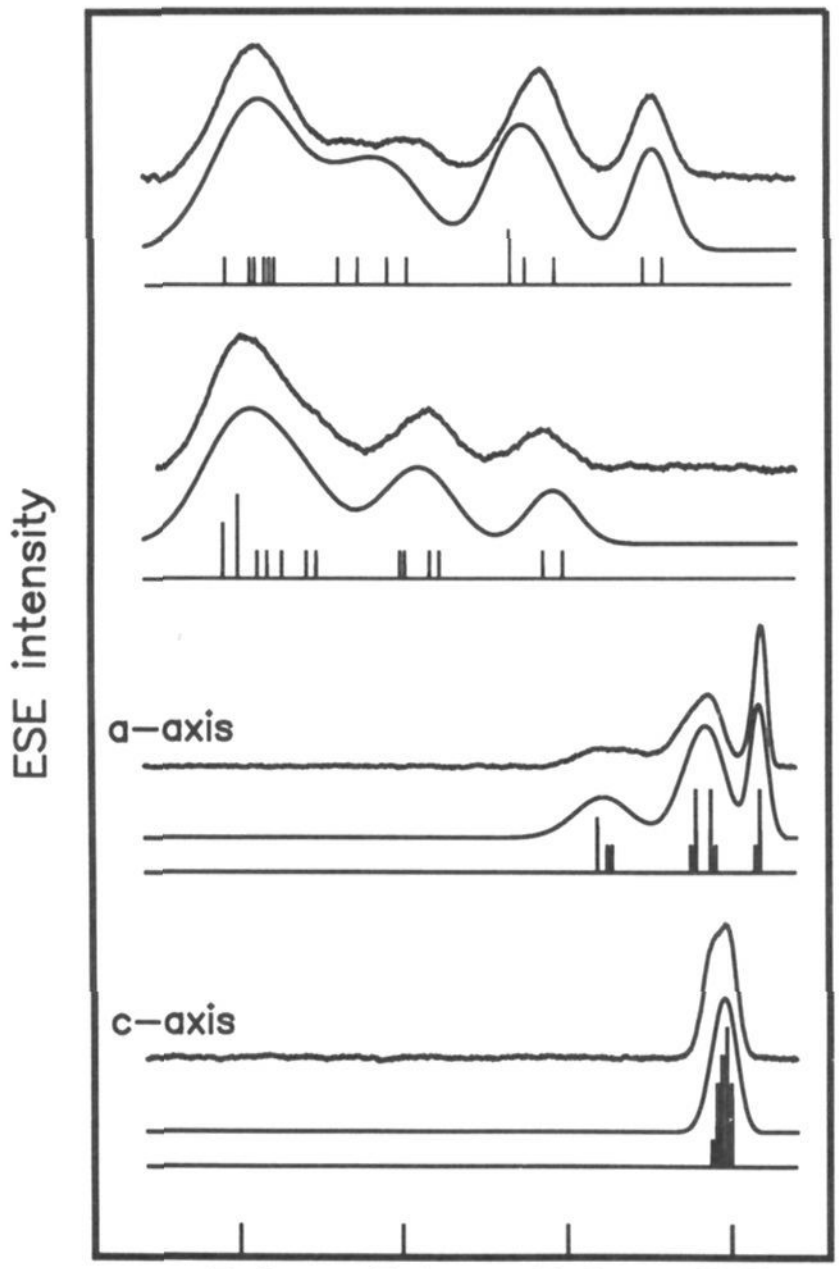

Figure 3. W-band ESE-detected EPR spectra of a single crystal of azurin at 1.2 K for four different orientations of the magnetic field with respect to the crystal. For the top two spectra, the magnetic field was directed along the principal z-axis of the g-tensor of one of the molecules in the asymmetric unit. The bottom two spectra correspond to orientations of the magnetic field along the crystallographic a- and c-axes. For further details see the legend of Figure 2.

corresponds to the direction of one of the principal x-axes. Because there are 16 molecules in the unit cell, 16 of such directions exist. We observed 14 distinct orientations for which B_0 is parallel to an x-axis, summarized in a Wulffnet projection in Figure 4. Within the accuracy of the experiment (2°), four orientations coincide two by two in the ac plane. We observed four different EPR spectra for orientations of B_0 parallel to the x-axes, represented in Figure 2, corresponding to the fact that the asymmetric unit contains four molecules. By varying the orientation of B_0 in the plane perpendicular to each x-axis we were able to localize the approximate directions of 10 z-axes, denoted by the shaded symbols in Figure 4. The accuracy of the directions of the z-axes is only 14°, much worse than for the x-axes for a reason that will be discussed in the simulations section. The orientations of the remaining six z-axes were found to be in an area of 15° around the crystallographic b-axis. Their orientation could not be established more precisely because the crystal happened to be mounted such that for these orientations of B_0 the external magnetic field and the microwave field almost coincided. Experimental determination of the directions of the y-axes was impeded by the large overlap of bands in the central part of the EPR spectrum. These directions, being perpendicular to both x- and z-axes, have been constructed. From the symmetric grouping of the x- and z-axes around the crystallographic a- and c-axes

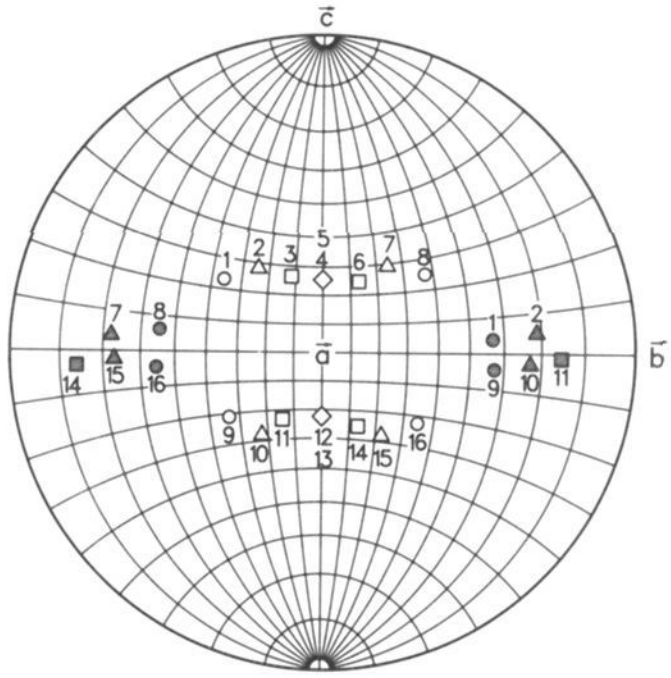

Figure 4. Wulffnet projection of the experimentally determined orientations of the principal axes of the g-tensors of azurin molecules with respect to the crystallographic a-, b-, and c-axes. The open symbols indicate the directions of the x-axes, the shaded symbols those of the z-axes. The different symbols refer to the four different molecules in the asymmetric unit, the numbers to the 16 molecules in the unit cell. All points lie at the front side of the globe. The accuracy of the x-axes is 2° and of the z-axes 14°.

approximate directions of the a- and c-axes were inferred. By varying the orientation of B_0 around these directions and looking for the most symmetrical spectra we determined the precise orientations of the a- and c-axes. The EPR spectra obtained for B_0 parallel to these directions are shown in Figure 3.

B. Simulations. Each EPR spectrum of the single crystal depends on the orientation of B_0 with respect to the g-tensor axes systems (x, y, z) of all 16 molecules in the unit cell. Simulations of the EPR spectra therefore not only offer the possibility to verify our interpretation of the data but also to improve the accuracy of the directions of the z-axes. The simulations were based on a spin Hamiltonian that includes only the electron Zeeman term. The copper hyperfine interaction is not considered explicitly as it was not resolved in the spectra. For a certain orientation of B_0, the field of resonance for a molecule is given by

$$B_{res} = \frac{h\nu}{\mu_\beta(\sum_i (g_i \cos \phi_i)^2)^{1/2}}, \qquad i = x, y, z$$

where h is Planck's constant, ν the microwave frequency, μ_β the Bohr magneton, g_i the principal values of the g-tensor obtained from the study on the random sample, and cos φ_i the direction cosines between the i-axes and B_0. The fields of resonance obtained in this way for all molecules in the unit cell result in a stick diagram. In order to allow a comparison of simulated and experimental spectra, a Gaussian line shape has been attributed to the resonance lines. The experimental line widths derive from g-strain and unresolved hyperfine interaction and vary along the EPR spectrum. The resulting line widths have been approximated by taking the width ΔB of the Gaussian function according to

$$\Delta B = (\sum_i (W_i \cos \phi_i)^2)^{1/2}, \qquad i = x, y, z$$

where the three parameters W_i have been optimized for each spectrum. This expression for ΔB is rigorously valid if the principal axes of the hyperfine tensor and the g-strain tensor coincide with the principal axes system of the g-tensor. In that case the line width shows the same dependence on the orientation of B_0 with

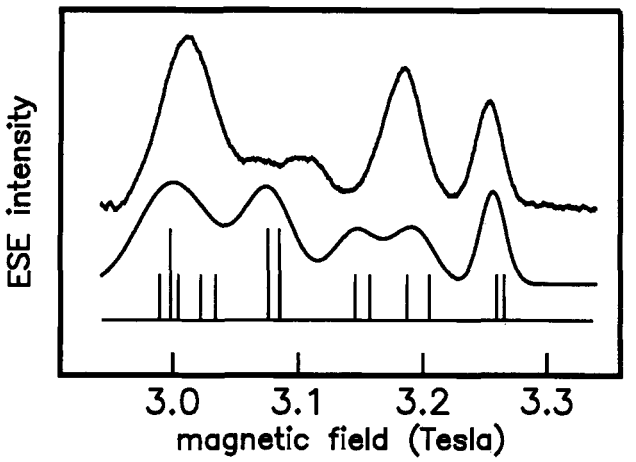

Figure 5. W-band ESE-detected EPR spectrum of a single crystal of azurin at 1.2 K for an orientation of the magnetic field along the principal z-axis of the g-tensor of one of the molecules in the unit cell. The simulated spectrum (middle) and the stick diagram (bottom) have been calculated with the set of experimentally determined directions of the x- and z-axes. For this starting set (see text), the agreement between the experimental and simulated spectrum is poor which is attributed to the large error in the experimentally determined directions of the z-axes. The stick diagram shows that the low-field line results from five overlapping transitions which underscores the difficulty in the experimental determination of the directions of the z-axes. The proper simulation based on the improved directions of the z-axes is represented in the top spectrum of Figure 3.

respect to x, y, z for the hyperfine interaction and the g-strain[19] which implies that both interactions can be included in one parameter set W_i. For azurin the mutual orientation of the principal axes systems is unknown. Intensity effects that derive from the use of the pulsed ESE method in recording the EPR spectra are not taken into account in the simulations.[20]

In order to simulate the EPR spectra the orientations of the principal axes of the g-tensor of all molecules in the unit cell are needed. As a starting set we took the experimentally obtained directions. In Figure 5 an EPR spectrum simulated with this set is shown and compared with the experimentally observed spectrum. The poor agreement between the experimental and simulated spectrum is attributed to the large error in the directions of the z-axes as determined experimentally. The stick diagram in Figure 5 reveals that not one but a number of molecules contribute to the resonance line at the lowest field, which explains why the directions of the z-axes could only be determined approximately from the experimental EPR spectra. In subsequent simulations we kept the directions of the x-axes fixed (as these were determined with great accuracy) and varied the orientation of the z-axes under two constraints: (1) the z-axes were rotated in the plane perpendicular to the corresponding x-axes and (2) the mutual symmetry of the z-axes was conserved. In this way a set of 16 z-axes has been obtained with which we could reproduce the experimental EPR spectra for B_0 parallel to the x-axes (see Figure 2) and those for B_0 parallel to the z-axes and the a- and c-axes (see Figure 3). These orientations of the z-axes differ on the average by 12° from those estimated experimentally, their final accuracy being about 2°. The average values used for the parameters W_i in the simulations of the spectra (W_x = 5 mT, W_y = 9 mT, W_z = 38 mT) are comparable to the line widths obtained at the canonical orientations in the spectrum of the frozen solution (5.3, 8.4, and 29.0 mT at x, y, z, respectively). This indicates that in the crystal no additional strain arises.

C. The Orientation of the g-Tensor in the Copper Site. From the EPR experiment, in combination with the spectral simulations, the orientations of all 16 principal axes systems of the g-tensors have been fixed with respect to the crystallographic a-, b-, and c-axes. The result is summarized in Table 2. In order to derive the orientation of the x-, y-, and z-axes within the azurin molecule, in particular with respect to the copper site, we make use of the structure of the unit cell as determined by X-ray diffraction. We define the orientation of each azurin molecule in the unit cell by the vectors pointing from the copper center toward the C-α atoms of residues isoleucine-7, glycine-58, and valine-59 and toward the sulfur atom of cysteine-26 because these directions are known with high accuracy. Then we calculate the angles between the principal axes of *all* g-tensors and these vectors for *all* molecules. In this way a unique assignment of the 16 axes systems to the molecules in the unit cell is found, which results from the asymmetry of the four molecules in the asymmetric unit. For this assignment the x-, y-, and z-axes have the same orientation in each molecule, apart from deviations within the error margin set by the X-ray (±1°) and EPR data (±2°). Having fixed the directions of the principal axes of the g-tensor with respect to the azurin molecule, we have calculated the angles between these axes and the copper–ligand bonds from the X-ray data. The latter calculation introduces an additional error in the angles due to the fact that in the X-ray data the vectors pointing from the copper center to the ligand atoms are less accurately determined than the vectors pointing to the C-α atoms. The result is summarized in Table 1 and represented in Figure 6. According to the X-ray structure of azurin the copper ion is almost in the plane spanned by the two coordinating nitrogen atoms (Nδ) of histidines-117 and -46 and the sulfur atom (Sγ) of cysteine-112.

Table 2. Orientation of the Principal Axes x, y, and z of the g-Tensors of the 16 Molecules in the Unit Cell with Respect to the Crystallographic a-, b-, and c-Axes[a]

x			y			z		
a	b	c	a	b	c	a	b	c
−0.762	0.532	0.369	−0.393	0.072	−0.917	−0.515	−0.844	0.154
0.741	−0.543	0.396	0.433	−0.065	−0.899	0.513	0.838	0.187
0.762	0.520	−0.387	0.422	0.056	0.905	0.492	−0.852	−0.177
−0.734	−0.520	−0.437	−0.470	−0.076	0.880	−0.491	0.851	−0.189
−0.810	0.362	0.462	−0.487	0.025	−0.873	−0.327	−0.932	0.156
0.811	−0.354	0.465	0.495	−0.007	−0.869	0.311	0.935	0.170
0.805	0.347	−0.481	0.502	0.032	0.864	0.315	−0.937	−0.149
−0.800	−0.348	−0.488	−0.511	−0.030	0.859	−0.314	0.937	−0.154
0.881	−0.236	−0.410	0.380	−0.165	0.910	−0.282	−0.958	−0.056
−0.872	0.206	−0.444	−0.404	0.210	0.891	0.276	0.956	−0.100
−0.874	−0.214	0.437	−0.397	−0.208	−0.894	0.282	−0.955	0.097
0.883	0.193	0.428	0.389	0.213	−0.896	−0.264	0.958	0.113
−0.926	0.001	0.378	−0.371	−0.193	−0.908	0.072	−0.981	0.180
0.905	−0.008	0.426	0.423	0.145	−0.895	−0.055	0.990	0.134
0.926	−0.001	−0.378	0.373	−0.150	0.916	−0.058	−0.989	−0.138
−0.905	0.008	−0.426	−0.421	0.149	0.895	0.071	0.989	−0.132

[a] The angles are expressed as direction cosines. The signs are chosen in correspondence with the assignment of the g-tensors to the respective copper sites.

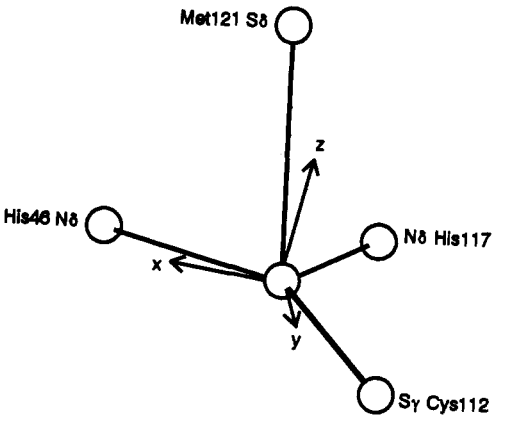

Figure 6. The orientation of the principal axes of the g-tensor with respect to the copper site.

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The x- and y-axes are found approximately in this NNS plane, while the z-axis is perpendicular to it and makes an angle of 15° with the bond between the copper and the sulfur atom (Sδ) of methionine-121.

IV. Discussion

Field-swept ESE experiments in W-band on a frozen solution and a single crystal of azurin have enabled the determination of the complete g-tensor for this protein. Both the principal values and the directions of the principal axes have been ascertained with great accuracy. The principal values of the g-tensor for azurin are in line with the general notion of an axial g-tensor for blue copper proteins: $g_z - {}^1/_2(g_x + g_y) = 0.225$, $g_y - g_x = 0.0175$. The values of g_x and g_y are close to earlier estimates based on simulations of X- and Q-band EPR spectra but the value of g_z turns out to be slightly larger than reported up to now.[13]

The deviation of the g-values from the free-electron value stems from spin-orbit coupling (SOC). Theoretical descriptions of the electronic structure of the copper site in blue copper proteins hint toward a considerable covalency of the copper–ligand bonds.[14] The near-axiality of the g-tensor for blue copper proteins has led to qualitative descriptions in the literature in terms of effective C_{3v} symmetry, with some rhombic distortion,[15] bringing out the $d_{x^2-y^2}$ character of the unpaired electron orbital. Besides this copper $d_{x^2-y^2}$ atomic orbital character, the wave function of the unpaired electron reveals delocalization mainly over the histidine-117, histidine-46, and cysteine-112 ligands because the axial ligands are significantly further away from the copper atom. A calculation of the SOC therefore has to take into account not only the contribution of copper (ζ = 829 cm^{-1}) but also that of the cysteine sulfur (ζ = 382 cm^{-1}) and the histidine nitrogens (ζ = 76 cm^{-1}).[21] For azurin this truncated site (cf. Figure 6) has approximately C_{2v} symmetry because the copper ion is almost in the NNS plane (distance 0.08 Å). Such a symmetry of the copper site would fix the directions of the axes of the g-tensor along the Cu–Sγ(Cys112) bond, perpendicular to this bond in the NNS plane, and perpendicular to this plane. This crude model correctly predicts the orientation of the z-axis. The x- and y-axes, although indeed in the NNS plane, are found to be rotated by more than 20° with respect to the C_2 axis of this model site.

In relation to experiments on plastocyanin, whose EPR spectrum in solution closely resembles that of azurin, spin-restricted SCF-Xα-SW calculations have been performed on a number of models for the copper site, in particular one indicated as the C_S(Met) site.[14] In this model the coordinating amino acids were replaced as follows: the histidines by ammonia, the cysteine by methanethiolate, and the methionine by dimethyl thioether. The position of the ligands was adjusted so as to obtain C_S symmetry with the two copper–sulfur bonds in the mirror plane. The calculations revealed a dominant role of the nitrogens in the bonding. The half-occupied molecular orbital was found to be strongly antibonding between the copper and the thiolate sulfur, with a 36% delocalization over a sulfur p_π atomic orbital. Starting from the wave functions obtained from this model, satisfactory fits to known g-values for plastocyanin could be obtained. However, in this description the direction of one of the principal axes of the g-tensor is of course perpendicular to the mirror plane, fixed by symmetry. This we do not observe for azurin (cf. Figure 6).

Neither C_S nor C_{2v} symmetry of the copper site is compatible with the observed directions of the principal axes of the g-tensor. The actual orientation of the x- and y-axes results from the relatively small rhombic components of the ligand field around the copper ion, and it, thus, provides for a subtle fingerprint of the electronic wave function of the unpaired electron. The present determination of the complete g-tensor for azurin supplies the data that a proper, more refined quantum-chemical approach should explain. Work in this direction is in progress in our laboratory.

Finally we emphasize that this study for azurin has shown the feasibility of W-band EPR experiments on single crystals of blue copper proteins. Experiments on mutated proteins are underway which will provide us with additional data that will be helpful to arrive at a reliable description of the unpaired electron orbital that is instrumental in the electron transfer in blue copper proteins.

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