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Danielsen, E.; Bauer, R.; Hemmingsen, L.; Andersen, M.L.; Bjerrum, M.J.; Butz, T.; ... ; Messerschmidt, A.

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Structure of Metal Site in Azurin, Met^{121} Mutants of Azurin, and Stellacyanin Investigated by ^{111m}Cd Perturbed Angular Correlation (PAC)*

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Eva Danielsen‡§, Rogert Bauer‡, Lars Hemmingsen‡, Marie-Louise Andersen‡, Morten J. Bjerrum¶, Tilman Butz‖, Wolfgang Tröger‖, Gerard W. Canters, Carla W. G. Hoitink**, Göran Karlsson‡‡§§, Örjan Hansson‡‡, and Albrecht Messerschmidt§§**

*From the ‡Department of Mathematics and Physics and the ¶Department of Chemistry, Royal Veterinary and Agricultural University, DK-1871 Frederiksberg, Denmark, the ‖Faculty of Physics and Geosciences, University of Leipzig, D-7010 Leipzig, Federal Republic of Germany, the **Leiden Institute of Chemistry, Leiden University, NL-2300 Leiden, The Netherlands, ‡‡Chalmers Technical University, S-41390 Göteborg, Sweden, and the §§Max Planck Institute for Biochemistry, D-8033 Martinsried, Federal Republic of Germany*

The geometries of the metal sites in cadmium-substituted azurins have been investigated by ^{111m}Cd perturbed angular correlation (PAC). The study includes wild type azurin as well as Met^{121} mutants of azurin, where methionine has been substituted by Ala, Asn, Asp, Gln, Glu, and Leu.

The nuclear quadrupole interaction of wild type azurin analyzed in the angular overlap model is well described as coordination of His^{46}, His^{117}, and Cys^{112} and cannot be described by coordination of Met^{121} and/or Gly^{45}.

For most of the mutants, there exist two coordination geometries of the cadmium ion. With the exception of the Glu and Asp mutants, one of the conformations is similar to the wild type conformation. The other coordination geometries are either best described by a coordinating water molecule close to the original methionine position or by coordination by the substituting amino acid. These experiments show that even though the methionine does not coordinate it plays an important role for the geometry of the metal site.

The nuclear quadrupole interaction of stellacyanin was also measured. The value resembles the most prominent nuclear quadrupole interaction of the Met^{121} → Gln mutant of *Alcaligenes denitrificans* azurin, indicating that the structures of the two metal sites are similar.

Azurin and stellacyanin belong to the family of blue copper proteins with copper in a type 1 geometry. From x-ray diffraction (1–3), it is known that in azurin 2 histidines and 1 cysteine are coordinating to the copper ion with the copper ion in the plane formed by the three ligands. This geometry is strained for Cu(II) as well as for Cu(I). Two additional amino acids are relatively close, namely methionine 121 with its sulfur at a distance of 3.1 Å and glycine 45 of which the backbone oxygen is at a distance of 3.0 Å.

The methionine residue is conserved not only in the azurins, but also in pseudoazurins, plastocyanins, and cucumber blue protein (see, *e.g.*, Ref. 4). It is therefore believed to play an important role in the function of the blue copper proteins. One possible function of the methionine could be a fine tuning of the redox potential (5). Stellacyanin is interesting in this context because it does not contain a methionine residue at all. The present paper addresses the question as to whether any conformational changes occur at the metal site in cadmium-substituted azurin upon substitution of the methionine residue with other amino acids.

The structure of the metal site in wild type azurin from *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* does not change when the copper ion is reduced (6, 7). In contrast, the structure of the metal site in the Met^{121} → Gln mutant of (*A. denitrificans*) azurin depends strongly on the oxidation state of the copper ion (8). For this azurin mutant Cu(II) is 4-coordinate (His^{46}, His^{117}, Cys^{112}, and Gln^{121}), whereas Cu(I) is almost linearly coordinated with Cys^{112} and His^{46} as strong ligands and two weak interactions with Gln^{121} and His^{117} (both at a distance of 2.7 Å). This indicates that part of the function of the methionine residue is to stabilize the conformation around the copper and thereby to facilitate the exchange of electrons (8).

The effect of substitution of copper with zinc in azurin from *P. aeruginosa* has been investigated previously (9). The movement of the atoms upon substitution was minor, but resulted in a distorted tetrahedral structure, where the zinc ion is now coordinating to the carbonyl oxygen from Gly^{45} with a binding distance of 2.3 Å. The distance to the Met^{121} Sδ is 3.4 Å, which means that a bonding interaction of methionine with zinc is negligible. The effect of substitution by cadmium has been studied for *A. denitrificans* azurin only.[1] The distance between cadmium and glycine was 2.8 Å and between cadmium and methionine 3.2 Å. This means that cadmium behaves more like copper than zinc in this protein.

It should be noted when comparing cadmium to copper that cadmium has the same valence as Cu(II) but a closed shell d_{10} configuration like that of Cu(I). Therefore, it is possible that cadmium in some situations will coordinate in a way similar to Cu(I), while in other situations it will coordinate like Cu(II).

The technique of perturbed angular correlations of γ-rays (PAC)[2] is described in detail in the review by Frauenfelder and

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§ To whom correspondence should be addressed: Dept. of Mathematics and Physics, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark. Tel.: 45-3528-2363; Fax: 45-3528-2350.

[1] E. Baker, private communication.

[2] The abbreviations used are: PAC, perturbed angular correlation of γ-rays; NQI, nuclear quadrupole interaction; tda, thiodiacetate; XAFS, x-ray absorption fine structure; Mrad, megaradians; AOM, angular overlap model.

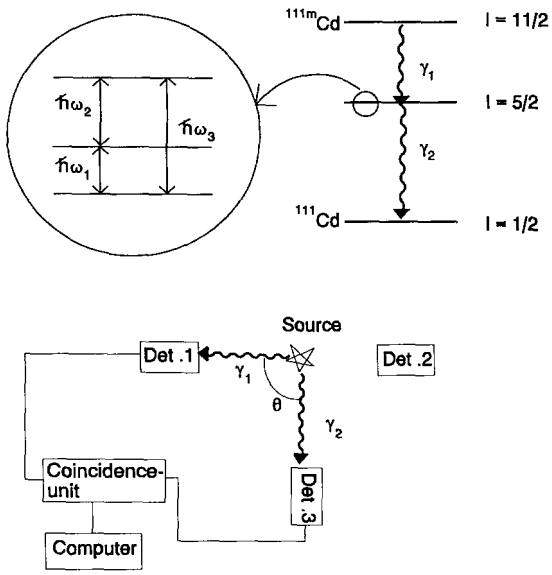

FIG. 1. **Schematic illustration of a PAC experiment.** The *top right* illustrates the decay of ^{111m}Cd. In the *top left* the effect of the electric field gradient on the intermediate energy level (I = 5/2) is illustrated. The electric field gradient causes the energy to split into three (double degenerate) levels depending on the quantum number for the z component of the nuclear angular momentum, m. (ħdenotes Planck's constant divided by 2π.) The *bottom part* illustrates the principle of the experiment. The present work was carried out with six detectors (only three are shown) in an octahedral arrangement whereby 6 combinations of 180° and 24 combinations of 90° are achieved. For each pair of detectors, the probability of detecting the second γ-ray at the angle θ is detected as a function of the time elapsed between the two γ-rays.

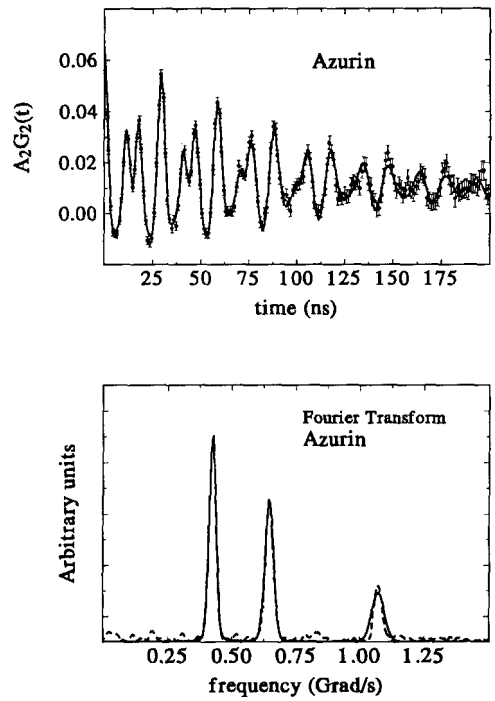

FIG. 2. **PAC spectrum (*top*) of cadmium-substituted wild type azurin from *P. aeruginosa* and Fourier transform (*bottom*).** The *solid line* in the PAC spectrum represents the result of a least squares fit of a theoretical perturbation function to the data points. The *solid* and *dashed lines* in the Fourier transform represent the theoretical and experimental PAC spectra, respectively.

Steffen (10) and with special emphasis on biological application by Bauer (11). An introduction to the technique applied to metal proteins is given in Ref. 12. Here, only a very schematic introduction will be given.

PAC provides a means of measuring the nuclear quadrupole interaction at the site of the nucleus. The NQI is the interaction of the electric quadrupole moment of the nucleus and the electric field gradient tensor, V_{ij}, due to the surrounding charge distribution. For samples of identical, randomly oriented molecules, with no rotational diffusion, two parameters can be measured: the magnitude of the electric field gradient, $|V_{zz}|$, and the asymmetry of the electric field gradient, η. (A more detailed description of the different parameters derived from the PAC spectrum is given under "Materials and Methods.")

PAC requires an isotope that decays by emitting two successive γ-rays. (The principle of the technique is illustrated in Fig. 1.) The detection of the first γ-ray in a specific direction selects an ensemble of nuclei with non-isotropic orientation of nuclear spin. The direction of the emission of the second γ-ray depends on the orientation of the nuclear spin, and the second γ-ray can therefore be emitted in a non-isotropic way relative to the first. For ^{111m}Cd this intrinsic probability of detecting the second γ-ray at an angle of 180° relative to the first γ-ray is about 26% higher than at an angle of 90°. If, however, the nucleus is under the influence of external forces (for example from ligands) during the time between the emission of the two γ-rays, this "angular correlation" is "perturbed." Classically the interaction of the electric field gradient with the nuclear quadrupole moment causes a torque on the nucleus resulting in a precession of the nuclear spin. Since the emission direction of the second γ-ray is related to the direction of the spin, this causes oscillatory variations in the angular correlation between the two γ-rays. Quantum mechanically, this interaction causes an energy splitting of the intermediate energy of the nucleus. This energy splitting of the intermediate level is reflected in a PAC spectrum. In the case of ^{111m}Cd, the intermediate level has spin 5/2 and the energy splits into three levels under the influence of an electric field gradient. Between these three levels, three energy differences exist. Each energy difference, ΔE_i, gives rise to an oscillation in the PAC spectrum, $\omega_i = 2\pi\Delta E_i/h$, where h is Planck's constant. In the case of identical, static, and randomly oriented molecules, the perturbation function measured by PAC, $G_2(t)$, is as shown by Equation 1.

$$G_2(t) = a_0 + a_1\cos(\omega_1 t) + a_2\cos(\omega_2 t) + a_3\cos(\omega_3 t) \qquad \text{(Eq. 1)}$$

Each frequency is proportional to $|V_{zz}|$ with a proportionality constant depending on η. The four amplitudes, a_0 to a_3, also depend on η. Thus, from $G_2(t)$, V_{zz} and η can be determined (11). An example of a PAC spectrum ($G_2(t)$) and the Fourier transform are shown in Fig. 2. In general, it is necessary to include rotational diffusion and frequency distributions in the description of the spectra. Further details can be found elsewhere (13).

It is the fact that the surrounding charge distribution is reflected in the electric field gradient, and in particular the possibility through the angular overlap model (AOM) (14) to calculate the electric field gradient for a given particular coordination sphere, that makes ^{111m}Cd PAC a useful tool for studying metal sites in proteins.

MATERIALS AND METHODS

Preparation of Proteins—All chemicals were of analytical grade. Stellacyanin was prepared from acetone powder of the Japanese lacquer tree (*Rhus vernicifera*) according to Ref. 15. The quality of the preparation was checked by SDS-electrophoresis and uv/vis absorption spectroscopy. A ratio for $A_{280\text{ nm}}/A_{604\text{ nm}} = 5$ was obtained. Wild type and methionine mutants of azurin were isolated from *Escherichia coli* cells transformed with specific plasmids for the *P. aeruginosa* azurin and its mutants (16) and with specific plasmids for the *A. denitrificans* Met[121] → Gln azurin (8). Apoproteins were prepared by dialysis for at least 12 h of approximately 1 ml of 0.5 mM copper protein in 0.1 M Tris, pH 7.2, against 5 ml of the same buffer containing 0.1 M KCN. The KCN was

removed by 3-fold dialysis against 100 ml buffer, each dialysis lasting 24 h. All dialyses were carried out at 5 °C. The apoproteins were frozen in liquid nitrogen and stored at −80 °C. For the experiments, the apoenzyme was quickly thawed.

The binding properties of cadmium to the apoprotein were investigated using ^{109}Cd with a half-life of 1.3 years, with nonradioactive cadmium acetate added as carrier. These experiments showed that the binding kinetics for cadmium are much slower than for copper, particularly if the cadmium was added at about pH 6. However, if the binding was performed at a pH of about 7.5, more than 90% of the metal sites were reconstituted by cadmium after 2 h of incubation time. Due to the slow binding kinetics of cadmium to the apoprotein and the short half-life of ^{111m}Cd (49 min), most of the PAC experiments were carried out without cadmium carrier. In the cases where cadmium carrier was used, ^{111m}Cd was added to cadmium chloride or cadmium acetate in a 1:1 stoichiometry of Cd^{2+} to protein. Protein-bound cadmium was separated from free cadmium by G25 chomatography. For the experiments without carrier, the G25 chomatography was omitted. In all cases the metal incubation was carried out at room temperature for 5–60 min.

In order to slow down the rotational diffusion, which would otherwise damp the oscillations in the perturbation function, sucrose was added at a concentration of 55 weight % and the sample was cooled to 4 °C. Buffers used were either Tris or HEPES, or a mixture of both, with a molarity between 10 and 200 mM. The pH was measured at room temperature and was between 7.2 and 7.9, except for three experiments where the pH was varied on purpose: Met^{121} → Asp, pH 10.0; and Met^{121} → Leu, pH 5.8 and pH 9.0. The actual pH values at 4 °C are about 0.7 pH units higher (estimated from the known temperature dependence of the buffers). For the experiments with pH 9 and 10, additional corrections for the presence of sucrose were made. The sample volumes varied from 25 μl to 2 ml.

Preparation of Cadmium(II) Thiodiacetate Crystals (Cd(tda)H_2O) for the Determination of ω_l of Methionine—A solution of thiodiacetate (2.0 mmol) in 0.7 ml water was mixed with 0.8 ml of 4 M sodium hydroxide. To this solution we added cadmium(II) nitrate (2 mmol) mixed with ^{111m}Cd (less than 1 pmol) in a total volume of 0.55 ml. Crystals formed within minutes. The mixture was cooled on ice. The precipitated crystals were separated by decantation. The crystals were washed once with ice-cold doubly deionized water. Yield was 88%.

Powder x-ray diffraction analysis of Cd(tda)H_2O was carried out with a Phillips x-ray diffraction unit using Co-K_α radiation and a XPLOT software program packet (CSIRO 1990). Analysis of the resulting pattern obtained from crystals of Cd(tda)H_2O used in the PAC experiment revealed 26 lines in the interval from 10° to 50°. All lines were indexed using the computer program TREOR giving a monoclinic unit cell with the dimension a = 8.008(7), b = 5.361(4), c = 9.143(7) (all in Å), and β = 115.92(6). This is in agreement with the unit cell determined previously in a single crystal x-ray study (17).

^{111m}Cd was produced by bombarding ^{108}Pd with α-particles at the cyclotron at either the Niels Bohr Institute or at the National Hospital, both located at Copenhagen. The sample was then transported to the Royal Veterinary and Agricultural University, where all chemistry was carried out. Palladium and cadmium were separated by the procedure described elsewhere (12). This reduces the amount of palladium to about 10^{-10} mol.

The Met^{121} → Glu experiment was repeated at the ISOLDE facility at CERN, Geneva, Switzerland. The sample preparation was similar to the one at Copenhagen. However, at CERN the solution was saturated with sucrose and the temperature was 0 °C. These differences are not expected to affect anything but the rotational diffusion time.

The PAC spectrometer consists of six BaF_2 scintillator detectors with conical fronts arranged such that each detector is situated at the face center of an imaginary cube. The sample is positioned at the center of this cube. It is a built-out version of the "PAC camera" described previously (18). In addition, the instrument has a facility for automatic adjustment of detector-sample distance, thus adjusting to an optimal counting rate until the sample-detector distance reaches its minimum value.

The temperature of the sample was controlled by a Peltier element in thermal contact with the sample. The temperature can be set between −10 °C and 40 °C with an accuracy of ±2 °C.

Six combinations of 180° coincidence spectra and 24 combinations of 90° coincidence spectra were collected simultaneously. In each coincidence spectrum, the background due to accidental coincidences is subtracted and the perturbation-function is formed according to

$$A_2G_2(t) = 2\,\frac{W(180°,t) - W(90°,t)}{W(180°,t) + 2W(90°,t)} \qquad \text{(Eq. 2)}$$

where $W(180°,t)$ denotes the sixth root of the product of the six 180° spectra after background subtraction and zero-point adjustment, and $W(90°,t)$ denotes the 24th root of the product of the 90° spectra. The time resolution was about 850 ps full-width at half-maximum at the energies of ^{111m}Cd.

Data Analysis—The function $A_2G_2(t)$ was analyzed by conventional least χ^2 fitting routines. Each NQI is described by the parameters: ω_0, η, $\Delta\omega_0/\omega_0$, τ_c, and A_2. Details of the perturbation functions are given elsewhere (13).

ω_0 is the magnitude of the nuclear quadrupole interaction and is proportional to the numerically largest diagonal element of the electric field gradient, $|V_{zz}|$, by the relation shown in Equation 3.

$$\omega_0 = 12\pi|V_{zz}eQ|/(40h) \qquad \text{(Eq. 3)}$$

η is the asymmetry parameter and is defined as shown in Equation 4.

$$\eta = (V_{xx} - V_{yy})/V_{zz} \qquad \text{(Eq. 4)}$$

(In the two equations, above the electric field gradient tensor is assumed diagonal, and $|V_{zz}| \geq |V_{yy}| \geq |V_{xx}|$.)

ω_0 and η depend on the coordinating ligands as well as their positions. If the partial nuclear quadrupole interaction is known for each ligand as well as the geometry of the metal site, they can be calculated in the AOM (14).

$\Delta\omega_0/\omega_0$ is used for describing small inhomogeneous frequency distributions due to variations from one molecule to another with respect to conformations of the probe sites. It is assumed that the variations can be described by identical asymmetry parameters, η, and a Gaussian distribution of ω_0 with the width $\Delta\omega_0/\omega_0$. Thus, deviations from zero indicate that the ^{111m}Cd nuclei are subjected to a distribution of surroundings. In general, shifts in angular position of a ligand by only a few degrees will be quite enough to show up as a frequency distribution of a few percent.

τ_c is the correlation time of the rotational diffusion induced by the Brownian motion. For a rigid spherical molecule with volume V, embedded in a solution with viscosity ξ, the correlation time is: $\tau_c = \xi V/(kT)$, where k is Boltzmann's constant and T is the absolute temperature (19).

A_2 is the amplitude of the perturbation function. It is a property of the radioactive nucleus, and for ^{111m}Cd it has a maximum value of +0.16 (20). The experimental value is normally significantly lower due to solid angle correction factors (sample volume, detector sizes, and sample-detector distance). In the case where inequivalent sites are present, each NQI must be included in $G_2(t)$ with the relative populations of the nuclear quadrupole interactions.

All PAC spectra were analyzed with both one and two nuclear quadrupole interactions, respectively. A single nuclear quadrupole interaction was chosen unless the reduced χ^2 of the fit with two NQIs was significantly better (the requirement was that the probability of the one-component fit was less than 5% based on an F-test). This nuclear quadrupole interaction was then analyzed in the angular overlap model.

The rotational diffusion time for the mutants was taken from the fit of wild type azurin and subsequently fixed.

In the case of the Met^{121} → Glu experiments, the ISOLDE experiment was used to establish all parameters except the amplitude, the relative populations, and the rotational diffusion time. The result of the experiment carried out at the Veterinary and Agricultural University was then analyzed with these parameters fixed (see Table I).

Angular Overlap Model Analysis—The measured nuclear quadrupole interactions were analyzed in the angular overlap model (AOM) (14, 21). This is a semiempirical model, assuming that each ligand contributes to the nuclear quadrupole interaction with an axially symmetric tensor of strength ω_l, which is the so called partial nuclear quadrupole interaction. ω_l depends only on the ligand and the z axis points along the ligand–cadmium bond. The total nuclear quadrupole interaction is then calculated by adding the different tensors for the different ligands with subsequent diagonalization (12). Thus, if the nuclear quadrupole interaction is known for a number of cadmium complexes with known crystal structure, it is possible to determine the ω_l values of all of the coordinating ligands. In this way ω_l has been determined previously for all the relevant ligands with the exception of methionine, which has therefore been determined as part of the present work.

The two parameters describing the experimental nuclear quadrupole interactions (ω_0 and η) of the proteins were analyzed in the following way. The theoretical values of ω_0 and η were calculated using the published crystal structure and the partial nuclear quadrupole inter-

TABLE I
Experimental nuclear quadrupole interactions (NQI) determined by least χ^2 analyses

Only the NQI of the most probable fit are given. (f) means that the parameter was fixed in the fit. The azurin mutants were from *P. aeruginosa* (P.a.) except Met^{121} → Gln, which was also from *A. denitrificans* (A.d.).

Protein	η	ω_0	$\Delta\omega_0/\omega_0$	% amp
		Mrad/s		
Wild type azurin	0.522 (1)	337.7 (2)	0.010 (1)	100
Met^{121} → Asn	0.485 (6)	325 (1)	0.042 (3)	100
Met^{121} → Asp	0.678 (7)	254 (1)	0.078 (3)	100
Met^{121} → Gln (A.d.)	0.392 (5)	304.0 (8)	0.028 (2)	72
	0.496 (6)	309.6 (9)	0.007 (4)	28
Met^{121} → Gln (P.a.)	0.612 (3)	280.2 (4)	0.018 (2)	100
Met^{121} → Ala	0.566 (4)	294 (1)	0.016 (4)	34
	0.532 (3)	278.4 (6)	0.023 (2)	66
Met^{121} → Leu	0.73 (1)	264 (2)	0.071 (1)	52
	0.485 (4)	332.3 (6)	0.014 (2)	48
Met^{121} → Glu	0.82 (1)	124.7 (7)	0.035 (4)	42
(ISOLDE exp.)	0.78 (1)	151.6 (6)	0.052 (4)	58
Met^{121} → Glu	0.82 (f)	124.7 (f)	0.035 (f)	26
	0.78 (f)	151.6 (f)	0.052 (f)	74
Stellacyanin	0.450 (3)	300.9 (5)	0.022 (2)	100

actions of the ligands. If the calculated NQI of one of the tested structures lies within the uncertainty of the experimental ω_0 and η, then this structure is considered probable for the cadmium coordination. If this is not the case, either the copper protein structure is different from the cadmium protein structure or another possible explanation is that the limited resolution of the x-ray structure and/or the limited accuracy in the partial nuclear quadrupole interactions leads to a small but significant difference. Since such differences between theory and experiment should not exclude a structure, they were taken into account by performing a least χ^2 AOM fit minimizing χ^2 defined as shown by Equation 5.

$$\chi^2 = \frac{(\omega_{0(c)} - \omega_{0(m)})^2}{\sigma^2(\omega_0)} + \frac{(\eta_c - \eta_m)^2}{\sigma^2(\eta)} + \sum_{ligands}\left[\frac{(\theta_f - \theta_m)^2}{\sigma^2(\theta)} + \frac{(\phi_f - \phi_m)^2}{\sigma^2(\phi)} + \frac{(\omega_{l(f)} - \omega_{l(m)})^2}{\sigma^2(\omega_l)}\right] \quad \text{(Eq. 5)}$$

In the equation above the indexes "*m*" and "*f*" refer to "measured" and "fitted", respectively. "c" refers to values "calculated" from the fitted parameters. σ refers to the standard deviation of the parameter (see below).

θ and ϕ are the polar and azimuthal angles of the different ligands. The measured values are taken from the x-ray structure of native azurin from *P. aeruginosa* at pH 5.5 and 9.0 (3), except for the experiment on Met^{121} → Gln from *A. denitrificans* where the structures published for Cu(I) as well as Cu(II) were used (8).

The coordinate system was chosen such that the coordinating nitrogen of His^{117} was positioned on the *z* axis ($\theta = 0°$ and $\phi = 0°$) and Nδ of His^{46} was lying in the *z*-*x*-plane ($\phi = 0°$). The rest of the polar and azimuthal angles were calculated from the average of the high pH and low pH structure (3) with an estimated standard deviation of 5° ($\sigma(\theta)$ and $\sigma(\phi)$). When a water molecule was placed at the position of the mutated amino acid, the standard deviations of the angles were chosen to be 10°. This value was also chosen as standard deviation for calculations including a coordinating atom from the substituting amino acid. The Met^{121} → Gln mutant of *A. denitrificans* azurin was analyzed based on the x-ray structure of this mutant with copper in the reduced and oxidized state, respectively (8). The coordinate system was chosen as described above, and $\sigma(\theta)$ and $\sigma(\phi)$ were chosen as 5°.

ω_l refers to the partial nuclear quadrupole interaction of the ligands. Here, we used the measured values published in Ref. 14 (water, imidazole, carbonyl, and carboxylate) and Ref. 22 (cysteine) and the partial nuclear quadrupole interaction of methionine determined from thiodiacetate. The values used are: (in Mrad/s): carbonyl oxygen, 161; monodentate carboxylate oxygen, 245; bidentate carboxylate oxygen, 175; water oxygen, 207; methionine sulfur, 102; cysteine sulfur, 300; imidazole nitrogen, 95; amine nitrogen, 139.

The experimental standard deviations of ω_0 are between 0.2 and 0.9 Mrad/s, and the experimental standard deviations of η are between 0.001 and 0.02. If these values had been used in the AOM fit, too much emphasis would be put on the experimental nuclear quadrupole interactions compared to the x-ray data. This is related to contributions to the nuclear quadrupole interaction that are not taken into account by the AOM model, such as more distant contributions to the NQI. A unit point charge at a distance of 5.4 Å, for example, will contribute with a partial nuclear quadrupole interaction of about 10 Mrad/s which is unaccounted for. Therefore, in the analyses, the experimental values of ω_0 and η were used with standard deviations of 10 Mrad/s and 0.05, respectively. For the same reason, the partial nuclear quadrupole interactions are taken into account with standard deviations of 10 Mrad/s, although the published standard deviations are generally on the order of 4–8 Mrad/s.

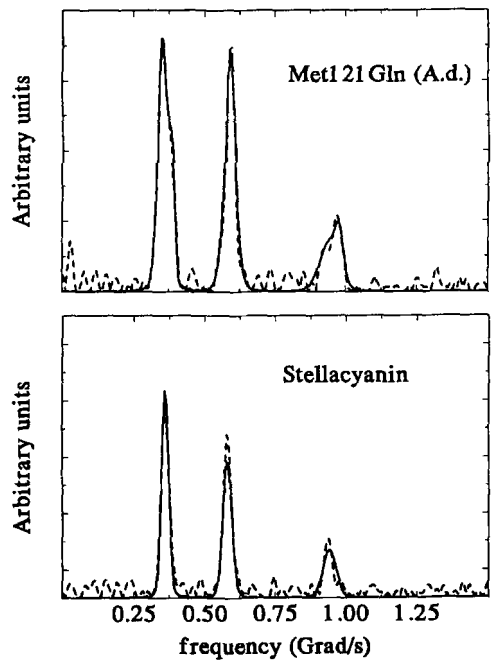

FIG. 3. **Fourier transform of PAC spectra of cadmium-substituted stellacyanin from Japanese lacquer tree (*R. vernicifera*) (*bottom*) and of Met^{121}Gln azurin from *A. denitrificans* (*top*).**

These choices will, of course, affect the absolute values of the χ^2. However, we do not believe that a different choice of standard deviations will have any major effect on the relative values of χ^2.

The AOM fits are a way of fitting the x-ray structure and partial nuclear quadrupole interactions to the measured nuclear quadrupole interaction, but at the same time ensuring that the structure and partial nuclear quadrupole interactions cannot deviate too much from the starting point. The number of degrees of freedom is 2, and so the reduced χ^2 is defined as $\chi^2/2$.

RESULTS

The PAC spectrum and the Fourier transform of wild type azurin from *P. aeruginosa* are shown in Fig. 2. The Fourier transforms of the PAC spectra of stellacyanin and Met^{121} → Gln from *A. denitrificans* are shown in Fig. 3. Fig. 4 shows the Fourier transforms of the PAC spectra measured for the Met^{121} mutants of azurin from *P. aeruginosa*. For the mutants Met^{121} → Asp and Met^{121} → Leu, the pH was varied (Met^{121} → Asp pH = 7.2 and 10.0; Met^{121} → Leu pH = 5.8, 7.2, and 9.0). Lower pH values were not applied due to slow uptake of cadmium by the proteins at low pH. The pH was varied in order to see whether the two different nuclear quadrupole interactions displayed any variation in NQI or in relative population as a function of pH. The experiments showed no detectable difference. Therefore spectra collected at different pH were added before the final least χ^2 analyses were carried out. The results of the least χ^2 analyses are given in Table I. All of the analyses listed in this table gave a satisfactory χ^2.

The total amplitude, A_2, was between 0.05 and 0.09. The

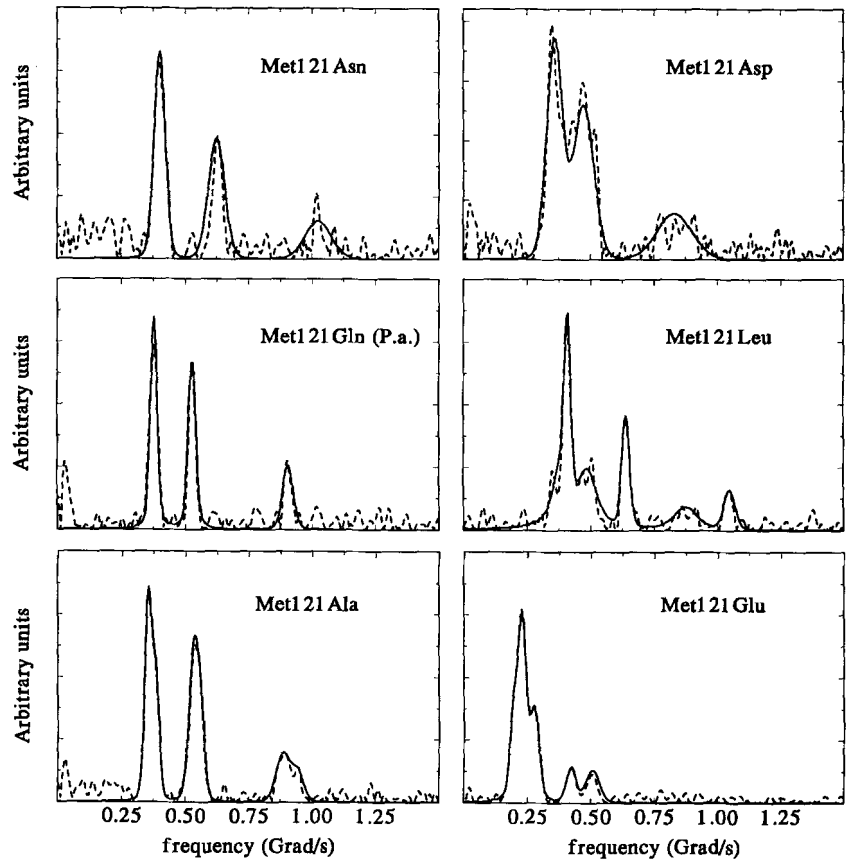

FIG. 4. **Fourier transforms of PAC spectra of various cadmium-substituted Met^{121} mutants of azurin from *P. aeruginosa*.**

rotational diffusion time of stellacyanin was 530(170) ns, that of the Glu mutant in saturated sucrose at 0 °C was 444(6) ns, and for wild type azurin in 55% sucrose at 4 °C the correlation time was 200(20) ns. The rotational diffusion time of wild type azurin was fixed in the fit of all other Met^{121} mutants.

Determination of ω_l for Methionine—Since the partial NQI of methionine is not known from the literature, it was determined from crystals of cadmium thiodiacetate. A least χ^2 analysis of the PAC spectrum for Cd(^{111m}Cd)(tda)H_2O gave a unique NQI with $\omega_0 = 72.3 \pm 0.3$ Mrad/s and $\eta = 0.642 \pm 0.006$. The angles used in the application of the AOM to this structure were taken from Ref. 17. As the only other ligating atoms are water and bidentate carboxylate, a fit to the partial NQI for the methionine-like sulfur atom was possible. The result was a unique solution with ω_l = 102 Mrad/s for the thioether sulfur. The standard deviation was estimated to be 10 Mrad/s. A detailed analysis including more cadmium complexes will be published later.

AOM Analysis—Table II summarizes results of AOM calculations of ω_0 and η based on ligand positions (3), published partial nuclear quadrupole interactions (14, 22), and the partial nuclear quadrupole interaction of methionine determined in the present work for a variety of possible coordinations. The results of the AOM fits of the measured nuclear quadrupole interactions are given in Tables III and IV. The Met^{121} → Gln mutant should also be analyzed with a coordinating nitrogen from Gln. However, since the partial nuclear quadrupole interaction of the amide nitrogen is not known, the calculation was instead carried out with the partial nuclear quadrupole interaction of an amine nitrogen (ω_l = 139 Mrad/s). This calculation can of course only give an indication of whether the amide nitrogen could be coordinating as judged from the measured NQI values. This AOM analysis gave almost the same reduced χ^2 as with a coordinating carbonyl oxygen (ω_l = 161 Mrad/s). We can therefore in this case not distinguish between a coordinating carbonyl oxygen and a coordinating amide nitrogen at position 121.

TABLE II

Calculated nuclear quadrupole interactions based on the x-ray structure of P. aeruginosa azurin (without "refinement")

The middle four rows should be compared to the measured wild type result (ω_0 = 337.7 Mrad/s and η = 0.522). The position of the ligands are given in the two top rows. (Only coordinating ligands contribute to the calculated nuclear quadrupole interaction.)

Polar angle (θ), azimuth angle (ϕ)					Calculated ω_0	Calculated η
0, 0	103, 0	122.5, −170	87.5, 75.0	88.5, −74.3		
					Mrad/s	
His^{46}	His^{117}	Cys^{112}	Met^{121}	Gly^{45}	207.9	0.916
His^{46}	His^{117}	Cys^{112}		Gly^{45}	240.7	0.337
His^{46}	His^{117}	Cys^{112}	Met^{121}		286.1	0.151
His^{46}	His^{117}	Cys^{112}			314.2	0.547
His^{46}	His^{117}	Cys^{112}	Water		279.8	0.287
His^{46}	His^{117}	Cys^{112}	Carbonyl oxygen		280.5	0.153
His^{46}	His^{117}	Cys^{112}	Carboxylate oxygen		283	0.396
His^{46}	His^{117}	Cys^{112}	Amide nitrogen		282.6	0.121

It should be noted that the number of degrees of freedom is only 2 for the fits in Tables III and IV. This means that the probability distribution is very wide. The median of reduced χ^2 is 0.693 (5% will be above 2.996) (23).

No satisfactory AOM fits of the Met^{121} → Glu mutant could be achieved when the structure of the metal site was based on the wild type structure (see Table III). The crystal structure of this mutant at pH 6–6.5 has, however, been determined recently.[3] This shows that the Glu coordinates by one oxygen.

[3] G. Karlsson, L. Tsai, H. Nar, V. Langer, and L. Sjölin, unpublished results.

TABLE III
AOM analysis of measured nuclear quadrupole interactions ("refined structures")

Reduced χ^2 of various cadmium-substituted mutants of azurin assuming different coordination spheres. The fits are based on the wild type structure (*P. aeruginosa*) (3). Water molecules are placed at the position of S^{δ} of Met^{121}. Numbers in the first column refer to measured ω_0 and η (Table 1). Ligands are abbreviated as follows: H ($N^{\delta 1}$ of histidine), C (S^{γ} of cysteine), G (carbonyl oxygen of glycine), M (S^{δ} of methionine), Q (amide oxygen of glutamine), N (carbonyl oxygen of asparagine), D (carboxylate oxygen of aspartate), E (carboxylate oxygen of glutamate). Only conformations for which the probability is more than 5% are acceptable and are in boldface.

ω_0 η	Reduced χ^2				
Wild type azurin	2H,C,M,G	2H,C,G	2H,C,M		2H,C
337.7 0.522	11.6	12.41	9.06		**0.81**
Met^{121} → Asn		2H,C,G	2H,C,N	2H,C,H_2O	2H,C
325 0.485		9.1	**2.38**	**1.68**	**0.26**
Met^{121} → Asp		2H,C,G	2H,C,D	2H,C,H_2O	2H,C
254 0.678		3.8	**0.342**	**0.9**	5.4
Met^{121} → Gln (*P. aeruginosa*)		2H,C,G	2H,C,Q(O)	2H,C,H_2O	2H,C
280.1 0.610		5.31	**1.90**	**1.13**	**1.71**
Met^{121} → Ala		2H,C,G		2H,C,H_2O	2H,C
294 0.566		6.2		**1.26**	**0.65**
278.4 0.532		3.6		**0.66**	**2.54**
Met^{121} → Leu		2H,C,G		2H,C,H_2O	2H,C
265 0.74		6.1		**1.46**	3.66
332.4 0.484		10.4		**1.95**	**0.49**
Met^{121} → Glu		2H,C,G	2H,C,E	2H,C,H_2O	2H,C
124.7 0.82		61.2	30.6	48.0	65.9
151.6 0.78		10.8	5.73	18.0	47.1

TABLE IV
AOM analysis of measured nuclear quadrupole interactions based on the x-ray structure of the Met^{121} → Gln mutant of A. denitrificans ("refined structure")

Reduced χ^2 of various cadmium-substituted mutants of azurin assuming different coordination spheres. The fits are based on the structure of the Met^{121} → Gln mutant with copper in the reduced and oxidized form, respectively (8). Abbreviations are listed in Table III.

ω_0	η	Met^{121} → Gln-Cu(II)		Met^{121} → Gln-Cu(I)	
		2H,C,Q(O)	2H,C	2H,C,Q(O)	2H,C
304.0	0.392	4.29	**1.25**	**0.44**	**1.76**
309.6	0.496	7.26	**0.02**	**1.63**	**0.81**

This structure was then used for an additional AOM analysis of one of the two NQIs determined for Met^{121} → Glu. The result of this analysis are given in Table V. Here, the fitted angles are also listed, since a rather significant change in structure is necessary compared to the copper structure in order to get a satisfactory fit.

TABLE V
AOM calculations of Met^{121} → Glu

Averaged polar coordinates from the x-ray structure of Cu(Met^{121} → Glu) azurin (pH 6) (28), calculated NQI (left) and the result of the AOM fit to ω_0 = 151.6 and η = 0.78 (right). The reduced χ^2 of the fit was 3.8.

Ligand	Met^{121} → Glu (x-ray structure)		Met^{121} → Glu AOM fit (+/− 10°)	
	θ	ϕ	θ	ϕ
His^{117}	0	0	0	0
His^{46}	99.8	0	119.2	0
Cys^{112}	119.6	−157.8	119.95	−162.5
Glu^{121}	102	81.3	96.7	93.1
Calculated ω^0	256.1			
Calculated η	0.71			
Fitted ω^0			160.2	
Fitted η			0.79	

DISCUSSION

NQI of Wild Type Azurin from P. aeruginosa—When the measured NQI of wild type azurin (ω_0 = 337.7 and η = 0.522) is compared to the different calculated values assuming different coordination spheres (see Table II), it is clear that the only conformation giving calculated values close to the measured ones is the 3-coordinate complex where the cadmium ion is coordinating to the two histidines and the cysteine. A more detailed AOM analysis shows that for the cadmium-substituted protein the measured NQI can only be described as this 3-coordinate complex (Table III). This means that a good match of experiment and calculation is achieved by assuming a 3-fold coordination; in other words, neither the glycine nor the methionine are binding with the partial nuclear quadrupole interactions known for these ligands from other cadmium complexes. No AOM calculations were carried out assuming fractions of binding as suggested by Lowery and Solomon (24).

This is the first time a 3-coordinate cadmium complex has been measured by PAC. The existence of 3-coordinate complexes has, however, been demonstrated recently (25–27).

NQIs of Met^{121} Mutants of P. aeruginosa azurin—The fact that many of the Met^{121} mutants have NQIs very close to the one measured for wild type azurin further supports that methionine does not bind to the cadmium ion. The calculated NQIs in Table II show that introduction of a fourth ligand at position 121 in addition to the two histidines and the cysteine with an ω_l of the fourth ligand between 102 to 245 Mrad/s changes ω_0 as well as η significantly (compare His-His-Cys with His-His-Cys-*X* in Table II where *X* is any methionine sulfur, water, carbonyl oxygen, carboxylate oxygen, or amine nitrogen). At the same time the substitution of one ligand at position 121 with another at the same position mainly affects η and leaves ω_0 almost unaffected (compare the different His-His-Cys-*X* in Table II). This is the main reason for the rather small differences in reduced χ^2 when a coordinating ligand at position 121 is replaced by a coordinating water molecule (column 4 and 5 in Table III).

The Met^{121} → Asn gives almost the same NQI as wild type but with a significantly larger $\Delta\omega_0/\omega_0$, indicating a less rigid structure. The NQI is interpreted as a 3-coordinate complex like the wild type (Table III). However, it cannot be excluded that the asparagine or a water molecule coordinates (Table III).

The Met^{121} → Asp data can be fitted with one NQI only. This NQI, however, is significantly different from the wild type NQI and cannot give a satisfactory fit by assuming 3-coordination by the wild type ligands. It is therefore assumed that cadmium in this case is 4-coordinate with aspartate or water as the fourth ligand. This is further supported by the AOM analysis in Tables III and IV. For this mutant, a significantly higher $\Delta\omega_0/\omega_0$ was also found, again indicating a less rigid site.

For the Met^{121} → Gln (*A. denitrificans*), Met^{121} → Glu, Met^{121} → Ala, and Met^{121} → Leu mutants, it was necessary to include two different NQIs in the data analysis. With the exception of the Glu mutant, one of the NQIs could be described as a wild type-like 3-coordinate complex.

For the Met^{121} → Gln mutant of *P. aeruginosa*, only one nuclear quadrupole interaction was found. This NQI was analyzed based on the wild type structure of (*P. aeruginosa*) azurin (Table III). The result of this analysis is that it is not possible based on this NQI to discriminate between 3-coordinate cadmium as in wild type azurin and 4-coordinate cadmium where the fourth ligand is either water or the amide oxygen of Gln.

For the Met^{121} → Gln mutant of *A. denitrificans* azurin, two geometries with quite close NQIs were found. The x-ray diffraction work (8) on the Cu(I) and Cu(II) crystals showed no

evidence of presence of more than one form in the same crystal but the metal site geometry depends strongly on the oxidation state of the copper ion. The two NQIs were analyzed based on the structures of the Met^{121} → Gln mutant of *A. denitrificans* (Table IV). The analysis shows that the cadmium ion cannot be 4-coordinate in the same geometry as Cu(II). For the NQI with ω_0 = 309.6 Mrad/s and η = 0.496, the fit with the lowest χ^2 is 3-coordinate with the same angular distribution of the ligands as in the Cu(II) crystal. Similarly, the NQI with ω_0 = 304.0 Mrad/s and η = 0.392 is best fitted with four ligands in the same angular positions as in the Cu(I) crystal (Table IV). Other explanations are also possible according to the table. It is important to note that the AOM does not give any information on the bonding distance of the ligands. We interpret the result of the AOM analysis as follows. For cadmium there exist two conformations; one of them could be 4-coordinate with 2 His, 1 Cys, and the amide oxygen or nitrogen of Gln, and the other probably 3-coordinate with 2 His and 1 Cys, but it cannot be excluded that a water molecule is binding in one of the two forms. Partly due to the lack of information on the ω_l of the amide nitrogen, the AOM analyses are not conclusive on whether the nitrogen or oxygen of Gln is coordinating.

The Met^{121} → Gln mutants of *A. denitrificans* and *P. aeruginosa* are significantly different. The difference is, however, not bigger than what could be expected by comparing nuclear quadrupole interactions based on the x-ray structure of wild type *A. denitrificans* azurin (not shown) with calculations based on wild type *P. aeruginosa* azurin (Table II). Thus the difference might simply reveal differences in angular distribution of the ligands and not necessarily differences in which ligands coordinate.

The two mutants Met^{121} → Ala and Met^{121} → Leu both have an NQI that is best described as a wild type-like 3-coordinate complex; however, they can also be described with an additional coordinating water molecule. The other NQI is in both cases best described by a coordinating water molecule. The NQI of Met^{121} → Leu that is best described by a coordinating water molecule has a much higher $\Delta\omega_0/\omega_0$ than wild type azurin. This further supports the coordination of a water molecule, which could be less restricted in its position than ligands from the protein.

The Met^{121} → Glu mutant could not be analyzed satisfactorily in the AOM model with any of the proposed structures (Tables III and IV) if the structure was taken from the wild type x-ray structure. This means that the structure must have changed more than the ±5° allowed for in the fit (± 10 for the mutated amino acid) or that the right coordination sphere (for example bidentate coordination by Glu) was not tested. The x-ray structure of the Met^{121} → Glu mutant of *P. aeruginosa* has been determined recently (28).[4] The crystals contain four subunits, and the variation in angles between pairs of ligands from one subunit to another subunit makes it necessary to use a standard deviation in the polar angles of ±10°. This structure is 4-coordinate with the carboxyl group of the Glu as the fourth ligand. The Met^{121} → Glu-NQI with the highest ω_0 was analyzed in the AOM model and the result is listed in Table V. It shows that this NQI can indeed be interpreted as 4-coordination by the 2 histidines, the cysteine, and the Glu, but also that this fit gives a rather big change in the angle between the two histidines, being the main reason for the very high reduced χ^2. The lower NQI measured could possibly be the Glu coordinating in a bidentate fashion. This hypothesis was not tested by AOM calculations.

Comparison to Other Measurements—Met^{121} → Leu and Met^{121} → Ala both show EPR spectra with only one detectable form. In both cases the EPR *g*-parameters changed a little and the parameter $A_\circ$ showed a small decrease when compared to wild type azurin (29, 30). Both mutants show, however, a significant increase in reduction potential from 308 mV (wild type) to 375 mV (Met^{121} → Leu) and to 373 mV (Met^{121} → Ala) (29, 30). Thus from the EPR parameters it seems that there is little change in the geometry for Cu(II) when Met^{121} is substituted by Ala or Leu. However, the substitution of Met with Ala or Leu disturbs the charge around Cu(II) since the partial charge in Met is missing in Ala and Leu. This probably explains part of the change in reduction potential upon substitution. When this is compared to the two forms found by Cd PAC, an explanation could be that because cadmium has the same oxidation state as Cu(II), but a d_{10} configuration like that of Cu(I), cadmium might bind as either. The NQI close to the wild type NQI could reflect Cd(II) in a Cu(II) conformation, and the other NQI, which is best described by an additional coordination water molecule, could then reflect Cd(II) in a Cu(I) conformation.

Met^{121} → Asp has been studied by x-ray absorption fine structure (XAFS), electron paramagnetic resonance, and optical spectra (31). The main conclusion by the XAFS is the occurrence of an additional oxygen ligand at a distance of 2.26–2.23 Å at pH 5 as well as pH 8. EPR, in contrast, shows no detectable difference at pH 5 compared to wild type and at pH 8 shows a rather big change in *g*-parameters as well as A_x and A_z. The PAC experiments were carried out at pH 7.2 and pH 10.0. There was no detectable difference between these two experiments, and the nuclear quadrupole interaction is well described by a coordinating carboxyl group from the aspartate but can also be explained by a coordinating water molecule (Tables III and IV). Thus, the results are in good accordance with the results at pH 8 achieved by XAFS and EPR. The pH in the PAC experiment was not lowered further due to the low affinity of cadmium ions to azurin at low pH.

Met^{121} → Asn shows a significant difference in EPR parameters as compared to wild type (30). This is in contrast to the PAC results. A possible explanation could be a difference in the coordination chemistry between Cu(II) and Cd(II) for this mutant.

Met^{121} → Gln from *A. denitrificans* was studied by Romero *et al.* (8). Their study showed that the mutant had ultraviolet-visible and EPR characteristics of a type I site but the spectroscopic details and midpoint potential differ significantly from wild type. This is generally in accordance with the changes observed by PAC. However, Romero *et al.* (8) do not report more than one metal site geometry with any of their techniques.

Met^{121} → Glu has the characteristic absorption spectrum of a blue copper protein at low pH but not at high pH (16). The p*K* of this change is 4.9. This pH behavior is also found in the EPR spectrum, where at low pH a rhombic EPR spectrum is observed, while at pH 7 the EPR characteristics are in between those of a type 1 and a type 2 site (16, 30). In particular, the hyperfine splitting changes dramatically. This is in good accordance with the drastic change in nuclear quadrupole interaction seen by PAC measured at pH above 7.2. These changes are all in good accordance with the coordination of the carboxyl group of Glu.

Nuclear Quadrupole Interaction of Cadmium-substituted Stellacyanin—Stellacyanin does not have any methionine in the amino acid sequence (32); it is believed to have a glutamine at this position (33). This is supported by comparison of optical spectra and EPR spectra for stellacyanin and Met^{121} → Gln azurin (8). Our PAC results further support this view when the NQI of stellacyanin is compared to the different Met^{121} → *X* mutants in Table I. The NQI of stellacyanin resembles closely the two NQIs of Met^{121} → Gln.

[4] G. Karlsson, L. Tsai, H. Nar, V. Langer, and L. Sjölin, unpublished data.

CONCLUSION

The use of Cd PAC on copper proteins has the advantage that cadmium with its d_{10} configuration might give some information on the behavior of Cu(I) not accessible by other spectroscopic techniques. Whereas many other techniques require low temperatures or crystals, PAC also has the advantage that it can be applied to molecules in solution without loss of resolution. Thereby the molecules studied by PAC can be in an environment that better mimics the natural environment of the protein.

The present work shows for the first time that detailed information on copper proteins can be obtained with the technique of perturbed angular correlations of γ-rays from ^{111m}Cd. In spite of the chemical difference between copper and cadmium, the active site of the wild type protein is apparently unaffected by the substitution of copper with cadmium. In contrast to this, the substitution of methionine 121 with other amino acids is generally characterized by the presence of two different conformations and/or wider linewidth, both characteristics indicating a less rigid structure. The comparison of the PAC spectrum of stellacyanin with the different Met^{121} mutants further supports that stellacyanin has a glutamine at this position.

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