X-ray Structure Determination and Characterization of the *Pseudomonas aeruginosa* Azurin Mutant Met121Glu[†,#]

B. Göran Karlsson,*[,‡] Li-Chu Tsai,[§] Herbert Nar,[||] Joann Sanders-Loehr,[⊥] Nicklas Bonander,[‡] Vratislav Langer,[§] and Lennart Sjölin[§]

Department of Biochemistry and Biophysics, Lundberg Laboratory, Göteborg University and Chalmers University of Technology, S-413 90 Göteborg, Sweden, Department of Inorganic Chemistry, Göteborg University and Chalmers University of Technology, S-412 96 Göteborg, Sweden, Max-Planck-Institut für Biochemie, Abteilung für Strukturforschung, Martinsried bei München, Federal Republic of Germany, and Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science and Technology, Portland, Oregon 97291-1000

Received September 24, 1996; Revised Manuscript Received January 15, 1997[⊗]

ABSTRACT: The Met121Glu azurin mutant has been crystallized and the structure determined at a resolution of 2.3 Å. In the crystal structure a carboxyl oxygen of Met121Glu is coordinated to the metal at a distance of 2.2 Å. Single-crystal resonance Raman spectroscopy was used to show that the glutamic acid residue in the copper site was in the protonated state. Titration of this residue gives rise to a number of unusual, pH-dependent properties: as the pH is increased from 4 to 8, the S(Cys)−Cu ligand-to-metal charge transfer bands are blue shifted and their intensity ratio is reversed, the EPR signal changes from type 1 copper to a new form of protein-bound copper, and the redox potential changes from 370 to 180 mV. The spectroscopic changes in this pH interval are consistent with a two-state model. From the pH dependence of the optical and EPR spectra, pK_a = 5.0 for the glutamic acid in the oxidized protein was determined.

Azurin is a member of the small blue copper proteins or cupredoxins, which are found in electron-transfer chains in plants and bacteria. Their molecular mass is between 9 and 18 kDa, and they contain a single redox-active copper ion per molecule. In the oxidized state, the type 1 copper site found in the cupredoxins is characterized by an intense transition near 600 nm in the optical spectrum and a unique hyperfine splitting in the EPR[1] signal. In addition, a reduction potential in the range between 184 and 680 mV has been found (Reinhammar, 1972). The spectroscopic and electrochemical properties of cupredoxins have been subject to a number of studies through the years, and recently the structures of several proteins in this class have been determined [for a review see Adman (1991)].

The azurin copper site geometry can be described as trigonal bipyramidal, with the three ligands, $N^{\delta 1}$ of His46, S^{γ} of Cys112, and $N^{\delta 1}$ of His117, in a plane with the copper ion at a distance of ∼2 Å and two weakly interacting atoms, the carbonyl oxygen of Gly45 and the S^{δ} of Met121, perpendicular to the plane at a distance of ∼3 Å. This geometry, the carbonyl oxygen excepted, is conserved in almost all determined structures of type 1 copper sites. The interaction between copper and the axial methionine ligand has been suggested as an important factor in the fine tuning of the reduction potential (Gray & Malmström, 1983) and is also important for the spectroscopic properties. The near C_{3v} geometry of the copper site is associated with an axial EPR spectrum and a very weak transition at 450 nm in the optical spectrum. A closer interaction with an axial ligand decreases the symmetry of the copper site, and as is found for pseudoazurin and cucumber blue protein, this interaction is associated with a rhombic EPR spectrum and an additional strong transition near 450 nm (Martinkus et al., 1980; Colman et al., 1977).

We have previously reported the cassette mutagenesis of the ligand methionine in azurin from *Pseudomonas aeruginosa* (Karlsson et al., 1991). The spectroscopic properties of the mutant proteins vary considerably, and the reduction potentials span a range of 250 mV (Pascher et al., 1993). The spectroscopic properties of the Met121Glu mutant show a strong pH dependence. At low pH, the properties are characteristic for a type 1 copper site, whereas at higher pH, properties for a new form of protein bound copper are observed. We have now further characterized this mutant, and in this paper the crystal structure of the Met121Glu azurin mutant is presented as well as its spectroscopic properties. The reduction potential is also discussed in view of the structure of the copper site.

MATERIALS AND METHODS

Expression of Azurin. We have previously cloned and expressed the azurin-encoding gene from *P. aeruginosa* in *Escherichia coli* (Arvidsson et al., 1989; Karlsson et al., 1989). The cassette mutagenesis, where the copper ligand methionine 121 was exchanged for all other amino acids and

[†] This work was supported by grants from the Swedish Natural Science Council and a grant from the National Institutes of Health (GM18865 to J.S.-L.).

[#] Coordinates have been deposited in the Brookhaven Protein Data Bank under the filename 1ETJ.

* Author to whom correspondence should be addressed at the Biochemistry and Biophysics Department, Lundberg Laboratory. Telephone: (+46)-31-7733917. Fax: (+46)-31-7733910. E-mail: Goran.Karlsson@bcbp.gu.se.

[‡] Lundberg Laboratory, Göteborg University and Chalmers University of Technology.

[§] Göteborg University and Chalmers University of Technology.

[||] Max-Planck-Institut für Biochemie.

[⊥] Oregon Graduate Institute of Science and Technology.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: EPR, electron paramagnetic resonance; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

S0006-2960(96)02416-6 CCC: $14.00

a preliminary characterization was performed, has subsequently been described (Karlsson et al., 1991).

Spectroscopy. Resonance Raman (RR) spectra were obtained as described previously (Andrew et al., 1994) using an ~150° backscattering geometry for data collection. Solution samples containing ~6 mM protein were frozen at 15 K, exposed to ~30 mW of laser power at 514 nm (Coherent Innova 90-6 Ar) or 580 nm (Coherent 599-01 dye), and analyzed with a Jarrell-Ash 25-300 spectrophotometer. A single crystal in a sealed capillary was analyzed at room temperature using ~15 mW of laser power at 568.2 nm (Spectra-Physics 2025-11 Kr) and a Dilor Z-24 spectrophotometer. Optical spectra were recorded between 350 and 750 nm in a Shimadzu 3000 spectrophotometer. EPR spectra were obtained on a Bruker ER 20D-SRC spectrometer equipped with an Oxford Instrument EPR-9 helium cryostat. Integrations were performed as described by Aasa and Vänngård (1975).

Crystallization. The crystallization procedures for the azurin Met121Glu mutant can be described in the following way. Following the last gel filtration step in the purification, at pH 5.7, the protein was brought to a final concentration of 15 g/L, dialyzed against distilled water, and subsequently centrifuged at 20000*g* to remove aggregates. After a large-scale screening, the optimum conditions were established, and the Met121Glu mutant crystals could be obtained from three different recipes. The largest crystals, about 2.0 × 0.4 × 0.1 mm^3 in size, were grown at room temperature from a solution containing 25% PEG 4000, 80 mM sodium acetate, and 80 mM $CaCl_2$. The crystallization took on the average about 14 days. Crystals could also be grown from 25% PEG 4000 at 25 °C, and in this case crystals appeared after only 7 days. The dimensions of these crystals were roughly 0.8 mm × 0.3 mm × 0.15 mm. Finally, well-formed prismatic crystals, around 0.8 mm × 0.5 mm × 0.2 mm in size, were also obtained in 10 days from a solution composed of 25% PEG 4000, 0.24 M $CaCl_2$, and 0.26 M $LiNO_3$, pH 6.0, in the temperature interval of 24−25 °C. These crystals were used in the data collection of the Met121Glu mutant. The final protein concentration in all crystallization experiments was 7.5 g/L.

Data Collection. X-ray intensity data for the Met121Glu mutant were collected on a micro Vax III controlled FAST television area detector diffractometer (ENRAF-NONIUS, Delft) in a thermostated room at 10 °C. The crystal was cooled by a stream of cold air to 4 °C. Cu Kα radiation from a rotating anode generator (RIGAKU) operating at 5.4 kW was used. Complete data sets were obtained by rotating the crystal 100° about the *c* axis. Measurements were recorded in frames of 0.1° with 80 s exposure time and evaluated on-line using MADNES (Messerschmidt & Plugrath, 1987). The structure factors were scaled and corrected for absorption effects by applying the program ABSCOR (Huber & Kopfmann, 1969; Messerschmidt et al., 1990) and merged by means of PROTEIN (Steigemann, 1974). One crystal was used to collect data extending to 2.3 Å resolution. The mutant Met121Glu crystals belong to the monoclinic system, and the space group has been determined to be $P2_1$. The cell parameters are $a = 50.58$ Å, $b = 60.94$ Å, $c = 81.60$ Å, and $\beta = 90.1°$. The final data set after internal scaling consisted of 17 964 reflections to 2.3 Å resolution. There are four molecules in the asymmetric unit, and the calculated V_m value is 2.25 $Å^3$/Da. The unique reflections were analyzed using the program LOAD from the PROTEIN system (Steigemann, 1974), and some of the data collection statistics are shown in Table 1.

Table 1: Statistics of Data Collection

unit cell constants	$a = 50.58$ Å $b = 60.94$ Å $c = 81.60$ Å $\beta = 90.10°$
space group	$P2_1$
crystal mosaicity	0.18
no. of measurements	47678
unique reflections	17964
nonrejected unique reflections	17222
data completeness	75.7%
r_{merge}[a]	11.5%
no. of atoms	4275
protein atoms	3904
solvent atoms	371
rms deviations	
bonds	0.013 Å
angles	3.18°
resolution range	8.0−2.3 Å
no. of reflections	15836
no. of parameters	17101
R-value[b]	18.4%

[a] $R_m = \sum_H \sum_{i=1}^{N} \langle I(H) \rangle - I(H)_i / \sum_H \sum_{i=1}^{N} I(H)_i$, where $I(H)_i$ is the *i*th measurement of reflection H and $\langle I(H) \rangle$ is its mean value, and the summation extends over all reflections measured more than once in the set. [b] $R = \sum |F_o - F_c| / \sum |F_o|$.

X-ray Structure Solution. The crystallographic solution of the Met121Glu mutant structure was obtained using rotation-translation functions. The azurin monomer used in the calculations was selected from the wild-type structure solution by Nar et al. (1991b). For the rotational search the model was placed in a triclinic cell with orthogonal axes of length 150 Å. The Fourier transform and the Patterson function were calculated from this model in the range of 8.0−3.5 Å resolution. Subsequently, the 6200 highest peaks of the Patterson synthesis with vector length 3.0−20.0 Å were selected, and the product correlation function of crystal and model Patterson functions was calculated at the Eulerian angles θ_1, θ_2, and θ_3 in the interval from 0° to 180° and with a $\Delta\theta$ of 5° using PROTEIN (Steigemann, 1974). In addition, a fine search using $\Delta\theta = 1$ was performed around the highest peaks.

The positions of the four correctly oriented monomers in the asymmetric unit were determined by calculating translation functions (Crowther, 1967) using programs written by Lattman and modified by Deisenhofer and Huber. The model Fourier transform (resolution 8.0−4.0) was calculated on the correctly oriented monomers shifted with their centers to the origin of the cubic cell with axes of 150 Å. The top four peaks in the peak list of the rotation function calculation proved to be the correct solutions. Consistent solutions could then be obtained from the output of the translation function calculations. The peak heights were in the range of 3.45−5.61 rms from the translation function calculations. The correctly oriented and positioned monomers for the Met121Glu mutant were now subjected to a rigid-body refinement using X-PLOR (Brünger, 1992). After 50 steps of refinement the initial *R*-value dropped from 44.3% to 29.6%, based on reflections in the resolution range of 8.0−3.0 Å resolution. After conventional energy-restrained positional refinement using X-PLOR and data between 10.0 and 2.3 Å resolution, the *R*-value dropped to 26.1%. Finally, individual isotropic

Table 2: Distance to Copper in the Four Molecules in the Asymmetric Unit

molecule	His46N$^{\delta 1}$	His117N$^{\delta 1}$	Cys112S$^{\gamma}$	Glu121O$^{\epsilon}$	O45
A	2.08	2.00	2.16	2.37	3.43
B	1.98	1.97	2.15	2.12	3.23
C	2.20	1.83	2.00	2.06	3.35
D	1.80	2.26	2.12	2.28	3.67
average	2.02	2.02	2.11	2.21	3.42

temperature factors were subjected to refinement as well, and the *R*-value decreased to 22.9%. After inclusion of 371 water molecules the final crystallographic *R*-value became 18.4% with an rms deviation on the bonds of 0.013 Å and 3.18° for the angles.

RESULTS

Crystal Structure. The final model consists of 3904 atoms in four molecules and, in addition, 371 water molecules. The final crystallographic *R*-value is 18.4% for 15 836 reflections to 2.3 Å resolution. The average temperature factor for the protein atoms is 19.7 Å^2, the average temperature factor for the solvent atoms is 33.6 Å^2, and the average temperature factors in the four monomers are 16.6, 17.6, 21.7, and 21.3 Å^2, respectively. Most of the structure is well-defined, especially the β-strands, the loop around the copper site, and all internal side chains. The regions around the N- and C-termini and the loops between the β-strands have, in general, higher temperature factors. Also, the side chain of Met121Glu is somewhat more mobile than are the other ligands to the copper atom. However, a comparison of the four monomers in the asymmetric unit suggests that the deviation from the main-chain atom positions is relatively small in the structure. The rms deviations for the α-carbons and all heavy atoms are 0.35 and 0.54 Å, respectively. This is similar to the values found in the wild-type crystal structure (Nar et al., 1991b). The distance between the side chains to the copper atom in the metal site for the four molecules in the asymmetric unit is given in Table 2.

The four crystallographically independent molecules in the asymmetric unit form a dimer of dimers. As in other azurin structures, the intermolecular contact is mediated via the hydrophobic patch (Norris et al., 1983; Nar et al., 1991a), but the molecules are differently packed compared to other reported structures. In this case, the contact takes place at Met64, Ala119, and Leu120, surrounding the copper ligand His117. Compared to the molecules in the wild-type azurin, the dimers in the Met121Glu mutant are rotated 140° relative to each other and tilted by approximately 15°, which is similar to the situation in the Phe114Ala mutant and also in the *Alcaligenes denitrificans* azurin (Figure 1).

Optical and EPR Spectroscopy. The pH dependence in the optical and EPR spectra of the Met121Glu azurin mutant is shown in Figures 2 and 3, respectively. The strong transition in the optical spectrum at 614 nm at low pH is shifted to a weaker 570 nm transition at high pH, and the 460 nm transition shifts to a strong, higher energy transition at 416 nm. Thus, at pH 4 the protein in solution is blue, at pH 5 it is greenish, and at pH >6 the color changes to brown. The EPR signal is strongly affected by changes in pH. At pH 3.5, a typical rhombic EPR spectrum for a type 1 copper is obtained. At higher pH, the spectrum changes, and the parameters are different from the combinations normally found for protein-bound copper (Vänngård, 1972). From the pH dependence in the optical and EPR spectra, pK_a^{ox} for the titration of the glutamic acid residue in the oxidized protein can be determined to be 5.0.

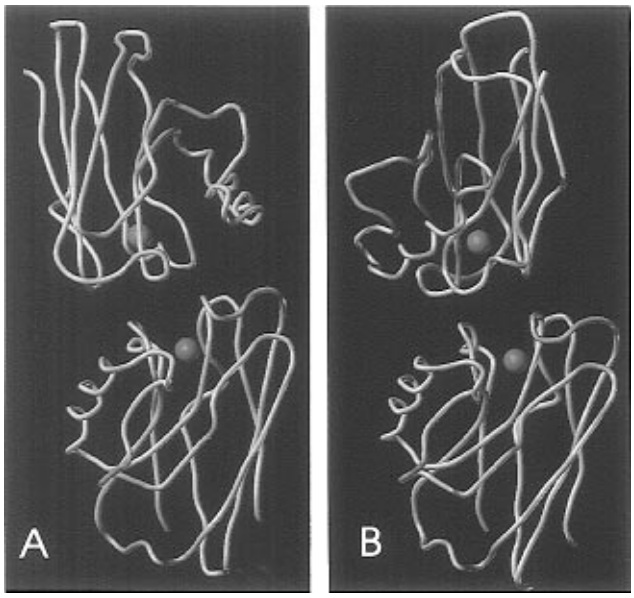

FIGURE 1: Trace of the backbone for two molecules located around the noncrystallographic 2-fold axis in the wild type (A) and the Met121Glu mutant (B).

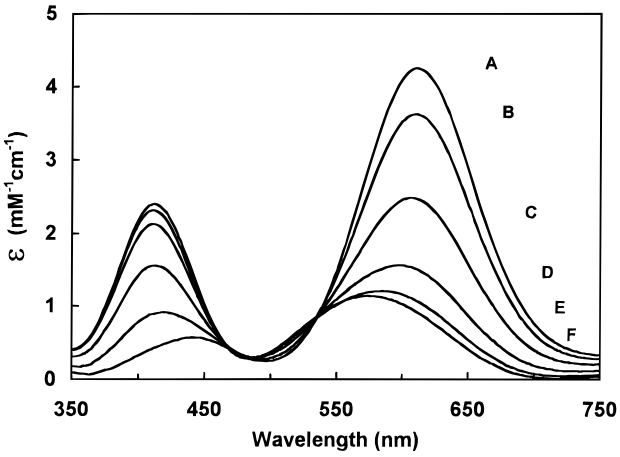

FIGURE 2: pH dependence of the optical spectrum of Met121Glu. Spectra were obtained in 10 mM HEPES buffer at different pH values in steps of 0.5 pH unit in the range between pH 4.0 and pH 6.5 accordingly: pH 4.0 (A), pH 4.5 (B), pH 5.0 (C), pH 5.5 (D), pH 6.0 (E), and pH 6.5 (F).

Optical and EPR parameters at pH 3.5 and 7 are summarized in Table 3. The absorption coefficients have an error of ∼10% because of the uncertainty in the determination of the copper concentrations from integration of the EPR spectra. The ratio of the experimentally determined absorption coefficients corresponds to the observed absorbance ratio at λ_{max} at pH 4 and 7 of the strong bands.

Resonance Raman Spectra of the Copper Site. Previous studies of Met121Glu azurin at pH 3.5 have shown it to have a resonance raman (RR) spectrum characteristic of a rhombic type 1 copper site (Andrew et al., 1994), in agreement with its optical and EPR spectral properties. Excitation within the 614 nm absorption band leads to intense RR features at 400 and 409 cm^{-1} (Table 4). The intensity arises from substantial Cu−S(Cys) stretching character in each of these vibrational modes (Dave et al., 1993). The high frequencies

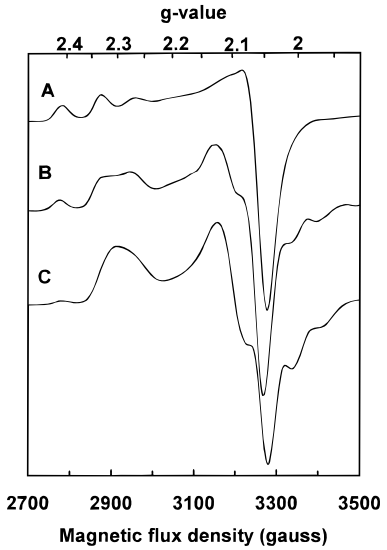

FIGURE 3: pH dependence of the EPR spectrum as obtained at pH 7.0 (A), 4.9 (B), and 3.9 (C) in 100 M MES.

Table 3: EPR and Optical Absorption Parameters of Azurin Met121Glu[a]

sample	$g_{\|\|}$	$A_{\|\|}$ (G)	$g_\perp$	g_x	g_y	ϵ_{416} (M^{-1} cm^{-1})	ϵ_{614} (M^{-1} cm^{-1})
Met121Glu (pH 3.5)	2.29	<30		2.09	2.03	200	4500
Met121Glu (pH 7)	2.298	90	2.060			2500	1100

[a] $g_{\|\|}$ and $g_\perp$ are g values in the parallel and perpendicular directions, respectively, and $A_{\|\|}$ is the hyperfine coupling in the parallel direction.

Table 4: Resonance Raman Spectral Properties of Azurin Met121Glu

sample	RR frequencies[a]				I_{360}/I_{410}[b]
Met121Glu (pH 7.5)	**345**	**358**	393	409	2.8
Met121Glu (pH 3.5)	348	359	**400**	**409**	0.4
Met121Glu (crystal)	348	359	**400**	**412**	0.5

[a] RR spectra obtained with excitation near 570 nm. Boldface numbers indicate peaks with greatest intensity. Frequencies are accurate to ± 1 cm^{-1} for solution samples and ± 2 cm^{-1} for the crystalline sample. [b] Relative intensity of peaks at ~360 and ~410 cm^{-1}.

are indicative of a short Cu−S(Cys) bond distance of ~2.16 Å (Andrew et al., 1994). In contrast, the RR spectrum of azurin Met121Glu at pH 7.5 is indicative of a species with a longer Cu−S(Cys) bond, due to the influence of the deprotonated carboxylate ligand. Excitation within either the 416 or 570 nm absorption bands produces maximal RR intensity at 345 and 358 cm^{-1} (Table 4). The lower frequencies for the Cu−S(Cys) stretches suggest a Cu−S(Cys) bond distance of ~2.21 Å. These properties are in keeping with a copper site in which copper is coordinated to four strong ligands.

The Met121Glu protein was crystallized in the oxidized state from a protein stock solution which was extensively dialyzed against distilled water. However, the presence of 25% PEG alters the effective pH of the crystallizing solution. The crystals used for X-ray structure determination were greenish blue in color, suggesting that they contained mainly the component with a protonated carboxylate ligand. This finding is supported by the RR spectrum obtained from a single crystal of this protein (Table 4). The appearance of two intense spectral features at 400 and 412 cm^{-1} is characteristic of the protonated form. The weak bands at 348 and 359 cm^{-1}, due to residual deprotonated species, yield an intensity ratio (~360/~410 cm^{-1}) of only 0.5. This is similar to the 0.4 value for the pH 3.5 form and markedly different from the 2.8 value for the pH 7.5 form. In addition, the major peak in the crystalline sample at 400 cm^{-1} is at the same frequency as the pH 3.5 sample and distinctly different from the 393 cm^{-1} frequency for the pH 7.5 sample. We conclude that the Glu121 ligand in the crystalline sample was ~80% protonated.

Crystal Structure of the Copper Site. In the crystal structure, the average distance between O^ϵ of the Glu121 and the copper is 2.21 Å, and the glutamic acid residue is directly coordinating the metal ion. It is similar to the value of 2.27 Å for the bond from Cu to the carbonyl oxygen in the Gln121 mutant of *A. denitrificans* azurin (Romero et al., 1993). The relative weakness of this bond is consistent with a predominantly protonated carboxylate ligand. The short Cu−S(Cys) average distance of 2.11 Å is not very different from that of the wild-type copper site, and also the interaction with the His46 $N^{\delta 1}$ and His117 $N^{\delta 1}$ is maintained. However, the distance to the Gly 45 carbonyl oxygen has increased to 3.4 Å, and a four-coordinated copper site geometry shown in Figure 4 is observed in this mutant. The temperature factors for Glu 121 indicate a slight increase in flexibility in the orientation of the side chain, possibly due to the presence of a small amount of deprotonated ligand in the crystals (Figure 5). The average temperature factor of the side chain of Glu121 is 20.1 $Å^2$, whereas the average temperature factors of the side chains of the liganding histidines 46 and 117 are 15.1 and 17.1 $Å^2$, respectively.

The short distance between the copper and the glutamic acid side chain is made possible through the movement of the copper 0.3 Å in the direction of the glutamic acid side chain, and copper is coordinated in a *syn* fashion with a glutamic acid O^ϵ, the copper being 0.36 (±0.09) Å above the plane defined by the three main ligands. The increase in distance to the carbonyl oxygen is explained by the movement of the copper ion, but an additional distance is gained by a slight conformational change in the peptide backbone, effectively rotating the carbonyl oxygen away from the copper (cf. Figure 6). The copper site distances are listed in Table 2.

DISCUSSION

Copper Site Geometry at Low pH. The pH dependence of the spectroscopic properties has previously been observed (Karlsson et al., 1991), and in a modeled copper site the distance between the glutamic acid residue and the copper was estimated to 2.4 Å. The shorter distance observed in the crystal structure depends on the movement of the copper atom in the direction of the fourth ligand. The mobility observed in the glutamic acid residue side chain may indicate a conformational heterogeneity in the copper site due to the limited protonation of the Glu121 side chain.

A carboxylate *syn* coordination in metal sites is often connected with the presence of a water molecule in a five-membered ring (Chakrabarti, 1990), but in this structure no water molecule is present. Instead, the side chain is here ordered by the hydrophobic environment in the outer coordination sphere, and a lone pair of the nonligating glutamic acid residue O^ϵ interacts with the π-electrons of the aromatic side chain of the Phe15 residue.

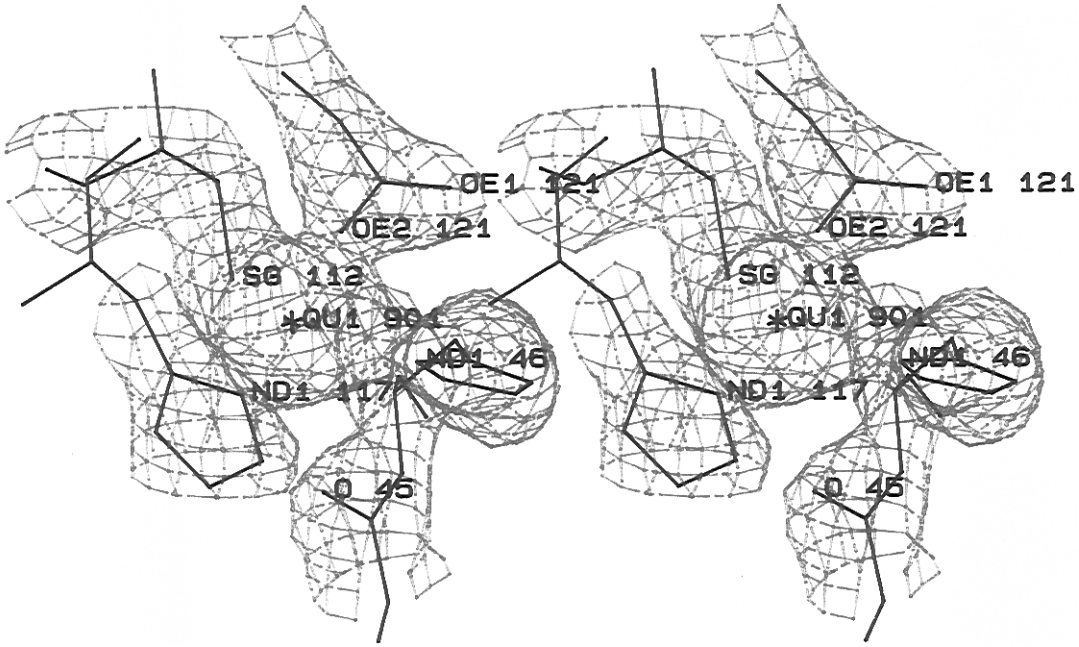

FIGURE 4: Stereoview of the electron density map of the copper site. The map is contoured on 1σ level.

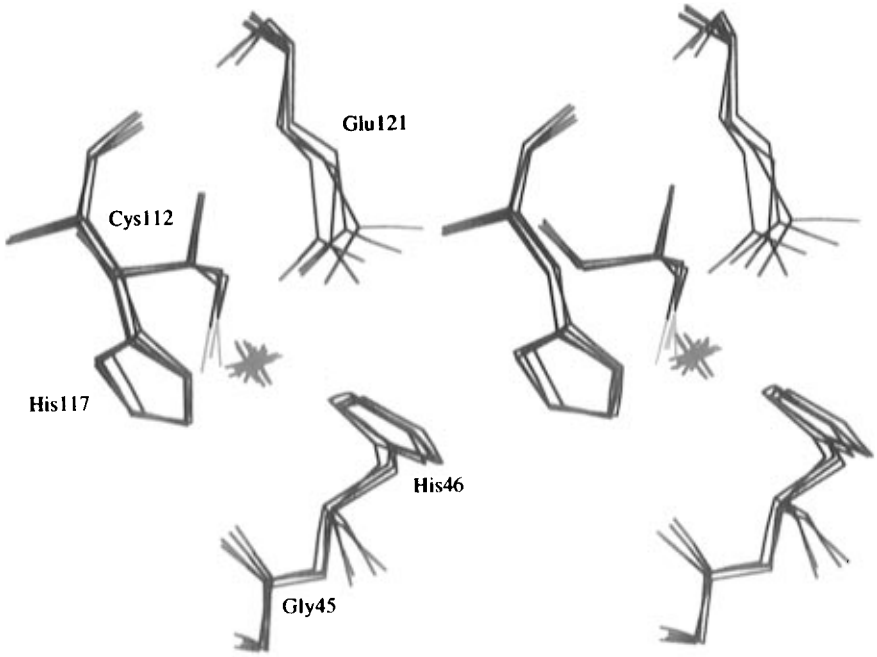

FIGURE 5: Stereoview of the copper site in the Met121Glu mutant. The four molecules in the asymmetric unit have been superimposed, illustrating the mobility of the glutamic acid residue side chain.

Copper Site Structure at High pH. The copper site of Met121Glu azurin at pH 4 and 8 has previously been investigated with EXAFS (Strange et al., 1996). At pH 4 the interaction between copper and the glutamic acid side chain could not be observed, probably due to a locally disordered side chain. At pH 8, however, a very close interaction is present between the copper and one of the oxygens of the glutamate side chain with a distance as short as 1.90 Å. To achieve this conformation, the copper has to be pulled up further, out of the plane, toward the glutamate side chain. This requires that the distance to Cys112 S^{γ} increases by about 0.06 Å as observed in RR.

Copper Site Structure Compared to Wild-Type Copper and Zinc Proteins and to the Met121Gln Copper Protein. The idea that the metal coordination in a protein is governed by the protein fold, the rack mechanism, was proposed by Eyring et al. (1956) and was extended to blue copper proteins by Malmström (1965) and Vallee and Williams (1968). Although the negative charge of the dipole of the glutamic acid carboxyl group tends to pull the copper out of the plane, the His_2-Cys interaction with Cu remains almost unchanged. Similar results have been observed in the zinc protein (Nar et al., 1992). In this case, the zinc ion favors a more tetrahedral conformation, and an interaction with the glycine 45 carbonyl oxygen is observed. The His_2-Cys ligation is also in this structure almost unchanged, which lends firm support to the rack mechanism. The difference between the structures lies primarily in the position of the metal ion along an axis perpendicular to the His_2-Cys plane. This is illustrated in Figure 6, where a stereoview of the superim-

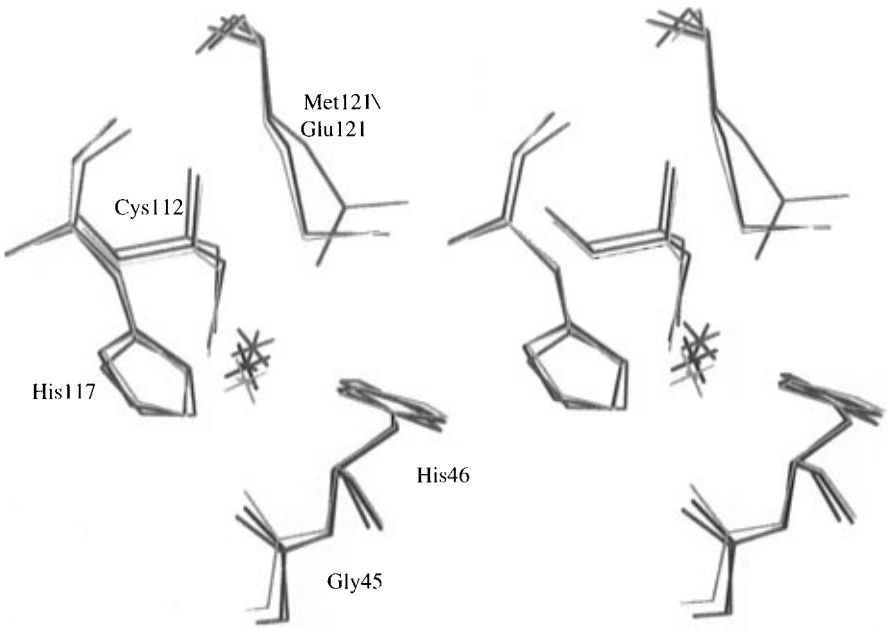

FIGURE 6: Stereoview of the superimposed metal sites in zinc azurin (green), wild type (blue), and the Met121Glu mutant (red).

posed metal sites in the zinc and wild-type azurin and the Met121Glu mutant is presented.

In the *A. denitrificans* Met121Gln mutant azurin the distance between the glutamine O^{ϵ} and the copper ion is 2.27 Å in the oxidized form. A copper site geometry, similar to the geometry observed in the protonated form of Met121Glu, is in addition observed (Romero et al., 1993).

Influence of Structure on Spectroscopic Properties. The experimental data for the pH dependence between pH 3.5 and 7.5 can be accounted for in a two-state model involving the two states of protonation of the Glu121 side chain. At even higher pH the titration of both His35 and His83, as well as of a number of basic side chains, would be expected to interfere.

At low pH the added proton to the side chain effectively neutralizes the charge, and as a consequence the interaction there is a weaker coordination than at high pH. The coordinating ligand in Met121Glu azurin at low pH induces the increase in the 450 nm region of the visible spectrum and the change from an axial into the rhombic EPR spectrum observed in this mutant. A free rotation of the Glu121 side chain then becomes more likely due to the geometric arrangements in the vicinity, which is indicated by the flexibility in the orientation of this side chain in Figure 5.

Almost identical spectral properties have been found in stellacyanin and also in the *A. denitrificans* azurin Met121Gln mutant. The properties at low pH of the Met121Glu mutant protein are also similar to the properties observed for cucumber blue protein (Colman et al., 1977) and pseudoazurin (Martinkus, 1980), and in these cases the distance to the corresponding ligand is in the range between 2.6 and 2.7 Å (Fields et al., 1992; Adman, 1991).

At high pH, however, the most striking change is the shift of the ligand-to-metal charge transfer (LMCT) band at 614 and 460 nm to higher energy, which effectively changes the color of the protein from blue to brown. Several other weaker transitions are also shifted as a result of the change in pH, and there is a very clear change in the EPR spectrum as well. The main reason for this strong effect can be explained by the interaction between the free electron pair and the copper atom. This interaction is in addition stabilized by the negative charge on the Glu121 side chain. The introduction of an extra free electron pair into the copper site consequently imposes a change in the Cu−Cys(S) interaction, thereby shifting the LMCT band to higher energy. A similar change is also observed in the previously studied Met121Ala mutant where the axial interaction is mediated through an exogenously added ligand (Bonander et al., 1996). The crystal structure of Ala121 + N_3^- (Tsai et al., 1996) shows that the azide binds in the cavity formed when the amino acid methionine is replaced with the smaller alanine residue and subsequently interacts with the copper atom from a similar position compared to Glu121. The copper in the Ala121−azide complex is coordinated by the N_3^- and the His117, Cys112, and His46 residues (Tsai et al., 1996), the copper being 0.3 Å from the major ligand plane. The similarities in optical and EPR parameters of the copper coordination between Ala121 + N_3^- and Glu121, pH 7.0, clearly indicate an analogous structure change in the Met121Glu mutant upon changing the pH from 3.5 to 7.0.

When this mutant protein is compared with the Met121Lys mutant, a similar very strong change in spectroscopic properties takes place at high pH where the lysine side chain would be expected to be uncharged (Karlsson et al., 1991). In the Met121Lys mutant, we also interpret the observed effect as a result of an interaction between the metal and the free electron pair of the nitrogen. This interaction is, in this case, supported by the geometry in the metal site not imposing any strains on the nonbranched lysine side chain. This is in contrast to the properties observed for most other Met121 mutants (Karlsson et al., 1991; Murphy et al., 1993), where a direct side-chain interaction with the metal is precluded for steric reasons.

Reduction Potentials. The pH dependence in the reduction potential is well-known in azurin (Pascher et al., 1993) and has been attributed to an electrostatic effect caused by the protonation state of the two nonliganding histidines, His35 and His83 (Nar et al., 1991b), but a contribution from pH-dependent structural changes to the reduction potential cannot be excluded in azurin (Lu et al., 1993). In *P. aeruginosa*

wild-type azurin, a change from ~350 mV at pH 4.5 to ~290 mV at pH 8.5, resulting in a total span of 60 mV, has previously been reported (Pascher et al., 1993; St. Clair, 1992). In the Met121Glu mutant, reduction potentials of 370 mV at pH 4 (unpublished results), 220 mV at pH 7, and 184 mV at pH 8 (Pascher et al., 1989) are found. The low reduction potential at pH 8 is an effect of the stabilization of the Cu^{2+} by the glutamate negative charge. An equally low reduction potential is observed when the protein is truncated before position 121 and the Cu^{2+} form is stabilized by a water molecule, which now has access to the copper ion (Murphy et al., 1993).

The pK_a, 5.0, for the glutamic acid residue is ~1 pH unit higher than for the free side chain, which is somewhat surprising since the close proximity to the polar metal ion would be expected to decrease the pK_a. The increase is probably due to the inaccessibility of the copper site in combination with a rather hydrophobic environment, which destabilizes the anionic form and favors a net increase in pK_a. The hydrophobic effect is even more pronounced in the Met121Asp mutant where pK_a^{ox} of the aspartic acid is 6.2 (Murphy et al., 1993), being ~2 pH units higher than for the free side chain.

CONCLUSION

The exchange of the conserved methionine residue of type 1 copper sites can induce large changes in geometry, spectroscopic properties, and reduction potential of the copper site. The concept of rack mechanism in the small blue copper proteins is again confirmed. In the azurin mutant Met121Glu, the glutamic acid residue is directly coordinating the copper ion at a distance of 2.2 Å. The blue shift of the optical transitions at 614 and 460 nm to higher energy and the strong pH dependence in the reduction potential are attributed to the introduction of a negative charge very close to the copper ion and a simultaneous movement of the copper atom out of the major ligand plane.

ACKNOWLEDGMENT

We thank Professors Bo G. Malmström and Tore Vänngård for valuable discussions. We also thank Drs. Thomas M. Loehr and Colin R. Andrew for assistance in obtaining RR spectra.

REFERENCES

Aasa, R., & Vänngård, T. (1975) *J. Magn. Reson. 19*, 308−315.
Adman, E. T. (1991) *Adv. Protein Chem. 42*, 145−197.
Andrew, C. R., Yeom, H., Valentine, J. S., Karlsson, B. G., Bonander, N., van Pouderoyen, G., Canters, G. W., Loehr, T. M., & Sanders-Loehr, J. (1994) *J. Am. Chem. Soc. 116*, 11489−11498.
Arvidsson, R. H. A., Nordling, M., & Lundberg, L. G. (1989) *Eur. J. Biochem. 179*, 195−200.
Bonander, N., Karlsson, B. G., & Vänngård, T. (1996) *Biochemistry 35*, 2429−2436.
Brünger, A. T. (1992) *X-PLOR, version 3.1*, Yale University Press, New Haven, CT.
Chakrabarti, P. (1990) *Protein Eng. 4*, 49−56.
Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., Venkatappa, M. P., & Vickery, L. E. (1977) *J. Mol. Biol. 112*, 649−650.
Crowther R. A., & Blow, D. M. (1967) *Acta Crystallogr. 23*, 544−549.
Dave, B. C., Germanas, J. P., & Czernuszewicz, R. S. (1993) *J. Am. Chem. Soc. 115*, 12175−12176.
Eyring, H., Lumry, R., & Spikes, J. D. (1956) in *Mechanism of Enzyme Action* (McElroy, W. D., & Glass, B., Eds.) pp 123−136, John Hopkins Press, Baltimore, MD.
Fields, B. A., Guss, J. M., & Freeman, H. C. (1992) *J. Mol. Biol. 222*, 1053−1065.
Gray, H. B., & Malmström, B. G. (1983) *Comments Inorg. Chem. 2*, 203−209.
Huber, R., & Kopfmann, G. (1969) *Acta Crystallogr. A25*, 143−150.
Karlsson, B. G., Pascher, T., Nordling, M., Arvidsson, R. H. A., & Lundberg, L. G. (1989) *FEBS Lett. 246*, 211−217.
Karlsson, B. G., Nordling, M., Pascher, T., Tsai, L.-C., Sjölin, L., & Lundberg, L.G. (1991) *Protein Eng. 4*, 343−349.
Lu, Y., LaCroix, L. B., Lowery, M. D., Solomon, E. I., Bender, C. J., Peisach, J., Roe, J. A., Gralla, E. B., & Valentine, J. S. (1993) *J. Am. Chem. Soc. 115*, 5907−5918.
Malmström, B. G. (1965) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) Vol. 1, pp 207−216, Wiley, New York.
Martinkus, K., Kennelly, P. J., Rea, T., & Timkovich, R. (1980) *Arch. Biochem. Biophys. 199*, 465−472.
Messerschmidt, A., & Plugrath, J. W. (1987) *J. Appl. Crystallogr. 20*, 306−315.
Messerschmidt, A., Schneider, M., & Huber, R. (1990) *J. Appl. Crystallogr. 23*, 436−439.
Murphy, L. M., Strange, R. W., Karlsson, B. G., Lundberg, L. G., Pascher, T., Reinhammar, B., & Hasnain, S. S. (1993) *Biochemistry 32*, 1965−1975.
Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991a) *J. Mol. Biol. 218*, 427−447.
Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991b) *J. Mol. Biol. 221*, 765−772.
Nar, H., Huber, R., Messerscmidt, A., Fillipou, A. C., Barth, M., Jaquinod, M., van de Kamp, M., & Canters, G. W. (1992) *Eur. J. Biochem. 205*, 1123−1129.
Norris, G. E. E., Anderson, B. F., & Baker, E. N. (1983) *J. Mol. Biol. 165*, 501−521.
Pascher, T., Karlsson, B. G., Nordling, M., Malmström, B. G., & Vänngård, T. (1993) *Eur. J. Biochem. 212*, 289−296.
Reinhammar, B. (1972) *Biochim. Biophys. Acta 275*, 245−259.
Romero, A., Hoitink, C. W. G., Nar, H., Huber, R., Messerschmidt, A., & Canters, G. W. (1993) *J. Mol. Biol. 229*, 1007−1021.
St. Clair, C., Ellis, W. R., & Gray, H. B. (1992) *Inorg. Chim. Acta 191*, 149−155.
Steigemann, W. (1974) Thesis, Technische Universität, München.
Strange, R. W., Murphy, L. M., Karlsson, B. G., Reinhammar, B., & Hasnain, S. S. (1996) *Biochemistry 35*, 16391−16398.
Tsai, L.-C., Bonander, N., Harata, K., Karlsson, B. G., Vänngård, T., Langer, V., & Sjölin, L. (1996) *Acta Crystallogr. D52*, 950−958.
Vallee, B. L., & Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 498−505.
Vänngård, T. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R., & Borg, D. C., Eds.) pp 411−447, John Wiley & Sons, Inc., New York.

BI962416O