Environment of Copper in *Pseudomonas aeruginosa* Azurin Probed by Binding of Exogenous Ligands to Met121X (X = Gly, Ala, Val, Leu, or Asp) Mutants†

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Received September 15, 1995; Revised Manuscript Received December 13, 1995⊗

ABSTRACT: The binding of small exogenous ligands to mutants of the blue copper protein azurin from *Pseudomonas aeruginosa,* altered in the axial position, Met121X (X = Gly, Ala, Val, Leu, or Asp), has been studied with optical and electron paramagnetic resonance (EPR) spectroscopy. The results show that small molecules can enter the pocket left by the side chain of Met121. For azide, the dissociation constants are Leu > Val > Ala, reflecting the increasing space available. The Gly and Asp mutants bind azide less strongly than the Ala mutant, due to competition with water (Gly) and the polar side chain (Asp). Similar trends are found for thiocyanate. Cyanide binds equally well to the Ala and Val mutants. A number of other small potential ligands were tried. Alcohols do not affect room-temperature optical spectra, but at low temperatures, the EPR spectrum is stellacyanin-like, indicative of a weak axial interaction. Ligands binding with a carboxyl group or nitrogen (e.g. acetate or azide) convert the metal center to a form intermediate between regular types 1 and 2, presumably by pulling the copper ion out of the trigonal plane formed by Cys(S) and two His(N). Cyanide interacts strongly as shown by the hyperfine coupling to the ^{13}C nucleus. With increasing strength of the axial interaction, the two major bands in the visible region (600 and 400−500 nm) shift in parallel to higher energy, and at the same time, the strength of the latter transition increases at the expense of the former. This demonstrates that these transitions have a common origin, namely S-to-Cu charge transfer transition.

Azurin belongs to the family of small, intensely blue copper proteins, the cupredoxins, which are characterized primarily by the presence of a strong absorption in the visible region around 600 nm (Adman, 1991). This arises from a charge transfer transition involving the so-called type 1 Cu(II) ion and a coordinating cysteine, the Cu−S distance being about 2.1−2.3 Å. There are two additional strong ligands, nitrogens at about 2.0−2.2 Å in two histidines. The copper ion is situated within 0.5 Å of the plane formed by these three ligands. The copper coordination is completed by amino acid residues in the axial direction which interact less strongly. The azurins all have a methionine with its sulfur at a distance of about 3.1 Å in one axial direction and a glycine carbonyl oxygen at 3.0 Å in the opposite direction. The cupredoxins function as electron transfer proteins, and the copper ion can readily exchange electrons with its redox partners, presumably through one of the liganding histidines (van de Kamp et al., 1990).

In spite of the weak coordination in the axial directions, small exogenous metal ligands like cyanide and azide do not bind to the copper ion. For azurins, only the neutral NO molecule can penetrate to the metal ion (Gorren et al., 1987; Ehrenstein & Nienhaus, 1992), forming a complex that can be dissociated by light. A number of mutants of azurin in which the liganding residues have been altered have been prepared. Many of these changes increase the accessibility of the copper ion for exogenous ligands. Canters and his co-workers (den Blaauwen & Canters, 1993; den Blaauwen et al., 1993; Canters & Gilardi, 1993) have performed a series of studies on a mutant where the strong ligand His117 was replaced with Gly. Then, exogenous ligands like imidazole could take the place left by reduction of the size of the side chain of residue 117. Various spectroscopic forms of the metal site could be formed this way.

In a very recent report (Vidakovic & Germanas, 1995), data on the binding of azide, thiocyanate, and cyanide to a mutant where the axial Met121 was replaced with Gly were reported. In our laboratory, mutants in which the axial Met121 residue has been changed to all other amino acids have been prepared (Karlsson et al., 1991). The purpose of the present work is to study the reactivity of a selection of these mutants against small metal ligands such as azide and cyanide. In addition, a number of other small potential ligands have been tested. An easily detected interaction also occurs with relatively inert substances, like methanol. The resulting spectral properties and binding affinities are presented. Depending on the type of ligand, modified type 1 centers arise.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The mutants were constructed using degenerate oligonucleotides *in vitro* with a mutagenesis system purchased from Amersham described earlier (Karlsson et al., 1991).

Expression of Azurin. Azurin was expressed in the bacterial strain RV308 which has an enhanced capacity for translocating proteins to the periplasmic space. The gene was induced by IPTG (isopropyl β-D-galactopyranoside), and the vector also contained the gene for *Amp* resistance described earlier (Karlsson et al., 1989). The bacteria were

† The work was supported by a grant from the Swedish Natural Science Research Council.

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

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grown at 37 °C in 1 L cultures of LB medium (Bertani, 1951) with ampicillin (100 μg/mL), induced with 0.3 mM IPTG at the start of the cultivation, and harvested 15−17 h later. The expression levels were 50−150 mg of azurin per liter of cultivation medium depending on the mutant.

Preparation of Azurin. The gene for azurin contains a signal peptide that is coding for translocation to the periplasmic space; this simplifies the purification of the azurin. The signal peptide is spliced off during the translocation. For purification of azurin, the protein was extracted from the periplasmic space and the crude preparation was subjected to pH 4.1, which denatures many of the *Escherichia coli* proteins, but not the azurin mutants. The sample was then applied to a CM52 ion exchange column and eluted with a pH 4.1 to 9.0 gradient. The fractions with azurin were concentrated and applied on a Sephacryl S-100 gel filtration column. For further details, see Karlsson et al. (1989). The samples were pooled and concentrated using an ultracentrifugation unit Macrosep (FILTRON). The purity of the azurin mutants was checked on a Pharmacia Phast-System using a Pharmacia sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gradient of 8 to 25% gels under reducing conditions. All preparations of the azurin mutants showed only one band on an SDS gel.

Optical and EPR Spectra. The optical spectra were recorded on a Cary 4.0 spectrometer. The temperature was 20 °C except for the studies of temperature dependence. The X-band EPR[1] spectra were recorded on a Bruker ER 200D-SRC spectrometer at 77 K. Other conditions were as follows: microwave frequency, 9.38 MHz; modulation amplitude, 2 mT; and microwave power, 2 mW. The protein samples were dissolved in 100 mM Hepes (pH 7) unless stated otherwise. The total protein concentration in the EPR samples was 0.2 mM, on the basis of $\epsilon_{280} = 9.0$ mM^{-1} cm^{-1} (Pascher et al., 1993; Karlsson, 1993).

RESULTS

Mutants Investigated in This Study. Optical and EPR spectral parameters of the WT and mutated proteins used in this study are compiled in Tables 1 and 2. EPR spectra at 77 K are shown in Figure 1 together with the spectrum of stellacyanin. The general appearance of the spectra agrees with what has been reported earlier (Karlsson et al., 1991). Thus, the WT protein as well as mutants with hydrophobic residues (Ala, Val, and Leu) have an optical spectrum with a rather weak secondary maximum in the 450−500 nm region and an axial EPR spectrum with $A_{\|}$ in the range of 50−60 G. The same is true for Asp at low pH, where the residue most likely is protonated and interacting less strongly with the metal center. The Gly mutant and the Asp mutant at pH 7 resemble stellacyanin both in the optical spectra with a stronger secondary maximum and in the more rhombic character of the EPR spectrum (Figure 1B,G,H) [the value of A_z for Met121Gly recently reported by Vidakovic and Germanas (1995) appears to be too large].

The earlier studies of the pH dependence of the mutants were extended to the Met121Gly mutant. Its spectroscopic properties were found to be essentially independent of pH in the range of 4.5−8.5. Similarly, Met121Ala was independent of pH in the range of 4.5−7 with only a small change in the EPR spectrum above pH 8.

EPR Spectra of Complexes. No binding to the WT protein of the small molecules used in this work could be detected.

Table 1: Optical Parameters and Dissociation Constants of Azurin Mutants and Their Complexes with Exogenous Ligands[a]

sample[b]	$\lambda_1{}^{max}$ (nm)	$\epsilon_1{}^{max}$ (mM^{-1} cm^{-1})	$\lambda_2{}^{max}$ (nm)	$\epsilon_2{}^{max}$ (mM^{-1} cm^{-1})	K_D (mM)
WT	628	5.5	480	0.04	−
stellacyanin[c]	604	4.1	450	0.96	−
Met121Gly	612	4.9	453	0.8	−
+ CN^-	550	0.9	432	3.4	4.5
+ N_3^-	550	1.9	422	3.2	65
+ SCN^-	550	1.4	415	3.5	28
Met121Ala	625[d]	5.6[d]	463	0.5	−
+ CN^-	520	1.1	413	5.1	2.5
+ N_3^-	520	2.7	409	5.2	6[e]
+ SCN^-	530	1.7	409	4.6	20
+ CH_3NH_2	580	2.3	431	3.4	55
Met121Val	626[d]	5.2[d]	460	0.8	−
+ CN^-	550	1	420	3.7	2.5
+ N_3^-	520	1.8	416	5.5	30
+ SCN^-	550	1.1	416	3.5	30
Met121Leu	630[d]	6[d]	480	0.1	−
+ CN^-	550	1.1	416	3.7	15
+ N_3^-	550	1.2	412	3.0	700
Met121Asp (pH 4)[f]	620	6.2	470	0.4	−
+ N_3^-	−	2.3	400	7.0	30
Met121Asp	614	5.9	450	1.2	−
+ CN^-	580	1.8	433	5.7	5
+ N_3^-	−	2.6	403	7.4	120
Met121Asp (pH 8)	611[d]	5.6[d]	450	1.5	−

[a] For comparison, stellacyanin is also included. [b] pH 7 if not stated otherwise. [c] Malmström et al. (1970). [d] Pascher et al. (1993). [e] Tsai et al. (1995). [f] 100 mM MES.

The mutants, on the other hand, interacted with a variety of small molecules, capable of coordinating to Cu(II). Representative spectra are shown in Figures 2−4, and parameters of the EPR spectra of the complexes are given in Table 2. In cases where it was difficult to precisely define the g_x and g_y, the g value at maximum absorption, g_0, is given instead. A A_z vs g_z plot of the type first introduced by Vänngård (1972) is presented in Figure 5. The WT and mutant proteins all fall close to the line in Figure 5, which connects ordinary blue type 1 proteins (Vänngård, 1972).

Earlier, it was reported that the anions Cl^- and Br^- coordinate to Cu in the His117Gly azurin mutant (den Blaauwen & Canters, 1993; den Blaauwen et al., 1993). In the present study, no effect was observed when 10 mM chloride, 20 mM nitrate, or 50 mM phosphate was added to the Met121Ala mutant. However, with larger concentrations of Cl^-, changes were observed, but the resulting spectrum was complex, arising from several species, and was not investigated further.

Methanol and ethanol caused a significant change in the EPR spectrum; compare, for example, spectra A and B of Figure 2. Similar changes indicating weaker binding were seen also with Met121Ala plus 100 mM propanol, whereas 2-propanol had no effect. The derivatives obtained upon alcohol binding are type 1 species, all grouping at that end of the type 1 line (Figure 5) where stellacyanin is located.

A set of other small potential ligands was tried which were found to interact more strongly with the copper, creating spectral forms which were intermediate between types 1 and 2. In a previous publication, such forms were called type 1.5, and this nomenclature will for simplicity be used also in the present work.[2] The ligands were small carboxylic

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

Table 2: EPR Parameters of Azurin Mutants and Their Complexes with Exogenous Ligands[a]

no[b]	sample[c]	g_z	g_y	g_x	A_z (G)	g_0
1	WT	2.261	2.052	2.035	53	
2	stellacyanin[d]	2.287	2.08	2.03	35	
3	Met121Gly	2.308	2.07	2.02	<25	
4	Met121Ala	2.258	2.04	2.04	50	
5	Met121Val	2.256	2.05	2.05	50	
6	Met121Leu	2.268	2.05	2.05	51	
7	Met121Asp (pH 4.5)[g]	2.268	2.05	2.05	56	
8	Met121Asp	2.295	2.06	2.02	<25	
−	Met121Gly					
9	+ CN^-	2.223	2.06	2.04	92	
10	+ N_3^-	2.242	2.04	2.04	98	
11	+ SCN^-	2.217	2.06	2.03	80	
12	+ CH_3NH_2	2.233	2.04	2.04	111	
13	+ imidazole-1[e]	2.238	−	−	118	2.06
14	+ imidazole-2[e]	2.267	−	−	170	2.06
15	+ CH_3OH	2.299	2.07	2.02	<30	
−	Met121Ala					
16	+ CN^-[f]	2.180	2.065	2.032	142	
17	+ N_3^-	2.262	2.06	2.06	112	
18	+ SCN^-	2.194	2.05	2.03	114	
19	+ CH_3NH_2	2.257	2.06	2.06	110	
20	+ imidazole-1[e]	2.235	−	−	127	2.05
21	+ imidazole-2[e]	2.266	−	−	170	2.05
22	+ CH_3CN	2.26	2.06	2.06	115	
23	+ NH_3	2.285	2.08	2.08	64	
24	+ CH_3OH	2.296	2.05	2.01	<25	
25	+ CH_3CH_2OH	2.31	−	−	<25	2.07
26	+ $HCOO^-$	2.258	2.04	2.04	132	
27	+ CH_3COO^-	2.263	2.04	2.04	130	
−	Met121Val					
28	+ CN^-	2.189	2.06	2.03	139	
29	+ N_3^-	2.238	2.04	2.04	106	
30	+ SCN^-	2.235	2.05	2.05	124	
31	+ CH_3OH	2.295	2.08	2.03	<25	
−	Met121Leu					
32	+ CN^-	2.175	2.06	2.03	135	
33	+ N_3^-	2.239	2.05	2.05	103	
34	+ SCN^-	2.23	2.04	−	128	2.06
−	Met121Asp					
35	+ CN^-	2.218	−	−	97	2.06
36	+ N_3^-	2.246	2.05	2.05	94	
−	Met121Asp pH 4.5[g]					
37	+ N_3^-	2.231	2.04	2.04	115	
38	+ CH_3NH_2	2.233	2.04	2.04	110	

[a] For comparison, stellacyanin is also included. [b] Refers to the numbering in Figure 5. [c] pH 7 if not stated otherwise. [d] Malmström et al. (1970). [e] The two components observed in EPR. [f] From simulation shown in Figure 4. [g] 100 mM MES.

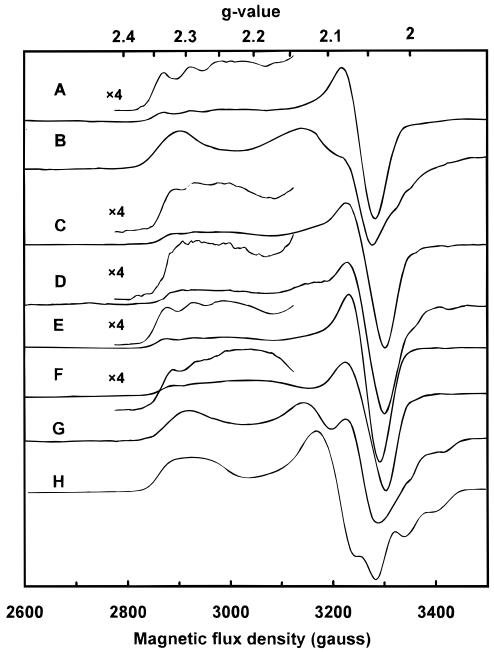

FIGURE 1: EPR spectra of WT azurin at pH 7 (A) and some of its Met121 mutants, Met121Gly at pH 7 (B), Met121Ala at pH 6 (C), Met121Val at pH 7 (D), Met121Asp at pH 4.5 (E), Met121Leu at pH 7 (F), and Met121Asp at pH 7 (G). The spectrum of stellacyanin at pH 7 (H) is included for a comparison.

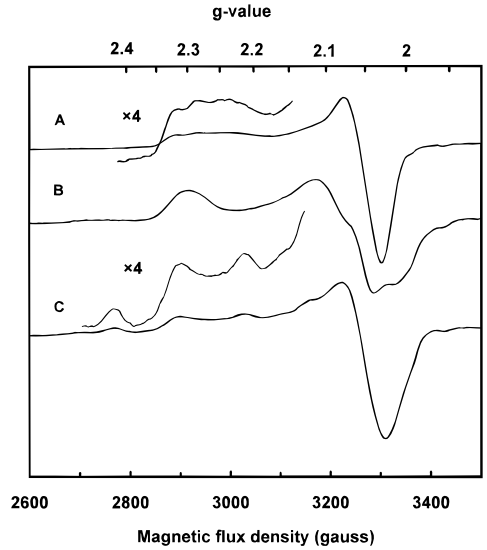

FIGURE 2: EPR spectra of the mutant Met121Ala at pH 6 after addition of oxygen-containing ligands: (A) no addition of ligand, (B) 100 mM CH_3OH, and (C) 100 mM CH_3COOH.

acids and small ligands providing a coordinating nitrogen. Experiments were also performed with CN^- (coordinating with its carbon, see below) and SCN^- (coordinating with S or N).

Ligands containing a carboxyl group (COOH or CH_3-COOH) induced a large change, cf. Figure 2C. From the A_z vs g_z plot (Figure 5), it appears that these derivatives are type 1.5 complexes. On the other hand, glycine, 100 mM, had no effect on the Met121Ala mutant.

Molecules coordinating to copper with a nitrogen, N_3^-, CH_3NH_2, and CH_3CN, also gave spectra of type 1.5 character, and Figure 3 shows some examples. Addition of imidazole induced two spectral forms; one form was similar to the one induced by the other nitrogen-containing ligands, whereas the other was of more pure type 2 character (Table 2).

Finally, interaction with SCN^- and CN^- gave spectra, also of type 1.5 character, but with significantly lower g_z values. The CN^- spectra were particularly well resolved (an example is shown in Figure 4A) and could be reproduced accurately in a simulation (Figure 4B). However, the Gly and Asp pH 7 mutants differ from the other mutants in that they gave complexes with higher g_z values, closer to those of regular type 1 proteins (Table 2 and Figure 5).

For the Met121Ala mutant, the effect of replacing $^{12}CN^-$ with $^{13}CN^-$ was studied. The interaction with the ^{13}C nucleus is clearly seen (Figure 4C, which is corrected for 13% remaining ^{12}C), and the corresponding hyperfine coupling is about 38 G in the g_z region. The hyperfine coupling is

[2] Although established, this nomenclature must not be understood literally as a type precisely in between type 1 and 2. A continuous range of intermediates between types 1 and 2 is likely to be found. Also, EPR and optical spectra may give a slightly different picture; see, for example, the SCN^- complex of Met121Gly which has an EPR spectrum close to those of type 1 proteins but which has an optical spectrum which is very shifted from type 1 spectra.

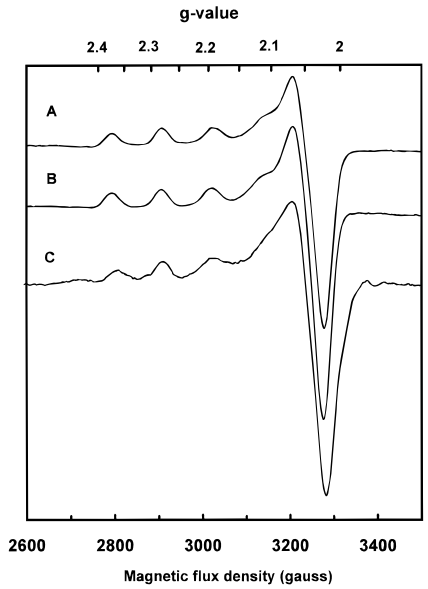

FIGURE 3: EPR spectra of mutants after addition of nitrogen-containing ligands: (A) Met121Ala plus 50 mM CH_3CN, (B) Met121Ala plus 10 mM N_3^-, and (C) Met121Val plus 90 mM N_3^-.

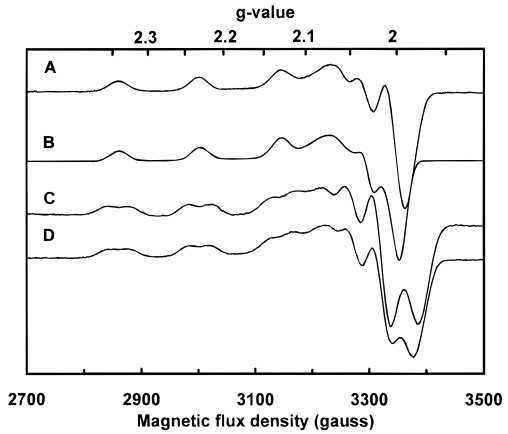

FIGURE 4: EPR spectra of the mutant Met121Ala after addition of CN^-. (A) 10 mM $^{12}CN^-$; (B) simulated spectrum using the parameters $g = 2.032$, 2.065, and 2.180, $A = 10$, 15, and 142 G, and peak-to-peak width = 20, 35, and 30 G (Gaussian shape); (C) 10 mM $^{13}CN^-$, with part of spectrum A subtracted to compensate for the presence of 13% ^{12}C in the sample; and (D) simulated ^{13}C spectrum obtained by shifting spectrum A 19 G to the left and right and summing the resulting spectra, thereby simulating an isotropic ^{13}C coupling of 38 G.

essentially isotropic, as shown in Figure 4D, which is obtained by addition of two ^{12}C spectra, displaced relative to each other by 38 G.

Optical Absorption of Complexes. Wavelengths and absorption coefficients at 20 °C of the two major maximums in the visible region are shown in Table 1, and some examples are given in Figure 6. Note that the alcohols and acids are missing in the table and figure; this is so because no effect was observed at room temperature upon addition of these reagents at concentrations up to 100 mM. The same was also true of CH_3CN.

The addition of the other reagents all resulted in rapid changes which were complete at the first observation time (30 s). Some spectra of derivatives of the Met121Ala mutant are presented in Figure 6. With CN^-, (Figure 6B), the 500−600 nm band is quite weak. The N_3^- and SCN^- complexes (Figure 6C,D) exhibit a somewhat stronger band in the 500−550 nm region (the band around 600 nm is due to uncomplexed protein still present at the ligand concentrations used). With CH_3NH_2 (Figure 6E), so much of the uncomplexed protein remains that the weaker band is obscured. To define its wavelength maximum (Table 1), an amount of the spectrum of the uncomplexed proteins was subtracted as calculated from the dissociation constants obtained from titrations (see below).

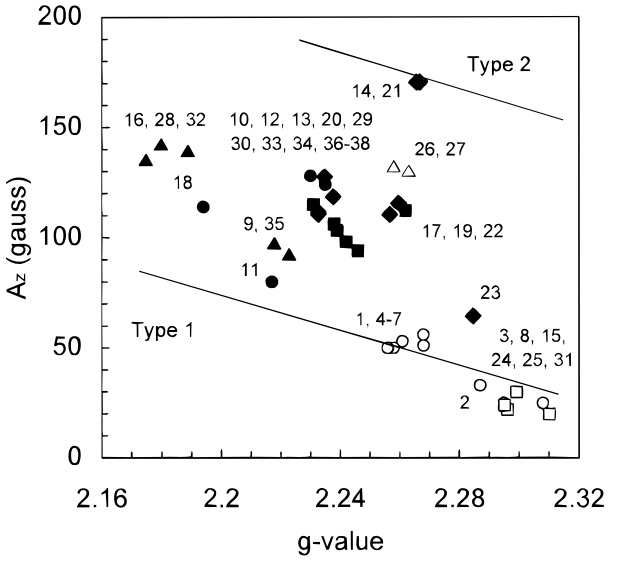

FIGURE 5: A_z against g_z for the investigated mutants and their derivatives: WT and mutants without exogenous ligands (○), complexes with alcohols (□), carboxylic acids (△), thiocyante (●), azide (■), remaining nitrogen-liganding molecules (◆), and cyanide (▲). The two lines indicate the parameter regions for the regular types 1 and 2, respectively (Vänngård, 1972). Numbering refers to Table 2.

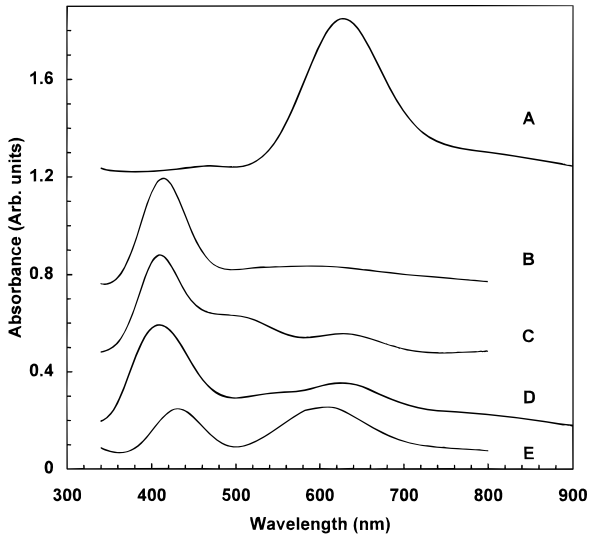

FIGURE 6: Optical spectra of the mutant Met121Ala with (A) no addition, (B) 15 mM CN^-, (C) 21 mM N_3^-, (D) 100 mM SCN^-, and (E) 175 mM CH_3NH_2.

Since the spectral properties of the mutant Met121Asp depend on pH, this mutant was studied at two pH values. As seen in Table 1, the addition of N_3^- converts the protein to a spectral form that is essentially identical at the two pH values. The Met121Glu mutant was also studied at pH 4.0 and 7.0. No binding was observed; the absorbance spectrum remained unchanged up to 30 mM CN^-.

The spectra had a common trend; as the ~600 nm band shifted to lower wavelengths, its intensity decreased, larger shifts accompanying lower intensity. Similarly, the 400−

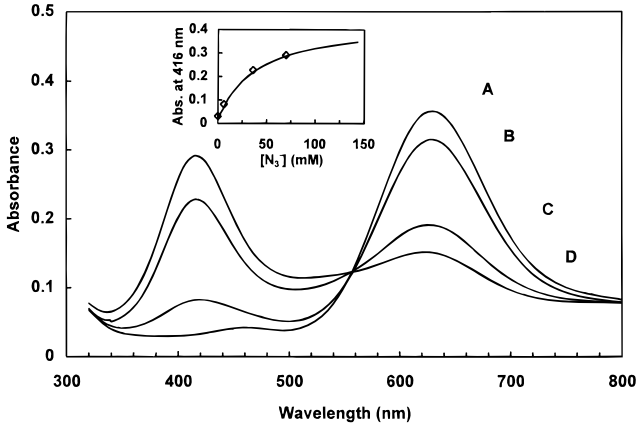

FIGURE 7: Optical titration of Met121Val with azide: (A) no addition and (B) 6 mM, (C) 36 mM, and (D) 70 mM N_3^-.

'500 nm was blue-shifted, and the larger the shift, the higher the intensity. The two bands were shifted by about the same amount of energy. This was clearly seen from a graph with the energies of the two transitions in Table 1 plotted against each other. The slope of a straight line fitted to the points was 0.96 with a correlation coefficient $r^2 = 0.95$.

For a number of ligands, dissociation constants were obtained from titrations. As an example, the titration of the Met121Val mutant with N_3^- is shown in Figure 7, and the resulting titration curve at 416 nm is seen in the inset in the same figure. The dissociation constants were determined by fitting the titrations curves at the 620 and 400−500 nm bands to calculated curves; the obtained values are given in Table 1.

Temperature Dependence of Spectra and Binding. As stated above, studies of the binding of alcohols were limited to temperatures below the freezing point of the samples. Complex formation was increased when the frozen samples were stored at temperatures not far from 0 °C. For example, whereas about 100 mM CH_3OH was needed to get the fully complexed Met121Ala mutant (Figure 2B) when the EPR sample was rapidly frozen by immersion into liquid nitrogen, incubation of the protein with 5 mM CH_3OH for 48 h at −20 °C resulted in approximately 50% formation of the complex.

The finding that temperature was an important factor in the determination of the binding strength of some ligands prompted a detailed study of the binding of azide. The dissociation constant for azide binding to the mutants Met121Ala and Met121Gly was measured at 5, 15, 35, and 50 °C and pH 7. The binding was much weaker at elevated temperatures; from 5 to 50 °C, the dissociation constant increased by about a factor of 20. From an Arrhenius plot, thermodynamic quantities were determined. For Met121Ala and Met121Gly, the binding free energy is $\Delta G° = -13.1$ and −7.4 kJ/mol at 25 °C, respectively. Both reactions had $\Delta H° = -50 \pm 5$ kJ/mol and $\Delta S° = -140 \pm 15$ J/mol K. The accuracy of the determinations was insufficient to tell if the difference in free energy changes of the two reactions arises from different enthalpy or entropy.

DISCUSSION

Axial Ligation in the Mutants in the Absence of an Exogenous Ligand. From the spectral data in Table 1 and the EPR spectra in Figure 1, it appears that the mutants Met121X (X = Ala, Val, or Leu) used in this study are very similar to the WT protein. Thus, it seems likely that they would have a small axial interaction like that in the WT protein. This has recently been confirmed in X-ray crystallography studies of the Met121Ala mutant, which show that the space left by the bulkier methionine residue appears empty (Tsai et al., 1995). Also, as predicted, there is no water near Cu in the Met121Leu mutant (L. Sjölin, V. Langer, L.-C. Tsai, and B. G. Karlsson, personal communication). The same is therefore very likely true for the Met121Val protein. Thus, we expect that there is a "cavity" available for ligands to copper, increasing in size in the order Leu, Val, Ala.

The Met121Gly mutant shows a distinctly different behavior. Its spectral properties are close to those of stellacyanin in which the axial ligand is Gln instead of Met. Most likely, a water molecule coordinates to the copper ion in Met121Gly, as is probably also the case (Murphy et al., 1993) in the mutant in which a stop codon has replaced the Met121 codon.

The spectral properties of the Met121Asp mutant vary with pH (Murphy et al., 1993). At low pH, the carboxylic group is protonated and the interaction with copper is weak. At neutral pH, the interaction is stronger, resembling more that in stellacyanin, possibly because a water molecule can enter between the side chain and Cu (Murphy et al., 1993). The Met121Glu mutants behave similarly, but with a stronger interaction in both the protonated and deprotonated forms, showing a rhombic type 1 and a type 1.5 EPR spectrum, respectively (Karlsson et al., 1991).

Correlations in Optical Spectra. It is generally agreed that the band at about 600 nm band is due to an S_{cys} $\pi \rightarrow$ Cu $d_{x^2-y^2}$ charge transfer transition [the term "charge transfer" might, however, be misleading, considering the strong delocalization (Larsson et al., 1995)]. Most workers also now assign the transition at a higher energy, around 450 nm, to another S-to-Cu charge transfer transition, namely S_{cys}-(pseudo) $\sigma \rightarrow$ Cu $d_{x^2-y^2}$ (Gewirth & Solomon, 1988; Han et al., 1993; Andrew et al., 1994).

Our results show that the energies of the two bands are shifted by the same amount upon complex formation. This would be the case if by increasing axial interaction the energy of the $d_{x^2-y^2}$ orbital increased, affecting the two transitions equally. Furthermore, as discussed for example also by den Blaauwen and Canters (1993), a stronger axial coordination, with displacement out of the plane formed by the Cys, His, and His residues, could lead to a lessened overlap between the S_{cys} π and the Cu $d_{x^2-y^2}$ orbitals accompanied by an increase of the overlap between S_{cys}-(pseudo) σ and the Cu $d_{x^2-y^2}$ orbitals. Thus, in line with our results, as the energy of the two transitions increases, the 600 nm absorption would decrease and the 400−500 nm absorption increase.

Recently, a different assignment for the 450 nm band has been presented (Larsson et al., 1995). Taking the *x*- and *z*-axes along Cu−Cys(S) and Cu−Met(S), respectively, these authors suggest the involvement of the Cu d_{yz} orbital. Also in this model, the 450 nm transition shifts in parallel with the 600 nm band upon increasing of the axial ligation. Thus, definite assignments have to await further work.

Correlations Related to EPR Spectra. For regular type 1 proteins, it has been suggested that strengthening the axial interaction results in an increased absorption at 400−500 nm. In addition, such changes have been related to an increase in the rhombicity of the EPR spectrum [see, for

example, Han et al. (1993) and Andrew et al. (1994)]. Often, with increasing rhombicity, the g_z value increases and A_z decreases, i.e. the corresponding point in a A_z vs g_z plot moves to the lower right. Such a correlation appears valid for the azurin mutants and derivatives that we have studied, but only for comparatively weak axial ligation, consistent with a true type 1 behavior, such as the carboxyl group of Met121Asp at high pH and the alcohol complexes reported in this work. It has been pointed out by one of the referees that such a correlation between axial interaction and EPR spectral parameters is not valid if in addition other proteins are considered. Pseudoazurin (Kakutani et al., 1981; Petratos et al., 1988), the cucumber basic protein (Colman et al., 1977; J. M. Guss, E. A. Merritt, R. P. Phizackerley, and H. C. Freeman, to be submitted) and the nitrate reductase type 1 copper site (Iwasaki et al., 1975; Gooden et al., 1991) have relatively small g_z values, although the bond distance to the axial ligand is comparatively short. In these proteins, the distance between the axial MetS^{δ} and Cu is 2.8, 2.6, and 2.5 Å, respectively.

With stronger axial interaction, when the derivatives studied here no longer can be classified as regular type 1 proteins, the optical high-energy absorption continues to increase at the expense of the 600 nm band. In EPR, the trend then is what is normally found for Cu complexes; the stronger the ligand, the smaller the g_z value. This is illustrated in Figure 5 by the changes observed going from oxygen to nitrogen ligands and finally to cyanide.

From the present data, it is clear that correlations between low-temperature EPR and room-temperature optical spectroscopic data may be weak. Equilibria may be shifted, not only because the affinity is temperature dependent (see Temperature Dependence of Spectra and Binding), but also because of altered (higher) effective concentrations of liganding molecules upon freezing (Peyratout et al., 1994). This is clearly illustrated by our finding that alcohols, carboxylic acids, and CH_3CN did not affect room-temperature optical spectra but changed the low-temperature EPR spectra. The binding of alcohols was even stronger if the sample was stored at about −20 °C, allowing the ligand to aggregate in the liquid-like layer surrounding the protein (Yang & Brill, 1991).

General Trend of the Binding Affinity. From Table 1, it appears that the binding of azide gets stronger as the pocket discussed above increases in size. For example, azide binds to the Met121Ala mutant about 100 times were more strongly than to Met121Leu. The Gly mutant, on the other hand, binds more weakly than both the Ala and the Val mutants. This is probably due to the competition with the water molecule. Binding to the Asp mutant is also relatively weak because of the limited space available and the competition with the carboxyl group and/or water. At high pH, the negative charge of the deprotonated group reduces the affinity further.

A similar trend is observed for thiocyanate. The binding to the Ala mutant is stronger than the binding to the Val mutant, which in turn is comparable to the binding to the Gly mutant. Cyanide behaves somewhat differently, probably because of its smaller size. With the Val side chain, the cavity is large enough to allow as strong a binding as with the Ala mutant. With the Leu mutant, the binding is less strong, indicating that this amino acid blocks the site to some extent.

Low-temperature EPR reveals also that methanol and ethanol can enter the metal site. Larger nitrogen-containing molecules, CH_3NH_2 and CH_3CN, are likewise capable of entering into the site in the Ala mutant. There is a limitation, however, on how bulky a molecule that can bind can be. For example, propanol does induce changes in the EPR, whereas the bulkier 2-propanol does not. Similarly, acetic acid but not glycine was found to bind.

Binding of Alcohols. Interaction between azurin and alcohols has been investigated earlier by Guzzi and Sportelli (1992). They saw only minor effects at high alcohol concentrations of the WT protein and concluded that there was no direct interaction with the metal site in contrast to our findings for the Met121Ala and Met121Val mutants. Binding of alcohols to copper is also known from several crystal structures. One example has been provided by Addison et al. (1983), with Cu tetragonally coordinated to one S, two N, and a water and with an axial methanol (d_{Cu-O} = 2.26 Å). The complex had an ordinary type 2 EPR spectrum. Methanol binding to protein-bound Cu is rare, however, and our findings illustrate that a protein, by forming a special environment around a metal ion, can induce rather unusual modes of binding. On the basis of EPR spectra, the interaction is not very strong; it is comparable to that in the Met121Asp mutant at high pH or Met121Glu at low pH (Andrew et al., 1994).

Binding of Carboxylic Acids. EPR spectra show that small carboxylic acids interact strongly with the metal ion, so that the copper acquires type 1.5 character (Table 2 and Figure 5). Since there is no restriction on how close to the metal the ligand can approach, the effect is about the same as in Met121Glu at pH 7 (Pascher et al., 1993) and stronger than in Met121Asp at the same pH.

Binding of Azide and Thiocyanate. The structure of the azide derivative of the Met121Ala mutant has recently been determined by X-ray crystallography (Tsai et al., 1995). The copper ion has moved about 0.3 Å out of the plane of the three strong protein ligands and is approaching a tetrahedral configuration. The Cu−azide distance is 2.01 Å [Cu−Met121S^{δ} in WT azurin is 3.1 Å (Nar et al., 1991)], and azide ion is stabilized through π-interaction with Phe15. However, the binding that is reported here is not exceptionally strong; however, the dissociation constants are on the same order as those of low-molecular weight complexes between Cu and azide. Presumably, thiocyanate binds in a similar manner. A direct interaction of azide and thiocyanide in Gly121 with the copper ion was demonstrated by Vidakovic and Germanas (1995), who found NN and CN Raman stretching modes at 2042 and 2072 cm^{-1}, respectively.

The binding of azide to Met121Ala and Met121Gly evidently is the result of competing effects between the fairly large enthalpy and entropy terms. Because other contributions are expected to be relatively small compared to the entropy (Murphy et al., 1994), it appears that there is a substantial decrease in configurational entropy upon binding. This is probably due to the fact that the ligands fill the empty space of the mutant, thereby reducing the degrees of freedom of the surrounding residues.

Spectroscopically, the azide and thiocyanate complexes fall well into the pattern formed by the other complexes (Table 1). The changes in optical spectra upon complex formation are somewhat larger than those reported by

Vidakovic and Germanas (1995) for reasons not understood. For the His117Gly mutant, azide and thiocyanate induced new transitions at 415−435 nm which were assigned to charge transfer from the liganding molecule to Cu(II) (den Blaauwen et al., 1993). Such transitions may in our case be hidden under the stronger S-to-Cu charge transfer transition. Also, in contrast to the His117Gly mutant, where azide and thiocyanate replace a protein histidine ligand and thereby retain the small A_z, the same molecules in the axial position induce a change to an altered form with a larger A_z (Figure 5).

The spectroscopic data indicate that azide binds in the same manner to the Met121Asp mutant irrespective of the pH (Tables 1 and 2). Thus, the ligand displaces the group provided by the protein (or the water molecule). Comparing thiocyanate binding to different mutants, there is a fairly large variation, particularly in the EPR parameters. Conceivably, this could be due to different modes of binding; SCN^- can coordinate to the metal with both S and N.

Binding of Acetonitrile, Methylamine, and Imidazole. Like the alcohols, acetonitrile does not bind at room temperature, but probably through the same mechanism, the observed interaction with the metal site in the frozen EPR samples is strong. Methylamine, which is about the same size as azide or thiocyanate, can enter the cavity, producing roughly the same changes in the spectroscopic properties (Figure 5). This indicates that the effect of azide and thiocyanate does not depend on the presence of a charge on these anions. The neutral methylamine is equally effective.

Imidazole is much more bulky. It does bind, however, but in one of the two forms created, a regular type 2 species is formed as judged from the EPR spectrum. The nature of this complex is not clear, but the copper site has been greatly modified, possibly to the extent that two imidazole molecules bind.

Binding of Cyanide. Cyanide binds most strongly of all ligands, and in line with this, the g_z value is the smallest observed in this work. From the EPR parameters, it appears that a somewhat different mode of binding is obtained in two cases where CN^- competes with other strong ligands, the carboxylic group (or water) in Met121Asp at pH 7 and in Met121Gly (H_2O). With Met121Ala, the observed ^{13}C splitting shows that the molecule binds in the normal way, with the carbon atom closest to the metal. The magnitude of the coupling constant, about 40 G, is somewhat less that what has been reported for other proteins capable of binding CN^-: fungal laccase type 2, 47 G (Malkin et al., 1968); and Cu(II) carbonic anhydrase, 50−56 G (Haffner & Coleman, 1975). In the last case, an equatorial binding was suggested. In the present case, the result indicates that there must be a substantial reorganization of the ligands, allowing strong mixing of the copper $d_{x^2-y^2}$ with the CN^- orbitals.

Vidakovic and Germanas (1995) reported a much stronger binding of cyanide than in our work. The reason for this is not clear.

Concluding Remarks. The function of the Met121 residue in azurin has attracted great interest. It has been proposed to regulate the reduction potential of the protein, and it has been shown that replacing it with other residues can change the potential considerably (Pascher et al., 1993). This work shows that it also serves as an effective barrier against unwanted reactions of the copper ion. When the residue Met121 is exchanged for an amino acid, hydrophobic or polar, which is not directly coordinated to the copper, the reactivity against exogenous ligands increases.

ACKNOWLEDGMENT

Useful discussions with Drs. Roland Aasa, David McMillin, and Lennart Sjölin are gratefully acknowledged. Dr. Örjan Hansson provided the computer program used for the simulations. Anita Samuelsson is acknowledged for purifying some of the azurin mutants.

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BI9522110