Creation of Type-1 and Type-2 Copper Sites by Addition of Exogenous Ligands to the *Pseudomonas aeruginosa* Azurin His117Gly Mutant

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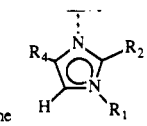
Abstract: The imidazole side chain of the copper-coordinating histidine117 of the type-1 copper protein *Pseudomonas aeruginosa* azurin protrudes through the surface of the protein where it has contact with the solvent. Replacement of this histidine by a glycine, using site-directed mutagenesis, makes the copper center amenable to direct manipulation through the addition of external ligands. Depending on the kind of the externally added ligand, different type-1 and type-2 copper centers are obtained with UV/vis and EPR spectroscopic characteristics that encompass the known range of proteins with one copper in their active sites. In a Vänngård-Peisach-Blumberg plot the $A_{\parallel}$ and $g_{\parallel}$ EPR parameters of the His117Gly type-1 sites appear to cluster in three distinct regions, possibly indicating a preference of the mutant protein for a few distinct configurations of the metal site. After addition of various substituted imidazoles, the His117Gly mutant obtains UV/vis and EPR spectroscopic characteristics that are virtually identical to those of wild-type (WT) azurin. This implies that the structure of the mutant protein and the geometry of the metal site are maintained despite the replacement of the histidine. The importance of His117 in WT azurin for electron transfer is illustrated by the observation that the reconstituted mutant is not functional in reversible electron transfer.

Introduction

The striking spectroscopic characteristics of mononuclear blue copper proteins (type-1 copper proteins) have made these proteins a favorite object of experimental and theoretical studies. Blue copper proteins can easily be discerned from nonblue copper proteins and low-molecular-weight copper complexes by their intense absorption (3-6 mM^{-1} cm^{-1}) at energies around 16 000 cm^{-1} (625 nm) and by their unusual small $A_{\parallel}$ value as measured from their EPR spectra ($\leq 90.10^{-4}$ cm^{-1}; see for a review ref 1). These copper sites are generally involved in mediating electron transfer and exhibit relatively high midpoint potentials[2] and rapid rates of electron transfer[3] as compared to the nonblue proteins. From the X-ray crystal structures of typical blue copper proteins like plastocyanin,[4] pseudoazurin,[5,6] and azurin[7-10] a prototypical copper site could be derived. The metal appears to be bound by a strongly binding pseudotrigonal N_2S donor set derived from the side chains of two histidines and a cysteine; in addition, there appear to be one or two weaker interacting axial groups.

The correct description of the Cu coordination in the azurins has been a matter of debate in the literature. After the 3-dimensional structures of the azurins from *Pseudomonas aeruginosa* and *Alcaligenese denitrificans* had been solved by X-ray diffraction techniques, the copper was seen to be coordinated in a trigonal bipyramidal fashion[6,10] by His117, His46, Cys112, and the axial ligands Met121 and the backbone carbonyl of Gly45.[7,10] Theoretical work by Solomon and co-workers[10a,b] has shown that it is probably preferable to use a C_{3v} description, according to which the Cu resides virtually in the plane of a strong N_2S donor set deriving from His46, His117, and Cys112, while there is an additional weak covalent axial interaction with the sulfur of Met121.

Chart I. Structural Formulae of the Imidazole Derivatives Used in This Study[a]



compound	R_n	group	name
1	-	-	imidazole
2	2	$-CH_3$	2-methyl imidazole
3	1	$-CH_3$	*N*-methyl imidazole
4	4	$-CH_3$	4-methyl imidazole
5	1	$-(CH_2)_3CH_3$	*N*-butyl imidazole
6	1	$-(CH_2)_4NH_2$	1-amino,4-[1-imidazolyl]bulane
7	1	$-(CH_2)_2Cl$	1-Cl,2-[1-imidazolyl]ethane
8	4	$-(CH_2)_2NHCOCH_3$	N_{ω}-acetylhistamine
9	4	$-(CH_2)_2NH_2$	histamine
10	4	$-(CH_2)CH(NH_2)(COOH)$	histidine
11	4	$-(CH_2)COOH$	4-imidazoleacetic acid
12	1	$-(CH_2\text{-}CH\text{-})_n$	*N*-polyvinyl imidazole

[a] Notice that the substituted 4- and 5-imidazoles are identical in the present case. [b] The n of R_n denotes the position of the side chain substituent. All R groups apart from R_n are hydrogen atoms.

The interaction of the Cu with carbonyl oxygen of Gly45 at the opposite axial position is almost exclusively Coulombic in nature. In the X-ray structures it can be seen that His117 protrudes through the surface in a shallow depression of the so-called hydrophobic patch of the protein and is directly accessible for the solvent.[9] This histidine is thought to mediate the electron transfer of azurin[11] in the electron self-exchange (ese) reaction as well as in the reactions with its physiological partners.[12] It has been shown that His117 can be replaced through site-directed mutagenesis by a glycine.[13,14] The absence of an amino acid side chain at position 117 creates an aperture in the surface of the protein which makes the copper center accessible to exogenous ligands. Here we report the binding of a variety of exogenous ligands to Cu in the metal site of His117Gly azurin. Several categories of exogenous ligands are able to reconstitute the type-1 spectroscopic (EPR and optical) features of wild-type (WT) azurin. One type of exogenous ligand induces a change in the geometry of the copper

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site which is reminiscent of type-2 copper proteins. The spectroscopic changes induced by the addition of the exogenous ligands are discussed.

Experimental Section

Materials and Methods. Imidazole (Im, **1**), thiazole (Thi), NaN_3, NaCl, NaBr, NaSCN, and KI were purchased from E. Merck AG, Darmstadt, Germany; *N*-methylimidazole (N-MeIm, **3**), 2-methylimidazole (2-MeIm, **2**), histidine (His, **10**), and 4(5)-imidazoleacetic acid (ImAc, **11**) were purchased from Sigma Chemical Co., St. Louis, MO, 4(5)-methylimidazole (4-MeIm, **4**) was purchased from BASF, Germany; histamine (Hista, **9**) was purchased from Janssen Chimica, Beerse Belgium; N^{α}-acetylhistamine (NacHista, **8**) was purchased from Aldrich (Milwaukee, WI); *N*-polyvinylimidazole (*N*-pVin, **12**) was a gift of Dr. G. Challa (Department of Chemistry, Groningen University, The Netherlands); and 1-amino-4-[1-imidazolyl]butane (**6**) and 1-chloro-2-[1-imidazolyl]ethane (**7**) were synthesized by I. M. M. Schellekens (Leiden University, The Netherlands). See Chart I for structural formulas (notice that the 4- and 5-isomers of the substituted imidazoles used in this study happen to be identical).

Cell Growth and Protein Isolation. The mutant His117Gly azurin was constructed as described.[13,14] The plasmid pTB46 containing the His117Gly *azu* gene was freshly transformed to *Escherichia coli* K12 JM101 cells grown overnight and diluted 1:100 in 30 L of LB medium[15] supplemented with 100 μM ampicillin (Ducheva, The Netherlands) and 30 μM isopropyl β-thiogalactopyranoside (Sigma) in a 40-L fermentor (MPP40, New Brunswick scientific, Edison, NJ). The cells were harvested immediately at the end of the exponential growth phase by filtration through a Pelicon cassette filter (Millipore Corporation, Bedford, MA), which resulted in 6 g of cell paste per liter of culture. The mutant was isolated as apo-His117Gly azurin, i.e., without addition of Cu^{2+} or potassium ferricyanide, but otherwise as described previously.[12] The yield was 8 mg of purified protein per liter of cell culture.

Preparation of His117Gly(ligand) Samples for UV/Vis and EPR Experiments. A stoichiometric amount of $Cu(NO_3)_2$ was added to a 0.1 mM apo-His117Gly solution in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (Sigma), pH = 6 (MES buffer). The absorption increase at 420 nm was monitored as a function of time, and when no further increase was observed a 10- or 30-fold (depending on the type of experiment) excess of ligand was added to the solution. When no further increase in the absorption was observed, a UV/vis spectrum was recorded at 293 K with a Varian Cary 219 spectrophotometer at a scan speed of 2 nm/s and a slit width of 1 nm. All spectra were recorded under identical conditions. Extinction coefficients of the visible absorption bands were calculated from peak heights by using the wild-type value of 9.8 mM^{-1} cm^{-1} for the extinction coefficient at 280 nm. NaCl (0.2 M) was added to the samples to remove adventitiously bound Cu, and the sample was concentrated in an ultrafiltration cell with PLGC 10000 NMWL Millipore membranes (Millipore). Subsequently, the NaCl was washed out by ultrafiltration with at least a 1000-fold excess of 10 mM ligand in MES buffer. Spectra in the near-infrared were measured in an Aminco–Chance DW-2 spectrophotometer.

Stopped-Flow Experiments. Stopped-flow kinetic experiments were performed at 293 K in 20 mM MES buffer, pH = 6, by using a Hi Tech Scientific stopped-flow apparatus (Hi Tech scientific PQ/SF-53, spectrophotometer unit SU-40, Photomultiplier PSU PPS-60) equipped with a 1-cm observation chamber (dead time 5 ms). Reactions of His117Gly(H_2O) with exogenous ligands were monitored at 420 and 630 nm for various methylimidazoles or at 400 and 629 nm for histidine. Absorbance changes were analyzed as a function of time by using a fitting procedure for first-order reactions (A. Braat, Technical University of Delft, The Netherlands).

NMR Spectroscopy. ^{1}H-NMR spectra were recorded at 298 K, pH = 6.4, and 3 mM protein concentration on a Bruker WM 300 spectrometer. The HDO resonance was suppressed by presaturation. About 400 free induction decays per spectrum were collected in 8 K memory, deconvoluted by Gaussian multiplication, and Fourier transformed. Chemical shifts are quoted in parts per million (ppm) from tetramethylsilane and calibrated with tetramethylammonium nitrate.

EPR Spectroscopy. EPR spectra were recorded at 77 K with a Jeol JES-RE2X spectrometer operating at X-band frequency and interfaced with an ES-Prit330 data manipulation system. Parameters for recording the EPR spectra were typically 12.5 mT/min sweep rate, 0.63 mT modulation amplitude, 8.950 GHz frequency, and 5 mW incident microwave power. The magnetic field was calibrated with α,α'-diphenyl-β-picrylhydrazyl (DPPH). S-Band spectra (4 GHz) were obtained at 15 K with a Bruker ER061SR microwave bridge plus a Bruker ER6102SR reentrant cavity in combination with a Varian E-line EPR spectrometer. A field modulation of 10 kHz was employed. Q-Band spectra (35 GHz) were recorded at 15 K on a Varian E-9 EPR spectrometer in combination with a Varian E-110 microwave bridge plus the E-266 cavity at a field modulation of 100 kHz.

Results

Characterization. For optimal purification the mutant was isolated as the apo-protein. On a 20% SDS-polyacrylamide gel the His117Gly azurin showed a molecular weight of 14 kDa, like the wild-type azurin (results not shown). The isoelectric points of His117Gly azurin as measured by IEF (Phastsystem, Pharmacia) are 5.0, 5.6, and 4.8 for apo-protein and denatured and Cu(II)-supplied His117Gly azurin, respectively (results not shown). For wild-type azurin these values are 6.2, 5.8, and 5.6, respectively.[12] The decrease in p*I* of about 0.6–0.8 for all three forms compared to the wild type could very well correspond with the loss of one histidine residue (apo-protein and denatured protein) or with the replacement of one histidine residue by a negatively charged ligand, like OH^- (Cu(II) form).

We have not been able to prepare a Cu(I) form of the His117Gly azurin. For instance, when His117Gly(4-MeIm) was reduced with a 23-fold excess of ascorbic acid at pH = 5.8 (20 mM MES buffer) or 8.5 (50 mM TrisHCl) and subsequently ultrafiltrated to remove excess reducing agent, the protein could neither be oxidized back nor be reconstituted. Apparently, the reduced copper is unstable at this protein site.

The ^{1}H NMR spectrum of apo-His117Gly azurin is almost identical to that of wild-type azurin at pH = 6.4 (Figure 1). The replacement of the His117 is clearly demonstrated by the absence of the C2H resonance of His117[12] (see His117 arrow in Figure 1). Another obvious difference is the shift of a number of methionine ϵ-CH_3 resonances (see Met arrows, Figure 1). When assuming that the assignment of the reduced holoazurin applies also to the apoazurin, the shifted resonances derive from Met44 and Met13.[12,16] Both methionines are situated next to His117 in the WT protein.[9] The ^{1}H NMR spectrum of oxidized Cu^{2+} His117Gly azurin with imidazole as exogenous ligand is again very similar to that of oxidized wild-type azurin (Figure 2). The resonances assigned to His83 apparently have broadened beyond detection in the His117Gly(Im)^{ox} spectrum. His83 is located at 15 Å from the copper center. It is conceivable that the His83 has bound a copper ion in combination with free imidazole and possibly water, and this might account for part of the adventitiously bound copper that is usually found in the EPR spectrum of the reconstituted protein before the NaCl treatment (see Experimental Section). The His117Gly amino acid replacement has apparently little effect on the structure of the protein, which is perhaps not too surprising in view of the fact that the protein mainly consists of tightly packed β-strands and His117 is located in a loop connecting two of these strands.[7,9] Besides, the backbone of the His117 amino acid residue is fixed by H-bonds to amino acid residues 120, 121, and 114, whereas the histidine side chain only entertains a bonding interaction with the copper.[9]

Optical Spectroscopy. His117Gly azurin was incubated with copper as described in the Experimental Section. The copper-containing mutant will further be referred to as His117Gly(H_2O) because no specific exogenous ligand was added and water is the most abundant ligand present in the solvent and therefore likely to coordinate the Cu at the vacant position of His117. The absorption spectrum of His117Gly(H_2O) shows two absorption maxima at pH = 6, at 420 and 628 nm (Figure 3, Table II). The intensities of the absorption bands suggest that they correspond to transitions with a substantial amount of ligand-to-metal charge-transfer (LMCT) character (vide infra).[16a]

Imidazole Derivatives. After addition of a 10-fold excess of an imidazole derivative (compounds **1–8** and **12**) to a His117Gly-(H_2O) solution the absorption at 420 nm decreased with a con-

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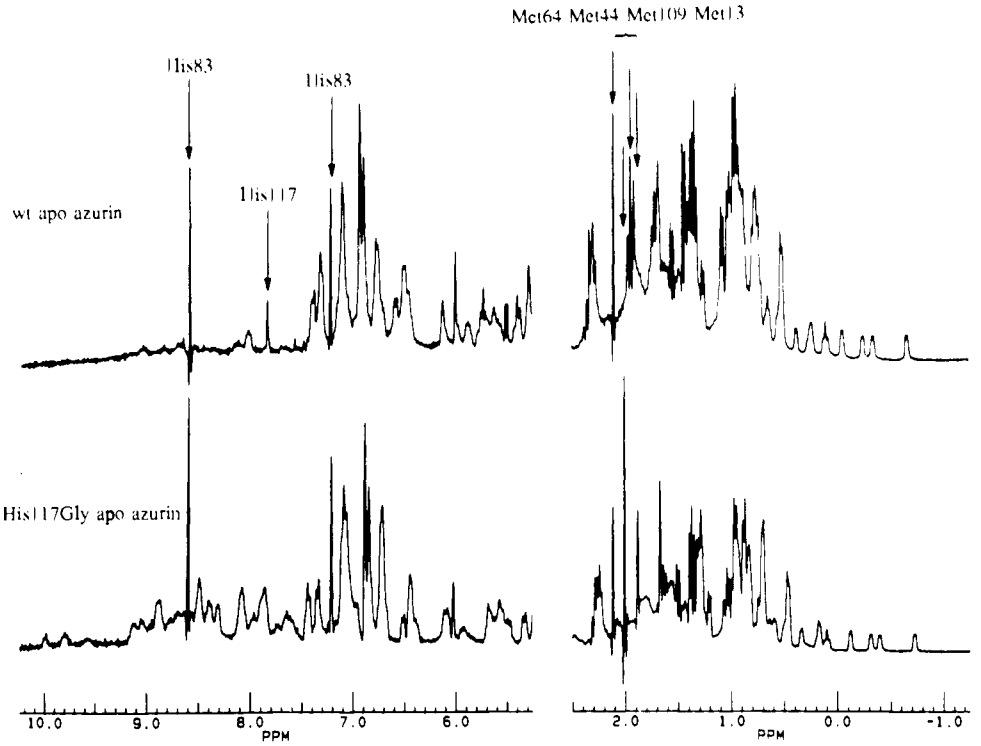

Figure 1. ^{1}H-NMR spectrum of a 4 mM solution of wild-type apoazurin and a 3 mM solution of His117Gly apoazurin, both in 10 mM phosphate buffer at pH* = 6.4, T = 298 K in D_2O (the asterisk indicates that the pH meter reading was not corrected for the deuterium isotope effect). Only the aromatic and aliphatic parts of the spectra are shown. The arrows indicate the position of relevant resonances of several amino acid residues (see text). The absence of several NH resonances in the 7.55–10 ppm region of the WT azurin spectrum is caused by amide exchange due to a longer incubation in D_2O compared to His117Gly.

Table I. EPR and UV/vis Parameters of Type-1 His117Gly with Exogenous Ligands

	ligand	$g_\perp$[a]	$g_\parallel$[a]	$A_\parallel$[a] (10^{-4} cm^{-1})	λ_{max} (nm)	ϵ ($\geq$ mM^{-1} cm^{-1})[b]
	His117 (WT)	2.058	2.260	58	628	5.7
A	imidazole	2.052	2.221	85	626	4.5
	thiazole	2.052	2.222	85	632	3.8
B	4(5)-MeIm	2.055	2.275	58	630	4.9
	2-MeIm	2.055	2.267	58	630	3.7
	N-MeIm	2.055	2.266	56	630	4.4
	N-BuIm	2.056	2.272	57	632	5.0
	N-pVin	2.055	2.263	59	628	4.2
	1-Cl,[1-Im]ethane	2.056	2.273	57	628	nd[d]
	1-NH_2,[1-Im]butane	2.056	2.269	57	632	5.0
	N^ω-Ac-Hista	2.057	2.270	57	637	4.4
	Br^-	2.061	2.275	52	683	5.5
C	N_3^-	2.041	2.317	21[c]	670	4.7
	SCN^-	2.042	2.340	17[c]	660	4.7
	Cl^-	2.042	2.340	17[c]	648	4.9

[a] $g_\perp$ ±0.001, $g_\parallel$ ±0.002, $A_\parallel$ ±0.002 cm^{-1}. [b] Lower limit for the extinction coefficient, calculated from the absorbance at the indicated wavelength by using the wild-type value of 9.8 mM^{-1} cm^{-1} for the extinction coefficient at 280 nm. [c] Values derived from spectra simulations. [d] Not determined.

Table II. EPR and UV/vis Parameters of Type-2 His117Gly with Exogenous Ligands

	ligand	$g_\perp$	$g_\parallel$	$A_\parallel$ (10^{-4} cm^{-1})	λ_{max} (nm)	A_{280},[a] %
a	H_2O	2.061	2.283	139	420	19
					628	14
b	histamine	2.056	2.220	164	402	40
					634	10
c	histidine	2.058	2.234	156	400	21
					634	4
d	4-ImAc	2.056	2.284	145	412	3
					628	4

[a] Since it could not be excluded that more than one absorbing species was present in solution, the absorption intensities are expressed as percentage of the 280-nm absorption.

Table III. First-Order Rate Constant of the Slow Phase of the Absorption Increase at 630 nm of His117Gly(H_2O) in the Presence of an Excess of Exogenous Ligand

imidazole[a]	k_{obs} (10^{-3} s^{-1})	4(5)-MeIm[a]	k_{obs} (10^{-3} s^{-1})
83	6.3	110	1.7
53	6.9	55	1.9
39	6.7	28	1.9
26	4.0	14	1.9
14	4.4		
5.3	4.2		

[a] Ratio of ligand-to-protein concentrations. The His117Gly(Im) concentration was 0.03 mM, the His117Gly(4(5)-MeIm) concentration was 0.09 mM. Experiments were performed at 302 ± 1 K.

comitant increase of the absorbance near 630 nm. The increase appeared to be biphasic as measured by stopped-flow absorption spectroscopy. The first phase comprised 80% of the total absorption increase and was completed in 0.2 s, whereas the second phase was much slower with a half-life of about 200 s. This was studied further by monitoring the absorption increase at 630 nm as a function of the concentration of added imidazole or of 4-MeIm. The slow phase of the absorption increase became ex-

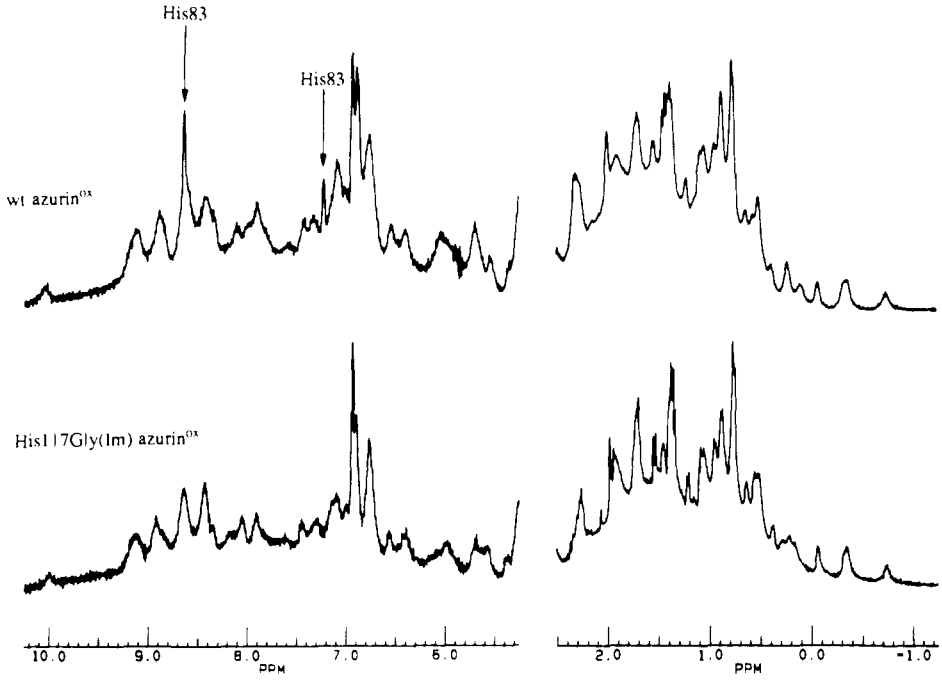

Figure 2. ^{1}H-NMR spectrum of a 4 mM solution of wild-type azurin^{ox} and a 3 mM His117Gly azurin^{ox}, both in 10 mM phosphate buffer, pH* = 6.2, at 298 K in D_2O. Only the aromatic and aliphatic parts of the spectra are shown. The arrows indicate the position of the His83 resonances.

ponential in time, and the rate became independent of ligand concentration when the latter exceeded the protein concentration by a factor of 5 or more (Table III). At lower concentrations the fast phase was slowed down sufficiently to become rate limiting. The fast phase probably reflects the binding of the ligand, whereas the slow phase may reflect a subsequent protein or active site conformational change. The imidazole derivatives had absorption spectra all very similar to that of the WT (see for an example 4MeIm in Figure 3). The λ_{max} and ϵ for the studied compounds are listed in Table I.

Anionic Ligands. After addition of a 30-fold excess of the anionic ligands SCN^- or N_3^- to His117Gly(H_2O) azurin, the solution turned again immediately blue with absorption maxima of 660 and 670 nm, respectively. The rate of the absorption increase of the slower phase was 10 times higher than in the case of the imidazole derivatives. After about 15 min the absorbance started to diminish slowly, possibly due to extraction of the copper by the excess ligand in solution. The anions Cl^- and Br^- coordinate the copper in the protein fully when present in a 1000- or 300-fold excess, respectively. His117Gly(Cl^-) has a normal type-1 copper center absorption spectrum with λ_{max} shifted 20 nm to lower energy compared to the wild-type (Table I, Figure 3). The visible spectrum of His117Gly(Br^-) shows an even larger shift of 55 nm to lower energy. No change in the absorption spectrum of His117Gly(H_2O) was observed after addition of up to a 1000-fold excess of F; an excess as low as 30-fold I^- immediately discolored the protein. The appearance of an absorption band at 353 nm in the latter case indicated that I^- had reduced the Cu(II) and that CuI was formed.[17,18]

Histidine-like Ligands. After addition of a 30-fold excess of histidine to His117Gly(H_2O) azurin (pH = 6), the solution turned blue within 1 s. The blue color was displaced by a greenish color within the next 5 s due to an increase of the absorbance at 400 nm and the change of the 629-nm absorption wavelength to 634 nm (Table II, Figure 3). Similar results were found for His117Gly(H_2O) after reaction with histamine (Table II). The initial absorption increase at 629 nm and decrease at 420 nm after addition of histidine to His117Gly(H_2O) and the subsequent replacements of these bands by absorptions at 634 and 400 nm (Figure 3) would suggest that the nitrogen of the imidazole binds first to the copper in a geometry characteristic of a type-1 site (absorption maximum at 629 nm) and that subsequently a second donor atom (possibly from the histidine peptide moiety) binds to the copper, forcing the copper to change its geometry into a more type-2-like coordination. Other exogenous ligands with bulky side chains such as 1-chloro-2-[1-imidazolyl]ethane (**7**), *N*-butylimidazole (**6**), and N^{ω}-acetylhistamine (**8**) have wild-type UV/vis and EPR features (Table I, see Chart I for structural formulas).

ImAc (**11**, see Chart I for structural formula) is potentially able to coordinate with its carboxy-terminal oxygen and with the imidazole nitrogen. It was added to His117Gly(H_2O) to allow us to discriminate between the possible binding of the carboxyl O and the amine N of the histidine. The optical spectrum of ImAc was clearly different from His117Gly(His), with absorption maxima at 412 and 628 nm and with absorption intensities more comparable with type-2 "nonblue" copper proteins (Table II). We therefore tentatively conclude that if the His coordinates with its peptide moiety, it will be with the amine group.

EPR Spectroscopy. Imidazole Derivatives. The EPR spectra recorded at 77 K of all imidazole derivatives (apart from histidine derivatives, vide infra) are very similar to that of the wild-type (Table I, and, e.g., 4-MeIm in Figure 4). Exceptions were imidazole and thiazole, which both had a relatively large hyperfine splitting in the parallel region and even some splitting in the perpendicular region with an $A_{\parallel}$ of 85.10^{-4} cm^{-1} and a splitting in the $g_{\perp}$ region of about 7.10^{-4} cm^{-1}, respectively (e.g., thiazole in Figures 4 and 5).

Anionic Ligands. Since His117Gly(X^-) azurin seemed to be unstable in the course of minutes to days depending on the used anion, all EPR spectra were recorded within 10 min after addition of the ligand to the protein to be sure that the major species in the EPR would correspond to that of the optical spectrum.

The EPR spectra of His117Gly(Cl^-), -(SCN^-), and -(N_3^-) all exhibited an unresolved, very small $A_{\parallel}$ (Table I, Figure 4 NaCl). The hyperfine splitting in the parallel region could also not be resolved by S-band EPR spectroscopy (data not shown) due to *g*-strain effects.[19] The width of the $g_{\parallel}$ region of the His117Gly(Cl^-) S-band EPR spectrum was used to obtain an

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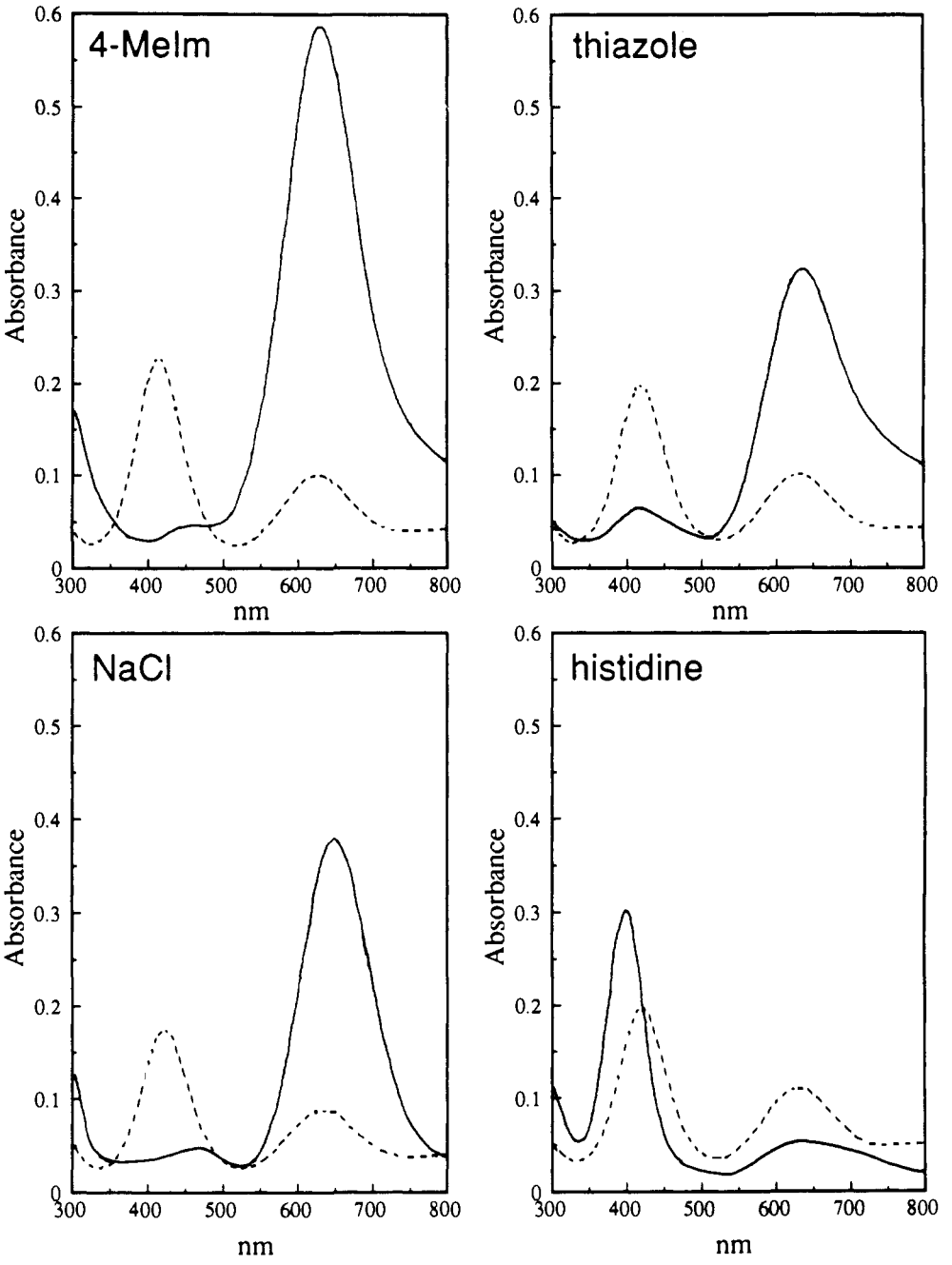

Figure 3. Room temperature UV/vis spectra (absorbance versus wavelength in nanometers) of a His117Gly azurin sample (approximately 0.1 mM) with a stoichiometric amount of $Cu(NO_3)_2$ added to it in 20 mM MES buffer, pH = 6 (dashed line), and the spectrum of the same sample after addition of 4 MeIm, thiazole, NaCl, or histidine, respectively (solid line). All ligands were added in a 30-fold excess apart from NaCl, which was added to an end concentration of 0.2 M.

upper limit for the magnitude of $A_\parallel$. A more precise estimate was obtained from simulations of the X-band spectra (results not shown). The Q-band spectrum of His117Gly(Cl^-) exhibits rombicity (g_x = 2.014, g_y = 2.044, and g_z = 2.340). No Q-band spectra were recorded of N_3^- and SCN^-. The unusually small hyperfine splitting in the parallel region has also been observed in CuN_4X compounds in which X is a halogen.[18,20,21]

His117Gly(H_2O), -(ImAc), -(His), and -(Hista) EPR Spectra. The X-band EPR spectra of His117Gly with either of these exogenous ligands showed $A_\parallel$ values in the range of (139–164) × 10^{-4} cm^{-1} which are typical for type-2 copper centers (Table II, e.g., Figure 4 His).

Discussion

Considerable efforts have been devoted over the past years to elucidating the composition and the geometry of the copper binding sites in metalloproteins. Especially the type-1 copper sites have preoccupied bioinorganic and coordination chemists because of their unusual spectroscopic characteristics, which until recently[22-24] were unknown for low-molecular-weight copper complexes. Resolution of the X-ray structure of several of these proteins[4,7,9,10] revealed a number of interesting and unique structural features of these sites. In the present study, site-directed mutagenesis of one of the copper ligands of azurin (His117) was used to look

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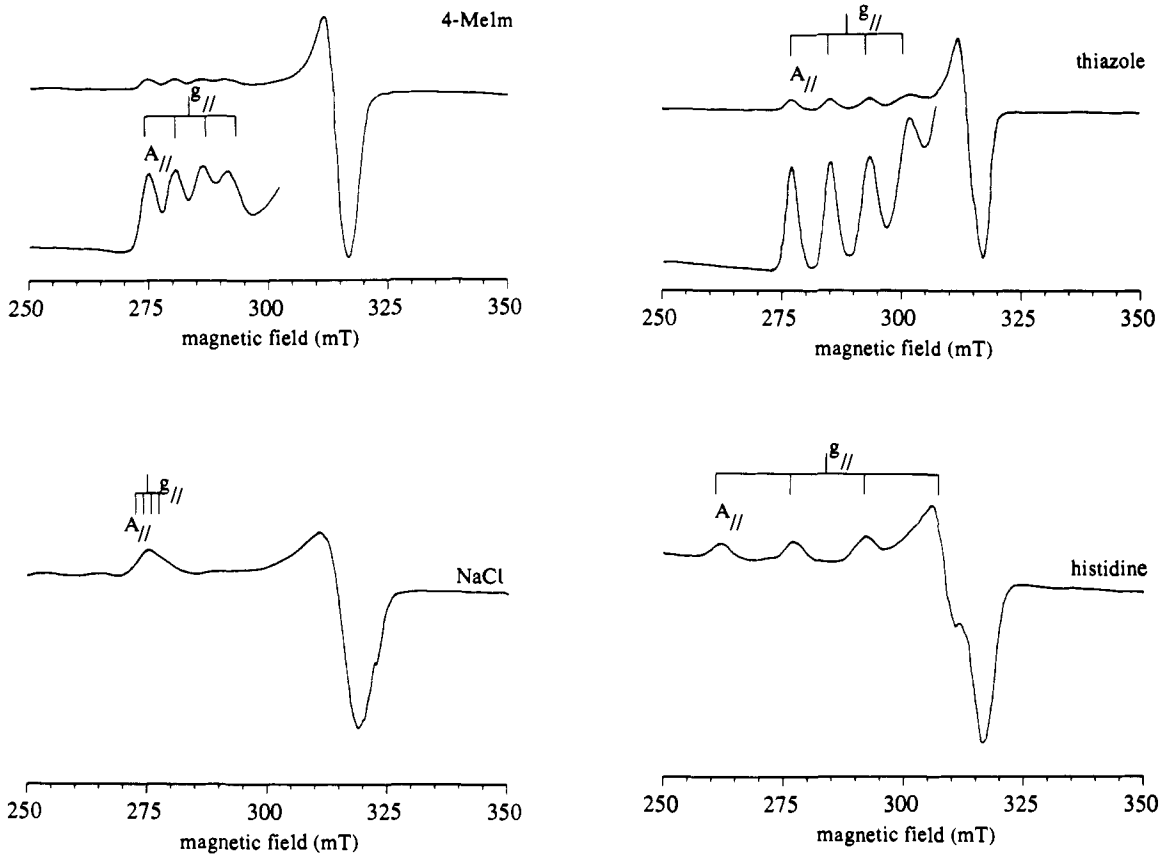

Figure 4. EPR spectra of a 2 mM His117Gly azurin sample at 77 K in 10 mM MES buffer, pH = 6.2, containing 40% glycerol and 1 mM 4-MeIm, 1 mM thiazole, 200 mM NaCl, or 1 mM histidine, respectively.

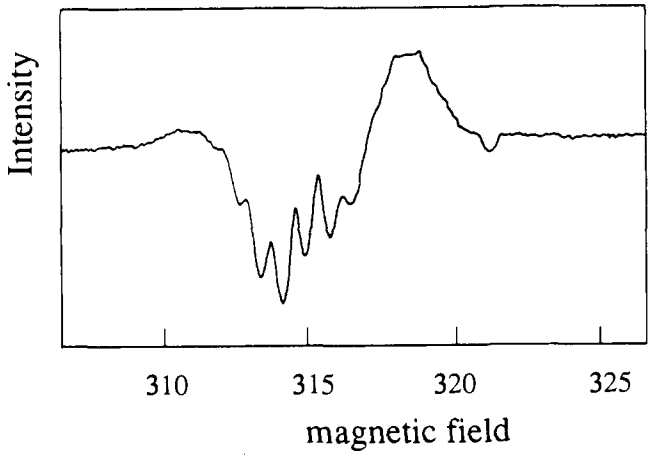

Figure 5. Second derivative of the perpendicular region of the thiazole EPR spectrum of Figure 4.

further into the details of the spectroscopy of these Cu sites.

Reduction of His117Gly Azurin. The His117Gly azurin mutant could be reduced by dithionite or ascorbic acid irrespective of the exogenous ligand but never oxidized back or reconstituted. The difference in p*I* between the oxidized and the reduced state of the wild type is 5.6(ox) − 4.7(red) = 0.9, whereas the difference for oxidized and reduced His117Gly azurin is only 0.1. It might, therefore, be that in the reduced His117Gly the copper center undergoes a modification. It has been stated[25] that the type-1 Cu centers have been "designed" such that their coordination is a compromise between the coordination geometries of the Cu(I) and the Cu(II) state. The lack of a covalent bond between the exogenous ligand and the protein in the His117Gly mutant may have disturbed this delicate balance in favor of the oxidized state, thereby causing the copper center to become unstable in the reduced state. It is conceivable, for instance, that after Cu reduction the external ligand is lost, converting the type-1 into what is essentially a reduced type-2 center like in the His117Gly(H_2O) example. The activity of naturally occurring type-2 sites in catalyzing radical reactions is well known. By analogy, in the present case the reduced type-2-like Cu center may assist in some chemical modification of the protein in the region around the Cu, thereby irreversibly altering the properties of the metal site. This is the subject of further study.

Optical Spectroscopy. His117Gly Type-1 Proteins. The small differences in wavelength of the absorption maxima of the imidazole derivatives and the larger differences observed in the case of the anionic ligands can be related to differences in ligand field strength. In the present series of experiments the Cu^{2+} ion is in a ligand environment with a varying external ligand and a permanent Cys–S ligand, the latter being responsible for the strong LMCT band in the optical spectrum. It is commonly accepted that the spacing of the d–d transitions correlates with the strength of the ligand field of the external ligand, which can be deduced from the spectrochemical series.[26] Assuming the energy of the Cys–S orbital remains approximately constant, the energy of the LMCT bands should increase as the ligand field increases.[26] Indeed, the increase in energy of the CT transitions observed thus far (683, 670, 649, and 625 nm, Table I) corresponds with an increase of the ligand field strength of the ligands used (Br^- < N_3^- < Cl^- < Im, respectively). The ligand field strength of the thiocyanate ion varies depending on whether SCN^- coordinates with its nitrogen or with its sulfur moiety. In the former case the ligand field strength is comparable to that of imidazole, and in the latter case the ligand field strength is lower.[27] Judged by

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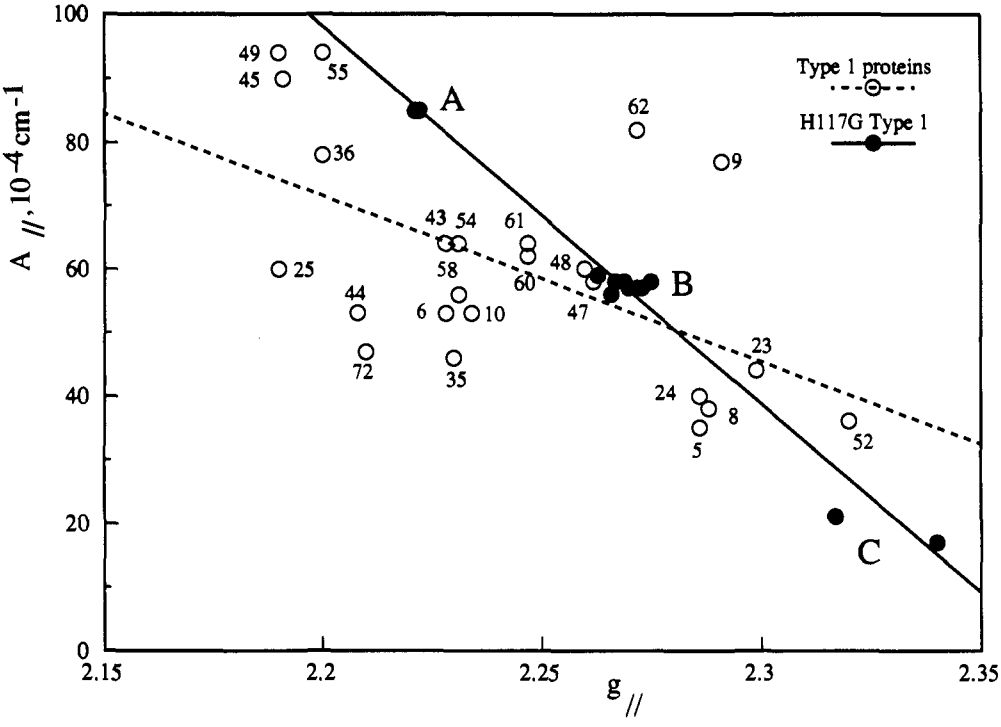

Figure 6. Map of $A_{\parallel}$ vs $g_{\parallel}$ for type-1 proteins (circles, dashed line) and His117Gly type-1 proteins (closed circles, solid line). The numbering corresponds with the numbering of A. W. Addison,[35] and the capital letters correspond to the following clusters of exogenous ligands: A, imidazole and thiazole; B, the *x*-methyl imidazoles **1**, **2**, and **4**, 1-amino-4-[1-imidazolyl]butane (**6**), 1-chloro-2-[1-imidazolyl]ethane (**7**), N^{ω}-acetylhistamine (**8**), *N*-*p*-vinylIm (**12**), and Br^-; and C: N_3^-, SCN^-, and Cl^-.

the 35-nm-longer wavelength of the CT transition (λ_{max} is 660 nm for SCN^- and 625 nm for imidazole coordination), SCN^- is expected to bind with its sulfur to the Cu in the His117Gly mutant. It is interesting to note that a λ_{max} closest to that of the wild-type azurin was not obtained by the histidine side-chain analogue 4(5)-methylimidazole (λ_{max} = 630 nm, Table I) but by *N*-polyvinylimidazole (*N*-pVin), which had a λ_{max} of 628 nm. Also in the EPR the similarity was greatest for *N*-pVin.

His117Gly Type-2 Proteins. The blue shift of the major absorption band in the optical spectra of the His117Gly type-2 proteins (Table II) of about 200 nm is typical for a change in geometry of copper complexes from tetrahedral to square planar or tetragonal[28-30] and a thiolate (S)–Cu bond length of 2.4 Å.[29,30] A lengthening of the Cu–(Cys)S bond of about 0.25 Å would require a movement of the copper in His117Gly out of the plane of the trigonal bipyramid to maintain a normal bond length to the N of His46, at least when assuming no change in the position of the Cys112. The binding of the majority of the imidazole derivatives used in this study to His117Gly reconstitutes the WT azurin spectroscopic characteristics, even in the case of N^{ω}-acHista (**8**) or 1-amino-4-[1-imidazolyl]butane (**6**), which have bulky side chains at the positions 1 and 4(5), respectively. Apparently, another type of ligand is needed to change the geometry of the copper center from type-1 to type-2. The exogenous ligands which cause the type-2 features all have the possibility to coordinate with their imidazole moiety and at the same time with their side-chain moiety,[31] i.e., to chelate in a bidentate fashion. One possibility is that the amino moiety of histidine (or histamine) has replaced the Met121 S, adding a fourth strong ligand and inducing a geometry change to square planar with the Cys(S) ligand as the axial ligand. In the case of His117Gly(H_2O) a second water molecule could replace the methionine ligand. A similar blue shift has been observed in the optical spectrum of an azurin mutant in which Met121 is replaced by a Glu, in which case the carboxyl group could supply the fourth strong ligand,[32] and in the optical spectrum of an insulin-stabilized Cu(II) complex after incorporation of an N,S bidentate chelator instead of a monodentate thiolate.[24]

The optical absorption spectrum of His117Gly(H_2O) exhibits strong bands at 420 and 628 nm. The two bands could be assigned on the basis of resonance Raman spectroscopy experiments to cysteine(S) to Cu CT transitions (den Blaauwen, T.; Canters, G. W.; Han, J.; Loehr, T. M.; Sanders-Loehr, J., manuscript in preparation). Their most likely assignment[10a,b] corresponds to the Cys(π) → d($x^2 - y^2$) (628 nm) and the Cys(pseudo-σ) → d($x^2 - y^2$) (420 nm) transitions. The variation in relative intensities of the two bands considered here as a function of the added ligand is remarkable.[33-35] According to the assignment suggested above, the bands carry a substantial LMCT character, and their intensity, thus, is sensitive to the overlap of the d($x^2 - y^2$) orbital with the Cys sulfur orbitals. As Solomon[10a,b] has pointed out, the orientation of the d($x^2 - y^2$) orbital is sensitively dependent on the overlap with the Cys S pπ orbital, which in turn depends on the length of the Cu–S(Cys) bond. In plastocyanin this bond is so short that the strong π-overlap orients the d($x^2 - y^2$) such that its lobes are bisected by the Cu–S bond axis. If the bond length increases sufficiently, however, the d($x^2 - y^2$) orbital may rotate by 45° so as to maximize its σ-interaction with the Cys pseudo-σ orbital. This will also have an effect on the amount of d(z^2) admixture into the half-occupied orbital. The shape and orientation of the d($x^2 - y^2$) orbital and thus the overlap with orbitals from which optical transitions are observed therefore depend sensitively on the Cu–S(Cys) bond length. Moreover, as the

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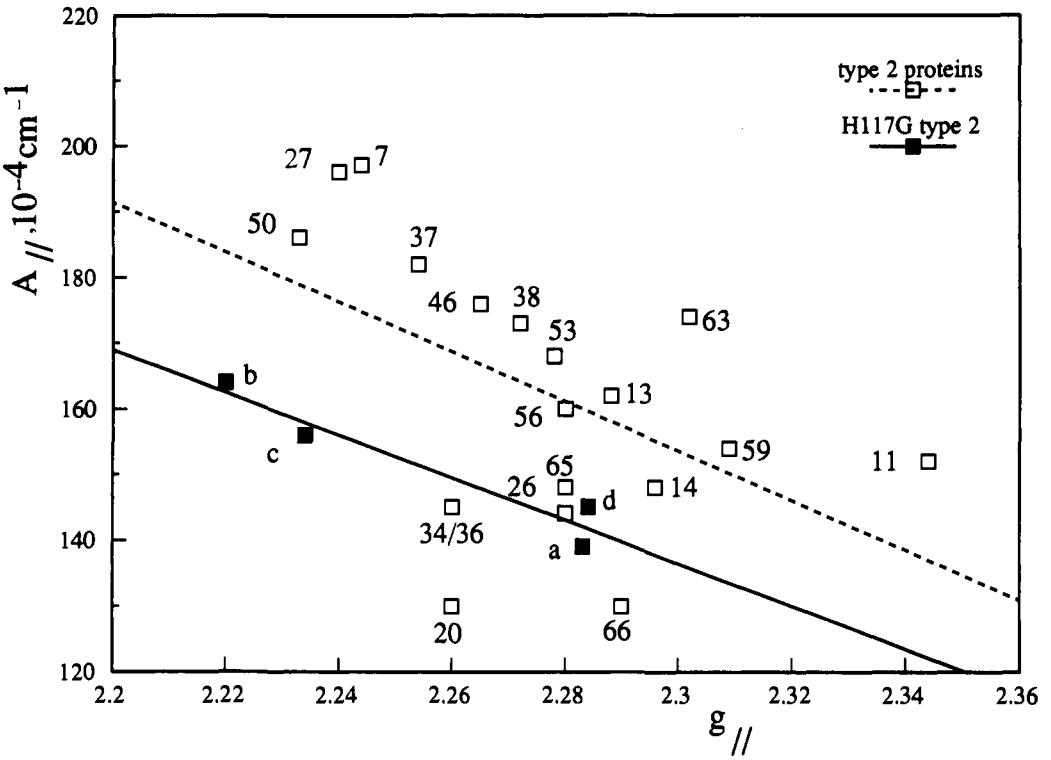

Figure 7. Map of $A_{\parallel}$ vs $g_{\parallel}$ for type-2 proteins (squares, dashed line) and His117Gly type-2 sites (closed squares, solid line). The numbering corresponds with the numbering of A. W. Addison;[35] a corresponds to His117Gly(H_2O); b corresponds to His117Gly(Hista); c corresponds to His117Gly(His); and d corresponds to His117Gly(ImAc).

Cu–S(Cys)–C_β(Cys) angle varies, the overlap between the d($x^2 - y^2$) orbital and the lower orbitals will vary. It is clear, therefore, that the intensities of the two bands discussed above must depend in a sensitive manner on the detailed structure of the Cu site and in particular on the details of the Cu–S(Cys) bond. Further speculation must await the elucidation of the structure of the Cu site in the H117G mutants.

EPR Spectroscopy. His117Gly Type-1 Proteins. When the values of the $A_{\parallel}$ and $g_{\parallel}$ parameters of the His117Gly azurin type-1 copper centers are plotted in a $g_{\parallel}$ versus $A_{\parallel}$ graph[36-38] together with literature data of known type-1 copper sites (Figure 6), a linear relationship is observed between $A_{\parallel}$ and $g_{\parallel}$ for the His117Gly data, similar to the other type-1 copper proteins. The His117Gly derivatives encompass the whole range of the known type-1 sites. Three clusters can be discerned; they are indicated by capital letters in Figure 6 and Table I. A includes imidazole and thiazole, B includes the majority of imidazole derivatives and Br^-, and C includes the other anionic ligands (Table I). The tendency for $g_{\parallel}$ to increase and for $A_{\parallel}$ to decrease with an increase in the degree of tetrahedral distortion has been reported previously for structurally well-characterized complexes of Cu(II).[39] When the general model of Brill,[40] which uses an (idealized) D_{2d} symmetry for the description of the copper site, is applied, the variation in $g_{\parallel}$ and $A_{\parallel}$ parameter values of the His117Gly type-1 copper centers can be explained in terms of small ligand displacements, as characterized by the tetrahedral angle β.[36,38,40,41] Values for β of 67°, 61°, and 56° can then be calculated for clusters A, B, and C, respectively, when using azurin as a calibration point with β = 60°. This would correspond to ligand displacements of 0.25, 0.035, and 0.14 Å, respectively. It illustrates that although the changes in the EPR parameters are considerable, the geometrical changes induced by the exogenous ligands are probably relatively small.

The increase of β in cluster A can perhaps be explained by diminished steric strain as a result of the coordination of a non-substituted imidazole to the copper. The fact that bromide falls close to the point of wild-type azurin (cluster B) shows that the small $A_{\parallel}$ value of the anionic ligands cannot be exclusively attributed to a charge effect. Part of the difference between Br^- and Cl^- can be explained by the fact that Br^- is more electron-rich than Cl^-, a property which often is reflected in smaller values of $g_{\parallel}$ and larger values of $A_{\parallel}$.[18,37] It has been suggested[21] that the magnitude of $A_{\parallel}$ is reduced when a ligand with a large spin–orbit coupling constant is present and when the bond between such a ligand and Cu(II) has a substantial covalent character. The spin–orbit coupling constant values of N and C are much smaller than those of Cl and S.[42] The position of the His117Gly(Cl^-) and -(SCN^-) EPR values in Figure 6 may, therefore, point to a substantial covalent character in the copper chloride and thiocyanate interaction. Although at present we do not have a plausible explanation for the small $A_{\parallel}$ value of His117Gly(N_3^-), it has been observed that replacement of the exogenous imidazole in Cu horse liver alcohol dehydrogenase by azide results in a similar increase in $g_{\parallel}$ and decrease in $A_{\parallel}$ as for His117Gly.[43]

His117Gly Type-2 Proteins. When the values of the $A_{\parallel}$ and $g_{\parallel}$ parameters of the His117Gly azurin type-2 copper centers His117Gly(H_2O), -(ImAc), -(His), and -(Hista) are plotted in a $g_{\parallel}$ versus $A_{\parallel}$ graph together with the data of known type-2 copper sites (Figure 7), the linear relationship between $A_{\parallel}$ and $g_{\parallel}$ is highly correlated (R^2 = 0.98) and similar to the other type-2 copper proteins.

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The correlation line of the His117Gly type-2 proteins runs below the line of the other type-2 copper centers in Figure 7. This can be attributed to the presence of the sulfur in the Cu coordination shell in the His117Gly type-2 proteins.[37,38] The smaller values of the $g_{\parallel}$ of His117Gly(His) and -(Hista) compared to those of His117Gly(H_2O) and -(ImAc) may reflect the aromaticity of the His and Hista ligands and the replacement of the less electron-rich oxygens of one or two water molecules or of the carbonyl of ImAc by one or two coordinating more electron-rich nitrogens.[37]

In the case of His117Gly(H_2O), one or two hydroxide ions could coordinate instead of water molecules. However, this seems less likely because the change in $g_{\parallel}$ upon incubation of His117Gly-(H_2O) by His is much larger than expected for a replacement of OH^- by His.[35]

Concluding Remarks

The replacement of the His117 ligand of azurin by a glycine apparently does not change the structure of the protein noticeably or the pocket which contains the copper ion. However, the important contribution of His117 to the biological function of azurin is best illustrated by the observation that the His117Gly(L) species that cluster around B in Figure 6 behave spectroscopically virtually identical to the wild type but are not functional in reversible electron transfer.

Geometrical changes of the copper center could be induced by various exogenous ligands and cause this one mutant to spectroscopically encompass the known range of type-1 and part of the range of the type-2 copper proteins. An interesting observation is that the His117Gly points in Figure 6 are not scattered more or less evenly along the correlation line connecting regions A and C but cluster in three areas. We tentatively interpret this as indicating a preference by the protein for a few discrete adaptations around the Cu site instead of a continuous variation.

In conclusion, it is interesting to note that a large variety of external ligands appear compatible with a metal center that retains the basic features of a type-1 or type-2 Cu site. It opens the attractive possibility of obtaining deeper insight into the correlation between copper site geometry and spectroscopic and mechanistic features by means of further detailed structural studies.

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Tetrafluorosulfate(1-) and Tetrafluorooxosulfate(1-) Radical Anions (SF_4^- and SF_4O^-)[†,‡]

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Abstract: The novel radical anions SF_4^- and *trans*-SF_4O^- were generated by γ-irradiation of $CsSF_5$ and $CsSF_5O$, respectively, at −196 °C and characterized by their isotropic EPR spectra at +27 °C. The SF_4^- anion (g = 2.0045, $a_{^{33}S}$ = 12.85 mT, $a_{^{19}F}$ = 9.75 mT) has a square-planar structure close to D_{4h} symmetry derived from a pseudo-octahedron in which the two axial positions are equally occupied by a total of three sterically active valence electrons. Accordingly, the greatest portion of the unpaired electron spin is localized in a sulfur p_z orbital. The *trans*-SF_4O^- anion (g = 2.0027, $a_{^{33}S}$ = 36.26 mT, $a_{^{19}F}$ = 18.95 mT) has a closely related, pseudo-octahedral structure of C_{4v} symmetry in which the equatorial positions are occupied by four equivalent fluorines, one axial position is occupied by a doubly bonded oxygen, and the second axial position is occupied by the sterically active free valence electron. Accordingly, the greatest portion of the unpaired electron spin is localized in an axial sp-hybrid orbital of sulfur. The structures and electron spin density distributions of SF_4^- and *cis*- and *trans*-SF_4O^- were analyzed by a local density functional study which also provided vibrational frequencies and charge distributions for these radical anions. For comparison, the closely related SF_5 radical, the *cis*- and *trans*-PF_4O^{2-} radical anions, and the SF_4, SF_4O, and SF_6 molecules were also calculated by this method. These calculations show that the exclusive formation of *trans*-SF_4O^- under our experimental conditions is probably due to both energetic and reaction mechanistic reasons and that the agreement between the LDF calculations and the experimental data is good.

Introduction

Previous studies have demonstrated that γ-irradiation of salts containing complex fluoro ions can result in the loss of one fluorine atom and in the formation of new complex fluoro radical ions that contain one fluorine atom less than the parent ion. Thus, the radical ions PF_5^-,[5-8] AsF_5^-,[8,9] and NF_3^+[10,11] have been obtained from PF_6^-, AsF_6^-, and NF_4^+ containing salts, respectively, and

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