Paramagnetic Cobalt and Nickel Derivatives of *Alcaligenes denitrificans* Azurin and Its M121Q Mutant. A ^{1}H NMR Study†

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Received July 28, 1995; Revised Manuscript Received December 6, 1995⊗

ABSTRACT: Using cobalt or nickel to replace copper in native azurin allows one to fingerprint the metal coordination site of the protein. The metal sites of wild type *Alcaligenes denitrificans* azurin and its M121Q mutant are clearly distinguishable through the paramagnetic ^{1}H NMR spectra of the Ni(II) and Co(II) derivatives. In the wild type azurin, Gly45 coordinates to nickel or cobalt, while Met121 appears as a weak metal ligand. On the contrary, in the M121Q azurin mutant, the metal exhibits a clear preference for the Gln121, which coordinates through the side chain carbonyl oxygen, and Gly45 is not a ligand. Changes in the isotropic shifts and relaxation properties of signals from the Cys112, His46, and His117 metal ligands suggest a movement of the metal ion out of the equatorial plane, indicating that the metal site is tetrahedral. These effects are less pronounced in the Ni(II) M121Q azurin than in the Co(II) metalloderivative. The similarity between the NMR spectra of the Co(II) derivatives of stellacyanin and the M121Q azurin is in agreement with a very similar metal site in both proteins and supports the existence of a coordinated Gln in stellacyanin.

Although its physiological function is still unclear (it is presumably part of the denitrification electron transfer chain in denitrifying bacteria), azurin continues to be one of the favorite subjects for the study of biological electron transfer (Adman, 1985; Sykes, 1991; Chapman, 1991; Canters & van de Kamp, 1992; Wuttke & Gray, 1993). Many reasons contribute to this. First, it is one of the best known electron transfer proteins, and its structural, chemical, and spectroscopic features have been investigated by almost all the suitable physical techniques (Chapman, 1991). Furthermore, since its gene was cloned and expressed in *Escherichia coli* (Canters, 1987; Arvidsson et al., 1989), many different site-directed mutants have been obtained and studied, allowing the understanding of the particular relevance of almost all of the potentially important residues for the functional, structural, and spectroscopic properties of the protein (van de Kamp et al., 1990a, 1993; Hoitink et al., 1992; van Pouderoyen et al., 1994; Pascher et al., 1993; Nar et al., 1991a; Romero et al., 1993; den Blaauwen & Canters, 1993; Mizoguchi et al., 1992; Germanas et al., 1993; Canters & Gilardi, 1993).

The crystal structures of azurins from different sources and in the two possible redox states of the copper ion, in the apo-protein as well as in various azurin metalloderivatives and site-directed mutants, have been solved in the last several years (Adman & Jensen, 1981; Nar et al., 1991a,b, 1992a,b; Romero et al., 1993; Baker, 1988; Shepard et al., 1990, 1993; Moratal et al., 1995; Blackwell et al., 1994). In the wild type copper protein, the metal ion is strongly bound to the Sγ of Cys112 and to the Nδ of both His46 and His117 (Baker, 1988; Nar et al., 1991b). This basic equatorial trigonal planar entity is claimed to be the essential part of the type 1 copper site, although mutagenesis studies of His46 (Germanas et al., 1993), His117 (den Blaauwen & Canters, 1993), and Cys112 (Mizoguchi et al., 1992) have demonstrated that Cys112 is the only coordinated residue which is absolutely essential for a blue copper center. The Sδ of Met121 and the carbonyl oxygen of Gly45 are weakly ligated, resulting in a distorted trigonal−bipyramidal geometry (Figure 1A) (Baker, 1988; Nar et al., 1991b). Although the role of these axial ligands is not completely understood, it is thought that they are responsible for modulating the redox potential of blue copper proteins (Gray & Malmström, 1983). Thus, azurins are unique in having a carbonyl glycine in the coordination sphere. Other blue copper proteins like stellacyanin, cucumber peelings cupredoxin, and umecyanin probably present an oxygen, from a glutamine side chain, in the axial position instead of the methionine sulfur which is common to the rest of the type 1 copper protein family (van Driessche et al., 1995).

Metal substitution has been frequently used for the structural and spectroscopic characterization of type 1 metal sites by different techniques (Nar et al., 1992a; Moratal et al., 1993a−c, 1995; Blackwell et al., 1994; McMillin et al., 1974; Tennent & McMillin, 1979; Strong et al., 1994; Di Bilio et al., 1992; Germanas et al., 1993; Hill et al., 1976; Blaszak et al., 1982; Dahlin et al., 1989; Piccioli et al., 1995; Salgado et al., 1995; Vila, 1994; Danielsen et al., 1995). Zinc, nickel, and cadmium azurins have been crystallized and their structures solved by X-ray diffraction (Nar et al., 1992a;

† This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO), by the Spanish "Dirección General de Investigación Científica y Técnica", (DGICYT, PB 91-0639), and by a fellowship from the Autonomous Government of Valencia (J.S.).

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

0006-2960/96/0435-1810$12.00/0

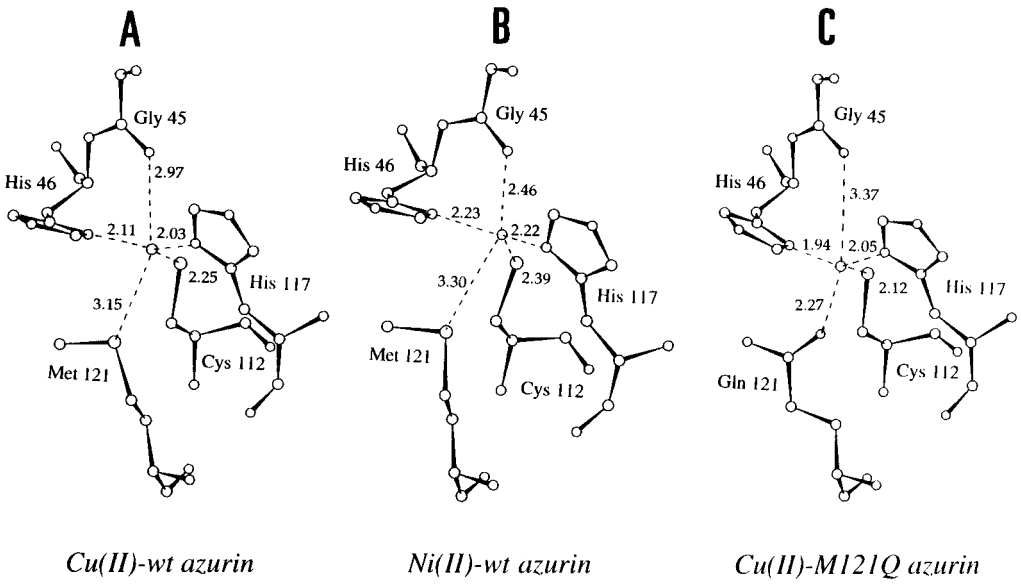

FIGURE 1: Schematic representations of the structure of different azurin metal sites as determined by X-ray crystallography. Data from Nar et al. (1991b) (A), Moratal et al. (1995) (B), and Romero et al. (1993) (C).

Moratal et al., 1995; Blackwell et al., 1994). In these cases, the metal site is better defined as four-coordinated due to the movement of the metal ion toward the carbonyl Gly45 ligand which leaves the Met121 sulfur practically out of the coordination sphere (Figure 1B). Substitution of copper by cobalt(II) and nickel(II) metal ions has been done because of the singular spectroscopic properties of their metal complexes. Early studies on cobalt-substituted blue copper proteins were used for understanding the physical properties of the type 1 copper site (McMillin et al., 1974; Tennent & McMillin, 1979). This strategy continues to be used to understand new type 1 copper sites (Strong et al., 1994; Di Bilio et al., 1994; Germanas et al., 1993). The application of NMR techniques to these paramagnetic metalloderivatives has proven to be very useful in fingerprinting the metal coordination site of the protein (Hill et al., 1976; Blaszak et al., 1982; Dahlin et al., 1989; Piccioli et al., 1995; Salgado et al., 1995; Vila et al., 1994). The relaxation properties of these metal ions permit the observation of relatively narrow, isotropically shifted signals in the ^{1}H NMR spectra. Signals from the coordinated residues are contact shifted outside the protein envelope, and the metal site can be selectively studied (La Mar et al., 1973; Bertini & Luchinat, 1986; Bertini et al., 1993).

The NMR spectra of the Ni(II) and Co(II) derivatives of *Pseudomonas aeruginosa* (*Pae*) azurin have been studied previously, and their paramagnetic signals have been assigned (Moratal et al., 1993a−c; Piccioli et al., 1995; Salgado et al., 1995). Here we present the results of the study of cobalt(II) and nickel(II) derivatives of *Alcaligenes denitrificans* (*Ade*)[1] azurin and its M121Q mutant by ^{1}H NMR. The M121Q azurin mutant is of great interest because it is supposed to be a good model of stellacyanin, a blue copper protein which lacks methionine and whose structure is still unknown (Romero et al., 1993). The structure of copper M121Q azurin in the oxidized and reduced states has been determined by X-ray diffraction studies. Cu(II) M121Q azurin is described as a distorted tetrahedron where the metal is coordinated to Gln121 O$^\epsilon$ but not to the Gly45 backbone carbonyl, which is now placed too far from the copper (Figure 1C). Upon reduction, the copper site changes to an almost linear coordination with His46 Nδ and Cys112 Sγ, leaving His117 Nδ and Gln121 Oϵ ~2.7 Å from the copper(I) (Romero et al., 1993). The present work can form the basis for characterization of a paramagnetic metallo-substituted type 1 copper site with Gln in the axial position, which helps the understanding of the stellacyanin- and stellacyanin-like metal sites through the study of their cobalt(II) and nickel(II) metalloderivatives.

EXPERIMENTAL PROCEDURES

Protein Preparation. Wild type and M121Q mutant azurins of *A. denitrificans* were obtained from the heterologous expression of the corresponding genes in *E. coli* as reported previously (Romero et al., 1993). For both wt and mutant azurins, a significant amount of colorless zinc protein is obtained together with the copper protein (van de Kamp et al., 1990b; Nar et al., 1992a; Romero et al., 1993). Separation of both metalloderivatives is an important step in the purification process, since any attempt made to demetallate the zinc protein was unsuccessful. The copper and zinc forms of wt azurin were separated by anion exchange chromatography under reducing conditions (van de Kamp et al., 1990b). The purification of the M121Q azurin was performed by using the published procedure (Romero et al., 1993) with some modifications for the separation of the contaminant zinc protein. After the DEAE chromatography, the pure azurin sample, consisting of a mixture of copper and zinc protein (5×10^{-5} M), was dialyzed against 0.5 M KCN in 0.1 M phosphate buffer at pH 8.5 and room temperature for 1 h (Salgado et al., 1995). This process can only extract the copper from the copper azurin while the zinc azurin remains intact. The mixture of apo- and zinc M121Q azurin was separated in a 10×2.5 cm CM Sepharose column (Pharmacia) equilibrated with 25 mM ammonium acetate buffer (pH 5) containing 0.5 mM

[1] Abbreviations: *Ade*, *Alcaligenes denitrificans*; *Pae*, *Pseudomonas aeruginosa*; NMR, nuclear magnetic resonance; WEFT, water-eliminated Fourier transformation; 1D, one-dimensional; 2D, two-dimensional; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; LMCT, ligand-to-metal charge transfer; LF, ligand field; wt, wild type.

ethylenediaminetetraacetic acid (EDTA) by elution with a 0 to 200 mM sodium chloride gradient. The apo-M121Q azurin can be reconstituted by the addition of $CuSO_4$, yielding a $A_{610}/A_{280} = 0.31$ spectral ratio. The Co(II) or Ni(II) metalloderivatives of wt and M121Q azurins were prepared by the addition of 5 mol equiv of $CoSO_4$ or $NiSO_4$ to the apo-protein solution at room temperature, and the metal uptake was followed by UV−vis spectroscopy. The formation of the Co(II) and Ni(II) metalloderivatives is very slow in the wild type protein (>48 h) but takes only a few minutes for the M121Q azurin. The Ni(II) wt azurin loses metal at pH <6.0, while all other metalloderivatives are stable at any pH value between 4.5 and 9.

Spectroscopic Measurements. Protein samples (4−6 mM) obtained by ultrafiltration in Centricon-10 concentration cells (Amicon) were used for the NMR experiments. NMR spectra were recorded in Varian Unity spectrometers operating at 300 or 400 MHz for ^{1}H. The super-WEFT sequence (Inubushi & Becker, 1983) was used to observe fast-relaxing signals in the diamagnetic region of the spectrum and to eliminate the solvent H_2O or HDO signal.

The reported methodology for the NMR study of paramagnetic metalloproteins was used (Bertini et al., 1993; La Mar & de Ropp, 1993). Nonselective longitudinal relaxation times were determined using the inversion recovery pulse sequence (180°-τ-90°-*Acq*). The T_1 values were calculated from the initial slope of semilogarithmic plots of the fractional deviation of the *z*-magnetization from the equilibrium versus the relaxation delay τ (La Mar & de Ropp, 1993). Saturation transfer and steady state 1D NOE measurements were made using the super-WEFT pulse sequence, irradiating the resonance of interest during the relaxation delay τ (20−100 ms) with a selective decoupler pulse. Spectra were acquired by interleaving blocks of 32 scans alternating on-resonance and off-resonance saturation. Data were analyzed according to the reported methodology (Neuhaus & Williamson, 1989; Banci et al., 1990).

NOESY experiments were performed in the hypercomplex mode (States et al., 1982). Recycle times ranged from 30 to 100 ms and mixing times from 3 to 30 ms, depending on the paramagnetic character of the signals of interest. To get NOESY correlations between paramagnetic signals in the diamagnetic region, the WEFT-NOESY sequence (Chen et al., 1982) was used. In these cases, the delay τ between the 180 and 90° pulses varied from 25 to 50 ms in experiments where the total effective relaxation delay (acquisition time plus relaxation delay d_1) was from 22 to 50 ms and the mixing time from 3 to 10 ms, respectively. NOESY and WEFT-NOESY spectra were Fourier transformed using 256, 512, or 1024 data points in both dimensions, zero filling the t_1 dimension when necessary, and square sine bell weighting functions shifted 45, 60, or 80°. Finally, the 2D spectra were base line corrected.

Electronic spectra were recorded on a Cary 1 spectrophotometer.

RESULTS

UV−Vis Spectra of Co(II) and Ni(II) Derivatives of Wild Type and M121Q Azurins

The UV−vis spectrum of the Co(II) wt *Ade* azurin (Figure 2A, Table 1) is essentially the same as that of the Co(II)

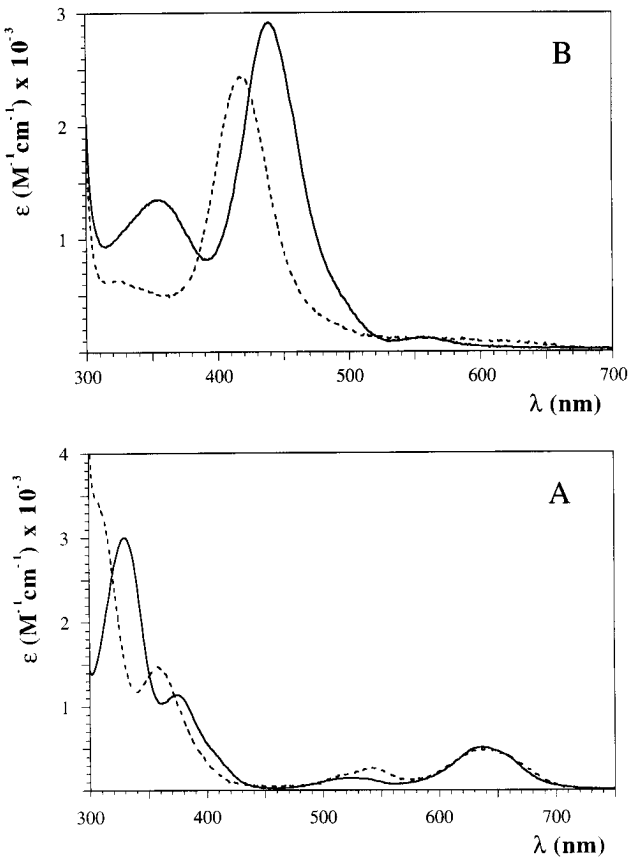

FIGURE 2: Uv−vis absorption spectra of cobalt (A) and nickel (B) derivatives of wild type (—) and M121Q (- - -) *A. denitrificans* azurins (pH 7.0 and 25 °C).

derivative of *Pae* azurin, the bands of which have been already assigned (McMillin et al., 1974; Tennent & McMillin, 1979; Di Bilio et al., 1992). It consists of two intense ligand-to-metal charge transfer transitions (LMCT) and various ligand−field (LF) or d−d transitions. The spectrum of the Ni(II) wt *Ade* azurin (Figure 2B, Table 2) is also very similar to the corresponding spectrum of *Pae* azurin (Tennent & McMillin, 1979).

The spectra of the Co(II) and Ni(II) M121Q proteins (Figure 2, Tables 1 and 2) are similar to those of the wild type metalloderivatives but present some significant differences. The LMCT bands are 10−30 nm blue shifted, being in agreement with the Co(II) and Ni(II) stellacyanin spectra (McMillin et al., 1974; Lum & Gray, 1981). A similar result is obtained from the UV−vis spectrum of the Cu(II) M121Q protein, in agreement with a similar coordination environment in both stellacyanin and the M121Q azurin (Romero et al., 1993).

^{1}H NMR Spectra

Ni(II) Metalloderivatives. The 1D ^{1}H NMR spectrum of the Ni(II) wt azurin from *A. denitrificans* (at pH 7.0) is shown in Figure 3B. The paramagnetic zone of the spectrum shows the same pattern of hyperfine-shifted signals as the spectrum of the Ni(II) derivative of *Pae* azurin (Figure 3A), and a similarity between both of them can be drawn. The assignment of the paramagnetic signals by means of 1D NOE and NOESY spectra (not shown) is in close agreement with the reported ^{1}H NMR study of the Ni(II) *Pae* azurin (Moratal et al., 1993b) (see the assignments in Table 3). In contrast to that of the *Pae* azurin, the NMR spectrum of the Ni(II)

Table 1: UV−Visible Bands (λ in Nanometers) for Co(II)-Substituted Wild Type Azurin, Azurin Mutants, and Stellacyanin

wt azurin[a]	stellacyanin[b]	M121Q azurin[a]	M121G azurin[c]	M121D azurin[c]	M121E azurin[c]	assignment[d]
330	310 (sh)	308 (sh)	305	303 (sh)	290 (sh)	LMCT
375 (sh)	365 (sh)	359	358	341	328	LMCT
403 (sh)		∼392 (sh)				
520		517 (sh)				LF
531	540	543	535	564	564	LF
635	625 (sh)	634	620 (sh)	625 (sh)	603 (sh)	LF
651 (sh)	655	∼648 (sh)	640	645	620	LF

[a] *A. denitrificans* azurin (this work). [b] Data from McMillin et al. (1974). [c] *P. aeruginosa* azurin mutants. Data from Di Bilio et al. (1992). [d] Assignments as in Di Bilio et al. (1992).

Table 2: UV−Visible Bands (λ in Nanometers) for Ni(II)-Substituted Wild Type Azurin and Azurin Mutants

wt azurin[a]	stellacyanin[b]	M121Q azurin[a]	M121G azurin[c]	M121D azurin[c]	M121E azurin[c]	assignment[d]
354	335	320	∼356	∼346	320	LMCT
440	410	416	418	414	392	LMCT
490 (sh)	470 (sh)	480 (sh)				
560	550	550	∼530		499	LF
	590	620	∼620		546	LF

[a] *A. denitrificans* azurin (this work). [b] Data from Lum and Gray (1981). [c] *P. aeruginosa* azurin mutants. Data from Di Bilio et al. (1992). [d] Assignments as in Di Bilio et al. (1992).

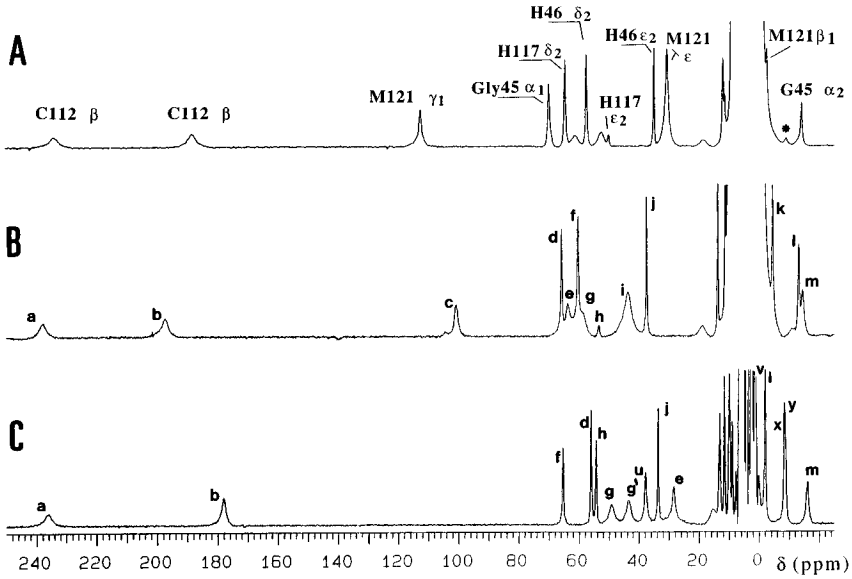

FIGURE 3: Comparison between the 300 MHz ^{1}H NMR spectra of *P. aeruginosa* Ni(II) wt azurin (A), *A. denitrificans* Ni(II) wt azurin (B), and *A. denitrificans* Ni(II) M121Q azurin (C). The conditions are H_2O solvent, 30 °C, 20 mM NaH_2PO_4, and pH 7.0 (A and B) or pH 5.2 (C). The assignments of the signals of spectrum A are taken from Moratal et al. (1993b). Signals mentioned in the text are labeled with letters.

wt azurin from *A. denitrificans* does not depend on the pH in the range 4.5−8.0. The only pH dependent effect is the increase of exchange rate of the His117 Nϵ_2H signal (signal h), whose intensity decreases at high pH as also seen in the case of the Ni(II) azurin from *P. aeruginosa* (Moratal et al., 1993a).

The corresponding spectrum of the Ni(II) M121Q azurin mutant (Figure 3C) shows some important differences with regard to the wild type azurin spectra. From a first comparison, it can be noticed that those signals assigned to the Met121 residue in the Ni(II) wt azurin are absent in the Ni(II) M121Q azurin spectrum, confirming previous assignments. Additionally, a similar pattern is found for the resonances of the three equatorial ligands: Cys112, His117, and His46 (signals a−d and f−j in Figure 3B,C). Thus, the two far downfield-shifted signals would correspond to the Cys112 β protons, and this is confirmed by the clear 1D NOE difference signals which are observed upon irradiation of both of them (Figure 4A,B). On the other hand, the two labile proton signals, corresponding to the imidazole Nϵ2H of the two coordinated histidines (signals h and j), can be paired with their vicinal imidazole Cδ2H protons (signals d and f, respectively) by means of 1D NOE (Figure 4C,D). The specific assignment of these signals is made according to the same criteria as in the case of the wt azurin spectra (Moratal et al., 1993a), i.e. taking into account the expected different exchange behavior of the two histidine Nϵ2H signals, due to their different exposition to the solvent according to the azurin crystal structure (Baker, 1988). So, since signal h enters fast exchange conditions as the pH is increased (not shown), the pair of signals d and h are assigned to the Cδ2H and Nϵ2H protons of His117 and signals f and j to the corresponding protons of His46.

The very broad signals g and g′ are also present in the spectra of the wt Ni(II) azurins. A similar pair of signals can be found in the spectra of the Co(II) derivatives of azurin

Table 3: Paramagnetic ^{1}H NMR Resonances of *A. denitrificans* Ni(II) Wild Type Azurin and Its M121Q Mutant[a]

	Ni(II) wt azurin				Ni(II) M121Q azurin			
assigned proton	signal	δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)	signal	δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)
Cys112 β_2	a	238	1.1	800	a	237	0.8	860
Cys112 β_1	b	197	1.4	750	b	178	1.6	470
Cys112 α					v	−2.1	17.6	[b]
Cys112NH						6.2	[c]	[c]
His46 ϵ_1	g	59	[b]	[b]	g′	43.5	1.5	450
His46 ϵ_2	j	37.5	14.0	78	j	33.5	17.5	62
His46 δ_2	f	60.3	10.3	160	f	65.1	12.5	68
His46 β_1[e]	m	−14.4	4.0	326	m	−16.3	3.0	300
His117 ϵ_1	g	59	[b]	[b]	g	49.0	1.6	560
His117 ϵ_2	h	53.4	[d]	150	h	54.1	12.2	72
His117 δ_2	d	65.7	14.2	120	d	55.7	12.5	100
Gly45 α_1	e	63.7	8.2	330	e	28.9	17.4	325
Gly45 α_2	l	−13.2	10.6	140	l	−2.3	19.4	[b]
Met121 ϵ	i	43.7	4.9	900				
Met121 γ_1	c	101.1	5.4	390				
Met121 γ_2		7.7	[c]	[c]				
Met121 β_1	k	−4.4	12.9	115				
Met121 β_2		−1.6	16.7	[b]				
Gln121 γ_1					u	37.7	10.1	225
Gln121 γ_2					x	−8.6	11.5	120
Gln121 β_1						2.4	[c]	[c]
Gln121 β_2						5.2	[c]	[c]
Gln121 α						3.9	[c]	[c]
Gln121 ϵ_{21}					y	−8.9	8.2	[b]

[a] Data from the Ni(II) M121Q azurin correspond to pH 5.5 (unbuffered sample) and 30 °C. Data from the Ni(II) wt azurin have been obtained at 30 °C and pH 7 (20 mM phosphate buffer) because the Ni(II) metal complex is not stable at low pH. No effects of the pH and buffer in the NMR spectra (δ and relaxation times) are observed for both wt and M121Q azurin Ni(II) metalloderivatives. [b] Overlap prevents measurement of this value. [c] Signal buried in the diamagnetic region of the spectrum. [d] Signal is in partial exchange with the solvent. [e] Tentative assignment.

and stellacyanin (Piccioli et al., 1995; Salgado et al., 1995; Vila, 1994), where they have been tentatively assigned to the imidazole Cϵ1H protons of the coordinated histidines. Irradiation of signal g′ (Figure 4E) confirms this assumption through a clear NOE difference signal corresponding to the His46 Nϵ2H proton (signal j). Although irradiation of signal g does not give an NOE with signal h, we assign signal g to the His117 Cϵ1H proton due to its close similarity with signal g′. Since signals h and g are quite close, and a strong decoupling has to be used in order to saturate the broad signal g, the absence of an NOE between both signals can be due to a cancellation effect produced during the off-resonance irradiation.

In the azurin structure, the Cys112 β protons are very close to the Met121 γ and β protons, and this is also the case when the Met121 is replaced by a glutamine (Romero et al., 1993). Irradiation of signal a gives very weak NOEs with both signals u and x (Figure 4A), but irradiation of signal b gives a clear NOE with signal u (Figure 4B). On the other hand, signals u and x are connected by a strong NOE, as expected for a pair of geminal protons (Figure 4F). This pattern is compatible with signals u and x being the Gln γ1 and γ2 protons, respectively, and signals a and b the Cys112 β2 and β1 protons. Additionally, these 1D NOE spectra allowed us to find the Cys112 CαH and NH protons (Figure 4A,B). As it was shown in a complete ^{1}H NMR study of the wt *Ade* Cu(I) azurin, the amide proton of Cys112 is in slow exchange with the solvent and it can be observed even in D_2O (Hoitink et al., 1994). No other signal shifted out

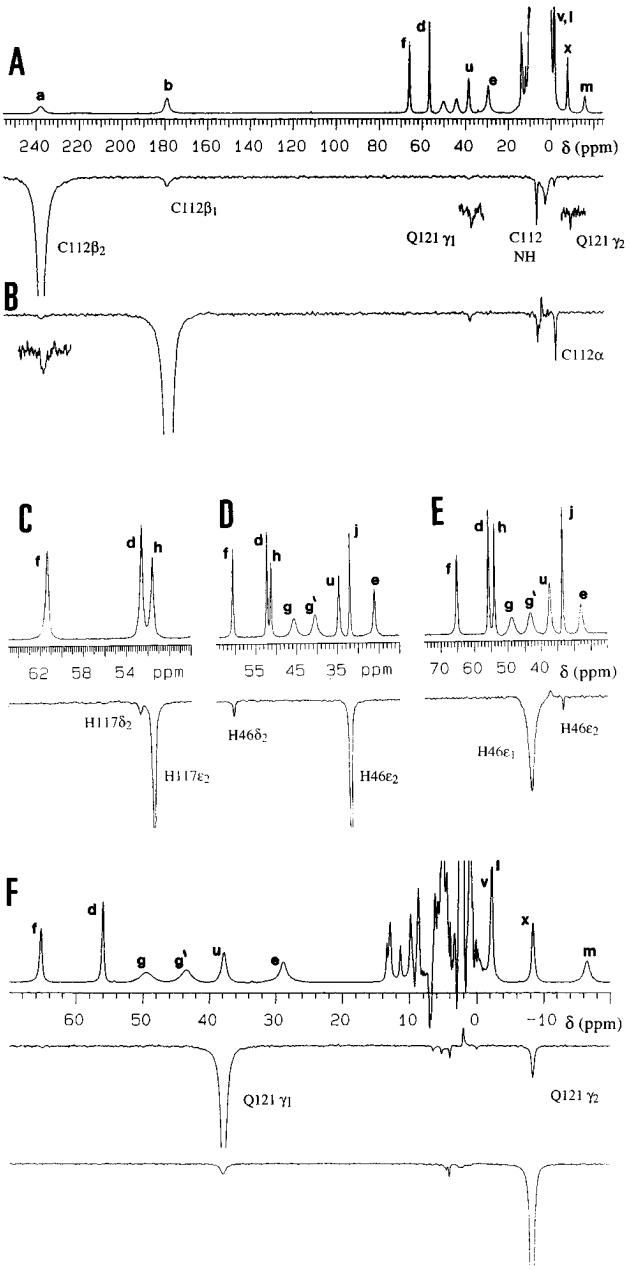

FIGURE 4: Difference and reference spectra from steady NOE experiments on Ni(II) M121Q azurin. The conditions are D_2O solvent, pH 5.2, and 30 °C (A, B, and F) or H_2O solvent, pH 5.2, and 50 °C (C−E). Please notice that signals h, j, and y have disappeared in A and F because of exchange with D_2O, and signal positions in C−E do not correspond with the values in Table 3 because the temperature conditions are not the same in both cases.

of the protein envelope is dipolarly connected with signals u and x, so the rest of the Gln121 signals must be buried in the diamagnetic region of the spectrum. A WEFT-NOESY spectrum (Figure 5) interpreted according to the M121Q azurin crystal structure allows us to find the Gln121 β and α protons.

A particularly characteristic resonance in the Ni(II) M121Q azurin spectrum is signal y (Figure 3C). This signal disappears in D_2O solvent, and it should be in principle assigned to one of the Gln Nϵ_2H$_2$ protons, since there is not a similar resonance in the Ni(II) wt azurin spectrum and it is shifted very close to the Gln Cγ_2H signal. The Gln121 Nϵ_{21}H proton is the most likely possibility for this assignment, since the Nϵ_{22}H is very close to the metal [about 3.3 Å in the Cu(II) protein] (Romero et al., 1993) and we would

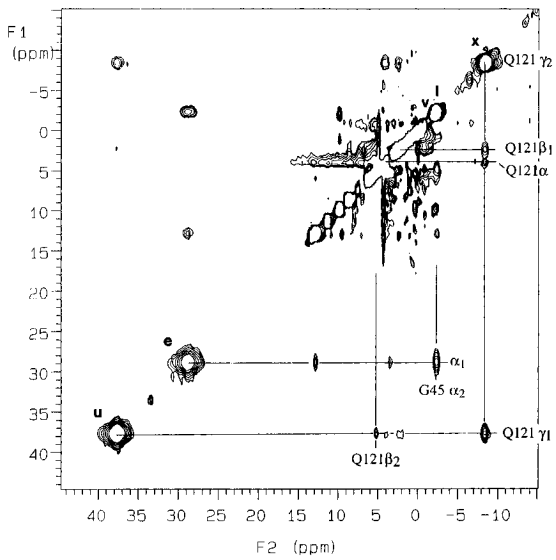

FIGURE 5: Phase-sensitive 400 MHz ^{1}H NMR WEFT-NOESY spectrum of Ni(II) M121Q azurin (4 mM protein concentration, D_2O solvent, pH 5.2, and 30 °C). The spectrum was obtained with a 6 ms mixing time, 512 t_1 values (1024 scans each), over a 56 kHz band width.

expect a very broad signal for this proton. According to the Cu(II) M121Q azurin crystal structure (Romero et al., 1993), an NOE between signals y and x (the Gln121 γ_2 signal) should be observed to confirm the above assignment of signal y, but the overlap between these two signals prevents the observation of this coupling.

On the other hand, signals e and l are another couple of geminal protons according to the intense NOESY connectivity observed between them (Figure 5). These signals are moderately paramagnetic as indicated by their relaxation times (table 3), so they should correspond to a CH_2 group placed at an intermediate distance from the metal center. According to this, the Gly45 CαH_2 protons are the best candidates for the assignment of signals e and l. In fact, the pattern of isotropic shifts of these signals, one being shifted upfield and the other downfield, resembles that of signals e and l from the spectrum of the wt Ni(II) azurin (Figure 3B), where they have been also assigned to the Gly45 α protons (see Table 3).

Finally, signal m is a characteristic signal in the ^{1}H NMR spectrum of the Ni(II) M121Q azurin which seems to be the same as signal m from the spectrum of the wt Ni(II) azurin (Figure 3B,C). The absence of NOE connectivities from these two signals makes their assignment difficult. The corresponding signal in the spectrum of Ni(II) wt azurin from *P. aeruginosa* (signal labeled * in Figure 3A) was tentatively assigned to the Cys112 α or the His46 β1 proton (Moratal et al., 1993b). These two candidates were chosen as the most probable according to their distance to the metal center [4−5 Å according to Nar et al. (1991b)] and the relaxation time of the signal. The Cys112 α proton has been assigned above in the case of the Ni(II) M121Q azurin to signal v, so signal m can be tentatively assigned as the His46 β1 proton.

Co(II) Metalloderivatives. The spectrum of the *Ade* Co(II) wt azurin is also very similar to the corresponding spectrum of the *Pae* azurin metalloderivative, and most of their signals are practically in the same position (Figure 6A−B). These signals have been assigned in agreement with the reported studies of Co(II) *Pae* azurin (Moratal et al., 1993c; Piccioli et al., 1995; Salgado et al., 1995), and the assignments are summarized in Table 4. Contrary to the *Pae* Co(II) azurin, the Gly45 amide signal is not clearly observed out of the protein envelope in *Ade* Co(II) wt azurin. Although this signal is important to assign the Gly45 α protons (Moratal et al., 1993c), here this assignment is supported on their clear dipolar connection with the Val86 Cδ1H$_3$ signal, as it was also observed in the *Pae* Co(II) azurin derivative (Piccioli et al., 1995; Salgado et al., 1995) (data not shown).

The spectrum of the Co(II) M121Q mutant protein is shown in Figure 6C. The two far downfield-shifted signals a and b can be assigned to the Cys112 β protons as in the Co(II) and the Ni(II) derivatives of the wt azurin. Signals corresponding to the two coordinated histidines are also assigned by means of NOESY correlations between the imidazole Cδ2H and Nϵ2H protons (Figure 7), following the same reasoning as in the case of the wt azurin metalloderivative (Moratal et al., 1993c) and the Ni(II) M121Q azurin (see above and Table 4).

On the other hand, when we compare the spectra of the Co(II) wt and Co(II) M121Q azurins, very clear differences are found in the signals corresponding to the two potential axial ligands. As shown in Figure 7, only a couple of well-shifted protons, one placed upfield and the other downfield, exhibit a strong NOESY connectivity (signals u and v). These can be assigned to a CH_2 group, and they are dipolarly connected with signals x and y, which in turn present a strong NOESY cross-signal between them. This pattern fits well with signals u, v, x, and y being the γ and β protons of the Gln121 residue. The Gly45 α signals can be found again through their NOESY connectivities with the Val86 γ1 methyl signal (Figure 7), as was shown for the Co(II) wt azurin (Piccioli et al., 1995; Salgado et al., 1995).

It is important to notice that, as we observed in the case of the nickel derivative, an upfield-shifted exchangeable signal is also observed in the Co(II) M121Q azurin spectrum (signal z). In this case, a weak NOESY cross-peak is found between this signal and signal v, assigned to the Gln121 γ2 proton (Figure 7). This cross-peak leads to the assignment of this signal to one of the Gln121 Nϵ_2H$_2$ protons as it is suggested above for the similar signal in the Ni(II) M121Q azurin. Since no NOE is observed between signals z and u (the Gln121 γ1 proton), we can confirm that signal z is most likely the Gln121 Nϵ_{21}H proton.

Finally, among the principal signals of the NMR spectrum, signals p and q are still to be assigned. Signal q is even broader than signals a−d and has to correspond to a proton very close to the metal (around 3 Å). The His117 β protons and the His46 α and β2 protons fit this condition according to the Cu(II) M121Q azurin structure (Romero et al., 1993). With regard to signal p, no NOE between this signal and the rest of the isotropically shifted signals was obtained. Again in this case, as we suggested for signal m from the Ni(II) M121Q azurin spectrum, the His46 β1 proton seems to be the best possibility.

DISCUSSION

Absorption Spectra. Studying the electronic spectra in Co(II) and Ni(II) azurins is an approach frequently used to discuss the electronic and structural properties of the type 1 metal site, since LMCT and LF absorption systems are normally well-separated in these metallosubstituted proteins

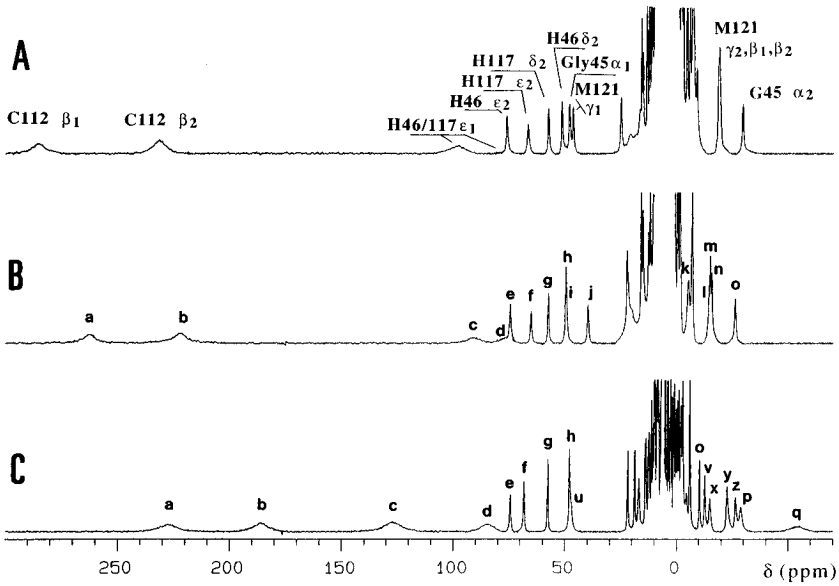

FIGURE 6: ^{1}H NMR 300 MHz spectra of *P. aeruginosa* Co(II) wt azurin (A), *A. denitrificans* Co(II) wt azurin (B), and *A. denitrificans* Co(II) M121Q azurin (C). The conditions are H_2O solvent, 37 °C, and unbuffered solutions at pH 4.5. The assignment of the signals of spectrum A are according to Moratal et al. (1993c). Only those signals mentioned in the text have been labeled.

Table 4: Paramagnetic ^{1}H NMR Resonances of *A. denitrificans* Co(II) Wild Type Azurin and Its M121Q Mutant[a]

	Co(II) wt azurin				Co(II) M121Q azurin			
assigned proton	signal	δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)	signal	δ (ppm)	T_1 (ms)	$\Delta\nu_{1/2}$ (Hz)
Cys112 β	a	262	0.5	1700	a	224	[d]	2100
Cys112 β	b	222	0.4	1900	b	183	[d]	2600
His46/117 ϵ_1	c	90	[d]	2000	c	125	[d]	2000
His46 ϵ_2	e	75.6	4.9	240	e	73.4	6.1	167
His46 δ_2	h	48.9	6.4	[b]	h	47.2	12.7	140
His46 β_1[e]					p	−28.6	2.2	430
His117/46 ϵ_1	d	76	[d]	1800	d	83	[d]	2600
His117 ϵ_2	f	64.4	7.3	230	f	67.4	7.8	138
His117 δ_2	g	56.7	12.4	180	g	56.8	15.0	100
Gly45 α_1	i	49.0	6.4	[b]		5.9	[c]	[c]
Gly45 α_2	o	−26.3	3.5	241	o	−10.5	22.5	77
Met121 ϵ	k	−5.7	2.5	439				
Met121 γ_1	j	39.2	4.8	245				
Met121 γ_2	l	−14.7	5.3	[b]				
Met121 β_1	m	−15.0	6.4	[b]				
Met121 β_2	n	−15.7	5.0	[b]				
Gln121 γ_1					u	46.4	4.2	320
Gln121 γ_2					v	−12.8	6.8	140
Gln121 β_2					x	−15.0	3.2	280
Gln121 β_1					y	−22.7	4.0	250
Gln121 α						−3.2	[b]	[b]
Gln121 ϵ_{21}					z	−26.4	3.2	260

[a] Data correspond to pH 4.5 (unbuffered solutions) and 37 °C in both cases. [b] Overlap prevents measurement of this value. [c] Signal buried in the diamagnetic region of the spectrum. [d] $T_1 \leq 0.2$ ms. [e] Tentative assignment.

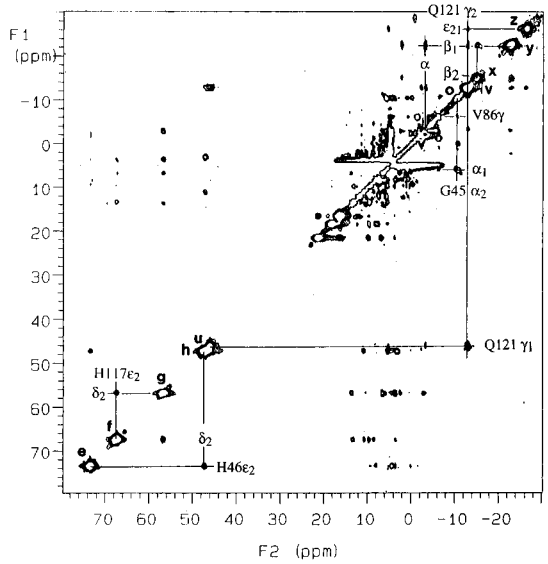

FIGURE 7: Phase-sensitive 400 MHz ^{1}H NMR WEFT-NOESY spectrum of Co(II) M121Q azurin (6 mM protein concentration, H_2O solvent, pH 4.7, and 37 °C). The map was collected with a 10 ms mixing time, 512 t_1 values (2024 scans each), over a 70 kHz band width.

(McMillin et al., 1974; Tennent & McMillin, 1979). In the case of the LF bands of the Co(II) derivatives, the magnitude of the extinction coefficients can be related with the coordination geometry of the metal center. For both wt and M121Q Co(II) derivatives, the intensity of these bands is very similar and is clearly in the typical range for distorted tetrahedral Co(II) complexes ($\epsilon_{max} > 250$ M^{-1} cm^{-1}) (Rosenberg et al., 1973; Bertini & Luchinat, 1984). This interpretation agrees with the crystal structures of the Zn(II) and Ni(II) wt azurins (Nar et al., 1992a; Moratal et al., 1995) and of the Cu(II) M121Q azurin (Romero et al., 1993). With regard to the LMCT transitions, their characteristics have been recently studied in detail by H. B. Gray and co-workers for various Met121 *Pae* azurin mutants, and they have found that the position of these bands depends on the nature of the 121 residue (Di Bilio et al., 1992). Thus, the blue shifts of the LMCT bands are interpreted as being due to an inner sphere carboxylate interaction in the case of the M121E mutant or an outer sphere interaction of the polar carboxylate group in the case of the M121D mutant (Di Bilio et al., 1992). For the M121Q azurin metalloderivatives studied here, LMCT bands are also blue shifted with regard to the wt azurin metalloderivatives (Figure 2). In the case of the Ni(II) M121Q azurin, the main LMCT band is placed almost in the same position as the corresponding band in the Ni(II) M121D azurin, while the second LMCT band is at 320 nm, as is the corresponding band in the Ni(II) M121E azurin (Table 2). The Co(II) M121Q azurin bands are less shifted than those from the Co(II) derivatives of both the M121D

and M121E azurin mutants, and their position is very similar to the bands of the Co(II) M121G azurin (Table 1). These results agree with the presence of an oxygen from the carbonyl of the Gln121 side chain in the metal coordination sphere, which would be the main cause of the blue shifts of the absorption bands (Di Bilio et al., 1992). The fact that these shifts are less dramatic than those observed for the M121E mutant, where oxygen is probably also coordinated (Di Bilio et al., 1992), can be due to the lower polar character of the glutamine carbonyl oxygen with regard to a glutamate carboxylic oxygen. On the other hand, the similarity between the Co(II) M121Q and Co(II) M121G azurin bands is compatible with the presence of an oxygen from a water molecule coordinated to the metal in the case of the second protein, as already suggested (Di Bilio et al., 1992).

^{1}H NMR Spectra of wt and M121Q Azurin Metalloderivatives. As discussed in the literature (La Mar et al., 1973; Bertini & Luchinat, 1986; Bertini et al., 1993), the NMR spectra of paramagnetic metalloproteins contain important structural information which can be extracted from an analysis of the isotropic shifts and the relaxation properties of previously assigned resonances. The ^{1}H NMR spectra of Ni(II) and Co(II) derivatives of azurins from two different bacterial sources (*P. aeruginosa* and *A. denitrificans*) are very similar. In other words, signals corresponding to analogous protons in the structures of the two molecules display almost the same isotropic shifts and relaxation times and, as in the case of the His117 Nϵ_2H signals, also show the same behavior, like the variations in the exchange rate with the solvent due to changes in the solution conditions. The structure of the metal site in both azurins is practically identical, and the close similarity between the paramagnetic NMR spectra is just an expression of such structural resemblance. The slight differences observed can be due to small differences in the first coordination sphere or variations in some amino acid positions in the second coordination sphere. So, the same conclusions derived from the studies of cobalt and nickel derivatives of *Pae* azurin (Moratal et al., 1993b; Salgado et al., 1995) can be stated here. Mainly, the existence of paramagnetic signals presenting large isotropic shifts and corresponding to the five possible coordinating residues leads to the conclusion that all of them exhibit contact interaction with the paramagnetic center. This interpretation can be safely accepted in the case of the three equatorial ligands, due to the dramatic shifts of the Cys112 β protons, and the pattern observed for the histidine signals which is typical of coordination to the paramagnetic center. However, evaluating the evidence for coordination of Met121 or Gly45 is problematic also because there seems to be disagreement with other spectroscopic data (as the intensity of the d−d transitions) and with the available structural information (which describes a tetrahedral metal site with the methionine sulfur placed 3.3 Å away from the metal) (Nar et al., 1992a; Moratal et al., 1995). The structural rigidity in the metal site would prevent the simultaneous approach of both axial ligands. Coordination by the Gly45 carbonyl oxygen is more probable according to the crystal structure. There are no examples in the literature of ^{1}H NMR spectra of Co(II) or Ni(II) complexes with a sulfur methionine as a ligand, but comparison of the Met121 signals with those from the strongly coordinated Cys112 permit the conclusion that the coordination of the Met121, if present, has to be very weak. The observed shifts for some of the Met121 signals [the γ1 proton signal in both Co(II) and Ni(II) derivatives and the methyl signal in the Ni(II) azurin] are ascribed primarily to the pseudocontact contribution, as expected for tetrahedral Co(II) and Ni(II) complexes [it should be larger for the Ni(II) ones].

In the case of the M121Q azurin metalloderivatives, we have quite a different scenario. First, we notice that the observed NMR results are only compatible with the coordination of the metal through the Gln121 side chain Oϵ1, since the Nϵ2H signal should be broadened beyond detection in the case of coordination by the amide Gln121 Nϵ2. Therefore, the two alternative axial ligands (the carbonyl oxygens of Gly45 and Gln121) are now chemically similar, and the choice for one of them must be dictated by the structural restrictions of the metal site and the geometrical preferences of the metal. Thus, if we look at the isotropic shifts of the Gln121 signals, we find that they are slightly larger than the Met121 signals of the wt protein in the case of the Co(II) metalloderivatives, but conversely, the Met121 signals are significantly more shifted than Gln121 signals in the case of the Ni(II)-substituted proteins. The γ and β protons from a methionine and a glutamine side chain which coordinate through their S_δ and O_ϵ atoms, respectively, are not exactly comparable in terms of their contact interaction with the metal center, since the glutamine protons are separated from the metal by an additional (double) bond. Thus, supposing we have the same pseudocontact contributions in both cases, the isotropic shifts should be larger in the case of the Met121 signals of the wt protein than in the case of the Gln121 signals of the M121Q protein. This could explain why the Gln121 signals are less shifted than the Met121 ones in the Ni(II) derivatives. On the other hand, in the case of the cobalt derivatives, we can conclude that the Gln121 residue interacts more strongly with the Co(II) ion than the Met121 does. Considering the Gly45 resonances, we find that for both Ni(II) and Co(II) derivatives of the M121Q azurin they are significantly less shifted than in the corresponding wt metalloderivatives. Moreover, their relaxation times are also larger, indicating a weaker interaction with the paramagnetic metal center in both cases.

Although signals from the equatorial ligands display a similar pattern in the wt and M121Q mutant metalloderivatives, some important differences can be observed. Signals from the two Cys112 β protons are less shifted in the Co(II) M121Q azurin than in the corresponding wt metalloderivative. Since the contact contribution to the isotropic shifts of these protons have to be clearly dominant, we conclude that the unpaired spin delocalization over the Cys112 ligand is smaller in the M121Q azurin. The origin of this effect can be a reduction in the orbital overlap between the cobalt and the cysteine sulfur due to the movement of the metal out of the equatorial plane because of a stronger axial coordination. Moreover, the relaxation times of these signals decrease significantly, indicating that these protons are closer to the paramagnetic center as a consequence of the above-discussed reorganization of the metal site. Additionally, changes are also observed in at least one of the Cϵ_1H histidine signals [signal c in the Co(II) M121Q azurin spectrum], which is now more shifted, although no other histidine signal is significantly displaced with regard to the positions in the spectrum of the wt protein. For the Ni(II) metalloderivatives, differences in the resonances from the equatorial ligands are less marked. Variations in the isotropic shifts affect only

one of the Cys121 β protons, whose relaxation time increases in the case of the Ni(II) M121Q azurin. With regard to the histidine signals, some changes are also observed which indicate that the structure of the metal site has changed, but they are not so significant as in the case of the cobalt derivatives. So one interpretation is that the tetrahedral character of the Ni(II) M121Q metal site is less pronounced than in the case of the Co(II) M121Q, the metal−O Gln121 bond being weaker in the Ni(II) derivative. The fact that the Gly45 α signals are more shifted in the Ni(II) M121Q azurin would agree with this conclusion.

The M121Q Azurin Mutant and the Stellacyanin Metal Site. The coincidence between the spectroscopic properties (visible and EPR spectra) of stellacyanin and the *A. denitrificans* M121Q azurin mutant is a solid argument in favor of the presence of a glutamine as the axial ligand in the former blue copper protein (Romero et al., 1993). Other studies like ^{111m}Cd-perturbed angular correlation (PAC) (Danielsen et al., 1995), structural modeling based on the structure of the cucumber basic protein (Fields et al., 1991), and ENDOR spectroscopy (Thonmann et al., 1991) also agree with the idea of a Gln side chain as the fourth ligand in stellacyanin. The absorption spectra of the Co(II) and Ni(II) derivatives of both stellacyanin and the M121Q azurin are very similar (Tables 1 and 2), supporting the above interpretation of the stellacyanin metal site.

It has been shown that the ^{1}H NMR spectra of azurin paramagnetic metalloderivatives are a very informative method of fingerprinting the metal site of the protein (Piccioli et al., 1995; Salgado et al., 1995), and we have demonstrated above that for the same coordination geometry of the paramagnetic metal a very similar ^{1}H NMR spectrum should be obtained. If we compare the Co(II) derivatives of the M121Q azurin and stellacyanin (Vila, 1994), we find many similarities. The resonances of coordinated cysteine and histidine residues are isotropically shifted to very similar positions, indicating that the position of the metal with regard to the three equatorial ligands has to be very similar for both cobalt derivatives. Although the axial ligand has not been assigned for the Co(II) stellacyanin, the signal at 71 ppm in its ^{1}H NMR spectrum (Vila, 1994) is a likely candidate. This signal can be cross-correlated by 1D NOE with an upfield-shifted signal (A. Vila, personal communication), thus resembling the pair of Gln121 CγH$_2$ protons of the Co(II) M121Q azurin spectrum. Finally, an important difference between the Co(II)-stellacyanin and the Co(II) M121Q azurin spectra is the upfield-shifted exchangeable signal z present in the latter (assigned here as the Gln121 Nϵ_{21}H proton), since no labile proton signal with these characteristics is reported in the NMR spectrum of Co(II) stellacyanin (Dahlin et al., 1989; Vila, 1994). It has been reported that the metal center in stellacyanin is more exposed to the solvent than the azurin metal center, as suggested on the basis of the higher exchange rate with the solvent of the Nϵ_2H protons from both coordinated histidines (Vila, 1994). A fast exchange rate also affecting the Gln97 NϵH$_2$ protons could explain the absence of the corresponding signal in the Co(II) stellacyanin spectrum.

CONCLUSIONS

The Ni(II) and Co(II) wt azurin metalloderivatives from both *P. aeruginosa* and *A. denitrificans* present almost the same ^{1}H NMR spectrum as expected for a similar coordination gometry. Signals from the five possible metal ligands are assigned. Cobalt(II) and nickel(II) are clearly coordinated to the Cys112, His46, His117, and Gly45 side chains. Additionally, some signals from the Met121 residue present large isotropic shifts, suggesting a weak coordination of this residue.

The spectra of Ni(II) and Co(II) M121Q azurin confirm the assignments of the Met121 signals in the wt metalloderivatives. The characteristics of the assigned resonances indicate that the metal center is tetrahedral for both Ni(II) and Co(II) M121Q azurins. The metal has moved toward the Gln121 axial position and coordinates to this residue through the carbonyl O side chain, while the Gly45 O is not coordinated. This tetrahedral character seems to be less clear in the case of the Ni(II) derivative than for the Co(II) one.

The spectra from both Co(II) stellacyanin and Co(II) M121Q are remarkably similar for those signals corresponding to the cysteine and histidine ligands. Although a more complete assignment of the former is needed to compare the signals of the fourth ligand, the data presented here constitute additional evidence for a glutamine side chain as the axial ligand in stellacyanin (Romero et al., 1993; Thomann et al., 1991; Fields et al., 1991).

ACKNOWLEDGMENT

Dr. A. Vila is gratefully acknowledged for helpful discussions.

REFERENCES

Adman, E. T. (1985) in *Topics in Molecular and Structural Biology: Metalloproteins* (Harrison, P. M., Ed.), Vol. 6, part 1, pp 1−42, Verlag Chemie, Weinheim.
Adman, E. T., & Jensen, L. H. (1981) *Isr. J. Chem. 21*, 8−12.
Arvidsson, R. H. A., Nordling, M., & Lundberg, L. G. (1989) *Eur. J. Biochem. 179*, 195−200.
Baker, E. N. (1988) *J. Mol. Biol. 203*, 1071−1095.
Banci, L., Bertini, I., Luchinat, C., & Piccioli, M. (1990) *FEBS Lett. 272*, 175−180.
Bax, A., & Davis, D. G. J. (1985) *J. Magn. Reson. 65*, 355−360.
Bertini, I., & Luchinat, C. (1984) in *Advances in Inorganic Biochemistry* (Eichorn, G. L., & Marzilli, L. G., Eds.) pp 71−111, Elsevier, New York.
Bertini, I., & Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, The Benjamin/Cummings Publishing Co., Menlo Park.
Bertini, I., Turano, P., & Vila, A. (1993) *Chem. Rev. 93*, 2833−2932.
Blackwell, K. A., Anderson, B. F., & Baker, E. N. (1994) *Acta Crystallogr. D50*, 263−270.
Blaszak, J. A., Ulrich, E. L., Markley, J. L., & McMillin, D. R. (1982) *Biochemistry 21*, 6253−6258.
Canters, G. W. (1987) *FEBS Lett. 212*, 168−172.
Canters, G. W., & van de Kamp, M. (1992) *Curr. Opin. Struct. Biol. 2*, 859−869.
Canters, G. W., & Gilardi, G. (1993) *FEBS Lett. 325*, 39−48.
Chapman, S. K. (1991) in *Perspectives on Bioinorganic Chemistry* (Hay, R. W., Dilworth, J. R., & Nolan, K. B., Eds.) Vol. 1, pp 95−140, Jai Press Ltd., London.
Chen, Z., de Ropp, J. S., Hernández, G., & La Mar, G. N. (1994) *J. Am. Chem. Soc. 116*, 8772−8783.
Dahlin, S., Reinhammar, B., & Ångström, J. (1989) *Biochemistry 28*, 7224−7233.
Danielsen, E., Bauer, R., Hemmingsen, L., Andersen, M. L., Bjerrum, M. J., Butz, T., Tröger, W., Canters, G. W., Hoitink, C. W. G., Karlsson, G., Hansson, Ö., & Messerschmidt, A. (1995) *J. Biol. Chem. 270*, 573−580.
den Blaauwen, T., & Canters, G. W. (1993) *J. Am. Chem. Soc. 115*, 1121−1129.

Di Bilio, A. J., Chang, T. K., Malmström, B. G., Gray, H. B., Karlsson, B. G., Nordling, M., Pascher, T., & Lundberg, L. G. (1992) *Inorg. Chim. Acta 198*−*200*, 145−148.
Fields, B. A., Guss, J. M., & Freeman, H. C. (1991) *J. Mol. Biol. 222*, 1053−1065.
Germanas, J. P., Di Bilio, A. J., Gray, H. B., & Richards, J. H. (1993) *Biochemistry 32*, 7698−7702.
Gray, H. B., & Malmström, B. G. (1983) *Comments Inorg. Chem. 2*, 203−209.
Hill, H. A. O., Smith, B. E., & Storm, C. B. (1976) *Biochem. Biophys. Res. Commun. 70*, 783−790.
Hoitink, C. W. G., & Canters, G. W. (1992) *J. Biol. Chem. 267*, 13836−13842.
Hoitink, C. W. G., Driscoll, P. C., Hill, H. A. O., & Canters (1994) *Biochemistry 33*, 3560−3571.
Inubushi, T., & Becker, E. D. (1983) *J. Magn. Reson. 51*, 128−133.
La Mar, G. N., & de Ropp, J. S. (1993) in *NMR of paramagnetic molecules* (Berliner, L. J., & Reuben, J., Eds.) Vol. 12, pp 1−78. Plenum Press Dir Plenum Publishing Corp., New York.
La Mar, G. N., Horrocks, W. D., & Holm, R. H. (1973) *NMR of Paramagnetic Molecules*, Academic Press, New York.
Lum, V. & Gray, H. B. (1981) *Isr. J. Chem. 21*, 23−25.
McMillin, D. R., Rosemberg, R. C., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4760−4762.
Mizoguchi, T. J., Di Bilio, A. J., Gray, H. B., & Richards, J. H. (1992) *J. Am. Chem. Soc. 114*, 10076−10078.
Moratal, J. M., Salgado, J., Donaire, A., Jiménez, H. R., & Castells, J. (1993a) *J. Chem. Soc., Chem. Commun.*, 110−112.
Moratal, J. M., Salgado, J., Donaire, A., Jiménez, H. R., Castells, J., & Martínez-Ferrer, Ma. J. (1993b) *Magn. Reson. Chem. 31*, 541−546.
Moratal, J. M., Salgado, J., Donaire, A., Jiménez, H. R., & Castells, J. (1993c) *Inorg. Chem. 32*, 3587−3588.
Moratal, J. M., Romero, A., Salgado, J., Perales-Alarcón, A., & Jiménez, H. R. (1995) *Eur. J. Biochem. 228*, 653−657.
Nar, H., Messerchmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991a) *J. Mol. Biol. 218*, 427−447.
Nar, H., Messerchmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1991b) *J. Mol. Biol. 221*, 765−772.
Nar, H., Huber, R., Messerschmidt, A., Filippou, A. C., Barth, M., Jaquinod, M., van de Kamp, M., & Canters, G. W. (1992a) *Eur. J. Biochem. 205*, 1123−1129.
Nar, H., Messerschmidt, A., Huber, R., van de Kamp, M., & Canters, G. W. (1992b) *FEBS Lett. 306*, 119−124.
Neuhaus, D., & Williamson, M. (1989) *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH Publications, New York.
Pascher, T., Karlsson, G. B., Nordling, M., Malmström, B., & Vänngård, T. (1993) *Eur. J. Biochem. 212*, 289−296.
Piccioli, M., Luchinat, C., Mizoguchi, T. J., Ramírez, B. E., Gray, H. B., & Richards, J. H. (1995) *Inorg. Chem. 34*, 737−742.
Romero, A., Hoitink, C. W. G., Nar, H., Huber, R., Messerschmidt, A., & Canters, G. W. (1993) *J. Mol. Biol. 229*, 1007−1021.
Rosenberg, R. C., Root, C. A., Wang, R.-H., Cerdonio, M., & Gray, H. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 161−163.
Salgado, J., Jiménez, H. R., Donaire, A., & Moratal, J. M. (1995) *Eur. J. Biochem. 231*, 358−369.
Shepard, W. E. B., Anderson, B. F., Lewandoski, D. A., Norris, G. E., & Baker, E. N. (1990) *J. Am. Chem. Soc. 112*, 7817−7819.
Shepard, W. E. B., Kingston, R. L., Anderson, B. F., & Baker, E. N. (1993) *Acta Crystallogr. D49*, 331−343.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286−292.
Strong, C., Harrison, S. L., & Wesley, Z. (1994) *Inorg. Chem. 33*, 606−608.
Sykes, A. G. (1991) *Adv. Inorg. Chem. 36*, 377−408.
Tennent, D. L., & McMillin, D. R. (1979) *J. Am. Chem. Soc. 101*, 2307−2311.
Thomann, H., Bernardo, M., Baldwin, M. J., Lowery, M. D., & Solomon, E. I. (1991) *J. Am. Chem. Soc. 113*, 5911−5913.
van de Kamp, M., Silvestrini, M. C., Brunori, M., van Beeumen, J., Hali, F. C., & Canters, G. W (1990a) *Eur. J. Biochem. 194*, 109−118.
van de Kamp, M., Hali, F. C., Rosato, N., Finazzi-Agró, A., & Canters, G. W. (1990b) *Biochim. Biophys. Acta 1019*, 283−292.
van de Kamp, M., Canters, G. W., Andrew, C. R., Sanders-Loehr, J., Bender, C., & Peisach, J. (1993) *Eur. J. Biochem. 218*, 229−238.
van Driessche, G., Dennison, C., Sykes, A. G., & van Beeumen, J. (1995) *Protein Sci. 4*, 209−227.
van Pouderoyen, G., Mazundar, S., Hunt, N. I., Hill, H. A. O., & Canters, G. W. (1994) *Eur. J. Biochem. 222*, 583−588.
Vila, A. J. (1994) *FEBS Lett. 355*, 15−18.
Wuttke, D. S., & Gray, H. B. (1993) *Curr. Opin. Struct. Biol. 3*, 555−563.

BI951748A