Studies of Individual Carbon Sites of Azurin from *Pseudomonas aeruginosa* by Natural-Abundance Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The environments of the aromatic residues (and of the single arginine residue) of azurin from *Pseudomonas aeruginosa* are investigated by means of natural-abundance ^{13}C Fourier transform NMR spectroscopy. In the case of the diamagnetic Cu(I) azurin, all 17 nonprotonated aromatic carbons (and C^{ζ} of Arg-79) yield narrow resonances. Furthermore, a single-carbon amide carbonyl resonance with an unusual chemical shift (peak *x*) is observed. The pH dependence of chemical shifts is used to identify the resonances of C^{γ} of titrating histidines, and of C^{γ} and C^{ζ} of the two tyrosines. The resonances of C^{γ} and $C^{\delta 2}$ of the single tryptophan residue (and C^{ζ} of Arg-79) are also identified. The pK_a values of the two tyrosines are different from each other and higher than typical values of "solvent-exposed" tyrosine residues. Two of the four histidine residues do not titrate (in the pH range 4 to 11). The resonance of C^{γ} of one histidine exhibits a pH titration with fast proton exchange behavior and a pK_a of 7.5 ± 0.2. The direction of the titration shift indicates that the imidazole form of this histidine is the $N^{\delta 1}$–H tautomer. The C^{γ} resonance of the other titrating histidine exhibits slow exchange behavior with a pK_a of about 7. The imidazole form of this histidine is the $N^{\epsilon 2}$–H tautomer. When going to the paramagnetic Cu(II) protein, only 11 of the 19 carbons mentioned above yield resonances that are narrow enough to be detected. Also, some of the observed resonances exhibit significant paramagnetic broadening. A comparison of spectra of fully reduced azurin, mixtures of reduced and oxidized azurin, and fully oxidized azurin yields the following information. (i) Peak *x* arises from an amide group that probably is coordinated to the copper. (ii) The two nontitrating histidine residues are probably copper ligands, with $N^{\delta 1}$ coordinated to the metal. (iii) The side chains of Arg-79 and the two tyrosine residues are not coordinated to the copper, and Trp-48 is probably not a ligand either. (iv) The γ carbons of Trp-48, the tyrosine with the lower pK_a, the titrating histidine with slow exchange behavior, and three or four of the six phenylalanine residues are sufficiently close to the copper to undergo significant paramagnetic broadening in the spectrum of oxidized azurin.

The copper environments of copper-containing proteins have been classified as "blue", "nonblue", or "EPR nondetectable" (Malkin and Malmström, 1970; Malkin, 1973; Fee, 1975). In recent years, considerable progress has been made toward an understanding of the unusual coordination environment of the "blue" copper centers (see Fee, 1975; Solomon et al., 1976; and references cited therein). It appears that the "blue" copper has a distorted tetrahedral coordination geometry (Siiman et al., 1976; Solomon et al., 1976). One of the ligands is sulfur (Solomon et al., 1975). In general, the sulfhydryl group of a cysteine residue is involved in the Cu–S linkage (Katoh and Takamiya, 1964; Finazzi-Agrò et al., 1970; Morpurgo et al., 1972; Graziani et al., 1974; McMillin et al., 1974a,b; Solomon et al., 1975), but there may be exceptions (Fee, 1975). A backbone peptide nitrogen or (less likely) oxygen is coordinated to the copper of bean plastocyanin (Hare et al., 1976; Solomon et al., 1976), and this type of coordination may be a property of "blue" copper centers of other proteins (Solomon et al., 1976; Siiman et al., 1974, 1976; Miskowski et al., 1975).

Azurin, the subject of this report, has a molecular weight of about 15 000 and contains one copper atom per molecule (Sutherland and Wilkinson, 1963; Ambler, 1963; Ambler and Brown, 1967). Several azurins from different bacterial strains have been sequenced (Ambler, 1971; Ryden and Lundgren, 1976). Research aimed at identification of the copper environment of azurin has not been as intense as in the case of plastocyanin (Solomon et al., 1976, and references cited therein). The single copper ion of azurin occupies an internal site, inaccessible to solvent (Brill et al., 1968; Finazzi-Agrò et al., 1970; Koenig and Brown, 1973; Boden et al., 1974). Various observations suggest that the single sulfhydryl group (of Cys-112) is a copper ligand (Fee, 1975; Finazzi-Agrò et al., 1970; McMillin et al., 1974b). Resonance Raman spectra strongly suggest that at least one ligand is a nitrogen or oxygen which is not part of an aromatic ring (Miskowski et al., 1975). The pH dependence of proton NMR[1] spectra of apo-, Cu(I), and Cu(II) azurins from *Pseudomonas aeruginosa* indicates that two of the four histidine residues are not copper ligands (Hill et al., 1976).

Proton-decoupled natural-abundance ^{13}C NMR spectra of small native proteins yield numerous narrow single-carbon resonances of nonprotonated aromatic carbons (Allerhand et al., 1973a; Oldfield et al., 1975a,b). In this report, we use ^{13}C NMR spectra of the reduced and oxidized forms of *P. aeruginosa* azurin to obtain information about properties of the aromatic amino acid residues of this protein.

Experimental Procedure

Materials. A culture of *P. aeruginosa* was kindly supplied by Dr. David C. Wharton of the Department of Biochemistry,

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[1] Abbreviations used are: Me_4Si, tetramethylsilane; PRFT, partially relaxed Fourier transform; NMR, nuclear magnetic resonance.

University of Texas, San Antonio, Texas. The organism originated from the Section of Microbiology, Cornell University, Ithaca, N.Y. It was grown in large scale by the Grain Processing Corp., Muscatine, Iowa, under the conditions described by Gudat et al. (1973). Azurin was prepared by a modification (Ugurbil and Bersohn, 1977) of the method of Ambler (1963). The purity of the preparation was monitored by means of the ratio of the absorption at 625 nm of a sample oxidized with ferricyanide to the absorption at 280 nm of an untreated sample (Ambler, 1963). This ratio was 0.58 for the protein samples used in this study. The final product was filtered through VM-type filters (from the Millipore Corp., Bedford, Mass.), lyophilized, and stored at −10 °C. Total azurin yield was 1.5 g from 8 kg of frozen wet paste of bacteria.

Methods. Azurin was reduced by addition of a small excess of solid sodium dithionite; the by-products of dithionite oxidation and decomposition were not removed. Samples of fully oxidized protein used for studying the effect of pH were prepared by addition of excess potassium ferricyanide. The ^{13}C NMR spectra of oxidized azurin shown in the figures were obtained after small molecules were removed from a sample of the Cu(II) protein on a Sephadex G-25 column (equilibrated with 0.05 M ammonium acetate buffer, pH 5.3), the eluted azurin solution was concentrated by ultrafiltration, and potassium ferricyanide was added (3:1 molar ratio of protein-ferricyanide). Samples of partly oxidized azurin were prepared by addition of sodium dithionite to this sample of oxidized azurin. The pH of each sample was measured at room temperature, in most cases both before and after the NMR experiment. The pH was adjusted with 0.5 M or 1.0 M HCl, or with about 2 M NaOH. Azurin concentrations were determined spectrophotometrically (after oxidation), using $E_{625}^{1\%} = 3500$ (Brill et al., 1968).

An azurin solution in D_2O was prepared as follows. About 15 ml of a protein solution in H_2O was diluted to 115 ml with D_2O and concentrated to about 10 ml by ultrafiltration at 25 °C. The solution was then diluted to 70 ml with D_2O and concentrated again to 10 ml. The latter procedure was repeated twice, and then the solution was diluted to 200 ml with D_2O and incubated at 36 °C for 1 day each at pH meter readings of 10.3 and 6. The protein solution was then concentrated to 10 ml, diluted to 60 ml with D_2O, and concentrated again to 10 ml (final protein concentration was 6 mM).

Natural-abundance ^{13}C Fourier transform NMR spectra were obtained at 15.18 MHz and 31 °C, with the use of 20-mm spinning sample tubes (Allerhand et al., 1973b), as described previously (Oldfield et al., 1975a). A spectral width of 3788 Hz was used. Time-domain data were accumulated in 4096 or 8192 addresses of a Nicolet 1085 computer. Fourier transformation was carried out on 16 384 time-domain data points by placing the appropriate number of addresses with a zero value at the end of each block of accumulated data points. Most spectra were recorded under conditions of noise-modulated off-resonance proton decoupling (Wenkert et al., 1969), as described previously (Oldfield et al., 1975a,b). Broad methine aromatic carbon resonances were removed from some spectra by means of the convolution-difference procedure (Campbell et al., 1973), as described previously (Oldfield et al., 1975b). The values of τ_1, τ_2, and K (defined as in Campbell et al., 1973) were 0.36 s, 0.033 s, and 0.9, respectively.

Chemical shifts were obtained digitally and are reported in parts per million downfield from the ^{13}C resonance of Me_4Si. Estimated accuracy is ±0.05 ppm for reduced azurin and ±0.1 ppm for the oxidized protein. The resonance of dilute aqueous dioxane (at 67.8_6 ppm) was used as an internal reference. Integrated intensities were measured digitally and have an estimated error of ±25% or ±0.3 carbons, whichever is greater.

TABLE I: Chemical Shifts of Some ^{13}C Resonances of *P. aeruginosa* Azurin at pH 5.2.[a]

Assignment	Peak Designation[b]	Chemical Shift Reduced	Chemical Shift Oxidized
Amide carbonyl	*x*	166.7_5	
Arg-79 C^{ζ}	1	158.5_0	158.4
Tyr C^{ζ}	2[c]	155.7_0	155.7
Tyr C^{ζ}	3[d]	154.8_4	155.2
	4[e,f]	138.5_2	138.1
	5	137.6_2	
6 Phe C^{γ}	6	137.2_4	
2 His C^{γ}	7[f,g]	136.8_8	136.9
Trp-48 $C^{\epsilon 2}$	8[f]	136.3_6	136.4
	9	135.6_6	
Tyr C^{γ}	10[d]	133.9_8	
His C^{γ}	11	130.6_9	
Tyr C^{γ}	12[c]	129.6_1	129.6
Trp-48 $C^{\delta 2}$	13	128.5_0	128.1
His C^{γ}	14	127.8_3	127.6
Trp-48 C^{γ}	15	113.2_7	112.5

[a] Values for reduced and oxidized azurin were obtained from the spectra of Figures 1A (pH 5.2) and 1B (pH 5.3), respectively. [b] Peak designations are those of Figures 1, 2, and 4. [c] Peaks 2 and 12 arise from the same tyrosine residue. [d] Peaks 3 and 10 arise from the same tyrosine residue. [e] Three-carbon resonance in the spectrum of reduced azurin (see Figure 1A and Table II); single-carbon resonance in the spectrum of the oxidized protein (Figure 1B). [f] One-to-one connections between peaks 4, 7, and 8 of oxidized azurin and the corresponding peaks of the reduced protein are tentative (see text). [g] Two-carbon resonance in Figures 1A and 1B.

The partially relaxed Fourier transform (PRFT) NMR spectrum (Allerhand et al., 1971) of the reduced protein was obtained on the sample of azurin in D_2O described above, with the use of 65 536 accumulations, a recycle time of 2.2 s, and an interval of 0.5 s between each 180° radiofrequency pulse and the following 90° pulse.

Spectrum of Reduced Azurin

The ^{13}C NMR spectrum of a native diamagnetic protein can be divided (Allerhand et al., 1973a; Oldfield et al., 1975a) into the region of aliphatic carbons (about 10 to 75 ppm downfield from Me_4Si), the region of aromatic carbons and C^{ζ} of arginine residues (about 100 to 160 ppm), and the region of carbonyl resonances (about 170 to 190 ppm). In the region of aromatic carbons and C^{ζ} of arginine residues, methine carbons yield broad bands, while nonprotonated carbons yield relatively narrow resonances (Allerhand et al., 1973a). The observation of these narrow resonances is facilitated by the use of noise-modulated off-resonance proton decoupling (Oldfield et al., 1975b), and by application of the convolution-difference procedure (Campbell et al., 1973) for the removal of the broad bands of methine aromatic carbons (Oldfield et al., 1975b). Figure 1A shows the aromatic region (and the upfield edge of the carbonyl region) in the convolution-difference spectrum of reduced azurin at pH 5.2. The single tryptophan (Trp-48), four histidines, two tyrosines, six phenylalanines, and single arginine (Arg-79) of azurin from *P. aeruginosa* (Ambler, 1971; Ryden and Lundgren, 1976) have a total of 18 nonprotonated side-chain carbons. These carbons give rise to peaks 1–15 of Figure 1A. Chemical shifts at pH 5.2 are given in Table I. The pH dependence of these chemical shifts (discussed

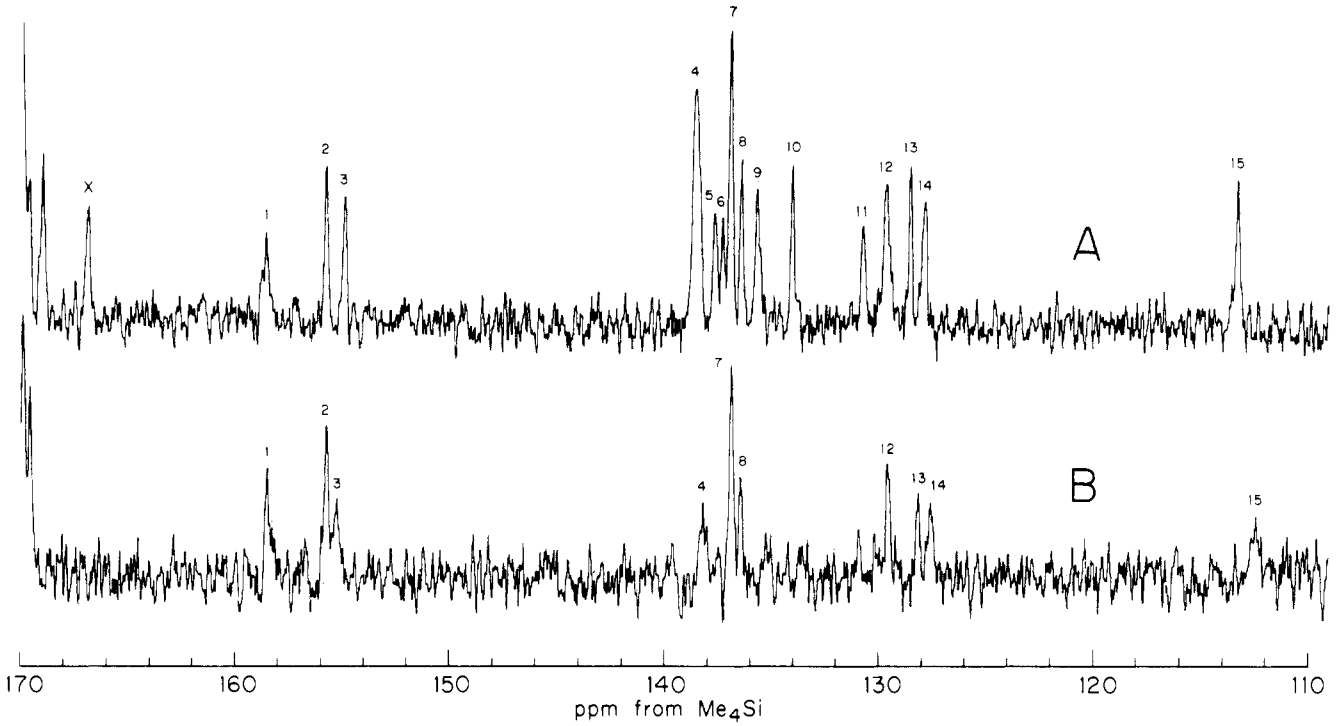

FIGURE 1: Region of aromatic carbons (and C^{ζ} of arginine) and upfield edge of the carbonyl region in convolution-difference natural-abundance ^{13}C NMR spectra of reduced and oxidized *P. aeruginosa* azurin in H_2O (0.05 M ammonium acetate, 31 °C). Each spectrum was recorded at 15.18 MHz, under conditions of noise-modulated off-resonance proton decoupling, with 8192 time domain addresses, and a recycle time of 1.105 s. (A) Reduced azurin (7.4 mM, pH 5.2) after 65 536 accumulations. Peaks assigned to C^{ζ} of Arg-79 and the 17 nonprotonated aromatic carbons are numbered consecutively. (B) Oxidized azurin, (5.8 mM, pH 5.3) after 131 072 accumulations. Peak designations are discussed in the text.

TABLE II: Integrated Intensities of Some Nonprotonated Carbon Resonances of Reduced *P. aeruginosa* Azurin at Various pH Values.

Peak Designation[a]	Integrated Intensity[b]							
	3.69	5.20	6.62	7.15	7.99	9.22	10.80	11.12
x	1.1	0.9	0.7	1.2	0.7	0.7	1.1	1.2
1	1.2	1.0	1.1	1.1	0.9	1.5	1.0	1.5[c]
2	1.1	0.9	1.3	1.2	0.9	1.0	1.2	1.2
3	1.0	0.9	1.1	1.2	0.9	1.1	1.1	*c*
4	3.2	3.0	2.7	3.1	3.0	2.9	3.0	2.5[d]
5	0.5	0.9	1.3	1.1	1.7	1.8	1.8	1.7
6	0.6	0.7	0.7					
7	2.0	2.0	2.0	2.3	3.0	2.6	2.7	2.9
8	0.8	1.0	0.9	1.1	1.0	1.2	1.1	1.5
9	1.0	1.2	1.2	0.9	1.7	0.8	0.7	1.3
10	1.3	1.0	1.3	1.2	1.2	0.9	0.8	1.1
11	1.0	0.7	1.0		*e*	*e*	*e*	*e*
12	1.2	1.5	0.9	1.4	1.0	1.0	1.4	1.2
13	1.0	0.9	0.9	0.7	0.8	1.0	0.8	1.0
14	1.2	1.1	0.9	1.0	0.8	0.8	0.9	1.0
15	0.9	1.1	0.9	1.0	1.1	0.9	1.2	1.0

[a] Peak designations are those of Figures 1, 2, and 4 and Table I. [b] Obtained digitally from the spectra whose chemical shifts are given in Figure 2, but after application of the convolution-difference method (see Experimental Procedure). Column headings indicate pH values. The arithmetic average of the intensities of the 15 carbons which give rise to peaks 1–6, 8–10, and 12–15 was set to unity in each case. The estimated error is ±0.3 or ±25%, whichever is greater. [c] Peak 3 moves downfield of peak 2 above pH 10 and probably merges with peak 1 at pH 11.1 (see Figure 2). [d] Consists of one single-carbon and one two-carbon resonances, with intensities of 0.8 and 1.7 carbons, respectively (see Figure 2). [e] Contributes to peak 7 (see text).

below) is shown in Figure 2. Integrated intensities at various pH values are given in Table II.[2] At pH $\lesssim$7, peaks 4 and 7 arise from three and two carbons, respectively, and the other 13 peaks designated by numbers are all single-carbon resonances (Table II). At pH $\gtrsim$8, peak 11 is not detected and peak 7 becomes a three-carbon resonance (Table II).

On the basis of their chemical shifts we can assign peaks 1–3 (Figure 1A) to C^{ζ} of Arg-79 and the two tyrosine residues (see Figure 5 of Oldfield et al., 1975b). Similarly, peaks 4–14 must arise from C^{γ} of the six phenylalanines, two tyrosines, and four histidines, and $C^{\epsilon 2}$ and $C^{\delta 2}$ of Trp-48. Peak 15 is assigned to

[2] It has been shown (Oldfield et al., 1975a) that, in general, the resonances of all nonprotonated aromatic carbons of a native globular protein should have about the same intensity, under the spectral and sample conditions of Figure 1.

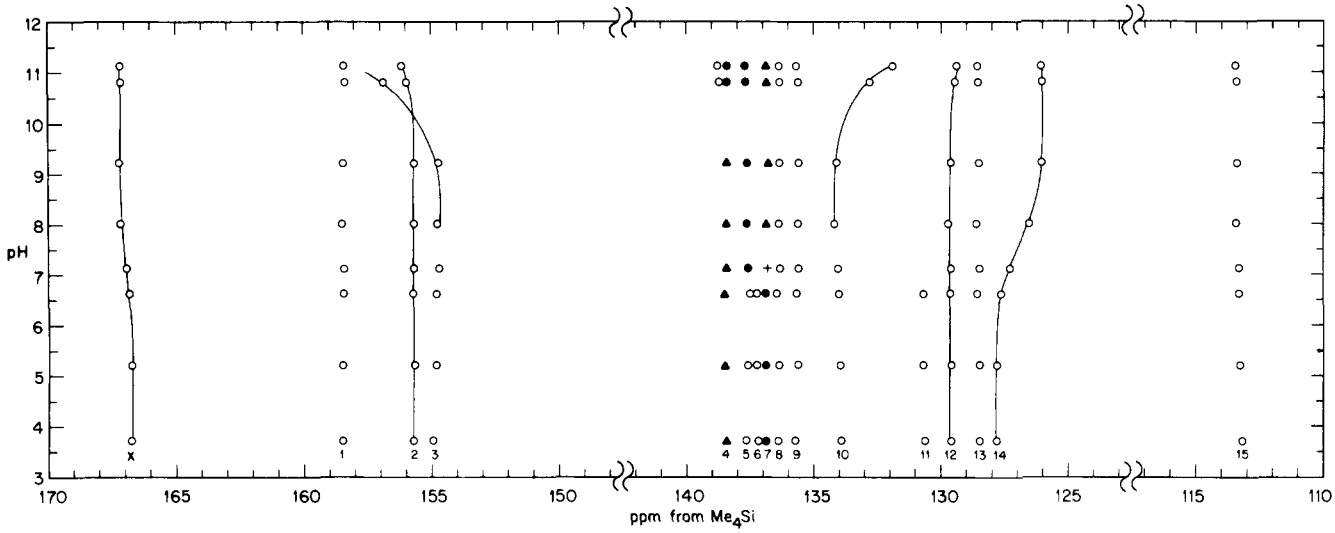

FIGURE 2: Effect of pH on the chemical shifts of some nonprotonated carbons of reduced *P. aeruginosa* azurin in H_2O (0.05 M ammonium acetate, 31 °C). Peak designations are those of Figure 1A and Tables I and II. Open circles, closed circles, and triangles indicate peaks that arise from 1, 2, and 3 carbons, respectively. The symbol + indicates a peak with an intensity intermediate between 2 and 3 carbons (see text). Protein concentrations were in the range 6.6–7.4 mM. Each spectrum was obtained at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, with 30 000 or more accumulations, a recycle time of 1.105 s, and a digital broadening of 0.88 Hz. The solid lines are best-fit titration curves, assuming a single pK_a in each case (the chemical shifts of C^ζ and C^γ of the phenoxy form of each tyrosine residue were constrained to values 10.4 ppm downfield and 6.2 ppm upfield, respectively, of the corresponding values of the phenolic form of the residue; see text).

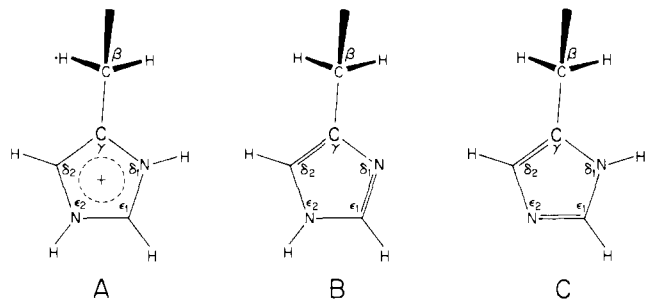

FIGURE 3: Histidine side chain in the imidazolium form (A), $N^{\epsilon 2}$–H imidazole form (B), and $N^{\delta 1}$–H imidazole form (C).

C^γ of Trp-48. The pH dependence of chemical shifts can be used to identify the resonances of titratable histidines (Oldfield et al., 1975b) and tyrosines (Norton and Bradbury, 1974; Wilbur and Allerhand, 1976). We shall consider the tyrosine residues first. On the basis of the pH dependence of their chemical shifts (Figure 2), we assign peaks 3 and 10 (Figure 1A) to C^ζ and C^γ, respectively, of one tyrosine residue, and peaks 2 and 12 to C^ζ and C^γ of the other tyrosine residue. By elimination, peak 1 is assigned to C^ζ of Arg-79. In order to obtain reliable pK_a values for the tyrosine residues, it would be necessary to continue the titration to pH values higher than 11.1. We obtained crude estimates of the pK_a values by applying the Henderson–Hasselbalch treatment (for a single pK_a value) to the chemical shift data at pH >8; titration shifts equal to those of tyrosine residues in small peptides (Norton and Bradbury, 1974) were assumed. This procedure yielded pK_a values of about 11.4 and 12.5 for the tyrosine residues which give rise to peaks 3 and 10, and peaks 2 and 12, respectively. It appears that the pK_a values of the two tyrosine residues in azurin are different from each other, and higher than those observed for tyrosine residues in small peptides (Norton and Bradbury, 1974).

On the basis of chemical shift considerations (see Figure 5 of Oldfield et al., 1975b) and the assignment of peaks 10 and 12 to the tyrosine residues (see above), we identify peaks 11, 13, and 14 as resonances of C^γ of two histidines and $C^{\delta 2}$ of Trp-48. At pH $\lesssim$9, the only resonances of Figure 1A which are strongly affected by changes in pH are peaks 11 and 14. Consider first peak 11. At pH $\lesssim$6 this peak has a pH-independent chemical shift (Figure 2) and an intensity of one carbon (Table II). Peak 11 becomes undetectable at pH $\gtrsim$7 (Figure 2). Also, peak 7 grows from a two-carbon resonance at pH $\lesssim$6 to a three-carbon resonance at pH $\gtrsim$8 (Table II). No other resonance appears as peak 11 disappears at high pH (Table II). We conclude that peak 11 shifts downfield to the position of peak 7 as the pH is raised. The direction and magnitude (6.2 ppm) of this shift are similar to those observed for C^γ of histidine residues in the imidazolium form (Figure 3A) upon deprotonation at $N^{\delta 1}$ (Reynolds et al., 1973; Deslauriers et al., 1974). We assign peak 11 (at pH $\lesssim$6) to C^γ of a histidine residue in the imidazolium form, and one component of peak 7 (at pH $\gtrsim$8) to the imidazole ($N^{\epsilon 2}$–H tautomer) form of this residue. The pH-dependent intensities of peaks 7 and 11 (Table II) indicate that the pK_a of this histidine is in the range 6.8–7.8.

We now consider the possible causes of our failure to detect the C^γ resonance of the histidine under consideration near neutral pH. One possible explanation is that a paramagnetic ion binds to the histidine in question (only at pH $\approx$7) and renders the C^γ resonance too broad for detection (Wasylishen and Cohen, 1974). A more attractive explanation is that exchange between the imidazole and imidazolium states is not sufficiently fast (relative to the difference in the chemical shifts of the two states) to yield a narrow exchange-averaged resonance (see section 2.17 of Dwek, 1973). If the rate of exchange is slow or comparable to the chemical shift difference between the exchanging species, then splitting and/or broadening of the C^γ resonance of the histidine may render this resonance undetectable (see Figure 2.17 of Dwek, 1973). In general, exchange between the imidazole and imidazolium forms of a histidine residue is fast (Eigen et al., 1960). However, there are precedents for slow overall interchange between the imidazole and imidazolium forms of some relatively "buried"

histidines in proteins (Markley, 1975, and references cited therein).

Peak 14 titrates *upfield* at pH $\gtrsim$6 (Figure 2). The total titration shift is about 1.8 ppm. The pH dependence of the chemical shift yields a pK_a of 7.5 ± 0.2, which is consistent with the assignment of this resonance to C^{γ} of a titratable histidine residue. However, the direction of the titration shift is opposite to that usually observed for C^{γ} of histidine residues (Reynolds et al., 1973; Deslauriers et al., 1974; Oldfield et al., 1975b). Most often, the imidazolium form (Figure 3A) of a histidine residue of a protein deprotonates at $N^{\delta 1}$ to yield the $N^{\epsilon 2}$–H imidazole tautomer (Figure 3B). Such deprotonation results in a downfield shift of the C^{γ} resonance (Reynolds et al., 1973). However, if deprotonation occurs at $N^{\epsilon 2}$ to yield the $N^{\delta 1}$–H tautomer (Figure 3C), then C^{γ} should undergo an *upfield* shift of about 2 ppm (Reynolds et al., 1973; Deslauriers et al., 1974). Thus, we believe that peak 14 arises from C^{γ} of a histidine residue which deprotonates at $N^{\epsilon 2}$, with a pK_a of 7.5. Titratable histidine residues in the $N^{\delta 1}$–H tautomeric form have been observed in other proteins, e.g., His-12 of bovine pancreatic ribonuclease A, which has $H^{\delta 1}$ hydrogen bonded to the carbonyl of Thr-45 (Patel et al., 1975). Proton NMR has been used extensively to study the titration of histidine residues of proteins (Markely, 1975). However, the titration behavior of the proton chemical shifts does not yield information about the tautomeric state of the imidazole ring (see, for example, Figure 4 of Markley, 1975).

By elimination, peak 13 can be assigned to $C^{\delta 2}$ of Trp-48. We used the PRFT method (Oldfield and Allerhand, 1975a; Oldfield et al., 1975b), to check this assignment. The PRFT method takes advantage of the fact that the T_1 value of $C^{\delta 2}$ of a tryptophan residue should be longer than the T_1 values of C^{γ} of tyrosine and histidine residues (Oldfield et al., 1975a). Comparison of a PRFT spectrum (see Experimental Procedure) with the normal spectrum (recorded with only 90° pulses) of azurin in D_2O (at pH meter reading 6.6) suggested that peak 13 has a significantly longer T_1 value than peaks 12 and 14 (as mentioned above, peak 11 was not observed near neutral pH). Note that peak 13 is narrower than peaks 11, 12, and 14 (Figure 1A). This observation provides further support for our assignment of peak 13, because the same factors which lead to a relatively long T_1 for $C^{\delta 2}$ of a tryptophan residue should also result in a relatively small natural line width for this carbon (Oldfield et al., 1975a).

Peaks 4–9 of Figure 1A (which arise from a total of nine carbons, see Table II) must represent the resonances of $C^{\epsilon 2}$ of Trp-48, C^{γ} of all six phenylalanines, and C^{γ} of the two remaining histidines. The absence of any major changes in the chemical shifts of peaks 4–9 as a function of pH (Figure 2) indicates that the two histidine residues under consideration do not titrate in the pH range 4–11. The chemical shifts of peaks 4–9 suggest that both histidines exist as the imidazole $N^{\epsilon 2}$–H tautomer (Reynolds et al., 1973; Deslauriers et al., 1974). In principle, it is possible to identify the resonances of $C^{\epsilon 2}$ of tryptophan residues with the use of PRFT spectra of proteins in D_2O (Oldfield et al., 1975b). Our PRFT spectrum of reduced azurin (see above) *suggests* that $C^{\epsilon 2}$ of Trp-48 contributes to one of the two multiple-carbon resonances (peak 4 or 7).

At the upfield edge of the carbonyl region there is a single-carbon resonance (peak *x* of Figure 1A) with interesting properties. This peak is slightly upfield of the typical carbonyl region in ^{13}C NMR spectra of diamagnetic proteins (see, for example, Oldfield et al., 1975a,b). The chemical shift of peak *x* shows a slight pH dependence (Figure 2), with a pK_a of 7.1 ± 0.3. However, the small change in chemical shift upon titration (0.4 ppm) and the value of the chemical shift are not consistent with an assignment to the side-chain carboxyl of an aspartic acid or glutamic acid residue (Keim et al., 1973). Also, the pK_a is much higher than that of a "normal" carboxylic acid. We conclude that peak *x* arises from an amide carbonyl of the backbone or a side chain. Although the "pK_a" of peak *x* is similar to typical values of N-terminal amino groups (Garner et al., 1973), it is unlikely that peak *x* arises from Ala-1, because the titration shift of only 0.4 ppm (Figure 2) is about $\frac{1}{20}$ of the reported value for the carbonyl of an N-terminal alanine residue (Christl and Roberts, 1972). The slight pH dependence of the chemical shift of peak *x* is probably caused by the ionization of a nearby functional group with a pK_a of about 7 (such as one of the titratable histidines).

The chemical shift of peak *x* is at least 3 ppm upfield from the nearest nonterminal amide carbonyl resonance in reported ^{13}C NMR spectra of diamagnetic proteins (see, for example, Figures 3, 4, and 15 of Oldfield et al., 1975b), and at least 6 ppm upfield from reported values for carbonyls of N-terminal alanine residues of small peptides (Christl and Roberts, 1972). This observation suggests that peak *x* arises from a carbonyl in an unusual environment. Information about the nature of this environment is obtained from spectra of oxidized azurin and mixtures of the two redox states of the protein (see below).

Spectrum of Oxidized Azurin

The convolution-difference ^{13}C NMR spectrum of fully oxidized azurin (at pH 5.3) is shown in Figure 1B. The spectrum of the oxidized protein contains fewer narrow resonances than that of the reduced protein, but we have retained the numbering system of Figure 1A for those peaks of oxidized azurin which can be correlated with peaks of the reduced protein (see below). The chemical shifts of clearly observable resonances in Figure 1B are given in Table I.

The chemical shift of peak 15 of Figure 1B is consistent with an assignment to C^{γ} of Trp-48. Some additional one-to-one connections between resonances in Figures 1A and 1B come from a comparison of the pH dependence of the chemical shifts of the Cu(I) and Cu(II) proteins: The effects of pH on the chemical shifts of peaks 2, 3, 12, and 14 of the oxidized protein are very similar to the effects observed for peaks 2, 3, 12, and 14, respectively, of reduced azurin (Figure 2). Also, by elimination, we assign peaks 1 and 13 of oxidized azurin to the same carbons as peaks 1 and 13, respectively, of the reduced protein. The one-to-one correspondence between peaks 7 (two carbons), 8 (one carbon), and 4 (one carbon) of oxidized azurin and peak 7, peak 8, and one contributor to peak 4, respectively, of the reduced protein are based on invariance of chemical shifts (Table I), and should be considered tentative.

Only 10 of the 17 nonprotonated aromatic carbons yield clearly detectable resonances in our convolution-difference ^{13}C NMR spectrum of oxidized azurin. Furthermore, some of these resonances are considerably broader than the corresponding ones of the reduced protein. We conclude that many aromatic residues are sufficiently close to the Cu(II) to undergo paramagnetic broadening (Dwek, 1973). If an amino acid residue is coordinated to the copper, scalar and dipolar contributions to the broadening must be considered (Espersen and Martin, 1976). If the residue is not a copper ligand, the dipolar contribution should be dominant, and the paramagnetic broadening should be proportional to the inverse sixth power of the carbon–copper distance (Dwek, 1973). The use of paramagnetic broadening to obtain accurate numerical values

of relative (and perhaps even absolute) carbon-copper distances must await much better signal-to-noise ratios than in Figure 1B. However, qualitative information about relative distances of aromatic residues to the copper can be extracted from Figure 1.

Arginine-79 and the tyrosine residue with the higher pK_a must be located far from the copper, because peaks 1, 2, and 12 are not broadened when going from reduced to oxidized azurin. The tyrosine residue with the lower pK_a must be closer to the copper, as judged from the disappearance of its C^{γ} resonance (peak 10) and the broadening of its C^{ζ} resonance (peak 3) in the spectrum of oxidized azurin (Figure 1B). The orientation of the phenolic ring must be such that the copper is closer to C^{γ} than to C^{ζ}. Our results eliminate the possibility that the hydroxyl group of either tyrosine residue is very close to the copper. The tryptophan residue is located sufficiently close to the Cu(II) for the C^{γ} resonance to be broadened by about 5 Hz. However, the paramagnetic broadening of the $C^{\delta 2}$ resonance (peak 13) is $\lesssim 2$ Hz, which implies that the copper is closer to C^{γ} than to $C^{\delta 2}$. The C^{γ} resonance of the titratable histidine which exhibits fast exchange behavior in spectra of reduced azurin (peak 14 of Figures 1 and 2) is clearly detectable in spectra of oxidized azurin (see Figure 1B). The C^{γ} resonance of the titratable histidine which shows slow exchange behavior in spectra of reduced azurin (peak 11 of Figure 1A) is not detected in our spectra of oxidized azurin. On this basis, we conclude that this histidine is closer to the copper than the one that gives rise to peak 14.

Now consider peaks 4–9 of reduced azurin (Figure 1A), which arise from $C^{\epsilon 2}$ of Trp-48 and from C^{γ} of the six phenylalanine and two nontitrating histidine residues. Only four of the nine carbons under consideration yield clearly discernible resonances in the spectrum of oxidized azurin (Figure 1B): One of the three carbons which contribute to peak 4 of reduced azurin yields a clearly detectable (but broadened) resonance in Figure 1B. Only peaks 7 and 8, which represent a total of three carbons, are not detectably broadened by the Cu(II) center. Results presented in the next section indicate that the histidine residues do not contribute to peaks 7 and 8. We conclude that two or three phenylalanines are far from the copper, and that three or four phenylalanines are near the copper.

Peak *x* of reduced azurin is not detected in the spectrum of oxidized azurin, as a result of either a downfield shift or a paramagnetic broadening, probably the latter (see below).

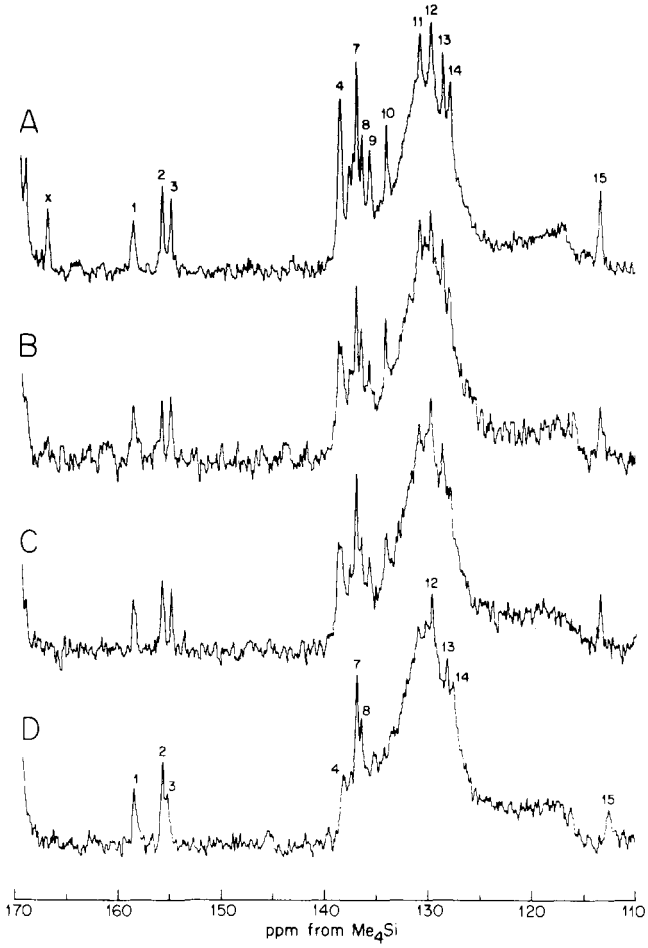

FIGURE 4: Region of aromatic carbons (and C^{ζ} of arginine) and upfield edge of the carbonyl region in natural-abundance ^{13}C NMR spectra of *P. aeruginosa* azurin in H_2O (0.05 M ammonium acetate, 31 °C). Each spectrum was recorded at 15.18 MHz, under conditions of noise-modulated off-resonance proton decoupling, with 8192 time-domain addresses, a recycle time of 1.105 s, and 1.47 Hz digital broadening. The convolution-difference procedure was not applied. Peak designations are those of Figure 1 and Tables I and II. (A) Reduced azurin (7.4 mM, pH 5.2) after 65 536 accumulations (same time-domain data as in Figure 1A); (B) 90:1 ratio of reduced to oxidized azurin, 5.1 mM total protein concentration, pH 5.3, after 49 152 accumulations; (C) 23:1 ratio of reduced to oxidized azurin, 5.2 mM total protein concentration, pH 5.3, after 65 536 accumulations; (D) 5.8 mM oxidized azurin, pH 5.3, after 131 072 accumulations (same time-domain data as in Figure 1B).

Spectra of Mixtures of Reduced and Oxidized Azurin

In our spectra of oxidized azurin, a paramagnetic contribution to the line width outside the range 2–6 Hz is not readily measurable. The lower limit is imposed by the other contributions to the line width (including an instrumental contribution of about 2 Hz), and the upper limit is determined by our signal-to-noise ratios. Thus, the difference between the shortest and longest measurable distance to the copper is only about 20%. As a result, in the previous section we could only classify the carbons as "close to" or "far away" from the copper. In order to get more quantitative information, it is obviously desirable to obtain signal-to-noise ratios that would extend the upper limit of measurable line widths. However, an alternative approach is to obtain a spectrum under conditions which yield a paramagnetic broadening that is only a fraction of the broadening in the spectrum of fully oxidized azurin. If the two redox states of the protein exchange in solution at a sufficiently fast rate (defined below), one can control the paramagnetic contribution to the line width by means of the ratio of concentrations of oxidized and reduced protein (Dwek, 1973).

Figures 4B and 4C show spectra of reduced azurin samples which contain about 1 and 4%, respectively, of the oxidized protein. We have not applied the convolution-difference procedure to these spectra, in order not to degrade the detectability of broad resonances. Figures 4A and 4D are the same spectra of fully reduced and fully oxidized azurin as in Figures 1A and 1B, respectively, but without application of the convolution-difference procedure. Clearly, the spectra of samples of reduced azurin which contain 1 and 4% oxidized protein (Figures 4B and 4C, respectively) differ from the spectrum of fully reduced azurin (Figure 4A). Therefore, exchange between the two redox states is taking place under the sample conditions of Figures 4B and 4C.

If the populations of two exchanging sites differ greatly, then the line width (W_A, in Hz) of the resonance of the dominant site (A) is given by (see eq 2.32 of Dwek, 1973):

$$W_A - W_A^{\circ} = P_B(\pi\tau)^{-1}(Q + R^2)(Q^2 + R^2)^{-1} \quad (1)$$

where $W_A°$ and $W_B°$ are the line widths (in Hz) of sites A and B in the absence of exchange, P_B is the mole fraction of species B, τ is the lifetime between exchanges (in seconds), δ_{AB} is the chemical shift difference between the sites (in Hz), $R = 2\delta_{AB}/W_B°$, and $Q = 1 + (\pi\tau W_B°)^{-1}$. In our case, species A and B are reduced and oxidized azurin, respectively. In general, in order to determine $W_B°$ from a measurement of W_A, it is necessary to know τ and δ_{AB}. However, it is reasonable to assume that, for a resonance which is significantly broadened in the presence of as little as 1% Cu(II) azurin, $W_B° \gg \delta_{AB}$, so that $R^2 \ll Q$. In this case, eq 1 becomes

$$W_A - W_A° = P_B W_B° (1 + \pi\tau W_B°)^{-1} \quad (2)$$

We shall also assume that the rate of exchange between the two redox states is fast enough to yield (for the resonances which are broadened beyond detection in the presence of 1% oxidized azurin) $\pi\tau W_B° \ll 1$. The resulting estimated values of $W_B°$ are tentative, pending experimental verification of this assumption (Dwek, 1973). Note that, although the rate of self-exchange between the Cu(I) and Cu(II) states is probably slow for azurin (see footnote 18 of Wherland et al., 1975) and plastocyanin (Beattie et al., 1975; Wherland et al., 1975), the rate of electron transfer between the two redox states of plastocyanin becomes fast in the presence of ferricyanide (Beattie et al., 1975). The spectra of Figures 4B and 4C were obtained with samples that contained ferricyanide (see Experimental Procedure).

Peak *x* is not detected in the spectra of mixtures of reduced and oxidized azurin (Figures 4B and 4C). Before concluding that this resonance is broadened beyond detection ($W_A \gtrsim 10$ Hz), it is necessary to eliminate the possibility that it has shifted into the main carbonyl band. The maximum possible change in chemical shift, relative to the value for 100% reduced azurin, is $P_B\delta_{AB}$ (Dwek, 1973). Thus, for the sample with 1% oxidized azurin, a shift into the main carbonyl band would require $\delta_{AB} \gtrsim 300$ ppm, an unreasonable difference between chemical shifts of the Cu(I) and Cu(II) proteins. We conclude that the lack of detection of peak *x* in Figure 4B is the result of line broadening ($W_A \gtrsim 10$ Hz). In the region of aromatic carbons of Figure 4B, only peak 6 and a one-carbon component of peak 4 have $W_A \gtrsim 10$ Hz. From eq 2 we estimate that $W_B° \gtrsim 800$ Hz for these two resonances and peak *x*. We have identified peak *x* as an amide carbonyl (see above). The very large value of $W_B°$ suggests that peak *x* arises from an amide group which is coordinated to the copper. Similarly, our values of $W_B°$ suggest that peak 6 and a one-carbon component of peak 4 arise from aromatic side-chains coordinated to the copper. Peaks 4–9 arise from C^γ of the six phenylalanines, $C^{\epsilon 2}$ of Trp-48, and C^γ of two nontitrating histidines in the $N^{\epsilon 2}$–H imidazole form (see above). Phenylalanine side chains cannot serve as metal ion ligands, and Trp-48 can be excluded from consideration as a copper ligand on the basis of various published experimental observations (see page 9 of Fee, 1975). Furthermore, although we have not specifically identified the resonance of $C^{\epsilon 2}$ of Trp-48 of either the Cu(I) or the Cu(II) protein, the values of $W_B°$ for C^γ and $C^{\delta 2}$ of Trp-48 are only ~5 Hz and $\lesssim$2 Hz, respectively (see above), which suggests that the tryptophan is not a copper ligand. Thus, we conclude that peak 6 and one component of peak 4 of reduced azurin arise from C^γ of coordinated histidine residues.

In contrast to the behavior of the three resonances discussed above, peak 11 is not significantly broadened in Figure 4B, even though it is not clearly detectable in spectra of fully oxidized azurin (see above). This indicates that the histidine that gives rise to peak 11 is not coordinated to Cu(II).

Structural Information

Coordinated Residues. Our results for peak *x* (see above) support published evidence for the involvement of an amide group as a copper ligand: The infrared studies of Hare et al. (1976) strongly implicate a backbone peptide nitrogen or oxygen as a copper ligand in bean plastocyanin. Resonance Raman studies of azurin (Miskowski et al., 1975) and other "blue" copper proteins (Siiman et al., 1974, 1976; Miskowski et al., 1975) indicate coordination of the "blue" copper centers to nitrogen (or oxygen) atoms of backbone or side-chain amide groups. Our results strongly suggest that two of the four histidines of azurin from *P. aeruginosa* are coordinated to the copper. There is strong evidence from proton NMR studies (Markley et al., 1975) that the two histidine residues of spinach plastocyanin, and two of the three histidine residues of plastocyanin from *Anabaena variabilis* are copper ligands.

The chemical shifts of C^γ of the two nontitrating histidines of reduced azurin strongly suggest that the coordinated histidine residues are in the $N^{\epsilon 2}$–H tautomeric form (Figure 3B), which implies that copper coordination involves $N^{\delta 1}$. In arriving at this conclusion, we have rejected the possibility that copper coordination to $N^{\epsilon 2}$ would shift the resonance of C^γ significantly downfield. In the case of the coordinated histidine of horse-heart ferrocytochrome *c*, which has $N^{\epsilon 2}$ linked to the heme iron (Takano et al., 1973), the C^γ resonance has a chemical shift of 122.7 ppm (Oldfield et al., 1975b). When corrected for an upfield shift of about 5 ppm caused by the ring current effect of the heme (Oldfield et al., 1975b), this resonance would still be 8 ppm or more upfield from peaks 4–9 of reduced azurin.

Our observation of fairly narrow resonances for C^ζ of both tyrosine residues of oxidized azurin rules out coordination of tyrosine hydroxyl oxygen atoms to the copper.

Residues Near the Copper. Our results indicate that Trp-48, one of the two tyrosines (the one with the lower pK_a), one of the two titrating histidines (the one with slow exchange behavior), and three or four of the six phenylalanines are close to the copper. We can use these results and the sequence analogies between azurins and plastocyanins (Dayhoff, 1976; Ryden and Lundgren, 1976) to make a tentative identification of the specific residues near the copper. Spinach plastocyanin contains only two histidine residues, at positions 37 and 87 (Scawen et al., 1975), and there is strong evidence implicating these residues as copper ligands (Markley et al., 1975). If the analogous residues of azurin from *P. aeruginosa* (His-46 and His-117, respectively[3]) are coordinated to the copper, and Cys-112 is also coordinated, then it is reasonable to propose that Trp-48, Tyr-108, Phe-110, Phe-111, and Phe-114 are sufficiently near the copper to undergo paramagnetic broadening in the spectrum of oxidized azurin. It then follows from our results that Tyr-72 is far from the copper. Furthermore, since our results indicate that Arg-79 is far from the copper, the histidine residue that is not close to the metal ion is probably His-83. Thus, His-35 should be relatively close to the copper, a suggestion consistent with the fact that this residue is part of a hydrophobic portion of the sequence (Ambler, 1971).

Our observation that Trp-48 is near the copper is consistent with the results of Finazzi-Agrò et al. (1973) and Grinvald et al. (1975). Our observation that the titrating histidine with slow exchange behavior is closer to the copper than the other

[3] Actually, His-87 of plastocyanins is considered aligned with Gly-116 of *P. aeruginosa* azurin (Dayhoff, 1976).

titrating histidine is consistent with the reported proton NMR spectra (Hill et al., 1976; Ugurbil, K., and Bersohn, R., to be published).

Effect of pH. Our results (Figure 2) indicate that two of the four histidines of reduced azurin are titratable, one with slow and one with fast exchange behavior. The imidazole form of the titrating histidine which has fast exchange behavior must be the uncommon $N^{\delta 1}$–H tautomer (Figure 3C). The pK_a of this histidine is not significantly affected by the redox state of the copper. Our results for the histidine residues of reduced azurin are consistent with those obtained from the histidine $H^{\epsilon 1}$ region in proton NMR spectra (Hill et al., 1976; Ugurbil, K., and Bersohn R., to be published): First, only one titrating histidine with fast exchange behavior is detected by ^1H NMR. The resulting pK_a of 7.6 (at 22 and 24 °C) is in agreement with our value for peak 14. As expected, the ^1H NMR spectra do not yield information about the tautomeric form of the imidazole state of this histidine. Second, at pH $\lesssim$7.0, the ^1H NMR spectrum of reduced azurin contains a resonance (at 9.4 ppm) which decreases in intensity as the pH is raised and is replaced by a new resonance (at 8.1 ppm) at pH $\gtrsim$7.0 (Hill et al., 1976).

The two tyrosine residues are titratable, with pK_a values that are much higher than that of a "solvent-exposed" tyrosine and are not significantly affected by the redox state of the copper. The pK_a of the tyrosine which has C^γ near the copper (probably Tyr-108) is significantly lower than that of the other tyrosine residue. Spectrophotometric titration data (Finazzi-Agrò et al., 1970) also indicated that the two tyrosine residues of azurin have abnormally high pK_a values ($\approx$12.4), but did not yield information about differences in pK_a values.

A number of resonances which arise from carbons near the copper undergo small pH-dependent chemical shift changes around pH 7 in the spectrum of reduced azurin (peaks *x*, 6, and 10 of Figure 2). Resonances from carbons that are distant from the copper are not affected (Figure 2). Thus, it appears that titration of some functional group affects the chemical shifts of some carbons near the copper, either via direct, through-space effects, or via a local conformational change. The "pK_a" of 7.1 $\pm$ 0.3 obtained for peak *x* (Figure 2) suggests that the functional group involved is either the N-terminal amino or the histidine which gives rise to peak 11. We know that the latter is close to the copper, and it is possible that the imidazole and imidazolium forms of this histidine may exist in different local environments.

The Question of Multiple Conformers. Heterogeneity of the tryptophan environment in holoazurin has been suggested on the basis of fluorescence lifetime studies (Grinvald et al., 1975). The existence of two conformations of reduced azurin has been inferred from temperature-jump experiments on electron exchange between azurin and cytochrome *c* (P551) (Wilson et al., 1975; Rosen and Pecht, 1976). Our results do not contain any evidence for the presence of multiple conformers. In fact, our spectra of reduced azurin are fully consistent with just one conformation. If azurin exists in more than one conformation under our sample conditions, then at least one of the following conditions applies: (i) only one conformer is present in sufficient concentration to yield detectable signals in our ^{13}C NMR spectra; (ii) the various conformers have indistinguishable chemical shifts; (iii) fast conformational rearrangement results in exchange-averaged resonances. Condition i is inconsistent with the reported proportions of the two conformers (Rosen and Pecht, 1976; Wilson et al., 1975). It seems unlikely that condition ii would apply to all resonances of Figure 1A. It is also unlikely that condition iii is applicable, because the line widths of Figure 1A would be consistent with the published rates of conformational rearrangement (Rosen and Pecht, 1976; Wilson et al., 1975) only if the chemical shift differences between the two conformers were $\lesssim$0.5 ppm. The only feature in our spectra which is consistent with two forms of reduced azurin (in slow exchange) is the change in chemical shift, around neutral pH, of C^γ of the titratable histidine which exhibits slow proton exchange. It is possible that the electron exchange efficiency between azurin and cytochrome *c* (P551) is affected by the ionization of this histidine, and/or that the pK_a of this residue is different in the oxidized and reduced azurins, resulting in complex equilibria and decay kinetics in the reported temperature jump experiments, which were carried out at neutral pH (Wilson et al., 1975; Rosen and Pecht, 1976).

Concluding Remarks

The results presented in this paper have provided some new information on the structure of azurin in solution, and, in particular, on the environment of the copper. More detailed structural information should result from extensions of this work. If accurate values of line widths and/or spin–lattice relaxation times of the ^{13}C resonances of oxidized azurin can be measured, and the value of the pertinent correlation time (Dwek, 1973) can be estimated, then accurate carbon–copper distances can be obtained (Dwek, 1973; Espersen and Martin, 1976). However, first it would be useful to assign most of the resonances to amino acid residues at specific positions in the sequence. Some techniques for this task have been described (Oldfield et al., 1975b; Oldfield and Allerhand, 1975b).

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References

Allerhand, A., Childers, R. F., and Oldfield, E. (1973a), *Biochemistry 12,* 1335.

Allerhand, A., Childers, R. F., and Oldfield, E. (1973b), *J. Magn. Reson. 11,* 272.

Allerhand, A., Doddrell, D., and Komoroski, R. (1971), *J. Chem. Phys. 55,* 189.

Ambler, R. P. (1963), *Biochem. J. 89,* 341.

Ambler, R. P. (1971), in Recent Developments in the Chemical Study of Protein Structure, Previero, A., Pechere, J.-F., and Coletti-Previero, M.-A., Ed., Paris, Inserm, p 289.

Ambler, R. P., and Brown, L. H. (1967), *Biochem. J. 104,* 784.

Beattie, J. K., Fensom, D. J., Freeman, H. C., Woodcock, E., Hill, H. A. O., and Stokes, A. M. (1975), *Biochim. Biophys. Acta 405,* 109.

Boden, N., Holmes, M. C., and Knowles, P. F. (1974), *Biochem. Biophys. Res. Commun. 57,* 845.

Brill, A. S., Bryce, G. F., and Maria, H. J. (1968), *Biochim. Biophys. Acta 154,* 342.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., and Xavier, A. V. (1973), *J. Magn. Reson. 11,* 172.

Christl, M., and Roberts, J. D. (1972), *J. Am. Chem. Soc. 94,* 4565.

Dayhoff, M. O., Ed. (1976), Atlas of Protein Sequence and Structure, Vol. 5, Suppl. 2, Silver Spring, Md., National Biomedical Research Foundation.

Deslauriers, R., McGregor, W. H., Sarantakis, D., and Smith,

I. C. P. (1974), *Biochemistry 13,* 3443.
Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry, London, Oxford University Press.
Eigen, M., Hammes, G. G., and Kustin, K. (1960), *J. Am. Chem. Soc. 82,* 3482.
Espersen, W. G., and Martin, R. B. (1976), *J. Am. Chem. Soc. 98,* 40.
Fee, J. A. (1975), *Struct. Bonding (Berlin) 23,* 1.
Finazzi-Agrò, A., Giovagnoli, C., Avigliano, L., Rotilio, G., and Mondovì, B. (1973), *Eur. J. Biochem. 34,* 20.
Finazzi-Agrò, A., Rotilio, G., Avigliano, L., Guerrieri, P., Boffi, V., and Mondovì, B. (1970), *Biochemistry 9,* 2009.
Garner, M. H., Garner, W. H., and Gurd, F. R. N. (1973), *J. Biol. Chem. 248,* 5451.
Graziani, M. T., Finazzi-Agrò, A., Rotilio, G., Barra, D., and Mondovì, B. (1974), *Biochemistry 13,* 804.
Grinvald, A., Schlessinger, J., Pecht, I., and Steinberg, I. Z. (1975), *Biochemistry 14,* 1921.
Gudat, J. C., Singh, J., and Wharton, D. C. (1973), *Biochim. Biophys. Acta 292,* 376.
Hare, J. W., Solomon, E. I., and Gray, H. B. (1976), *J. Am. Chem. Soc. 98,* 3205.
Hill, H. A. O., Leer, J. C., Smith, B. E., and Storm, C. B. (1976), *Biochem. Biophys. Res. Commun. 70,* 331.
Katoh, S., and Takamiya, A. (1964), *J. Biochem. (Tokyo) 55,* 378.
Keim, P., Vigna, R. A., Morrow, J. S., Marshall, R. C., and Gurd, F. R. N. (1973), *J. Biol. Chem. 248,* 7811.
Koenig, S. H., and Brown, R. D. (1973), *Ann. N.Y. Acad. Sci. 222,* 752.
Malkin, R. (1973), in Inorganic Biochemistry, Eichhorn, G. L., Ed., Amsterdam, Elsevier, p 689.
Malkin, R., and Malmström, B. G. (1970), *Adv. Enzymol. 33,* 177.
Markley, J. L. (1975), *Acc. Chem. Res. 8,* 70.
Markley, J. L., Ulrich, E. L., Berg, S. P., and Krogmann, D. W. (1975), *Biochemistry 14,* 4428.
McMillin, D. R., Holwerda, R. A., and Gray, H. B. (1974a), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1339.
McMillin, D. R., Rosenberg, R. C., and Gray, H. B. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4760.
Miskowski, V., Tang, S.-P. W., Spiro, T. G., Shapiro, E., and Moss, T. H. (1975), *Biochemistry 14,* 1244.
Morpurgo, L., Finazzi-Agrò, A., Rotilio, G., and Mondovì, B. (1972), *Biochim. Biophys. Acta 271,* 292.
Norton, R. S., and Bradbury, J. H. (1974), *J. Chem. Soc., Chem. Commun.,* 870.
Oldfield, E., and Allerhand, A. (1975a), *J. Am. Chem. Soc. 97,* 221.
Oldfield, E., and Allerhand, A. (1975b), *J. Biol. Chem. 250,* 6403.
Oldfield, E., Norton, R. S., and Allerhand, A. (1975a), *J. Biol. Chem. 250,* 6368.
Oldfield, E., Norton, R. S., and Allerhand, A. (1975b), *J. Biol. Chem. 250,* 6381.
Patel, D. J., Canuel, L. L., Woodward, C., and Bovey, F. A. (1975), *Biopolymers 14,* 959.
Reynolds, W. F., Peat, I. R., Freedman, M. H., and Lyerla, J. R. (1973), *J. Am. Chem. Soc. 95,* 328.
Rosen, P., and Pecht, I. (1976), *Biochemistry 15,* 775.
Ryden, L., and Lundgren, J.-O. (1976), *Nature (London) 261,* 344.
Scawen, M. D., Ramshaw, J. A. M., and Boulter, D. (1975), *Biochem. J. 147,* 343.
Siiman, O., Young, N. M., and Carey, P. R. (1974), *J. Am. Chem. Soc. 96,* 5583.
Siiman, O., Young, N. M., and Carey, P. R. (1976), *J. Am. Chem. Soc. 98,* 744.
Solomon, E. I., Clendening, P. J., Gray, H. B., and Grunthaner, F. J. (1975), *J. Am. Chem. Soc. 97,* 3878.
Solomon, E. I., Hare, J. W., and Gray, H. B. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1389.
Sutherland, I. W., and Wilkinson, J. F. (1963), *J. Gen. Microbiol. 30,* 105.
Takano, T., Kallai, O. B., Swanson, R., and Dickerson, R. E. (1973), *J. Biol. Chem. 248,* 5234.
Ugurbil, K., and Bersohn, R. (1977), *Biochemistry 16* (following paper in this issue).
Wasylishen, R. E., and Cohen, J. S. (1974), *Nature (London) 249,* 847.
Wenkert, E., Clouse, A. O., Cochran, D. W., and Doddrell, D. (1969), *J. Am. Chem. Soc. 91,* 6879.
Wherland, S., Holwerda, R. A., Rosenberg, R. C., and Gray, H. B. (1975), *J. Am. Chem. Soc. 97,* 5260.
Wilbur, D. J., and Allerhand, A. (1976), *J. Biol. Chem. 251,* 5187.
Wilson, M. T., Greenwood, C., Brunori, M., and Antonini, E. (1975), *Biochem. J. 145,* 449.