Nuclear Magnetic Resonance Study of Exchangeable and Nonexchangeable Protons in Azurin from *Pseudomonas aeruginosa*†

Kamil Ugurbil*,‡ and Richard Bersohn

ABSTRACT: Proton nuclear magnetic resonance (^{1}H NMR) spectra of the carbon-bound protons in apoazurin and native oxidized, native reduced, and Hg(II)-substituted *P. aeruginosa* azurin are presented. The pH dependence of chemical shifts is used to assign C(2)-H and C(4)-H protons of a titratable histidine in the fast-exchange domain and another which exhibits slow exchange. The pK* at 24 °C of the histidine with fast-exchange behavior is 7.57 and 7.35 in reduced and oxidized azurin, respectively. Two peaks seen in the reduced but not in the oxidized spectra are assigned to C(2)-H of the two histidines coordinated to copper. Several well-resolved ~3 proton resonances are observed in the methionine S-CH_3 region. Difference spectra obtained from oxidized, reduced, and Hg(II)-substituted azurin spectra show that (1) there are local conformational differences between the Cu(I)- and Cu(II)-bound proteins, (2) the Hg(II)-substituted protein is very similar in structure to oxidized azurin, and (3) paramagnetic Cu(II) broadens beyond detection the histidine with the slow-exchange behavior, 2 of the ~3 proton resonances in the Met S-CH_3 region, and a large number of resonances which stem from other methyl protons. ^{1}H NMR spectra in H_2O exhibit several well-resolved resonances between −9.5 and −12.5 ppm in reduced azurin spectra; the number of detectable resonances in the same chemical-shift region is fewer in the paramagnetic protein. Possible assignments to Trp-48 NH and imidazole NH's are discussed.

Because of their small molecular weight (~14 000), single "blue" copper, and the extensive information on their sequence (Ambler, 1971), azurins from *Pseudomonas* have been the subject of investigations aimed at understanding both the structure (Brill et al., 1968; Finazzi-Agro et al., 1970, 1973; Kocnig and Brown, 1973; McMillin et al., 1974a,b; Miskowski et al., 1975; Solomon et al., 1976; Hill et al., 1976; Ugurbil and Bersohn, 1977; Ugurbil et al., 1977a,b) and the mechanism of electron transfer (Antonini et al., 1970; Wilson et al., 1975; Rosen and Pecht, 1976; Goldberg and Pecht, 1976) of aqueous "blue" copper proteins. ^{13}C NMR[1] spectroscopy of *P. aeruginosa* azurin (Ugurbil et al., 1977b) has yielded extensive information about the proximity to Cu^{2+} of aromatic groups and the guanidinium moiety of the single arginine and the titration behavior of the ionizable aromatic side chains (histidines and tyrosines). ^{1}H NMR results presented here and previously reported (Hill et al., 1976) have been useful mainly in the study of the histidine residues.

Experimental Procedure

Materials. A culture of *Pseudomonas aeruginosa* and a sample of its azurin were kindly supplied by Dr. David C. Wharton of the University of Texas. *P. fluorescens* (ATCC 13430) culture was purchased from the American Type Culture Collection. The organisms were grown in large scale by the Grain Processing Corp., Muscatin, Iowa.

Azurin was isolated as previously described (Ugurbil and Bersohn, 1977). The stock solution of azurin was stored as a ~10^{-3} M, pH 3.9 solution at 1 °C. The A_{625}^{ox}/A_{280}, where A_{625}^{ox} is the absorption of a ferricyanide-treated sample and A_{280} the absorption of an untreated sample, of the *P. aeruginosa* samples used in this study was 0.58.

Methods. Apoazurin was prepared by dialyzing the native oxidized protein at 4 °C against 0.5 M KCN in 0.1 M ammonium acetate buffer. Hg(II)-substituted azurin was prepared by the addition of a small excess of $HgCl_2$ to the apoprotein solution. In the presence of excess Cu(II), Hg(II)-bound protein did not show the strong absorption at 625 nm characteristic of the Cu(II)-bound azurin.

Native azurin samples contain ~1 to 5% reduced protein. Therefore, they were oxidized with ferricyanide in order to obtain full oxidation, and reduced when necessary with the addition of a small excess of solid sodium dithionite. The small amount of iron-cyanide complexes in fully oxidized azurin samples and by-products of dithionite oxidation and decomposition in reduced azurin solutions were not removed prior to obtaining spectra.

Azurin solutions in D_2O were prepared by dissolving a lyophilized protein sample in 99.8% D_2O (Thompson-Packard) and incubating it at ambient temperature (~23 °C) and pH* 3.0 (uncorrected for deuterium isotope effect) for 24 h. This solution was then concentrated to approximately 1 mL by ultrafiltration (Millipore Corp., Bedford, Mass.) and diluted to approximately 25 mL with D_2O and reconcentrated; this procedure was repeated several times.

pH was measured at 24 °C using either a Corning Model 12 or Radiometer-Copenhagen meter equipped with an Ingold combination electrode and calibrated using buffers in H_2O. The pH readings were taken within the NMR sample tubes both before and after spectra were obtained. The notations pH* and pK* are used to indicate values uncorrected for the deuterium isotope effect.

The ^{1}H NMR spectra of the nonexchangeable protons (for samples in D_2O) were recorded in the Fourier transform mode at 220 MHz using a Varian spectrometer. Convolution difference spectra (CDS) were calculated according to Campbell et al. (1973). The values of τ_1, τ_2, and K (defined as in

† From the Department of Chemistry, Columbia University, New York, New York 10027. *Received December 28, 1976.* This research was supported by a grant (GM-19019) from the National Institute of Health, United States Public Health Service.

‡ Present Address: Bell Laboratories, Murray Hill, N.J. 07974.

[1] Abbreviations used are: NMR, nuclear magnetic resonance; CDS, convolution difference spectrum; PMB, *p*-mercurobenzoate; ODMR, optical detection of magnetic resonance; TSP, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4.

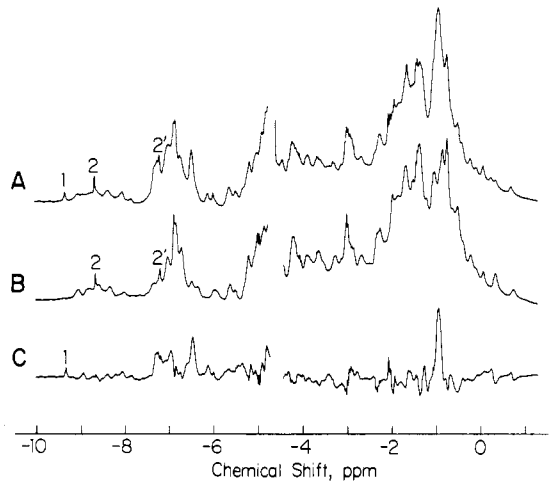

FIGURE 1: 220-MHz ^{1}H NMR spectra of *P. aeruginosa* azurin in D_2O, 0.1 M NaCl, at pH* 5.0 (pH* measured at 24 °C) and 34 °C. (A) Reduced azurin. (B) Oxidized azurin. (C) Reduced azurin spectrum minus 0.95 times the oxidized azurin spectrum. Amplitudes of the low-field spectra (downfield of −5.5 ppm) are twice that of the upfield spectra.

Campbell et al., 1973) are specified in the figure captions. Chemical shifts were measured digitally from internal TSP. D_2O samples were 3 to 5 mM in protein concentration, unbuffered, and in 0.1 M NaCl. ^{1}H NMR spectra taken to follow pH titration of histidine C(2) and C(4) protons were obtained at a probe temperature of 24 °C (measured using chemical shifts of methanol proton resonances), the same temperature at which the pH measurements were carried out. All other spectra of nonexchangeable protons were recorded at a probe temperature of 34 °C.

^{1}H NMR spectra in H_2O of the exchangeable protons were measured on the same Varian 220-MHz instrument, operated in the CW mode. There were 125 to 250 scans of the spectra accumulated in 2048 addresses of a Nicolet 1080 computer. Large intrinsic line widths of exchangeable resonances allowed 200–400 Hz/s sweeps through the spectral region without distorting the line shapes. All spectra, unless otherwise specified, were obtained at 22 °C probe temperature (determined from the chemical shifts of methanol proton resonances). H_2O solutions were 7 to 10 mM in azurin and 0.1 M in NaCl.

Results and Discussion

^{1}H NMR of Azurin in D_2O. ^{1}H NMR spectra of *P. aeruginosa* azurin in its reduced and oxidized forms at pH* 5.0 are shown in Figure 1A,B. Identical spectra were previously reported by Hill et al. (1976). Resolution can further be improved by the application of the CDS procedure as seen in Figure 2A,B. The CDS procedure has the additional advantage that it weights more heavily narrower lines, thus discriminating against the broad resonances of unexchanged NH protons. The CDS of apoazurin and Hg(II)-bound azurin are also presented (Figure 2C,D).

Aromatic Protons. P. aeruginosa azurin contains four histidines (Ambler, 1971). We concur with Hill et al. (1976) on the assignment of two resonances which appear both in the oxidized and reduced azurin spectrum (peaks 2 and 2′, Figures 1A,B, and 2A,B) to the C(2)–H and C(4)–H of one of the histidines (designated as His-2) on the basis of their pH behavior at 24 °C (Figure 3). The pK* obtained from the titration curves is 7.57 and 7.35 (24 °C) for reduced and oxidized azurin, respectively. The range of the total shifts with pH (1.1 ppm for peak 2 and 0.52 ppm for peak 2′), the chemical shifts

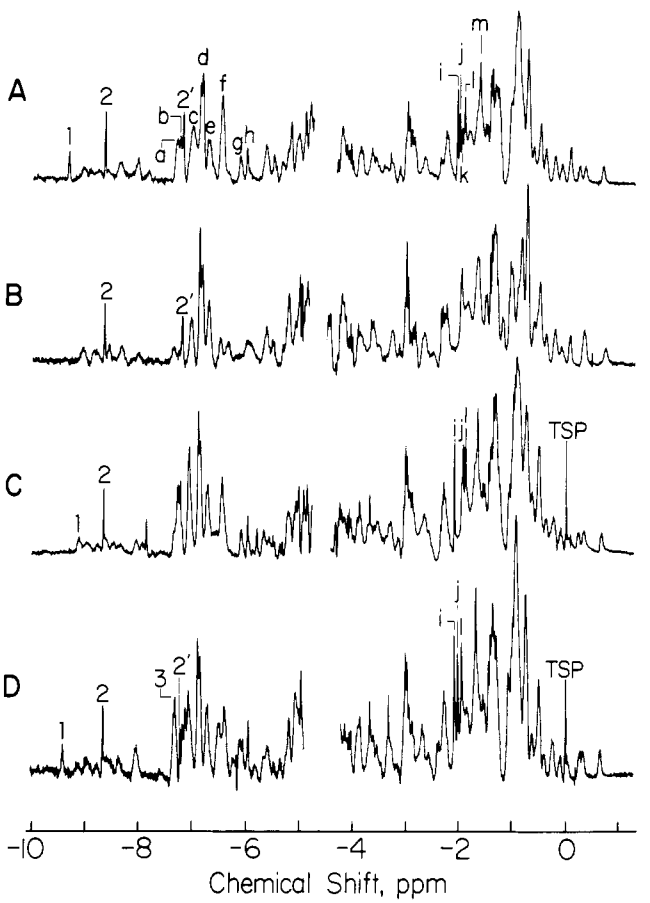

FIGURE 2: 220-MHz convolution-difference ^{1}H NMR spectra of various forms of *P. aeruginosa* azurin, 0.1 M NaCl, at pH* 5.0 (measured at 24 °C) and 34 °C. τ_1 and τ_2 (as defined by Campbell et al. (1973)) are 0.64 and 0.03 s, respectively, for all spectra; K is given for individual spectra. (A) Reduced azurin, K = 0.9; (B) oxidized azurin, K = 0.9; (C) apoazurin, K = 0.67; (D) Hg(II)-substituted azurin, K = 0.83. Amplitudes of the low-field spectra are twice that of the upfield spectra.

outside the titration range, and the pK* obtained are characteristic of histidine residues in proteins (Markley, 1975a; and references cited therein). An anomalous aspect of the peak 2 titration is that in both reduced and oxidized azurin its intensity decreases to less than unity at pH* ~7 to 8. This is shown for the reduced protein in Figure 4. The same phenomenon appears to be present in spectra reported by Hill et al. (see Figure 3 of Hill et al., 1976), although these investigators make no mention of it. On the other hand, the ^{13}C resonance assigned to His-2 C$^\gamma$ (discussed further on) in the ^{13}C NMR spectrum of reduced azurin (Ugurbil et al., 1977b) titrates with constant intensity (within experimental error of ±25%) over a 27.3 Hz (1.8 ppm) range. These observations imply that a pH-dependent interaction prevents a fraction of the His-2's from rapidly averaging over 242 Hz but not 27.3 Hz (the difference in the C(2)–H proton and C$^\gamma$ carbon-13 frequency, respectively, for the protonated and neutral forms of His-2).

At low pH*, another resonance at −9.39 ppm (peak 1, Figures 1A and 2A) is evident in the reduced but not in the oxidized protein spectra. In the CDS, this resonance appears smaller in area compared to peak 2 (Figure 2A); this, however, is expected because the width of peak 1 is greater (peak 1 $\Delta\nu_{1/2} \simeq 10$ Hz; peak 2 $\Delta\nu_{1/2} \simeq 4$ Hz, 34 °C, pH* 5.0) and, consequently, the loss in area upon application of the CDS procedure is larger than that of peak 2. The intensity of peak 1 decreases with increasing pH concomitant with the appearance of a new

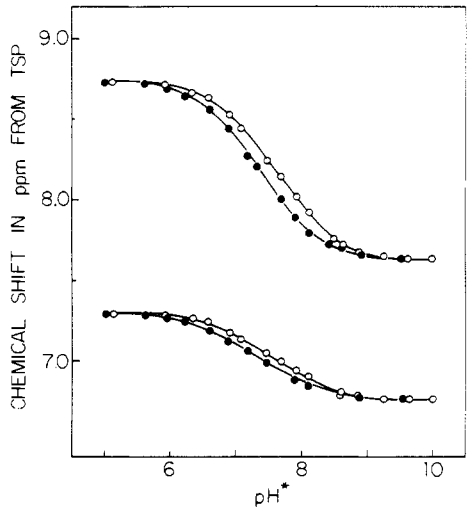

FIGURE 3: Chemical shifts of resonances designated 2 and 2′ (see Figures 1 and 2) vs. pH* in D_2O (0.1 M NaCl, 24 °C); (○) reduced azurin; (●) oxidized azurin.

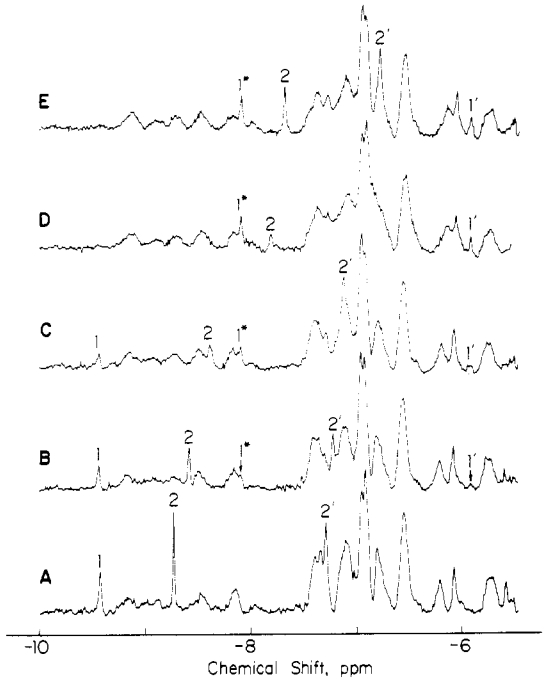

FIGURE 4: Effect of pH* on the aromatic region of the reduced *P. aeruginosa* azurin convolution-difference ^{1}H NMR spectrum, 220 MHz, 24 °C, in 0.1 M NaCl; $K = 0.83$, $\tau_1 = 0.4$ s, $\tau_2 = 0.03$ s; (A) pH* 4.90; (B) pH* 6.95; (C) pH* 7.30; (D) pH* 8.40; (E) pH* 9.0.

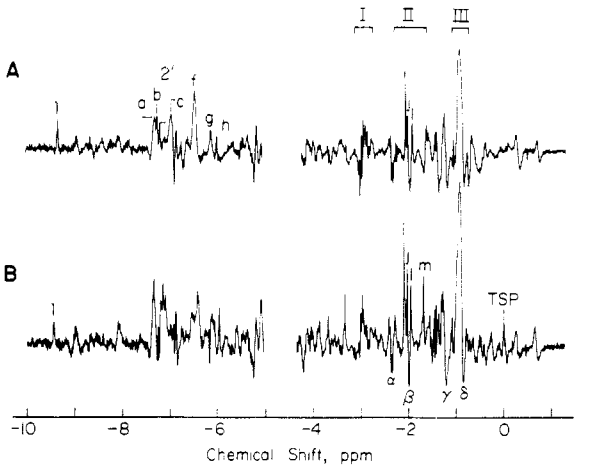

FIGURE 5: Difference of the convolution-spectra difference ^{1}H NMR spectra of diamagnetic native reduced, Hg(II)-substituted and the paramagnetic-oxidized *P. aeruginosa* azurin. Convolution-difference spectra used are those shown in Figure 2. (A) Reduced azurin CDS minus 0.98 times the oxidized azurin CDS. (B) Hg(II)-substituted azurin CDS minus 0.98 times the oxidized azurin CDS. Low-field spectra (aromatic region) amplitude is twice that of the upfield (aliphatic) region.

resonance at −8.09 ppm (peak 1*, Figure 4). These peaks are assigned, in agreement with Hill et al. (1976), to the C(2)–H of a histidine (designated His-1) whose overall exchange rate between its imidazole and imidazolium forms is slow relative to a chemical-shift difference of 1.3 ppm. Concomitant with the increase in area of peak 1*, the appearance of a second resonance at −5.91 ppm (peak 1′, Figure 4) is detectable in the reduced azurin CDS. This suggests that the new resonance arises from the C(4)–H of the histidine to which peak 1 (and 1*) has been ascribed. The simultaneous disappearance of peaks 1 (at acid pH), 1*, and 1′ (at basic pH) in the oxidized azurin spectrum is consistent with this suggestion. The assignment remains tentative, however, because the chemical shift of the proton which gives rise to peak 1′ is not known at acidic pH; consequently, possible assignment to an abnormally deshielded α-CH which undergoes a discontinuous shift via a pH-dependent change in its local environment cannot be ruled out. Peak 1′ is hardly detectable prior to the application of the CDS procedure because of overlapping broad resonances, and, as can be deduced from Figure 4, its large intrinsic half-width.

Spectra obtained at several different pH values showed that two peaks at −8.72 and −7.27 ppm, respectively, in the apoprotein and Hg(II)-bound protein spectrum at pH* 5.0 (Figure 2C,D) titrate upfield with increasing pH. Therefore, they are assigned to the C(2)–H and C(4)–H protons of His-2 and are labeled 2 and 2′. A resonance at −9.29 ppm in the apoazurin spectrum and at −9.47 ppm in the Hg(II) complex spectrum showed a similar behavior to that of peak 1 in reduced protein spectra. This resonance, however, was very broad in our apoazurin spectra at pH* 5.0 (Figure 2C) and sharpened only when pH* was lowered to 4 or less. It is assigned to the C(2)–H of the same histidine which gives rise to peak 1 in reduced azurin spectra.

The presence of the C(2) and C(4) protons of His-2 (peaks 2 and 2′) in the apoazurin and reduced, oxidized, and Hg(II)-bound azurin spectra indicates that this residue is far from the copper ion. The removal of the copper ion or its substitution with Hg(II) does not alter the chemical shifts of peaks 2 and 2′. The His-2 C(4)–H resonance (peak 2′) appears a little broader in the presence of Cu(II) than it does in the reduced azurin spectra (Figure 2A,B). This leads to the appearance of a small sharp peak in the difference spectrum (peak 2′, Figure 5A). Because the increase in the line width is small, it cannot unquestionably be ascribed to a paramagnetic interaction with the Cu(II). Closer proximity of other protons to His-2 C(4)–H in the oxidized protein could be an alternative explanation.

His-1, on the other hand, is sufficiently close to the Cu(II) to become undetectable in the oxidized azurin spectrum (and, consequently, appear in the difference spectrum (Figure 5A,B)). The titratability of this histidine with a pK* around 7 makes this residue an unlikely candidate for a Cu(I) ligand; the possibility that it can be a Cu(II) ligand cannot be ruled out from this or previously presented data by Hill et al. (1976). However, based on spectra obtained with partially oxidized

samples, ^{13}C NMR work (Ugurbil et al., 1977b) has shown that the histidine with the slow-exchange behavior (i.e., His-1) is not coordinated to Cu(II) but is located near it. Removal of the copper ion, or its replacement with Hg(II), alters the chemical shift of peak 1 (Figure 2). Since His-1 appears not to be a ligand, the chemical-shift changes indicate a metal-ion-dependent perturbation of the local environment about this residue in going from Cu(I) to Hg(II)-bound azurin.

The proton resonances of the two remaining histidines could (1) be present but buried within the aromatic packet (−6 to −7.5 ppm) in both the reduced and oxidized azurin spectra, and because the residues are nontitratable their proton resonances cannot be recognized as those of histidines; (2) appear in the −6- to −7.5-ppm region of the reduced protein spectra and are broadened beyond detection in the oxidized azurin spectrum; or (3) are undetectable under our experimental conditions as is the case for the His-48 C(2)-H proton resonance in bovine pancreatic ribonuclease A at pH $\gtrsim$ 5 (Markley 1975b). The alternative explanations cannot be distinguished by this or similar (Hill et al., 1976) ^{1}H NMR work alone. However, in the ^{13}C NMR spectra of reduced azurin (Ugurbil et al., 1977a,b) C$^{\gamma 2}$ resonances of all four histidines were observed. Two of the histidines were nontitratable in the pH range 4 to 11 and two were titratable (one in the fast-exchange domain with a pK of 7.5 (in H_2O, 31 °C) (corresponding to His-2, peaks 2 and 2′ in the ^{1}H NMR spectra) and the other in the slow- to intermediate-exchange domain with a pK between 6.8 and 7.8 (corresponding His-1, peaks 1 and 1* in the ^{1}H NMR spectra)). Furthermore, the ^{13}C NMR study of azurin (Ugurbil et al., 1977b) has suggested that the two nontitratable histidines in *P. aeruginosa* azurin are bound to the copper at the imidazole δ_1 nitrogen. This implies that the aromatic resonances affected by the copper-ion oxidation state (peaks a, b, c, f, g, and h, Figures 2A and 5A) should contain the C(2)-H and C(4)-H resonances of these two histidines. If binding to Cu(I) does not lead to large chemical-shift perturbations, the two C(2) protons of the histidines should appear downfield of the aromatic protons of the phenylalanines, Tyr-72, Tyr-108, and Trp-46. On this basis, we suggest the assignment of peaks a and b (Figures 2A and 5A) to the C(2)-H of the two nontitrating histidines. Similarly, the composite resonance peak 3 in the Hg(II)-bound azurin spectrum (Figure 2D) should contain the C(2)-H proton resonances of the same two histidines because these two histidines are also nontitratable in the Hg(II)-apoazurin complex. A new sharp resonance appears at −7.91 ppm in the apoazurin spectrum. This resonance shows a total of about 0.7-ppm upfield shift with increasing pH. Such a resonance has also been observed by Hill et al. (1976) and assigned to a third titrating histidine on the basis of its titration behavior and pK* of 7.8 (Hill et al., 1976). The intensity of this resonance was less than unity in our apoazurin spectra, suggesting that the residue which gives rise to this resonance exists in two or more different local environments.

Aliphatic Protons. Of the six methionines contained in *P. aeruginosa* azurin, two (Met-44 and Met-109) are expected to be not far from the copper ion because Tyr-108, Phe-110, and Phe-111 are located near the copper and His-46 is probably bound to it (Ugurbil et al., 1977b) The ^{1}H NMR results presented here support this expectation. Unless abnormally shifted, Met S-CH_3 proton resonances should be present in the −1.7- to −2.5-ppm region of the ^{1}H NMR and the difference spectra. One prominent 3-proton resonance (peak i, Figure 5A,B) is present in this region in both reduced/oxidized and Hg(II)-bound/oxidized azurin difference spectra. Two sharp and possibly 3-proton resonances (peaks j and l, Figure 5A,B) are also present; their intensities appear to have been decreased in the difference spectra by the overlapping and broad resonance which appears at −1.99 ppm in the oxidized azurin spectrum and which gives rise to the negative resonance at the same location in the difference spectra (peak β, Figure 5A,B). In the absence of similar 3-proton peaks with negative intensities, two (assuming that peak β results from a small shift of either peak j or l) or three (assuming that another resonance, such as peak m (Figure 5B), shifted into this location and broadened in oxidized azurin) 3-proton resonances appear to have been broadened beyond detection by the Cu(II). Peak k, which appears in the reduced azurin CDS (Figure 2A), is absent in the apoazurin-Hg(II) CDS and therefore cannot be the source of peak β in Figure 5B. The possibility that some of the Met S-CH_3 resonances have chemical shifts around −1.4 ppm (where the reduced/oxidized azurin difference spectrum is complicated) and appear broader (hence, less easily discernable in the difference spectrum) cannot be ruled out. However, the presence of sharp resonances of less than ~3-proton intensity in this region of the Hg(II)-bound/oxidized azurin difference spectrum makes it unlikely that *more than* two or three methionines are located near the copper ion.

Both difference spectra exhibit a prominent peak at −0.95 ppm which, judged by its large intensity, contains several overlapping resonances. The proton resonances of leucine, isoleucine, and valine CH_3 groups and possibly isoleucine CH_2 are expected to have chemical shifts which fall in this region (McDonald and Phillips, 1969). *P. aeruginosa* azurin contains 24 such residues (14 isoleucines, 10 leucines, and 10 valines (Ambler, 1971)). Thus, several of these hydrophobic Leu-CH_3, Ile-CH_3 (and possible -CH_2), and Val-CH_3 moieties must be located sufficiently near the copper to have their proton resonances wiped out of the oxidized spectrum. This observation further supports earlier conclusions derived from various spectroscopic studies (Brill et al., 1968; Finazzi-Agro et al., 1970; Koenig and Brown, 1973; Boden et al., 1974) that the copper ion is located at a hydrophobic site in the interior of the protein. That such groups are present in the tightly packed environment of Trp-48 was also concluded on the basis of excited triplet-state studies of Trp-48 (Ugurbil et al., 1977a); it has already been shown that Trp-48 is located relatively near the copper ion (Ugurbil et al., 1977b).

The aliphatic region of the oxidized/reduced azurin difference spectrum (Figure 5A) exhibits a number of (unidentified) sharp negative peaks adjacent to sharp positive peaks; these are particularly clear at or near −3.0 ppm (region I), −2.0 ppm (region II), and between 0 and 1 ppm. In the Hg(II) complex/oxidized azurin difference spectrum (Figure 5B), the total number of negative resonances is much smaller. For example, groups of negative peaks present at −3.0 ppm, around −1.5 ppm, and between −0.5 and 2 ppm in Figure 5A are absent from Figure 5B. This suggests that the conformation of the apoazurin-Hg(II) complex is similar to that of oxidized azurin. Moreover, in contrast to apoazurin, only two histidines are titratable in Hg(II)-bound azurin, as in the native protein. Finazzi-Agro et al. (1970) had earlier reported that Hg(II) quenches the fluorescence emission of azurin to the same extent as Cu(II) and that Hg(II) when bound to the apoprotein cannot be displaced by Cu(II). All these results point to the conclusion that Hg(II), a sulfhydryl binding agent, binds to the same site as Cu(II).

[2] Imidazole C$^{\gamma}$, N$^{\delta 1}$, and N$^{\epsilon 2}$ in histidine are alternatively labeled as C5, N1, and N3, respectively.

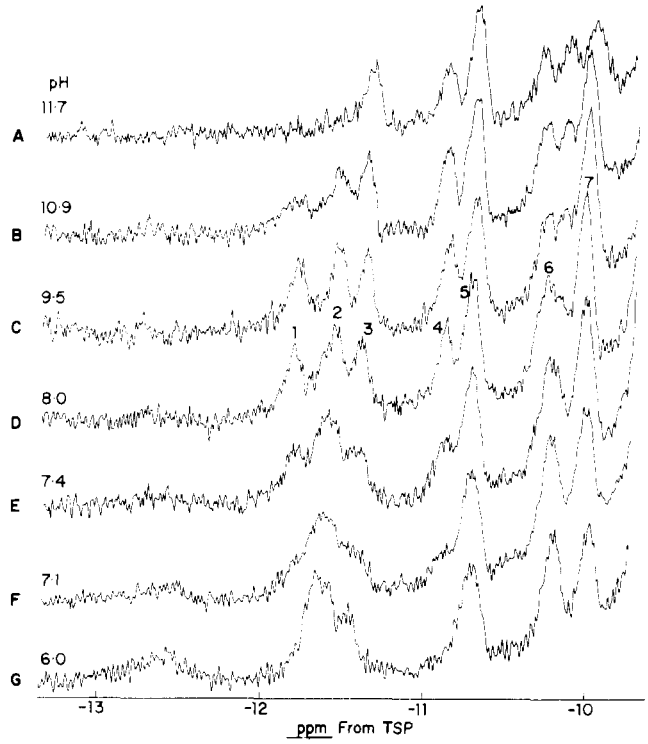

FIGURE 6: Downfield ^1H NMR spectra of reduced *P. aeruginosa* azurin as a function of pH at 220 MHz, 22 °C, in H_2O containing 0.1 M NaCl.

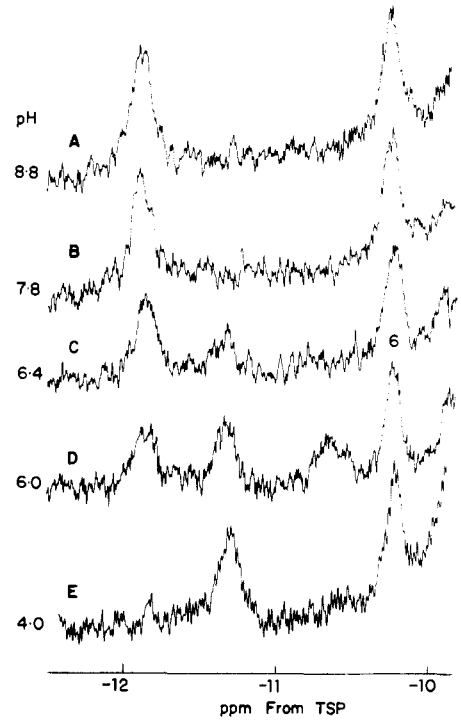

FIGURE 7: Downfield ^1H NMR spectra of oxidized *P. aeruginosa* azurin as a function of pH, at 220 MHz, 22 °C, in H_2O containing 0.1 M NaCl.

^1H NMR of Azurin in H_2O. Protons bound to atoms more electronegative than carbon, such as nitrogen and oxygen, have resonances at lesser fields which are observable if the protons are sufficiently protected by the protein structure and/or hydrogen bonding so that they exchange only slowly with H_2O. Tryptophan N-H resonances have been observed between −10 and −11 ppm in lysozyme (Glickson et al., 1969) and histidine NH resonances between −11.3 and −18 ppm (Markley, 1975; Patel et al., 1975; Robillard and Shulman 1974a,b; Stellwagen and Shulman, 1973) in a variety of proteins.

The −9.6- to −10.6-ppm region of the ^1H NMR spectra of reduced azurin in H_2O at 22 °C is shown as a function of varying pH in Figure 6. Chemical shifts of the observed resonances showed little dependence on pH. Peaks 2 and 3 showed small (≤0.2 ppm) upfield shifts about pH 7.5 and peak 7 shifted 0.1-ppm downfield with increasing pH. Line widths at half-height for peaks 1 to 5 at pH 9.5 and peaks 6 and 7 at pH 6.0 are ~25 Hz and show little variation with temperature between 12 and 35 °C. A very broad resonance is discernable at −12.5 ppm for pH ≲ 7.5. Comparison with a spectrum (Figure 8) obtained within 30 min after a sample of azurin (lyophilized powder) was dissolved in D_2O showed that resonances 1 to 4 and one-half the intensity of both peak 6 and 7 stem from protons readily accessible to the solvent (i.e., complete exchange with D in less than 30 min). The remaining intensities of peaks 6 and 7 (Figure 8) exchanged at 45 °C and pH* 6.8 (pH* measured at 22 °C) with a rate constant of 4×10^{-7} s^{-1} (±10%). Peak 5 remained unexchanged after 8 h of incubation, but was not detectable after 4 days of incubation at 45 °C at pH* 6.8 (22 °C measurement).

The absolute intensities of resonances shown in Figure 6 were calibrated by comparison with the His-12 NH resonance at −13 ppm (pH 5.0) (Patel et al., 1975) of a ribonuclease A sample of known concentration. Results (approximated to the nearest integer) showed that peaks 1 to 4 are single-proton resonances at pH ≳ 8, and peaks 5 (at all pH values studied), 6 (at pH ≲ 8), and 7 (at pH ≲ 11) exhibit two proton intensities. Peak 6 splits into two single-proton resonances at pH > 8 and peak 2 is a double-proton peak at pH ≲ 7. Thus, both peak 6 (at pH ≲ 8) and 7 (at pH ≲ 11) appear to represent overlapping resonances of two different protons, one of which is more readily accessible to the solvent (see above). Whether peak 5 represents the sum of single-proton resonances of two different groups or it stems from the identical proton of a two-proton group cannot be distinguished on the basis of this work.

Oxidized azurin spectra (Figure 7) are radically different in the same chemical-shift region. All of the resonances except one-half the intensity of the resonance at −10.2 ppm were undetectable in spectra obtained at pH* 8, 6, and 5.5 ~30 min after dissolving a lyophilized powder of oxidized azurin in D_2O; the resonance at −10.2 ppm exchanged with deuterium at 40 °C and pH* 6.8 (pH measured at 22 °C) at the same rate as peaks 6 and 7 of the reduced azurin spectra (Figure 6). When a sample of oxidized azurin was reduced after an 8-h incubation at 45 °C and pH* 6.8 (22 °C measurement), a two-proton resonance at −10.6 ppm (i.e., peak 5 of Figure 6D) was observed. Chemical-shift considerations and identical exchange rates with deuterium in D_2O strongly suggest that the −10.2-ppm resonance of oxidized azurin stems from the same two protons which give rise to peak 6 in the diamagnetic protein spectra; therefore, we label this resonance as 6 in Figure 7. It is not possible to establish a one to one correlation between the oxidized and reduced azurin resonances which deuterate readily in D_2O on the basis of Figures 6 and 7.

Because the single tryptophan (Trp-48) is located in a hydrocarbon-like environment (Ugurbil et al., 1977a, Grinvald et al., 1975, Finazzi-Agro et al., 1970), its nitrogen-bound proton should exchange slowly enough with the solvent protons to permit detection of its resonance. ^{13}C NMR experiments showed that Trp-48 $C^{\delta 2}$ and C^{γ} resonances were broadened but

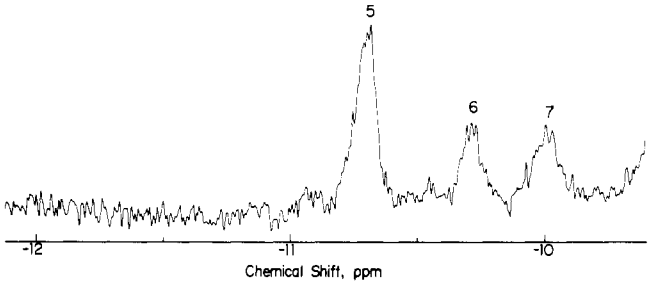

FIGURE 8: ^{1}H NMR spectra of the downfield region of reduced *P. aeruginosa* azurin obtained immediately after a lyophilized powder of azurin was dissolved in D_2O to give a 10 mM solution at 220 MHz, 45 °C and pH* 6.8 (measured at 22 °C).

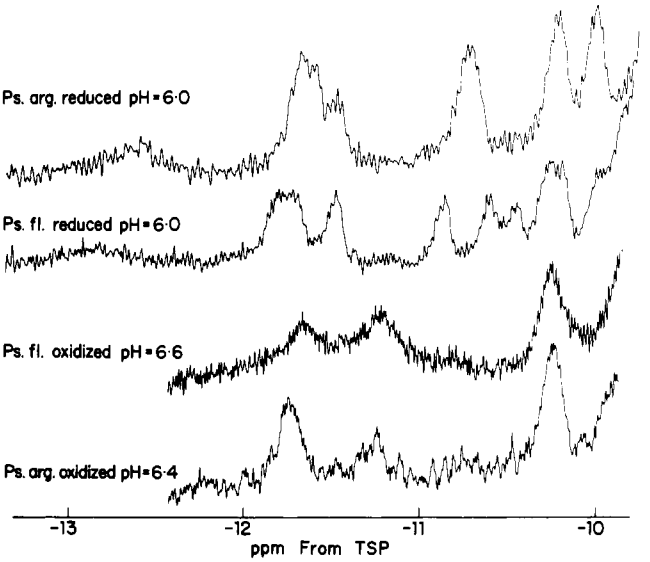

FIGURE 9: Comparison of the downfield ^{1}H NMR spectra of *P. aeruginosa* and tryptophan-less *P. fluorescens* azurins in the oxidized and reduced forms, at 220 MHz, 22 °C.

detectable in the paramagnetic protein spectrum (Ugurbil et al., 1977b). A more drastic effect is expected on proton resonances due to the larger gyromagnetic ratio of the proton. On this basis, peak 6 (Figures 6 and 7) cannot be assigned to Trp-48 NH. *P. fluorescens* (ATCC 13430) azurin lacks the single Trp residue (Ugurbil and Bersohn, 1977). The ^{1}H NMR spectra of the two proteins in the region of the exchangeable protons are very similar both for the oxidized and reduced forms (Figure 9). The comparison shows that peaks 2 and 3 as well as 6 (Figure 6) cannot be assigned to Trp NH. Assignment of either peak 1 or 4 to Trp-48 NH is also unlikely because on the basis of fluorimetric data (Finazzi-Agro et al., 1970) drastic changes in the Trp-48 environment are unlikely in the neighborhood of pH 7.5. Therefore, either peak 5 or 7 contains the Trp-48 NH resonance. Grinvald et al. (1975) concluded that water molecules may have access to the tryptophan residue in *P. aeruginosa* apoazurin. If the same is true for the native reduced or oxidized protein, peak 5 can also be excluded from consideration.

Two titratable histidine resonances are expected to be observed (see section on ^{1}H NMR of Azurin in D_2O) provided rapid exchange of the imidazole nitrogen proton with the solvent does not occur. Only peaks 2 and 3 show continuous shifts with pH. However, the magnitudes of the shifts (~0.2 ppm for peak 2 and 0.1 ppm for 3) are much smaller than expected for titratable histidine residues (1.8 ppm in ribonuclease A (Patel et al., 1975), ~3 ppm in chymotrypsin (Robillard and Shulman, 1974a)). The pH-dependent upfield shifts of these two resonances, therefore, are ascribed to either local conformational perturbation caused by or direct effects of a nearby titratable group whose pK is ~7 (22 °C). Small pH-dependent shifts of resonances around pH 7 were also observed for nonprotonated carbons located near the copper in the same protein (Ugurbil et al., 1977b). The only resonances which could arise from the titratable histidines of azurin are peaks 1 and 4. Assignment of these peaks to the imidazole NH of the two titratable histidines would require (1) that the histidine NH protons slowly exchange with the solvent only in the imidazole form and (2) that the rate of exchange between the imidazole and the imidazolium forms be slow (relative to the difference in the frequency of the protonated and neutral histidine NH) so that broadening and/or shifting of detectable imidazole NH resonances does not occur as the pH is lowered. The observation that peaks 1 and 4 are at one-half their unit intensity around pH 7.4 ± 0.3 would be consistent with their assignment to the titratable histidines. A similar behavior for a resonance in the oxidized azurin spectra would also be expected because one of the titratable histidines is far from the copper (see section on ^{1}H NMR of Azurin in D_2O and Ugurbil et al., 1977b). Such a single-proton resonance is not observed in the oxidized azurin spectra. The ~2-proton resonance at −11.8 ppm increases in area with increasing pH, and attains one-half of its full intensity at a pH of ~6, less than the pK of 7.35 measured for the titratable histidine which is far from the copper ion. It is possible, however, that the −11.8-ppm peak is a composite resonance of one histidine NH and another proton both of which are undetectable in the acid pH range. Even in the absence of concrete assignments to titratable histidines, we can conclude that either (1) the two titrating histidines are not hydrogen bonded and are exposed to the solvent so that their NH resonances are not detectable, or (2) are detectable only at basic pH when the histidines are in the imidazole form (assignment to peaks 1 and 4). It appears unlikely for option 1 to be true for the histidine (His-1) which displays a slow-exchange behavior between its protonated and neutral forms.

The two nontitrating histidines have been implicated in copper coordination (Ugurbil et al., 1977b). As spectroscopic evidence indicates that the copper site is buried within the protein, the NH resonances of the two nontitratable histidines are also expected to be detectable. The most plausible assignment is peaks 2 and 3 or 5, although the chemical shift of peak 5 is upfield by ~1 ppm of imidazole NH resonances previously observed in proteins. Specific assignment of these histidine NH resonances would yield important information about the accessibility of the copper site to solvent molecules.

Further insight into the assignment of these resonances could come from a knowledge of the x-ray structure of this protein, which is not yet available, and chemical labeling studies.

Summary

Recent spectroscopic studies have generated numerous structural data on azurin. This information is summarized in Table I. The calculation of the numerical values for the upper limits of distances from the Cu(II) are based on the paramagnetic broadening undergone by the ^{13}C NMR resonances of Trp-48 C^{γ} and $C^{\delta 2}$ in the presence of the Cu(II), and are described below. Trp-48 has been extensively studied because of its unusually blue-shifted fluorescence emission and the sensitivity of its fluorescence quantum yields to the copper ion presence or absence. This chromophore is definitely not a

TABLE I: Structural Features of Azurin.

Amino acid	Structural Data	Source of information
Cys-112	Cu ligand	PMB binding[a] and metal replacement studies;[b] analogies with plastocyanin[c]
Cys-3 to Cys-26	Buried disulfide bridge	Not reduced by CO_2^- [d]
His-35, -83	C^γ of one $\lesssim$7.5 Å from Cu, other is remote from Cu	^1H[e] and ^{13}C NMR[f]
His-46, -117	Cu ligand, imidazole $N^{\delta 1}$ coordination	^{13}C NMR[f]
Trp-48	$C^\gamma \lesssim 8.4$ Å, $C^{\delta 2} \lesssim 9.8$ Å from Cu; located in a hydrocarbon-like environment	^{13}C NMR[f]; ODMR and phosphorescence[g]
Tyr-72	Remote from Cu; $\gtrsim$20 Å from Tyr-108, inaccesible to the solvent, not adjacent to the disulfide bridge, and not hydrogen bonded	^{13}C NMR[f]; fluorescence[h] and phosphorescence[g]
Tyr-108	$C^\gamma \lesssim 7.5$ Å from Cu, C^ζ further away; inaccessible to the solvent, not adjacent to the disulfide bridge, and not hydrogen bonded	^{13}C NMR[f]; fluorescence[h] and phosphorescence[g]
Phe-110, -111, -114	Close to Cu	^{13}C NMR[f]
Arg-79	Remote from Cu	^{13}C NMR[f]
Two methionines, probably Met-44 and -109	$S-CH_3 \lesssim 8$ Å from Cu	^1H[e] and ^{13}C NMR[f]
Unknown amide moiety	Cu ligand	^{13}C NMR[f]
Several Ile, Leu and/or Val	Methyl groups close to Cu	^1H NMR[e]

[a] Finazzi-Agro et al. (1970). [b] Finazzi-Agro et al. (1973); McMillin et al. (1974b); this work. [c] Katoh and Takamiya (1964); Graziani et al. (1974); McMillin et al. (1974b). [d] Fukuda, R., and Bersohn, R., to be published. [e] This work. [f] Ugurbil et al. (1977b). [g] Ugurbil et al. (1977a). [h] Ugurbil and Bersohn (1977).

copper ligand, as evidenced by its photoemissive properties (Ugurbil et al., 1977a). Consequently, the only contribution by the paramagnetic Cu(II) to the resonance line widths of Trp-48 nuclei will come from the magnetic dipole-dipole interaction. Thus, using the relationship $\Delta W \sim r^{-6}$, and the Cu(II)-induced increase in line widths of Trp-48 C^γ and $C^{\delta 2}$ resonances ($\sim$5 and $\lesssim$2 Hz, respectively, Ugurbil et al., 1977b), we obtain $r_{\delta 2} \simeq 1.17\, r_\gamma$, where $r_{\delta 2}$ and r_γ are the distances from the copper to Trp-48 $C^{\delta 2}$ and C^γ, respectively, and ΔW is the paramagnetic contribution in Hz by Cu(II) to the line widths at half-height. From $|r_{\delta 2} - r_\gamma| \leq 1.42$ Å, where 1.42 Å is the tryptophan C^γ-$C^{\delta 2}$ bond length (Marsh and Donohue, 1967), we calculate $r_{\delta 2} \lesssim 9.8$ Å and $r_\gamma \lesssim 8.4$ Å. The C^γ resonance of the histidine, which displays slow exchange between its neutral and protonated forms (His-1), and one of the two tyrosines (the one with the lower pK) exhibit $\Delta W \gtrsim$ 10 Hz (i.e., undetectable) in the ^{13}C NMR spectra (Ugurbil et al., 1977b). Comparison with $\Delta W/r_\gamma{}^6$ for the Trp-48 C^γ yields an upper limit of 7.5 Å for the separation of these nuclei from the copper ion. These numbers should be regarded as rough approximations because of the relatively poor signal to noise of the ^{13}C NMR spectra from which the ΔW are obtained. The upper limit calculated for r_γ and the ΔW for the Trp-48 C^γ used in conjunction with the equation for the dipole-dipole contributions to the line widths[3] (Dwek, 1973) yields $\tau_c \lesssim 1.6 \times 10^{-9}$ s, where τ_c is the correlation time for the dipole-dipole interaction. This upper limit for τ_c, which is exactly in the range expected, allows us to put more confidence in the numerical values presented, and further suggests that the Cu(II)-carbon nuclei distances for Trp-48 C^γ and $C^{\delta 2}$ may not be very much different from the calculated upper limits. The rotational correlation time of azurin, considering its molecular weight, should be between 10^{-8} and 10^{-9} s, and the electron-spin relaxation rates of Cu(II) complexes are usually in the 10^{-8} to 10^{-10} s range. Therefore, τ_c should be in the range 10^{-8} to 10^{-10} s. Using $\tau_c \lesssim 1.6 \times 10^{-9}$ s, we also calculate that the protons undetectable in the ^1H NMR spectra ($\Delta W \gtrsim 30$ Hz) lie within 8 Å of the copper ion; these protons include the C(2)-H and C(4)-H of His-1, two (or possibly three) methionine $S-CH_3$, and methyl protons of several Ile, Leu, and/or Val residues.

^1H NMR spectra presented here and previously by Hill et al. (1976) show large Cu(II)-induced paramagnetic effects on the aromatic region. Besides Trp-48, the lower pK tyrosine and one titratable histidine (His-1), which are relatively near the copper and have already been discussed, at least three phenylalanines have been shown to be very near the copper ion (Ugurbil et al., 1977b). In view of the chemical and spectroscopic evidence for Cys-112 ligation to the copper ion, the three phenylalanines and the tyrosine close to the metal center were identified as Tyr-108, Phe-110, Phe-111, and Phe-114. It appears that this part of the peptide chain (Tyr-108 to His-117) folds around the copper, not only providing ligands in the sulfhydryl of Cys-112 and imidazole $N^{\delta 1}$ of His-117 but also creating part of a hydrophobic cage which results in solvent inaccessibility to the metal ion.

[3] The g value for the azurin bound Cu(II) electron was calculated from the $g_\perp$ and $g_\parallel$ previously reported for *P. aeruginosa* azurin (Brill et al., 1968).

Acknowledgments

We thank Dr. David C. Wharton for supplying us a culture of *P. aeruginosa* and a sample of its purified azurin and Kathleen Ugurbil and Michael Shulder for their help in the isolation and purification of the proteins.

References

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

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

Antonini, E., Finazzi-Agro, A., Avigliano, L., Guerrieri, P., Rotillio, G., and Mondovi, B. (1970), *J. Biol. Chem. 245*, 4847.

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.
Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry, London, Oxford University Press.
Esperson, W. G., and Martin, R. G. (1976), *J. Am. Chem. Soc. 98,* 40.
Fee, J. A. (1975), *Struct. Bonding (Berlin), 23,* 1.
Finazzi-Agro, A., Giovagnoli, C., Avigliano, L., Rotilio, G., and Mondavi, B. (1973), *Eur. J. Biochem. 34,* 20.
Finazzi-Agro, A., Rotillio, G., Avigliano, K., Guerrieri, P., Botti, V., and Mondavi, B. (1970), *Biochemistry 9,* 2009.
Freeman, H. C. (1973), in Inorganic Biochemistry, Vol. 1, Eichhorn, G. L., Ed., Amsterdam, Elsevier, p 69.
Glickson, J. D., McDonald, C. C., and Philips, W. D. (1969), *Biochem. Biophys. Res. Commun. 35,* 492.
Goldberg, M., and Pecht, I. (1976), *Biochemistry 15,* 4197.
Graziani, M. T., Finazzi-Agro, A., Rotillia, G., Burra, D., and Mondavi, B. (1974), *Biochemistry 13,* 804.
Grinvald, A., Schlessinger, J., Pecht, I., and Steinberg, I. Z. (1975), *Biochemistry 14,* 1921.
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.
Koenig, S. H., and Brown, R. (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 Malmstrom, B. G. (1970), *Adv. Enzymol. 33,* 177.
Markley, J. L. (1975a), *Acc. Chem. Res. 8,* 70.
Markley, J. L. (1975b), *Biochemistry 14,* 3554.
Markley, J. L., Ulrich, E. L., Berg, S. P., and Krogmann, D. W. (1975), *Biochemistry 14,* 4428.
Marsh, R. E., and Donohue, J. (1967), *Adv. Protein Chem. 22,* 235.
McDonald, C. C., and Philips, W. D. (1969), *J. Am. Chem. Soc. 91,* 1513.
McMillin, D. R., Holwerda, R. A., and Gray, H. B. (1974a), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1339.
McMillin, D. K., Rosenberg, R. C., and Gray, H. B. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4760.
Miskowski, V. Tung, S. P. W., Spiro, T. G., Shapiro, E., and Moss, T. H. (1975), *Biochemistry 14,* 1244.
Patel, D. J., Canuel, L. L., Woodward, C., and Bovey F. (1975), *Biopolymers 14,* 1959.
Robillard, G., and Shulman, R. G. (1974a), *J. Mol. Biol. 86,* 519.
Robillard, G., and Shulman, R. G. (1974b), *J. Mol. Biol. 86,* 541.
Rosen, P., and Pecht, I. (1976), *Biochemistry 15,* 775.
Siiman, O., Young, N. M., and Caray, 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.
Stellwagen, E., and Shulman, R. G. (1973), *J. Mol. Biol. 75,* 683.
Ugurbil, K., and Bersohn, R. (1977), *Biochemistry 16,* 895.
Ugurbil K., Maki, A., and Bersohn, R. (1977a), *Biochemistry 16,* 901.
Ugurbil, K., Norton, R. S., Allerhand, A., and Bersohn, R. (1977b), *Biochemistry 16,* 886.
Wilson, M. T., Greenwood, C., Brunori, M., and Antonini, E. (1975), *Biochem. J. 145,* 449.