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# High-Resolution Proton Nuclear Magnetic Resonance Studies of the Nickel(II) Derivative of Azurin†

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ABSTRACT: High-resolution (360 and 470 MHz) $^1$H NMR studies of Ni(II) azurin, the nickel(II) derivative of the blue copper protein azurin, are reported. The aliphatic resonances of Ni(II) azurin closely parallel those of apoazurin and Cu(I) azurin and indicate that no major structural changes are associated with the binding of nickel(II). The magnetic moment of Ni(II) azurin ($\mu_{eff}$ = 3.2 $\mu_B$) is in keeping with a pseudotetrahedral coordination environment like that of Cu(I) azurin. Resonances of protons from the ligand moieties are shifted as far as 125 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate and as far as 20 ppm upfield by internal fields due to the nickel center. One of these strongly shifted resonances is assigned to the methyl protons of the methionine ligand. From spectra of Ni(II) azurin as a function of pH, the p$K_a'$ values of histidine-35 and histidine-83 have been measured to be ~6.0 and 7.5, respectively. Histidine-35 titrates in a discontinuous fashion, and, significantly, so do several of the isotropically shifted ligand protons, also within experimental error with the same $pH_{mid}$. This result reinforces the suggestion that the conformational change coupled to the protonation of histidine-35 plays an important role in regulating electron transfer reactions of native azurin [Silvestrini, M. C., Brunori, M., Wilson, M. T., & Darley-Usmar, V. M. (1981) *J. Inorg. Biochem. 14*, 327–338].

Azurin is a soluble, type I, blue copper protein. The azurin from anaerobically cultured *Pseudomonas aeruginosa* has been purified to homogeneity (Ambler & Brown, 1967), and its primary sequence has been determined (Ambler, 1971). Azurin has been studied extensively by a variety of physical methods (Fee, 1975; Gray & Solomon, 1981), including electronic spectroscopy (Brill et al., 1968; McMillin et al., 1974; Solomon et al., 1976, 1980; McMillin, 1978; Tennent & McMillin, 1979; McMillin & Morris, 1981), NMR spectroscopy (Hill & Smith, 1976, 1978, 1979; Hill et al., 1976; Ugurbil & Bersohn, 1973; Ugurbil et al., 1977), and X-ray crystallography (Adman et al., 1978; Adman & Jensen, 1981); these studies have examined the nature and disposition of the copper ligands as well as the protein fold. Various roles have been envisaged for the protein structure, including (i) con-

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trolling the coordination geometry about copper in order to tune the reduction potential (Gray & Solomon, 1981) as well as to minimize the Franck-Condon barrier to electron-transfer processes (Holwerda et al., 1976), (ii) engendering a selectivity for copper, and (iii) regulating the velocity of electron transport in vivo (Ugurbil & Bersohn, 1973; Silvestrini et al., 1981).

The copper in native azurin is believed to have a distorted tetrahedral geometry (McMillin et al., 1974; Solomon et al., 1976; McMillin, 1978; Tennent & McMillin, 1979; Ugurbil & Bersohn, 1973; Adman et al., 1978). Lum & Gray (1981) have pointed out that Ni(II) may prefer a planar coordination geometry, although they did not establish the actual geometry about nickel in $Ni^{II}Az$.[1] Whether planarity can be achieved will depend on the flexibility of the protein binding site; hence, resolving this issue should give some indication of how rigid the binding site of azurin is. Our $^1H$ NMR studies reported here establish that $Ni^{II}Az$ is paramagnetic, contrary to expectation for a planar binding geometry. Furthermore, the pH dependence of the $^1H$ NMR spectrum of $Ni^{II}Az$ demonstrates the existence of a structural change that is dependent on the state of protonation of His-35.

## Materials and Methods

### *Materials*

The bacterial culture of *Pseudomonas aeruginosa* was obtained from the American Type Culture Collection (strain no. 10145). Horse heart cytochrome *c*, grade IIA, was purchased from Sigma, as was ribonuclease S (grade XII-S). All buffers were prepared from reagent grade chemicals and passed through a Chelex-100 (Bio-Rad) column before use. NMR samples were dissolved in $^2H_2O$ from Thompson Packard. The pH was adjusted with $^2HCl$ and $KO^2H$ from Merck & Co., Inc. The metal ion solutions were atomic absorption grade (Matheson Coleman and Bell). Standard 5-mm NMR tubes were obtained from Wilmad.

### *Methods*

The bacteria were cultivated in the medium described by Lenhoff & Kaplan (1956), and native azurin was isolated from the cell paste by the method of Ambler & Brown (1967). Apoazurin was prepared by using $CN^-$ or thiourea to remove Cu(I) (J. A. Blaszak and D. R. McMillin, unpublished experiments). This was followed by dialysis against 4 L of pH 7 phosphate buffer to remove the copper complexing agents. All dialyses were carried out in a fiber-dialysis device under nitrogen. $Ni^{II}Az$ was prepared from the apoprotein by the addition of 1 equiv of Ni(II); metal uptake was allowed to proceed for at least 24 h (Tennent & McMillin, 1979). $Cu^{I}Az$ was prepared by reducing native azurin with ascorbate followed by dialysis to remove the ascorbate.

Samples for NMR studies were first dialyzed against a dilute phosphate buffer and then lyophilized from 99.8% $^2H_2O$ 1 or more times depending on the degree of exchange required. After the final lyophilization, the sample was dissolved in 0.5 mL of 100% $^2H_2O$ so that the final concentration of phosphate was 0.1 M and the protein concentration was ~2 mM. The pH was adjusted and the sample centrifuged before spectra were taken. Oxidized horse heart cytochrome *c* was introduced as an intensity standard for the peaks subject to large isotropic shifts. All chemical shifts are measured from internal DSS (4,4-dimethyl-4-silapentane-1-sulfonate).

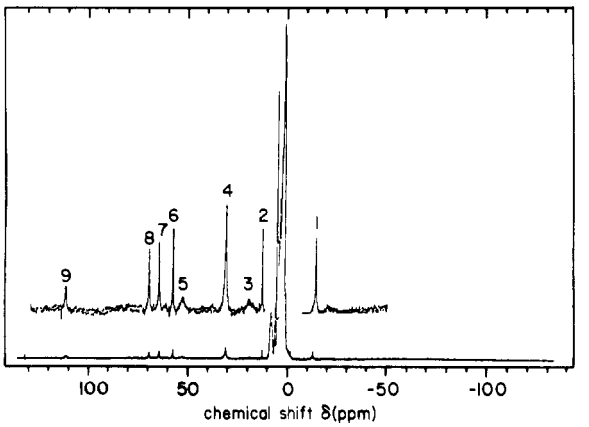


FIGURE 1: $^1H$ NMR spectrum of 1.5 mM $Ni^{II}Az$ at 25 °C, pH* 9.0. The spectrum was measured at 360 MHz, taking 10 000 16K scans by using 90° pulses at a fast repetition rate where the total acquisition time was about 0.26 s. This has the effect of enhancing the intensities of the isotropically shifted resonances (which have relatively short relaxation times) compared with the unshifted resonances.

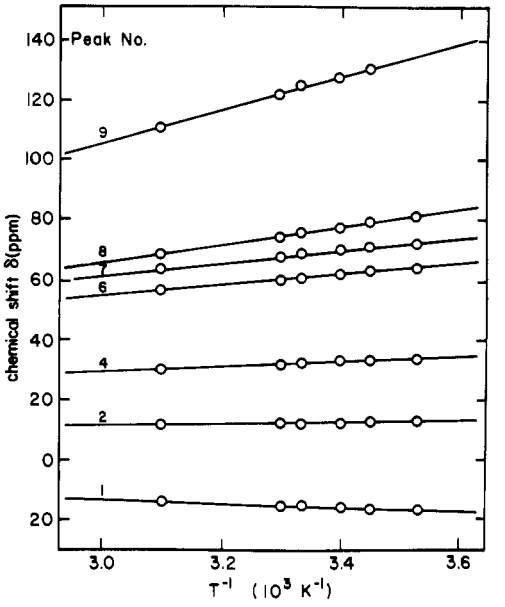


FIGURE 2: Temperature dependence of selected isotropically shifted resonances of Ni(II) azurin obtained with a 360-MHz spectrometer as described in Figure 1.

*Instrumentation.* Absorption spectra were recorded on either a Cary 17 or a McPherson EU-700 spectrophotometer. $^1H$ NMR spectra were obtained by using a Nicolet 8.5 T (360 MHz) or 10.7 T (470 MHz) NMR spectrometer equipped with probes that accommodate 5-mm (o.d.) sample tubes and operated in the high power, pulse Fourier-transform mode. The pH values of the samples were measured with an Orion Model 601 A digital ionizer or a Corning 112 digital pH meter; the pH and $pK_a'$ of the samples were not corrected for the deuterium isotope effect. Samples were centrifuged in a Brinkmann 3200 centrifuge. Calculations of $pK_a'$ and $pH_{mid}$ were made by using a program written by W. R. Finkenstadt (Markley, 1973) and modified for the Nicolet 1180 computer by D. E. Neves.

## Results

*$^1H$ NMR Spectrum of $Ni^{II}Az$.* As can be seen from Figure 1, the proton resonances of $Ni^{II}Az$ span a wide range of chemical shifts from 125 ppm downfield to ~20 ppm upfield from the DSS standard. Nine resonances are clearly resolved

[1] Abbreviations: $Ni^{II}Az$, azurin in which the copper has been replaced by nickel(II); DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; apoAz, azurin from which the copper has been removed; pH*, pH reading determined by using a glass electrode with a sample dissolved in $^2H_2O$ and uncorrected for the deuterium isotope effect.

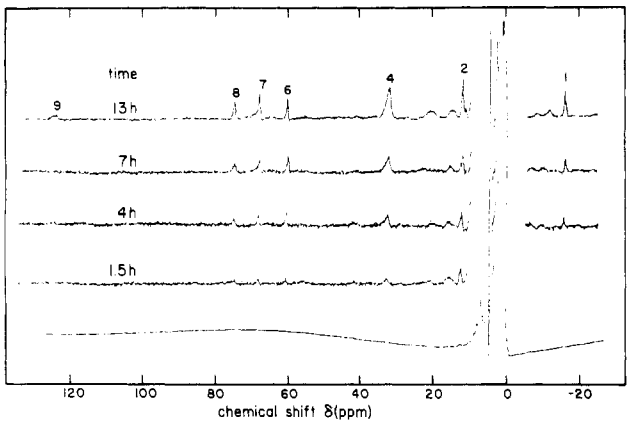


FIGURE 3: Time-resolved 360-MHz $^1$H NMR spectra of a solution of 1.5 mM apoazurin and 1.4 mM nickel(II) in 0.1 M $(NH_4)_2SO_4$, pH* 9.0, at 25 °C. The metal was added at $t$ = 0 h.

Table I: Relative Peak Intensities[a]

| peak | intensity | peak | intensity |
|---|---|---|---|
| 1 | 2.2 | 6 | 1.3 |
| 2 | 1.0 | 7 | 1.4 |
| 3 | 0.8 | 8 | 1.4 |
| 4 | 3.0 | 9 | 0.9 |
| 5 | 1.1 | | |

[a] Using 1.4 mM $Ni^{II}Az$ and 3.9 mM cytochrome $c$ as the intensity reference.

in positions outside of the usual chemical shift range of 0–10 ppm (Figure 1). The chemical shifts of these resonances are very temperature dependent, moving toward the DSS standard as the temperature increases (Figure 2).

The isotropically shifted resonances grow in over a period of hours when Ni(II) is added to apoazurin (Figure 3) in accord with previous studies of Ni(II) uptake by electronic absorption spectroscopy (Tennent & McMillin, 1979). Approximate values of the relative areas of these signals are given in Table I. The aliphatic regions of the spectra of $Ni^{II}Az$ and apoazurin are compared in Figure 4 and are seen to be practically identical.

*Magnetic Moment of $Ni^{II}Az$.* The Evans method (Evans, 1959; Phillips & Poe, 1972) was used to determine the magnetic moment of $Ni^{II}Az$. The difference in chemical shift experienced by a marker molecule in an experimental environment and a reference environment was measured. Concentric NMR tubes were used for the two environments, with the inner tube containing buffer and marker molecule only and the outer tube containing buffer, marker molecule, and either $Ni^{II}Az$ or apoAz. We found that a 1.80 mM solution of $Ni^{II}Az$ induced a downfield shift of 6 Hz in the methylene protons of ethylene glycol (Figure 5). A 1.0 mM solution of apoazurin was used to determine the induced diamagnetic shift which was found to be 5 Hz upfield for the same protons of ethylene glycol. This diamagnetic correction was checked by using a 2.0 mM solution of ribonuclease S, a small globular protein with a molecular weight similar to that of azurin, which was found to induce a diamagnetic shift of 11 Hz in the methylene protons of ethylene glycol. The total paramagnetic susceptibility of $Ni^{II}Az$ was calculated by adding the diamagnetic (apoAz) and total ($Ni^{II}Az$) contributions ($\chi_i$) to the molar susceptibility ($\chi$) of the nickel center, as determined from eq 1, where $\Delta f$ represents the shift of the marker signal

$$\chi_i = \frac{3}{4\pi}\frac{\Delta f}{FC} \quad (1)$$

in hertz, $F$ is the spectrometer frequency, and $C$ is the concentration of nickel in moles per cubic centimeter. Equation 2 was then used to estimate the value of the effective magnetic

$$\mu_{eff} = 2.828(\chi T)^{1/2} \quad (2)$$

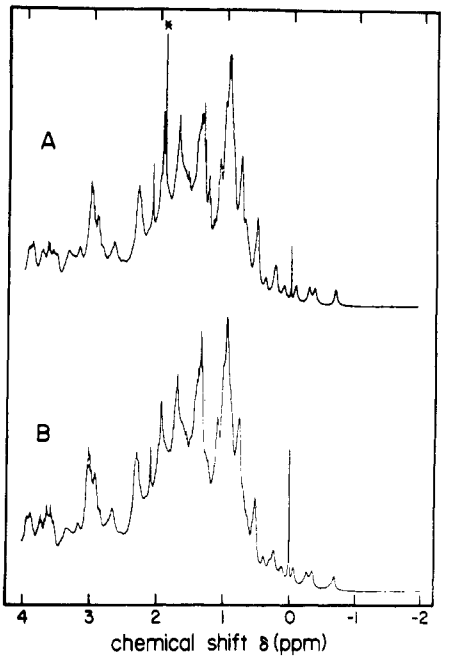


FIGURE 4: (A) Aliphatic region of the 360-MHz $^1$H NMR spectrum of 1.6 mM apoazurin. (B) The analogous region of the spectrum of Ni(II) azurin. In both cases, the pH* was 9, and the temperature was 25 °C. [The sharp spike (*) in (A) is most likely from an impurity.]

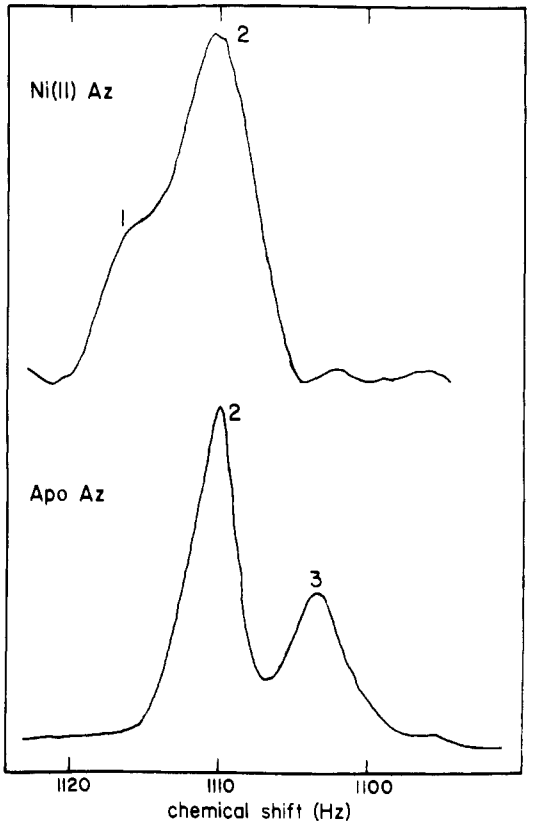


FIGURE 5: $CH_2$ resonances of ethylene glycol for concentric samples containing buffer and either 1.8 mM Ni(II) azurin or 1.0 mM apoazurin. Spectra were taken on a 470-MHz spectrometer. The sample pH* was 9.0, and the temperature was 23 °C. Peaks designated (2) are the ethylene glycol resonances in buffer only, and (1) and (3) are in the presence of protein.

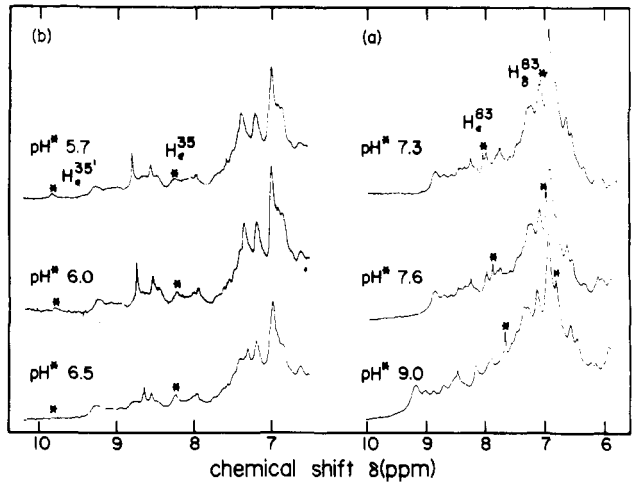


FIGURE 6: pH dependence of the 360-MHz $^1$H NMR aromatic region of 1.5 mM Ni(II) azurin (a) between pH* 5.7 and 6.5 and (b) between pH* 7.3 and 9.0. The sample temperature was 25 °C.

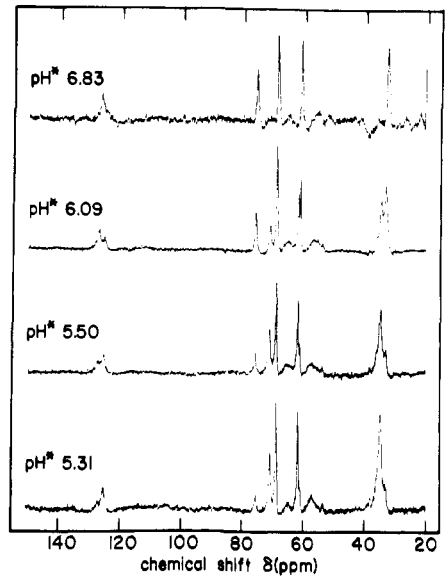


FIGURE 7: pH dependence of signals in the extreme downfield region of the 360-MHz $^1$H NMR spectrum of 1.5 mM Ni(II) azurin at 25 °C.

moment of the isolated nickel center ($\mu_{eff}$), where $\chi$ is the difference between the magnetic susceptibilities of Ni$^{II}$Az and apoAz and $T$ is the absolute temperature. The magnetic moment of the nickel center in Ni$^{II}$Az was found to be approximately 3.2 $\mu_B$ at 296 K.

*Studies of pH-Dependent Phenomena.* Selected spectra of the aromatic region of Ni$^{II}$Az are presented in Figure 6 as a function of pH*. In Figure 6b, two relatively sharp resonances are seen to shift smoothly upfield with increasing pH*. A plot of chemical shift vs. pH* indicates that both signals titrate with a $pK_a' = 7.5$ (±0.2). On the basis of the $pK_a'$ value and the chemical shift range involved, the signals can be assigned to $C_\epsilon$-H (lower field of the two) and $C_\delta$-H (higher field of the two) protons of His-83, which undergoes rapid exchange between the imidazole and imidazolium forms (Ugurbil et al., 1977). As the pH* is lowered below 7, a relatively broad signal at 8.2 ppm decreases in intensity while a similarly broad signal grows in intensity at 9.7 ppm (Figure 6a). The latter pair exhibit roughly equal intensities at pH* 6.0. These signals are most reasonably assigned to the $C_\epsilon$-H of His-35, whose imidazole and imidazolium forms in Cu$^{I}$Az interconvert slowly on the NMR time scale by proton exchange (Ugurbil et al., 1977). As can be seen in Figure 7, several of the resonances in the extreme low-field region also undergo a discontinuous shift in position as the pH* changes. Peaks that appear at 62, 32, 12.5, and 11.5 ppm (the latter two are not shown) are displaced downfield as the pH* decreases, whereas peaks at 125 and 75 ppm are displaced upfield. The signal at 68 ppm is unaffected in the same pH* range. From the relative intensities, one can associate the shifts with the protonation of a group having a $pH_{mid}$ of 6.0.

## Discussion

*Magnetic Properties.* One goal of the current investigation has been to clarify the structure about the metal center in Ni$^{II}$Az. McMillin (1978) analyzed the charge-transfer spectrum of Ni$^{II}$Az by assuming the geometry to be pseudotetrahedral as is the case in Cu$^{II}$Az. A preliminary $^1$H NMR study of Ni$^{II}$Az revealed resonances with large isotropic shifts which are consistent with paramagnetic nickel(II) (McMillin & Tennent, 1980). More recently, Lum & Gray (1981) examined the near-infrared spectrum of Ni$^{II}$Az and the nickel analogue of stellacyanin and could not locate transitions that would be expected from pseudotetrahedral chromophores containing nickel(II). While negative, this result raised the possibility that a planar, diamagnetic form of nickel(II) might occur in the protein derivatives. In line with this possibility, small molecule studies revealed that nickel(II) tends to prefer a planar environment in the presence of strong-field sulfur donors (Bertini et al., 1972; Engeseth et al., 1982).

The current data demonstrate that the nickel(II) ion is paramagnetic when bound at a single site in azurin. Time-resolved spectra in Figure 3 indicate that nickel(II) is taken up slowly by the protein and is not in rapid exchange with a free form. Some of the paramagnetically shifted resonances are independent of pH*, which is also an unlikely outcome of a model involving two environments for the metal center. The evidence for only a one to one metal–protein complex formation for Ni$^{II}$Az has already been established (Tennent & McMillin, 1979). The fact that nickel(II) is retained at pH 9 when Ni$^{II}$Az samples are dialyzed or eluted through gel columns proves there is a substantial binding constant for the metal ion; hence, in our experiments, the nickel(II) can be assumed to be bound to the protein. The temperature dependencies of the paramagnetic shifts are of the form expected for a well-behaved paramagnetic species in solution (Figure 2).

The measured magnetic moment ($\mu_{eff} = 3.2\ \mu_B$) also reveals the paramagnetic nature of Ni$^{II}$Az. The magnetic moment of "tetrahedral" nickel(II) complexes varies from 3.0 to 4.0 $\mu_B$, depending on the symmetry (Cotton & Wilkinson, 1980; Engeseth et al., 1982). Somewhat smaller magnetic moments are generally observed in five-coordinate environments, e.g., 2.9–3.4 $\mu_B$ for pseudooctahedral systems. Because of the low symmetry in the azurin binding site, $\mu_{eff}$ for Ni$^{II}$Az would be expected to occur at the low end of the tetrahedral range, consistent with our findings. Five- or six-coordinate nickel(II) could give rise to a similar magnetic moment, but the smooth correlation of the charge-transfer bands observed for the cobalt(II), nickel(II), and copper(II) derivatives of azurin (Tennent & McMillin, 1979) is more easily explained by assuming a common coordination environment (Lever, 1974).

*Structure-Sensitive Regions of the Spectra.* The aliphatic proton resonances depicted in Figure 4 should be indicative of any major structural changes that might be associated with the uptake of nickel(II) since these resonances reflect a pattern of ring-current shifts characteristic of the peptide structure. The close agreement between the spectra of apoazurin and

$Ni^{II}Az$ in this region suggests that no major structural differences exist between the two forms. This finding is all the more significant because approximately six aromatic residues of $Cu^{II}Az$ are found near the metal center (Ugurbil et al., 1977). As previously noted, the spectra of $Hg^{II}Az$, $Cu^{II}Az$, and apoazurin are all similar in this region (Hill & Smith, 1979; Ugurbil & Bersohn, 1973). Thus, the peptide appears to adopt a three-dimensional structure which is substantially determined exclusive of the metal ion.

The signals exhibiting very large chemical shifts are, in principle, also structure sensitive since they are due to protons near the nickel(II) center. However, the relative shifts expected for the ring protons of the imidazole ligands and the $\beta$-$CH_2$ protons of the His, Cys, and Met residues involved in binding the metal to the peptide cannot be predicted at this time. Peak 4 of Figure 1 which is due to three equivalent protons (Table I) can be assigned because of its chemical shift and intensity to the $CH_3$ group of Met-121, which donates its sulfur to the metal. It seems unlikely that peak 4 corresponds to some other methyl group which happens to reside near the metal center and experiences a large dipolar shift, although it is worth noting in this regard that a second methionine residue is located near the metal (Hill & Smith, 1979; Ugurbil & Bersohn, 1973; Adman & Jensen, 1981).

*Histidine Titrations and Conformational Effects.* On the basis of previous work (Hill & Smith, 1979; Ugurbil & Bersohn, 1973; Ugurbil et al., 1977), the continuously titratable histidine resonance can be associated with His-83, and the signal that undergoes a discontinuous shift in position with changing pH can be assigned to His-35. Although proton-transfer reactions of the imidazole group are usually rapid on the NMR time scale, slow exchange processes, evidenced by discontinuous titration curves, have been observed in a number of protein systems (Markley, 1975). In such cases, the histidine is not exposed to the solvent, and either the proton must penetrate slowly into the protein to reach the histidine (Woodward & Rosenberg, 1971; Richards, 1979) or a structural change must occur to expose the histidine (Englander et al., 1980; Wüthrich et al., 1980). In the latter case, a *net* conformational change in the protein need not be associated with the protonation; a transient fluctuation in structure which exposes the histidine will suffice. Mechanistic arguments aside, several of the isotropically shifted resonances in the spectra of $Ni^{II}Az$ titrate in a discontinuous fashion like the $C_\epsilon$-H resonance of His-35 and, within experimental error, exhibit the same $pH_{mid}$. This is proof that a structural change accompanies the protonation of His-35; a simple change in the charge of a neighboring residue would be unlikely to affect the ligand resonances so prominently.

A priori, the alteration in ligand resonances could be explained by a rearrangement of the metal binding site or by a reorientation of a neighboring aromatic residue(s); either possibility could influence the kinetics of electron-transfer reactions of the metal center (Colman et al., 1978). However, the fact that $pH_{mid}$ of His-35 is metal dependent, i.e., $\sim$7.3 for $Cu^{I}Az$ (Hill & Smith, 1976) vs. $\sim$6.0 for $Ni^{II}Az$, argues that some reorganization of the metal binding site is involved.

That direct evidence for the conformational change has been found is of interest because just such an effect has been postulated to explain the kinetics of electron transfer between azurin and cytochrome *c*-551 and, potentially, to have a regulatory role in vivo (Silvestrini et al., 1981; Farver et al., 1982). [A referee has pointed out that the data of Ugurbil et al. (1977) are also indicative of a conformational transition; however, those authors do not appear to discuss this point.] When the structural change can be described in more detail, it should be possible to decide whether the electron-transfer kinetics are affected by a change in the intrinsic reactivity of the metal site or by alterations in the interactions controlling the binding/recognition of a redox partner.

Finally, after this manuscript was accepted for publication, Adman et al. (1982) published a $^1$H NMR study which, based on pH and temperature studies, concluded that two conformations of azurin exist and that in the high-pH form the Cu–S(Met-121) bond is lengthened and perhaps broken. Our data indicate that—at least in the case of $Ni^{II}Az$—the metal–methionine bond is retained in both forms of the protein.

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# Effects of Reduction and Alkylation on Ligand Binding and Cation Transport by *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: The effects of sulfhydryl group modification on ligand binding and functional properties of the membrane-bound acetylcholine receptor from *Torpedo californica* have been investigated. Agonist binding kinetics were monitored by changes in fluorescence of the probe 5-(iodoacetamido)-salicylic acid which was covalently bound to the receptor after reduction of a reactive disulfide bond(s) by low concentrations of dithiothreitol. These labeling procedures did not affect either the equilibrium binding constant for [$^3$H]acetylcholine or the number of high-affinity binding sites measured in centrifugation experiments. Further reduction of these labeled receptor preparations by higher concentrations of dithiothreitol and subsequent alkylation by excess iodoacetamide resulted in a more than 10-fold decrease in the affinity of the receptor for [$^3$H]acetylcholine. This reduction and alkylation did not, however, radically alter the observed kinetics of acetylcholine binding. The fluorescence signal change on binding consisted of at least three phases similar to those observed for the control preparations, and the ligand concentration dependencies of the measured rate constants could be described by the same kinetic mechanism involving sequential binding of two ligand molecules and three conformational changes. Variation in the values of some of the kinetic parameters describing the formation of the monoliganded complex adequately accounted for the measured decrease in affinity for [$^3$H]acetylcholine. Stopped-flow fluorescence experiments showed that extensive reduction and alkylation resulted in an apparent loss of the ability of the acetylcholine receptor to mediate agonist-induced cation flux. These results show that reduction of disulfide bonds by high concentrations of dithiothreitol followed by alkylation with iodoacetamide seriously perturbs receptor function although the receptor can still undergo its characteristic conformational changes on the binding of acetylcholine but with altered concentration dependence accounting for the reduced affinity for agonist.

Chemical modification of sulfhydryl groups of the nicotinic acetylcholine receptor results in altered pharmacological responses and ligand binding properties. Treatment of *Electrophorus* electroplax with dithiothreitol (DTT)[1] inhibited the permeability response of the postsynaptic membrane to applied acetylcholine, and this inhibition was rendered irreversible by subsequent reaction with *N*-ethylmaleimide (Karlin & Bartels, 1966). After DTT reduction, the dose-response curves showed a decrease both in the affinity for agonist and in the apparent cooperativity of the response (Karlin, 1969). It has also been suggested from noise analysis experiments using the frog neuromuscular junction that DTT causes a reduction in the lifetime and conductance of single channels (Ben-Haim et al., 1975).

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[1] Abbreviations: AcCh, acetylcholine; AcChR, acetylcholine receptor; ANTS, 8-amino-1,3,6-naphthalenetrisulfonic acid; $\alpha$-BuTx, $\alpha$-bungarotoxin; Carb, carbamylcholine; DTT, dithiothreitol; HTX, histrionicotoxin; IAS, 5-(iodoacetamido)salicylic acid; MBTA, [4-(*N*-maleimido)-benzyl]trimethylammonium diiodide; NEM, *N*-ethylmaleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MPTA, [4-(*N*-maleimido)phenyl]trimethylammonium.

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