NMR Spectroscopic Identification of a Hexacyanochromate(III) Binding Site on *Pseudomonas* Azurin†

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ABSTRACT: Studies of redox reactions between the blue copper protein azurin and inorganic reagents have suggested the formation of discrete complexes between the reaction partners prior to the actual electron-transfer step. To get an insight into the structural nature of the complexes formed, we have studied the interaction (i) between oxidized *Pseudomonas aeruginosa* azurin and the anion $Fe(CN)_6^{3-}$ and (ii) between reduced azurin and $Cr(CN)_6^{3-}$. At low ionic strengths, stoichiometric binding of one $Fe(CN)_6^{3-}$ ion to the oxidized protein is observed. In the high-resolution proton magnetic resonance spectra of the reduced protein, specific broadening of the assigned residues is observed upon titration with the redox-inert $Cr(CN)_6^{3-}$ ion. Analysis of this paramagnetic spectral broadening in terms of the three-dimensional structure of the protein has led to the proposal that the binding site of the anions lies approximately midway between lysine residues 85 and 92. Evidence in support of this conclusion is provided by parallel studies on *Alcaligenes faecalis* azurin, which lacks these lysine residues. A similar site on the surface of *Pseudomonas* azurin has recently been identified by affinity labeling with chromous ions as an electron-transfer locus [Farver, O., & Pecht, I. (1981) *Isr. J. Chem. 21*, 13–17]. The results presented here suggest that this region on the protein surface also may be employed by anionic electron-transfer agents.

A central problem in the study of electron-transfer processes in biological systems is the understanding of the relationship between the rate of electron-transfer and the spatial arrangement of the redox partners involved. Insights into this problem may be obtained from studies of electron-transfer reactions between metalloproteins and inorganic redox agents. Hence, extensive studies have been made on the binding of inorganic redox agents to metalloproteins (Cookson et al., 1980; Handford et al., 1980; Eley et al., 1982; Hopfield & Urgubil, 1982) and on the kinetics of electron transfer within these complexes (Stellwagen & Shulman, 1973; Goldberg & Pecht, 1976; Segal & Sykes, 1978; Lappin et al., 1979; McCray & Kihara, 1979).

Azurin from *Pseudomonas aeruginosa* (*Ps. azurin*)[1] is a small ($M_r \simeq 14000$) electron-mediating blue copper protein. Its structure has been elucidated recently by X-ray crystallography at 2.7-Å resolution (Adman et al., 1978; Adman & Jensen, 1981). The three-dimensional structure analysis of azurin provides an opportunity to probe the electron-transfer loci on this interesting protein via studies of its reactivity with inorganic redox agents. *Ps.* azurin and the hexacyanoferrate(II)/(III) ions furnish a convenient system for such a study. Complex formation between the protein and hexacyanoferrate anions with a 1:1 stoichiometry and binding constants of about 1 mM have already been reported (Goldberg & Pecht, 1976). The intramolecular electron-transfer rates within these complexes have also been determined (Goldberg & Pecht, 1976; Lappin et al., 1979).

Here, we report ^1H NMR studies of the interaction between *Ps.* azurin and the $Fe(CN)_6^{3-}$ and $Cr(CN)_6^{3-}$ anions. Both $Fe(CN)_6^{3-}$ and its chromium isomorph $Cr(CN)_6^{3-}$ are expected to bind to the protein at the same site. However, unlike $Fe(CN)_6^{3-}$, the latter anion is essentially redox inert and may therefore be used in studies of the reduced protein. We expect $Cr(CN)_6^{3-}$ to induce large line-width changes in the ^1H NMR resonances of the protein. Since this spectral broadening arises from magnetic dipolar coupling between the protons of the protein and the Cr(III) electron spin, these $Cr(CN)_6^{3-}$-induced changes in the spectrum can be used to estimate the distances between the $Cr(CN)_6^{3-}$ binding site and the affected protons. Measurements of the line widths of the resonances due to His-83 and His-35 upon titration of the protein with $Cr(CN)_6^{3-}$ suggest that the Cr(III) complex is bound to a pair of lysine residues, namely, Lys-85 and Lys-92, at a distance of ~17 Å from the copper site. One of these lysyl residues (Lys-85) has been identified as a reductive labeling site by aqueous Cr(II) ions. This has led to the assignment of His-35, which lies within the opening defined by the peptide Val-80 to Lys-92, as part of an extended electron relay system from the protein–water interface to the copper ion (Farver & Pecht, 1981).

Materials and Methods

Azurin from *Pseudomonas aeruginosa* and *Alcaligenes faecalis* was prepared according to procedures described elsewhere (Rosen & Pecht, 1976; Rosen et al., 1981). Stock solutions of azurin were reduced by ascorbic acid, followed by extensive dialysis against 1 mM ammonium acetate buffer, pH 5.4, in water for 24 h under purified N_2. The protein was then lyophilized.

Samples for NMR measurements were prepared by dissolving the lyophilized protein in ^2H_2O containing 1 mM sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSP) and 1

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[1] Abbreviations: *Ps.* azurin, *Pseudomonas aeruginosa* azurin; NMR, nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate.

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mM sodium acetate buffer, pH 5.0, under purified N_2. A trace amount of ascorbic acid (0.1 mM) was also added to the protein solution to ensure complete reduction and to prevent reoxidation by O_2 in the course of the NMR measurements. The protein concentration was ~2 mM, as determined by using the extinction coefficient of 5700 M^{-1} cm^{-1} at 625 nm for *Ps.* azurin in the oxidized state (Goldberg & Pecht, 1976). Due to dilution during the titration with $Fe(CN)_6^{3-}$ or $Cr(CN)_6^{3-}$, the concentration of azurin decreased by about 10% by the end of the titration.

$K_3Fe(CN)_6$ was analytical-grade reagent and was used without further purification. Its concentration was determined by using an extinction of 1020 M^{-1} cm^{-1} at 420 nm. $K_3Cr(CN)_6$ (Alfa Inorganics) was dissolved in ^2H_2O under nitrogen immediately before the experiment; any undissolved material was removed by centrifugation. The pH of the stock solutions of the inorganic complexes was adjusted to 5 by the addition of ^2HCl. All pH values quoted were pH meter readings uncorrected for the deuterium isotope effect.

^1H NMR spectra were acquired at a field strength of 11.7 T (500.13-MHz proton resonance frequency) at 25 °C on a Bruker WM 500 spectrometer equipped with an Aspect 2000 data system. A spectral width of 10 kHz, recycling time of 1.7 s, and flip angle of 75° were used. Each spectrum consisted of a total of 32K points. Signal-to-noise enhancement was achieved by exponential multiplication of the free-induction decay signal with a line broadening of 2 Hz. Typically, 300 scans were acquired for sufficient signal averaging.

Chemical shifts are reported in parts per million (ppm) downfield from TSP. $Cr(CN)_6^{3-}$-induced line broadenings ($\Delta\nu$) were measured as the change in full width at half-maximum height. Due to the uncertainty in determining the base line, we estimate that $\Delta\nu$ is only accurate to within ±2 Hz.

Results

In detailed studies of the electron-transfer reaction between the hexacyanoferrate anions and azurin, clear evidence has emerged for formation of discrete complexes between the anions and the protein (Stellwagen & Shulman, 1973; Goldberg & Pecht, 1976; Segal & Sykes, 1978). At the ionic strength employed in these electron-transfer measurements (I = 0.22 M), the affinity of the interaction between $Fe(CN)_6^{3-}$ and reduced azurin was deduced to be 610 M^{-1} at 25 °C and that between $Fe(CN)_6^{4-}$ and oxidized azurin was found to be 54 M^{-1} (Goldberg & Pecht, 1976).

We have now obtained evidence for the existence of a tight binding site for $Fe(CN)_6^{3-}$ on *oxidized Ps.* azurin as well. In experiments wherein we attempted to dialyze the oxidized protein in the presence of excess $Fe(CN)_6^{3-}$ ions against distilled water, we consistently found that a stoichiometric amount of $Fe(CN)_6^{3-}$ was retained with the protein even after several days of dialysis. This was deduced from monitoring the sample absorbance at both 420 and 625 nm. As the interaction between the protein and the $Fe(CN)_6^{3-}$ is expected to be electrostatic in nature, the binding affinity should decrease with increasing ionic strength. In accordance with this expectation, when the samples were dialyzed against 100 mM NaCl, the bound $Fe(CN)_6^{3-}$ was completely removed within 12 h.

The foregoing observations clearly indicate that both reduced and oxidized *Ps.* azurin can undergo complex formation with $Fe(CN)_6^{3-/4-}$, particularly under low ionic strength conditions. Since the structure of such complexes may define the electron-transfer loci between kinetically competent redox partners, we have decided to further characterize and try to identify the site of interaction in the case of one of the four possible complexes by ^1H NMR spectroscopy. The very nature of the NMR experimental protocol limits these studies to the reduced protein only; in addition, considerations of redox inertness preclude measurements with the $Fe(CN)_6^{3-}$ ion. However, the hexacyanochromate(III) ion may be exploited in these studies. Not only is $Cr(CN)_6^{3-}$ isomorphous with $Fe(CN)_6^{3-}$, it is essentially redox inert. It is also paramagnetic, and the influence of its paramagnetism on the ^1H NMR spectrum of the reduced protein may be used to define the locus of interaction between the protein and the anionic complex.

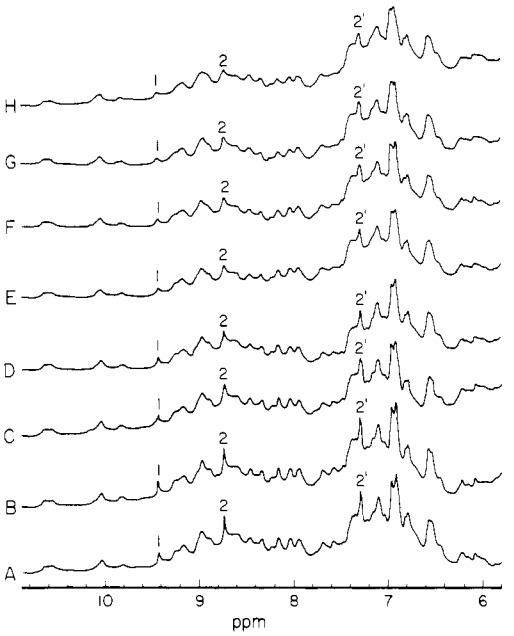

FIGURE 1: Titration of the aromatic region in the 500-MHz ^1H NMR spectrum of reduced *Ps.* azurin by $Cr(CN)_6^{3-}$ ions. Protein concentration was 2 mM in ^2H_2O solution, pH 5.0, containing 1 mM sodium acetate and TSP. A–H correspond to 0, 0.04, 0.10, 0.16, 0.30, 0.50, 0.90, and 1.33 molar equiv of $Cr(CN)_6^{3-}$ to protein, respectively.

Figure 1 shows the ^1H NMR spectra in the aromatic region of *reduced Ps.* azurin recorded after each successive addition of small aliquots of $K_3Cr(CN)_6$. Three resonances of the reduced azurin spectra in this region have previously been assigned (Urgubil & Bersohn, 1977; Hill & Smith, 1979). They are labeled 1, 2, and 2′ in Figure 1 and correspond to the C-2 proton of His-35, C-2 proton of His-83, and C-4 proton of His-83, respectively. Even at an extremely low concentration of $Cr(CN)_6^{3-}$, all three resonances were noticeably broadened. The broadening increased with the addition of $Cr(CN)_6^{3-}$ and reached a plateau at about 1 equiv of $Cr(CN)_6^{3-}$. In addition to the broadening of the histidine resonances, specific changes were noted throughout the whole spectrum. The resonance at 6.89 ppm, for example, was broadened more than its neighboring resonance at 6.93 ppm. Minor changes were also observed in the aliphatic region. Since no significant changes were observed for any nonoverlapping resonances and since definite assignments were not available in this region, these results are not further presented or discussed here.

The above $Cr(CN)_6^{3-}$-induced line-broadening data for the histidine resonances are summarized in Figure 2. The limiting increase in line widths is about 12.5 Hz for resonances 1 and 2 and 5.5 Hz for resonance 2′. The observation of saturation behavior at a stoichiometry of ~1:1 suggests that the affinity of the protein for $Cr(CN)_6^{3-}$ is high under our experimental conditions of low ionic strengths.

As controls, titrations of oxidized *Ps.* azurin with $Fe(CN)_6^{3-}$

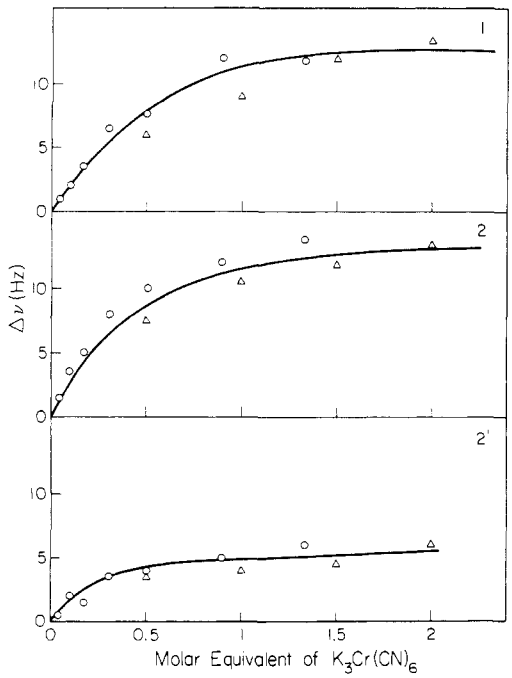

FIGURE 2: Variations in line widths of resonances 1, 2, and 2′ with increasing $Cr(CN)_6^{3-}$ concentration. (○) and (△) denote data obtained in two separate sets of titrations. The solid line is a smooth line joining the data.

were also carried out in the same manner as with $Cr(CN)_6^{3-}$. The ^1H NMR spectrum of oxidized *Ps.* azurin is already broad, and as expected, the spectral perturbations introduced by addition of $Fe(CN)_6^{3-}$ were very small. At 1 molar equiv of $Fe(CN)_6^{3-}$, the line broadenings observed are generally less than 2 Hz, and the chemical shifts do not vary by more than 0.01 ppm for all the observed resonances.

Parallel titrations with $Cr(CN)_6^{3-}$ were also carried out on samples of reduced azurin derived from *Alcaligenes faecalis*. Both His-35 and His-83 residues are conserved in *Alcaligenes* azurin, and their NMR resonances have recently been assigned by Mitra & Bersohn (1982). At 1 molar equiv of $Cr(CN)_6^{3-}$, the increase in line widths for all three histidine resonances (1, 2, and 2′) was less than 2 Hz. Also, no other resonances were significantly broadened.

Discussion

The specific and stoichiometric $Cr(CN)_6^{3-}$-induced line broadening of the histidine resonances observed in the ^1H NMR spectra of *Ps.* azurin suggests that this anion binds to a particular site on this protein. The line widths of the NMR resonances due to the C-2 proton of His-35 and C-2 and C-4 protons of His-83 reach saturation values at a $Cr(CN)_6^{3-}$ to azurin molar ratio of approximately 1:1, indicating that only one $Cr(CN)_6^{3-}$ is bound per azurin molecule and that the binding is strong.

The $Cr(CN)_6^{3-}$-induced broadening of the histidine resonances is a consequence of the enhancement in the nuclear relaxation due to magnetic dipolar coupling of these protons with the Cr(III) ion. This enhancement in the nuclear relaxation (Dwek, 1973) takes on the form

$$\Delta\nu_i = f(\tau_c)/r_i^6 \quad (1)$$

where $\Delta\nu_i$ is the induced broadening observed for the ith nucleus, r_i is the distance between this nucleus and the Cr^{3+}, and τ_c is the correlation time of the motional process modulating the magnetic dipolar relaxation. By using this result, relative values of the distances r_i and r_j can be obtained by taking ratios of $\Delta\nu$'s, namely

$$\Delta\nu_i/\Delta\nu_j = r_j^6/r_i^6$$

From the results of the titration, we deduce that to a very close approximation, the $Cr(CN)_6^{3-}$ ion must be bound to a site on the protein that is essentially equidistant from the C-2 protons of residues His-35 and His-83. Accordingly, a search for the binding site on the basis of the above constraints was conducted by using the three-dimensional coordinates available for *Ps.* azurin (Adman et al., 1978; Adman & Jensen, 1981) with the aid of the graphic display system available at the Molecular Biology Institute of the University of California at Los Angeles. This search revealed only one binding site that meets the constraints imposed by the NMR data. This binding site is situated between the ϵ-amino groups of Lys-85 and Lys-92. The number of possible binding sites is limited largely as a consequence of the fact that azurin carries a net negative charge [having a p*I* of about 4.9 (Goldberg, 1979)]. The side chains of Lys-85 and Lys-92 are exposed to solution and, therefore, are not resolved in the X-ray diffraction data. This introduces an uncertainty of about ±1 Å in the Cartesian coordinates of the bound $Cr(CN)_6^{3-}$ ion. Even then, assuming an ionic radius of 3 Å for $Cr(CN)_6^{3-}$, we arrive at a distance of 14.5 ± 0.6 Å between the center of the Cr(III) ion and the C-2 protons of His-35 and His-83. A stereo view showing the position of the $Cr(CN)_6^{3-}$ binding site on the protein is presented in Figure 3.

The titration results for the C-4 proton of His-83 provide an independent check on the reliability of the above assignment. From the X-ray coordinates, this C-4 proton should be about 1.4 Å further away from the Cr(III) ion than the C-2 proton. This is to be compared with the increased distance of ~2 Å predicted by using the saturation value of the C-4 line broadening. The presence of overlapping peaks does complicate measurements of the 2′ resonance; our method of determining the base line tends to underestimate the line width of this resonance and may account in part for this difference. In view of the uncertainty in the determination of $\Delta\nu$, we consider the agreement sufficiently satisfactory to furnish support for the proposed binding site.

The titrations of *Alcaligenes* azurin with $Cr(CN)_6^{3-}$ provide further independent corroboration for the above assignment of the anion binding site on *Ps.* azurin. The lack of appropriately placed lysine residues (vide supra) in conjunction with the absence of broadening of the His-35 and His-83 resonances serves as a control and supports our conclusion that the changes seen in ^1H NMR of *Ps.* azurin are associated with specific binding rather than with the mere presence of $Cr(CN)_6^{3-}$ in solution. In addition, these negative results indicate that $Cr(CN)_6^{3-}$ is not bound to *Alcaligenes* azurin at a concentration of about 2 mM. Since both Lys-85 and Lys-92 are absent in *Alcaligenes* azurin, this observation constitutes additional support for our proposal. Further tests of this proposal will, of course, require a more complete assignment of the *Alcaligenes* azurin ^1H NMR spectra.

The region defined by the peptide extending from Val-80 to Lys-92 has recently been shown to be labeled specifically by Cr(III) ions at the side chains of Lys-82 and Glu-91 upon reduction of *Ps.* azurin by Cr(II) aquo ions (Farver & Pecht, 1981). Examination of the protein crystal structure (Adman et al., 1978; Adman & Jensen, 1981) showed that the surface peptide forms an open loop providing access to His-35, which has been proposed to be part of an electron-transfer pathway to the copper. The present work suggests that this particular locus on the *Ps.* azurin surface also may be utilized in elec-

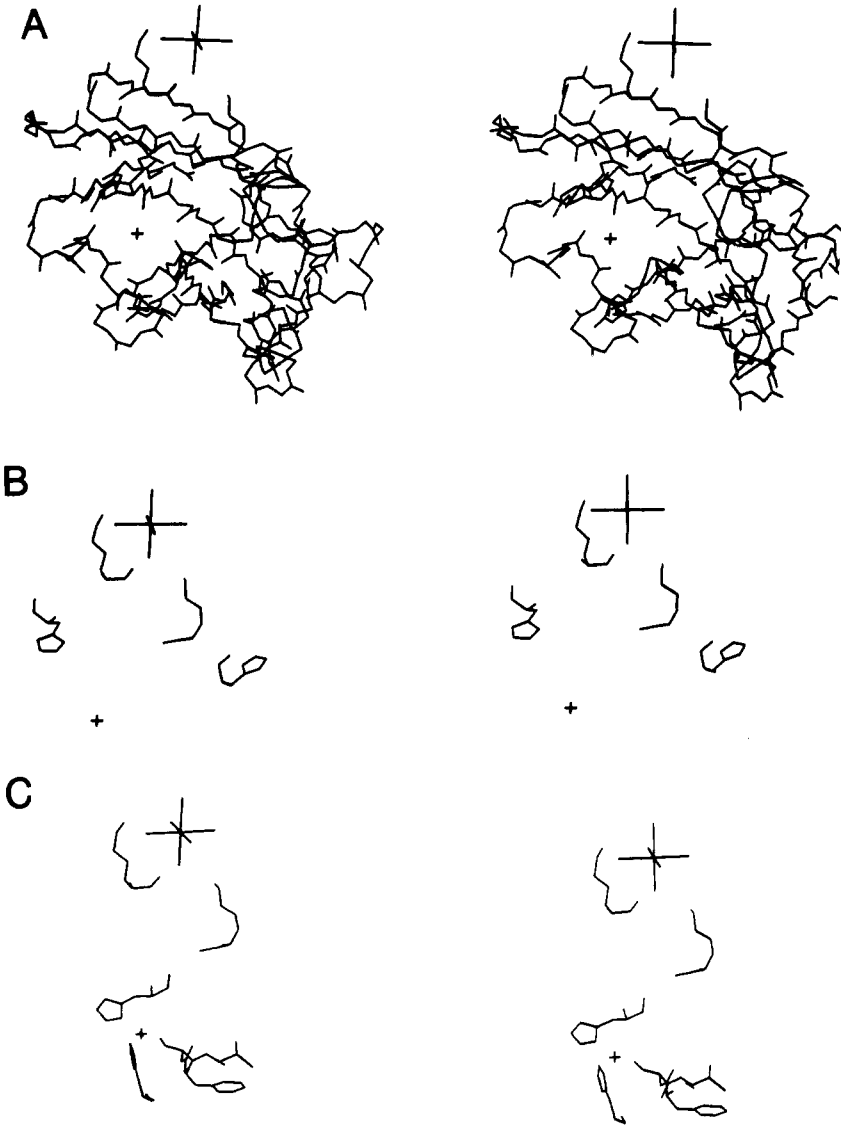

FIGURE 3: (A) Stereoscopic projection showing the peptide backbone of azurin, the proposed binding site of the $Fe(CN)_6^{3-}$ ion (large octahedron), and the type 1 copper site (small octahedron). (B) Stereoscopic projection showing the relationship of the proposed anion binding site to residues His-35 and His-83. The lysine residues thought to be involved in binding (residues 85 and 92) are also shown. Note that the bound ion is very nearly equidistant from the two histidine residues. (C) Stereoscopic projection showing the relationship of the ion binding site to the residues close to the type 1 copper site. Note that the histidine ligands to the type 1 copper are not expected to enhance the intersite electron-transfer rate significantly since they are not situated between the metal sites.

tron-transfer reactions involving anionic reagents.

The pH dependence of electron-transfer reactions between *Ps.* azurin and the hexacyanoferrate(II/III) couple has been interpreted in terms of protein residue(s) protonation equilibria most probably involving imidazole(s) (Segal & Sykes, 1978; Lappin et al., 1979). The above assignment of the anion binding site suggests that the origin of the pH dependence lies in residues intervening between the copper and the protein surface. It has repeatedly been proposed that it is the association step between the redox partners that is affected by pH rather than the actual electron-transfer step (Segal & Sykes, 1978; Lappin et al., 1979). That this is clearly *not* the case has been well established for plastocyanin where the protonation of the imidazole ligated to the copper was found to affect markedly the potential of the redox center (Freeman, 1981). On the other hand, the presence of His-35 in the neighborhood of the proposed hexacyanoferrate(II/III) binding can also provide the explanation for the pH dependence of the electron-transfer reaction with that redox couple. That a conformational transition involving that region is responsible for modulation of the electron-transfer reaction of *Ps.* azurin has been suggested earlier (Rosen & Pecht, 1976; Rosen et al., 1981). More recently, this was also shown to be coupled with the protonation equilibrium of His-35 (Farver et al., 1982; Corin et al., 1983).

In view of the identical charge and ligands and the similar size, $Fe(CN)_6^{3-}$ is expected to bind to the same site on reduced azurin as does $Cr(CN)_6^{3-}$. We propose in conclusion, then, that $Fe(CN)_6^{3-}$ is bound to the ϵ-amino side chains of Lys-85 and Lys-92 in *Ps.* azurin during the electron-transfer process involving the two redox partners.

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Registry No. $Cr(CN)_6^{3-}$, 14875-14-0; $Fe(CN)_6^{3-}$, 13408-62-3; L-lysine, 56-87-1.

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Metal Ion and Drug Binding to Proteolytic Fragments of Calmodulin: Proteolytic, Cadmium-113, and Proton Nuclear Magnetic Resonance Studies†

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ABSTRACT: Tryptic fragmentation of Ca^{2+}-saturated calmodulin (CaM) takes place mainly at Lys-77; however, proteolysis can occur instead at Arg-74 or Lys-75. This cleavage pattern results in the production of three peptides each of the amino- and carboxy-terminal halves of CaM of slightly different length. A purification scheme for the three carboxy-terminal half-peptides is reported. Proton nuclear magnetic resonance (^1H NMR) studies of peptides comprising the amino- or carboxy-terminal half of CaM reveal the great structural similarity between these two proteolytic fragments and the intact protein. Since this was observed for the apoprotein as well as the Ca^{2+}-saturated protein, this means that the two halves of the protein are independently folded. A comparison of the changes in the ^1H NMR spectra observed for the intact protein and the fragments upon addition of Ca^{2+} clearly identified sites III and IV as the two high-affinity binding sites. Furthermore, addition of Ca^{2+} or Cd^{2+} induces qualitatively similar changes in the spectra, thus indicating that Cd^{2+} is a reliable replacement for Ca^{2+} in these studies. Subsequent ^{113}Cd NMR studies of trifluoperazine (TFP) binding to tryptic and thrombic fragments of calmodulin revealed the presence of two distinct drug binding sites, one located in the amino-terminal half and one located in the carboxy-terminal half. The spectral changes, induced upon addition of the antipsychotic drug, were similar to those observed upon binding of TFP to intact calmodulin. The strongest TFP binding site is located in the carboxy-terminal half.

Hormonal or nerve impulses can cause the level of cytosolic Ca^{2+} in eukaryotic cells to rise from 10^{-7} to 10^{-5} M. During such an influx, the metal ion binds to regulatory calcium binding proteins like troponin C (TnC)[1] and calmodulin (CaM) which in response undergo large conformational changes. The completion of the amino acid sequence of CaM revealed its high homology to skeletal TnC (Watterson et al., 1980); both proteins contain four calcium binding domains each arranged in a continuous helix–loop–helix sequence, often called the EF hand (Kretsinger, 1976). TnC is the regulatory calcium binding subunit of the troponin complex that triggers contraction of striated muscles (McCubbin & Kay, 1980). Its soluble analogue CaM exposes hydrophobic regions upon Ca^{2+} binding (LaPorte et al., 1980; Tanaka & Hidaka, 1980; Vogel

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; TFP, trifluoperazine; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; 2D, two dimensional; EDTA, ethylenediaminetetraacetic acid. Peptide nomenclature: TR_1C, fragment 1–77; TR_2C, fragment 78–148; TM_1, fragment 1–106; TM_2, fragment 107–148 [see Kuznicki et al. (1981), Wall et al. (1981), and Andersson et al. (1983a)].

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