Alkaline Transition of Pseudoazurin from *Achromobacter cycloclastes* Studied by Paramagnetic NMR and Its Effect on Electron Transfer

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Paramagnetic NMR studies on the Cu(II) form of pseudoazurin have been used to demonstrate that the alkaline transition of this protein results in an active site in which the axial Cu−S(Met) interaction is considerably decreased. This observation confirms the conclusion made from various other spectroscopic methods. Furthermore, we show that the alkaline transition of pseudoazurin coincides with a dramatic increase in the electron self-exchange rate constant of the protein. The self-exchange rate constant (25 °C) at pH 8.2 is 3.5×10^3 M^{-1} s^{-1} ($I = 0.10$ M), consistent with a previously determined value (25 °C) of 2.9×10^3 M^{-1} s^{-1} ($I = 0.10$ M) at pH 7.5. Upon increasing the pH value to 10.9 the self-exchange rate constant (25 °C) increases to 1.7×10^4 M^{-1} s^{-1} ($I = 0.10$ M). The increased self-exchange reactivity at high pH is due to the deprotonation of a number of lysine residues that surround the hydrophobic patch of the protein, the most likely docking surface for the self-exchange process. The concomitant active site changes indicate that the deprotonation of one or more surface lysine residues is responsible for the alkaline transition in pseudoazurin.

Introduction

The type 1 blue copper (cupredoxin) family of electron transfer proteins has been the subject of many structural,[1,2] spectroscopic,[3−8] and kinetic[9,10] investigations. The huge interest has arisen due to the unique properties of the copper center in these proteins. Structural studies have demonstrated that all cupredoxins have a very similar overall structure (Figure 1), and in most cases the single copper atom is coordinated in a distorted tetrahedral geometry.[11−16] The copper ion is found slightly displaced from the plane of three strong equatorial ligands: the S^{γ} of a cysteine and the N^{δ} atoms of two histidines. A weak axially ligating atom is usually provided by the thioether sulfur of a methionine. In the phytocyanin subclass of the cupredoxins, the axial methionine ligand is usually replaced by a glutamine[16−18] which coordinates to the copper via its side chain amide oxygen atom.[17]

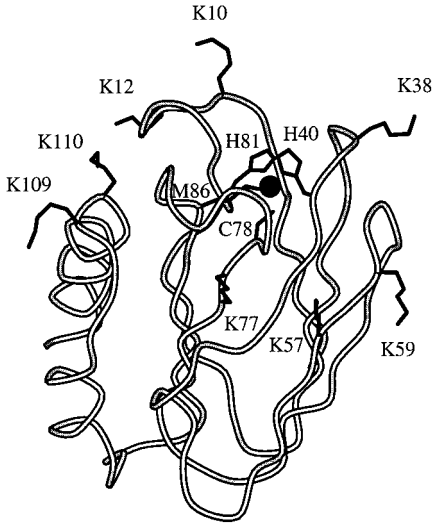

Figure 1. Representation of the structure of Cu(II) pseudoazurin from *A. cycloclastes*.[43] The copper ion is shown as a black sphere, and the side chains of the ligating amino acids are included. Also shown are the surface lysine residues which surround the hydrophobic patch of the protein.

The visible spectra of cupredoxins are dominated by a Cys(S)→Cu(II) ligand to metal charge transfer (LMCT) band at

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approximately 600 nm ($\epsilon \sim 2000-5000$ M^{-1} cm^{-1})[4] which gives rise to the characteristic intense blue color of the oxidized protein. A second, usually weaker transition, also due to a LMCT band involving the cysteine ligand, is found at around 460 nm.[8] The electron paramagnetic resonance (EPR) spectra of cupredoxins are easily identified by a small hyperfine coupling ($\sim 0.0035-0.0065$ cm^{-1}) in the $g_{||}$ region of the spectrum.[4] Varying degrees of rhombicity are observed in the EPR spectra of different cupredoxins.[4] Increased rhombicity has been associated with a shorter bond between the copper and the axial ligand and a concomitant displacement of the copper ion from the plane of the three equatorial ligands.[19−24] Cupredoxins possessing more rhombic EPR spectra also have stronger absorption at around 460 nm in their visible spectra.[19−24] Resonance Raman studies have demonstrated that these so-called type 1 rhombic copper sites have a longer Cu−S(Cys) bond length.[19,23,24] Recently it has been proposed that the spectral features of type 1 copper sites are also related to angular changes in the positions of the cysteine and methionine ligands.[8]

Alkaline transitions are a common phenomena in cupredoxins. The phytocyanin subclass of the cupredoxins have all been shown to undergo such a transition.[25−31] In the phytocyanins the transition is manifest by a blue shift of the main visible absorption band upon increasing pH, accompanied by a slight increase in the rhombicity of the EPR spectrum.[26] Recent resonance Raman[31] and paramagnetic NMR studies[32] have confirmed that the alkaline form of the phytocyanin called stellacyanin possesses a weaker Cu−S(Cys) interaction. It was originally thought that the alkaline transition of the phytocyanins was due to a change in the coordination mode of the axial glutamine ligand, i.e., from the O^{ϵ} atom at neutral pH to the deprotonated N^{ϵ} atom at more alkaline pH values.[33,34] However, cucumber basic protein, a phytocyanin with an axial methionine ligand, also undergoes a similar transition,[28,30] as does the Gln99Met variant of stellacyanin.[31] Tentative proposals have been made that the alkaline transition observed in the phytocyanins may be due to the protonation/deprotonation of a surface lysine residue situated close to the protein's active site.[22,35,36] This includes a recent theoretical study of the phytocyanin called stellacyanin from which it was suggested that the change in the electrostatic field around the copper caused by the deprotonation of a surface amino acid residue could be the cause of the alkaline transition in this protein.[36] Recently, it has been suggested that deprotonation of a surface amino acid triggers a change in the protein's secondary structure, which results in the observed effect at the active site.[32]

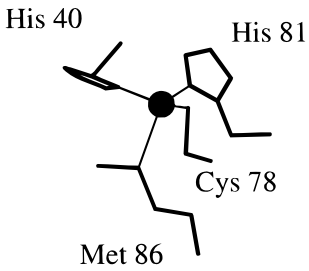

Figure 2. Representation of the active site of Cu(II) pseudoazurin from *A. cycloclastes*. The copper ion is indicated by a black sphere.

Pseudoazurin, which is not a phytocyanin, is found in denitrifying bacteria,[37−39] where it is the electron donor to a nitrite reductase.[38−40] At the active site of pseudoazurin the copper ion is coordinated by His40, Cys78, His81, and Met86 (see Figure 2).[14,41−44] The spectroscopic features of pseudoazurin indicate that it has a type 1 rhombic copper site.[39,45] Consistent with this fact the copper ion is displaced by ~0.3 Å from the plane of the three equatorial ligands.[14,41−44] A Cu−S(Met) distance of 2.76 Å is found in pseudoazurin from *A. faecalis* S-6,[14] which is slightly shorter than the corresponding distance in amicyanin (2.84 Å)[13] and plastocyanin (2.82 Å).[46] Pseudoazurin has previously been shown to undergo an alkaline transition similar to that observed in the phytocyanins.[47] Spectroscopic (EPR and visible) studies at pH values of 7.0 and 11.3 all suggest that the alkaline form of the protein possesses an active site which is less rhombic. This conclusion is confirmed by an increase of 8 cm^{-1} in the frequency of the main Cu−S(Cys) vibration in the resonance Raman spectrum of pseudoazurin at pH 11.3, as compared to its position at pH 7. Herein, we further characterize the alkaline transition of Cu(II) pseudoazurin utilizing paramagnetic ^{1}H NMR studies. Such

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investigations of cupredoxins were, until very recently, thought to be impossible due to the slow electronic relaxation of the metal ion.[48] However, recent studies on amicyanin have demonstrated that isotropically shifted resonances can be observed and assigned for cupredoxins.[49] In this paramagnetic NMR study we clearly show the effect of the alkaline transition on the structure of the active site of Cu(II) pseudoazurin.

The crystal structure of nitrite reductase from *A. cycloclastes*, the physiological electron acceptor of pseudoazurin, has been solved and shows that the protein contains both type 1 and type 2 copper centers.[50] The protein is trimeric both in the crystal and in solution.[51] The interaction of pseudoazurin from *A. faecalis* S-6 with its nitrite reductase has been shown to involve a number of surface lysine residues on the cupredoxin (see Figure 1).[52,53] These lysine residues surround the hydrophobic patch of pseudoazurin, a region common to all structurally characterized cupredoxins, through which the imidazole ring of His81 protrudes.[14,41−44] The lysine-encircled hydrophobic patch of pseudoazurin is thought to dock with an acidic patch on nitrite reductase, close to its type 1 copper center.[54] The hydrophobic patch of azurin has been shown to be the site of association prior to the electron self-exchange reaction of this cupredoxin.[55−58] In the case of pseudoazurin the nearby lysine residues are thought to hinder association and result in the protein having a very small self-exchange rate constant for a cupredoxin.[59,60] In this paper we also study the effect of the alkaline transition on the self-exchange reactivity of pseudoazurin. The results obtained clearly implicate the deprotonation of the surface lysine residues, close to the hydrophobic patch of pseudoazurin, in the alkaline transition of this protein.

Experimental Section

Protein Isolation and Purification. Pseudoazurin from *Achromobacter cycloclastes* was isolated and purified as described previously.[61] Pure pseudoazurin has an A_{277}/A_{593} ratio of 1.4 and gives a single band on an SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis) gel. The protein used for NMR studies was repurified by loading onto a CM column (Pharmacia) equilibrated with 5 mM potassium phosphate buffer at pH 6.0. The protein was eluted using a linear NaCl gradient (0−200 mM).

Protein Samples for Paramagnetic ^{1}H NMR Studies. For paramagnetic NMR experiments the protein was fully oxidized using a solution of $K_3[Fe(CN)_6]$. The excess oxidant was removed either using an ultrafiltration cell (Amicon) or with a centrifugal concentration unit (Centricon 10, Amicon). The protein was usually exchanged into 35 mM potassium phosphate buffer. Samples were prepared in both 90% H_2O/10% ^2H_2O and 99.9% ^2H_2O and typically contained 2 mM protein. The pH of the sample was adjusted using NaOD or DCl. All pH values quoted are uncorrected for the deuterium isotope effect. Spectra were also acquired in 50 mM phosphate buffer (99.9% ^2H_2O) at pH 7.0 to enable a direct comparison with the published isotropic shifts of other cupredoxins.[49]

Protein Samples for Electron Self-Exchange Rate Constant Measurements. For the electron self-exchange rate constant determinations the protein was exchanged into 99.9% deuterated 35 mM potassium phosphate buffer, pH 8.2 ($I = 0.10$ M). The reduced sample was obtained by adding approximately 1 equiv of sodium ascorbate to the protein. The excess reductant was removed from the sample by ultrafiltration using degassed buffer. The reduced sample was transferred to an NMR tube, flushed with nitrogen, and sealed. The oxidized sample was obtained in a manner similar to that described in the previous section (vide supra). NMR spectra (vide infra) were acquired of the reduced protein in the presence of various concentrations of the oxidized sample (40−965 μM). The concentration of oxidized pseudoazurin was measured by transferring the NMR sample, under air-free conditions, to either a 1 or 2 mm path length UV/vis cuvette, and monitoring the absorbance at 593 nm ($\epsilon = 3700$ M^{-1} cm^{-1}) on a Perkin-Elmer λ6 spectrophotometer at 25 °C. The concentration was measured immediately prior to acquiring the NMR spectrum and immediately after acquisition. The values used in the subsequent calculations were an average of these two readings. Values of T_2^{-1} for the C$^{\epsilon 1}$H resonance of one of the histidine ligands (at 7.55 ppm)[61,62] were obtained by multiplying the peak width at half-height by π ($\nu_{1/2} = (\pi T_2)^{-1}$) and were plotted against the concentration of oxidized protein in the sample. From the slope of this plot the self-exchange rate constant was obtained.

The dependence of the electron self-exchange rate constant on pH in the range 7.5−11.3 was investigated using an approximately 50:50 mixture of oxidized and reduced protein (ca. 1 mM of each). In all cases the line width of the C$^{\epsilon 1}$H histidine ligand resonance at 7.55 ppm was used to determine the self-exchange rate constant. The line width of this resonance was also measured in a fully reduced pseudoazurin sample at the various pH values. The concentration of oxidized pseudoazurin in the NMR tube was again measured before and after acquisition of the NMR spectrum (vide supra). The average of the two values was used in the calculations. The ionic strength of the buffer used changed only slightly over the pH range investigated. Previous studies on pseudoazurin[59] have shown that the self-exchange rate constant of the protein is almost independent of ionic strength in the range $I = 0.04$−0.30 M. The self-exchange rate constant measured at pH 10.9 was verified by performing a full titration of reduced pseudoazurin with oxidized protein (23−364 μM) at this pH value.

NMR Spectroscopy. All the ^{1}H NMR spectra were acquired on a Varian Unity 500 spectrometer at 25 °C (spectra of the protein in 50 mM phosphate buffer at pH 7.0 were acquired at 32 °C), either using a standard one pulse sequence employing presaturation of the H_2O or HDO resonance during the relaxation delay or using the super-WEFT (water-suppressed equilibrium Fourier transform) pulse sequence.[63] Diamagnetic 1D spectra were acquired with a spectral width of 8 kHz. Paramagnetic spectra were acquired with spectral widths ranging from 20 to 100 kHz and were processed with 10−50 Hz exponential line broadening as apodization. Spin lattice (T_1) relaxation times of hyperfine shifted resonances were determined at 32 °C with the protein in 50 mM phosphate buffer (pH 7.0), using the super-WEFT sequence as described previously.[64]

Results

Paramagnetic ^{1}H NMR Spectrum of Pseudoazurin. The paramagnetic ^{1}H NMR spectrum of Cu(II) pseudoazurin (Figure

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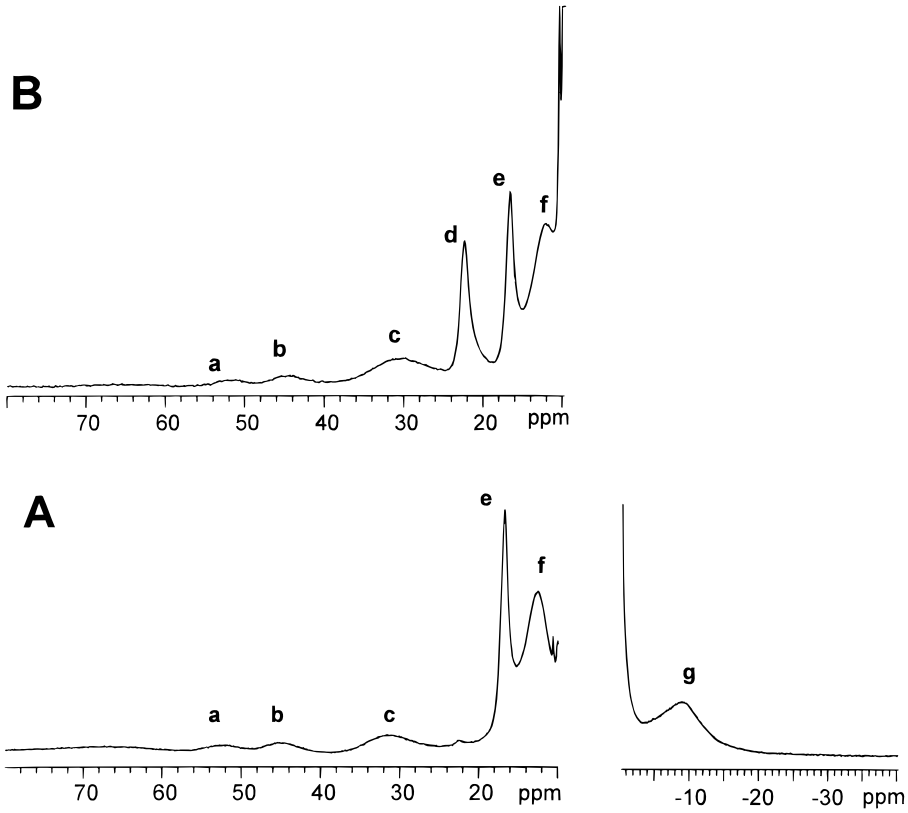

Figure 3. WEFT spectra (25 °C) of Cu(II) pseudoazurin (A) at pH 7.5 in ^2H_2O and (B) at pH 7.5 in H_2O.

3) possesses seven resonances (a−g), whose line widths and isotropic shifts identify them as belonging to protons associated with the four copper ligands (Figure 2). Previous studies[49] have demonstrated that only protons >4 Å from the copper ion in a cupredoxin will be observed in such spectra. The very close similarity of this spectrum to the assigned spectrum of Cu(II) amicyanin greatly simplifies the assignment process. The comparison between the spectra of these two cupredoxins is further justified by their very similar active site structures. The comparison of the spectrum of Cu(II) pseudoazurin in ^2H_2O (Figure 3A) and H_2O (Figure 3B) identifies the presence of the exchangeable isotropically shifted peak d. In previous paramagnetic NMR studies on Cu(II), Co(II), and Ni(II) cupredoxins and also Cu_A centers a similar resonance has been found,[49,64−66] at neutral pH, and clearly identifies peak d as belonging to the exchangeable $N^{\epsilon 2}$ proton of the more buried His40 ligand. The two very broad peaks a and b can be assigned to the $C^{\delta 2}H$ resonances of the two histidines ligands. Peak c can be tentatively assigned to a resonance belonging to one of the histidine ligands. In studies on Cu(II) amicyanin a corresponding resonance was tentatively assigned to a proton belonging to His96.[49] Signal e is assigned to the $C^{\alpha}H$ resonance of Cys78. The assignment of this latter resonance is supported by the fact that the corresponding resonance has been found at very similar chemical shift values in paramagnetic ^1H NMR spectra of all Cu(II) cupredoxins studied.[32,49,67,68] This is as you would expect when you consider the similarity of the conformation of the side chain of the cysteine ligand in the different cupredoxins.[69] The broader signal f can be assigned to one of the $C^{\gamma}H$ resonances of the axial Met86 ligand. The assignment of signals e and f is supported by their T_1 relaxation times of 5.2 and 0.5 ms, respectively. The T_1 value for resonance e (5.2 ms) is similar to those found for the ligand Cys $C^{\alpha}H$ resonances in amicyanin (8.7 ms) and azurin (9 ms at 600 MHz).[68] The shorter T_1 value of peak f (0.5 ms) clearly indicates that the proton responsible for this resonance must be much closer to the copper ion and therefore identifies resonance f as the $C^{\gamma 1}H$ of Met86, which is only 4.21 Å from the metal ion (the Cys78 $C^{\alpha}H$ is 4.82 Å from the copper ion and the Met86 $C^{\gamma 2}H$ is 4.96 Å away) (Note: all distances are taken from the crystal structure of oxidized *A. cycloclastes* pseudoazurin[42,43]). Further confirmation of the assignment of resonances e and f is obtained from the pH dependence of their chemical shifts (vide infra). Finally, signal g can be assigned as a C^{β} proton of one of the two histidine ligands. Table 1 lists the observed resonances along with the assignments made. Also included are the calculated pseudocontact and Fermi contact contributions to the observed shifts for resonances d, e, and f (vide infra).

Determination of the Fermi Contact Contributions to the Isotropic Shifts. The observed isotropic shifts (δ_{obs}) listed in Table 1 arise from three contributing factors as shown in eq 1;

$$\delta_{obs} = \delta_{dia} + \delta_{pc} + \delta_{Fc} \quad (1)$$

where δ_{dia} is the shift that would be observed for a similar diamagnetic molecule, δ_{pc} is the pseudocontact (through-space) contribution to the observed shift, and δ_{Fc} is the Fermi contact (through-bond) contribution. The pseudocontact contribution to the isotropic shifts, which is known to be small for such

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Table 1. Hyperfine Shifted Resonances of Cu(II) Pseudoazurin at 25 °C

resonance	assignment	δ_{obs}[a,b] (ppm) pH 7.5	δ_{obs}[a] (ppm) pH 10.8	δ_{pc}[c] (ppm)	δ_{Fc}[c] (ppm) pH 7.5	δ_{Fc}[c] (ppm) pH 10.8
a	His $C^{\delta 2}H$	51.8 (51.1)	51.9	n.d.	n.d.	n.d.
b	His $C^{\delta 2}H$	44.8 (44.0)	45.4	n.d.	n.d.	n.d.
c	His	31.4 (31.0)	32.0	n.d.	n.d.	n.d.
d	His40 $N^{\epsilon 2}H$	22.5	23.3	−0.9	11.9	12.7
e	Cys78 $C^{\alpha}H$	16.8 (16.4)	17.2	−1.1 (−1.0)[d]	12.7 (12.2)[e]	13.1
f	Met86 $C^{\gamma 1}H$	12.6 (12.2)	<10	1.8 (1.7)[d]	8.6 (8.3)[e]	<6.0
g	His $C^{\beta}H$	−9.3 (−8.8)	−8.5	n.d.	n.d.	n.d.

[a] For samples in H_2O. [b] Values shown in parentheses were obtained for a sample in 99.9% deuterated 50 mM phosphate buffer (pH 7.0) at 32 °C. [c] Pseudocontact and Fermi contact contributions to the isotropic shifts were calculated as described in the text. [d] Calculated pseudocontact contributions to the isotropic shifts at 32 °C. [e] Fermi contact shifts at 32 °C with the protein in 99.9% deuterated 50 mM phosphate buffer (pH 7.0).

systems,[49] can be estimated using eq 2;

$$\delta_{pc} = \left(\frac{\mu_0}{4\pi}\right)\frac{\mu_B{}^2 S(S+1)}{9kT}[g_{||}{}^2 - g_{\perp}{}^2]\left(\frac{3\cos^2\theta - 1}{r^3}\right) \quad (2)$$

where μ_0 is the vacuum permeability, μ_B is the Bohr magneton, S is the electron spin, k is the Boltzmann constant, $g_{||}$ and $g_{\perp}$ are the parallel and perpendicular components of the g-tensor,[45] θ is the angle between the z-axis of the g-tensor and the vector connecting the copper ion with a particular proton, and r is the distance between the proton and the copper ion. Since the orientation of the z-component of the g-tensor with respect to the molecular framework is not known for pseudoazurin, it was assumed that it is at an angle of 0° to the Cu−S(Met86) bond. Studies on cupredoxins have shown that the z-axis of the g-tensor makes an angle of 4−20° with the bond between the copper and the axial ligand,[70−73] and so any errors introduced into our calculations are small. The pseudocontact shifts calculated using the structure of *A. cycloclastes* pseudoazurin are shown in Table 1. Using δ_{dia} values typical for the particular type of proton in NMR studies on Cu(I) cupredoxins, the Fermi contact contributions to the observed isotropic shifts can be calculated using eq 1.

The Effect of pH on the Paramagnetic ^{1}H NMR Spectrum. The effects of increasing pH value on the visible, EPR, and resonance Raman spectra of Cu(II) pseudoazurin have been reported previously. The observed effects clearly point to an increased Cu−S(Met) distance in the alkaline form of the protein and an increased Cu−S(Cys) interaction.[47] The effect of pH on the paramagnetic ^{1}H NMR spectrum of Cu(II) pseudoazurin is shown in Figure 4. Upon increasing the pH value from 8.5, the Met86 $C^{\gamma 1}H$ resonance shifts in an upfield direction. At pH 10.8 (data obtained in D_2O, not shown) this resonance is no longer observable outside the diamagnetic envelope of the protein. These observations clearly point to a dramatic decrease (>30%) in the Fermi contact contribution to the isotropic shift of this resonance. The pseudocontact contribution to the observed isotropic shift of this resonance is very small (see Table 1) and would not be dramatically affected by a subtle structural change.

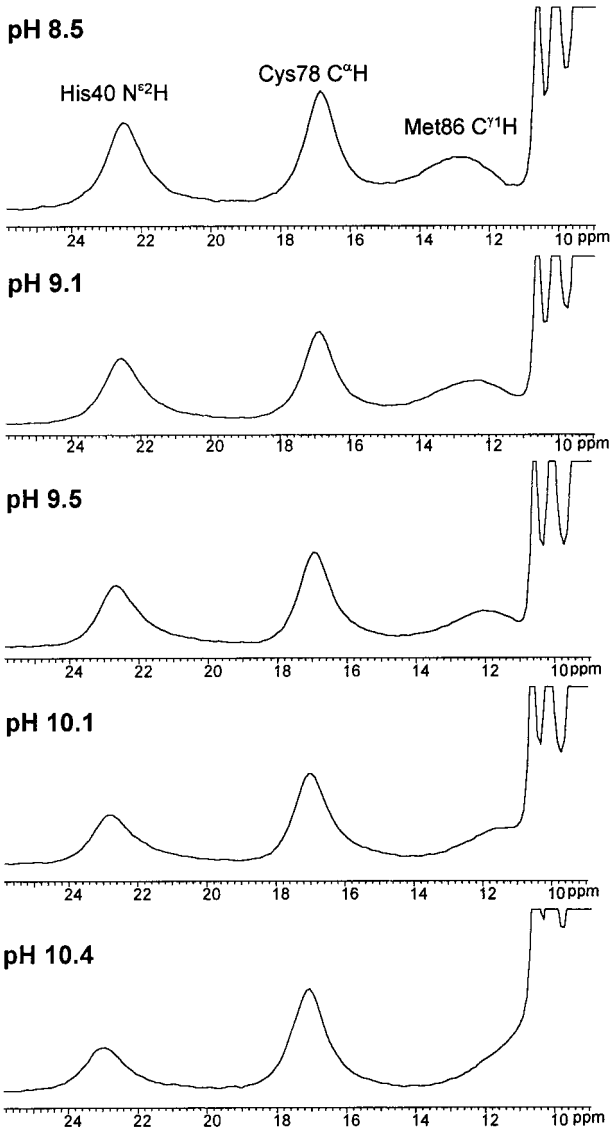

Figure 4. WEFT spectra (25 °C) of Cu(II) pseudoazurin in H_2O at various pH values. The two resonances between 10 and 11 are exchangeable resonances probably due to amide groups situated close to the metal site.

Upon increasing the pH, not only does resonance f shift in an upfield direction but there are concomitant downfield shifts of the Cys78 $C^{\alpha}H$ (peak e) and His41 $N^{\epsilon 2}H$ (signal d) resonances. These shifts are smaller than that experienced by the Met86 $C^{\gamma 1}H$ resonance. The observed effects are due to an increase of ∼3% in the δ_{Fc} of the Cys78 $C^{\alpha}H$ and ∼6% increase in δ_{Fc} of the His41 $N^{\epsilon 2}H$ signal. At values above pH 11 the Cys78 $C^{\alpha}H$ resonance (peak e) shifts in an upfield direction. At these pH values oxidized pseudoazurin is relatively unstable, and so this effect is not considered further. The pH dependences of the very broad signals a, b, c, and g are too difficult to quantify, and even though chemical shift values are quoted at pH 10.8 in Table 1, they are not considered in any detail here.

The influence of pH on the isotropic shifts of the His40 $N^{\epsilon 2}H$, Cys78 $C^{\alpha}H$, and Met86 $C^{\gamma 1}H$ resonances was fitted (three

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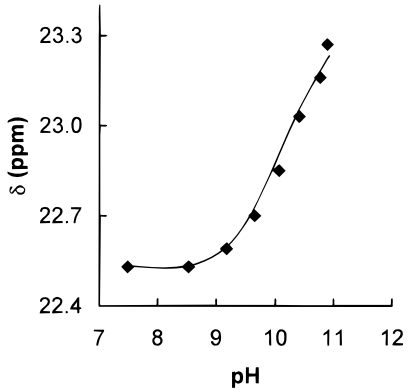

Figure 5. Dependence on pH of the chemical shift of the His40 $N^{\epsilon 2}H$ in 35 mM phosphate buffer (90% H_2O/10% ^2H_2O) at 25 °C. The fitting of the data to the line shown is explained in the text.

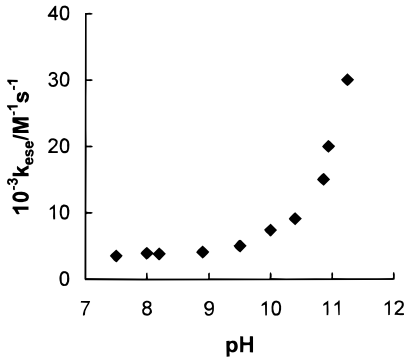

Figure 6. Dependence of the electron self-exchange rate constant (25 °C) of pseudoazurin on pH ($I \sim 0.10$ M).

parameters nonlinear least-squares) to an equation corresponding to a two-state pH-dependent equilibrium. In all cases pK_a values of 10.2−10.4 were obtained. The data, and the subsequent fit obtained for the His41 $N^{\epsilon 2}H$ signal, are shown in Figure 5. The pK_a values calculated indicate that the pH-induced conformational change at the active site of pseudoazurin is most probably caused by the deprotonation of a surface lysine residue.

The observed effect of pH on the δ_{Fc} values of the Met86 $C^{\gamma 1}H$, Cys78 $C^{\alpha}H$, and His41 $N^{\epsilon 2}H$ resonances is consistent with a more axial copper site in the alkaline form of the protein (see Discussion). The fact that this conclusion agrees completely with that made from various other spectroscopic measurements further confirms the assignments made for these resonances in the paramagnetic ^1H NMR spectrum of Cu(II) pseudoazurin.

The Self-Exchange Reaction of Pseudoazurin and Its Dependence on pH. The effect of pH on the self-exchange rate constant of pseudoazurin is shown in Figure 6. The measured self-exchange rate constant (25 °C) of 3.5×10^3 M^{-1} s^{-1} at pH 8.2 ($I = 0.10$ M) is very similar to a value of 2.9×10^3 M^{-1} s^{-1} reported previously for the protein at pH 7.5 under similar conditions.[59] As the pH value is increased, the self-exchange rate constant remains almost constant until pH 9. After this pH value a dramatic increase in the self-exchange rate constant is observed. At pH 11.3 a self-exchange rate constant of 3×10^4 M^{-1} s^{-1} is found, a value almost 10 times larger than that obtained at around neutral pH.

It should be noted that the observed pH dependences of both the paramagnetic ^1H NMR spectrum of Cu(II) pseudoazurin and the self-exchange rate constant of the protein are completely reversible. The paramagnetic NMR spectrum at pH 9.5 (see Figure 4) was obtained on a sample that had previously been taken to pH 11. Similarly, the self-exchange rate constants at pH 10.0 and 8.9 were measured on a 50:50 sample of oxidized and reduced pseudoazurin which had been used to determine the self-exchange rate constant at pH 11. Additionally, the diamagnetic ^1H NMR spectra of Cu(II) pseudoazurin at pH 8.5 and 11.0 are almost identical (data not shown). This demonstrates that the protein is not denatured at the high pH values and would tend to indicate that there are no dramatic changes in the overall structure of the protein over this pH range.

Discussion

The paramagnetic ^1H NMR spectrum of Cu(II) pseudoazurin shows a striking similarity to the assigned spectrum of Cu(II) amicyanin.[49] This is what one would expect considering the very similar structures of the active sites of these two proteins. Subtle differences do exist with respect to the isotropic shifts of certain resonances. The Met86 $C^{\gamma 1}H$ resonance of pseudoazurin experiences a larger Fermi contact shift than the corresponding resonance in amicyanin (8.3 ppm versus 6.7 ppm).[49,74] This indicates that there is slightly greater spin density on this ligand in pseudoazurin, consistent with the ca. 0.1 Å shorter Cu−S(Met) distance.

The effect of pH on the hyperfine shifted resonances in the spectrum of Cu(II) pseudoazurin provides a detailed picture of the structural consequences of the alkaline transition on the protein's active site. The drastic decrease (>30%) in the Fermi contact contribution to the isotropic shift of the Met86 $C^{\gamma 1}H$ resonance clearly points to a decreased interaction of the copper with this ligand at high pH. The concomitant increase in the isotropic shifts of the His40 $N^{\epsilon 2}H$ (∼6%) and Cys78 $C^{\alpha}H$ (∼3%) resonances clearly identifies that the interaction of these ligands with the copper increases. Collectively, the data show that in the alkaline form of the protein the copper ion has moved away from the axial methionine ligand toward the plane of the three equatorial ligands. This conclusion is completely consistent with previous spectroscopic studies carried out on the alkaline transition of pseudoazurin.[47] The small increase in the isotropic shifts of protons associated with the equatorial ligands as compared to a large decrease in the hyperfine shift of a proton associated with the axial ligand is what one would expect for a conformational change of this kind.

The hydrophobic patch of pseudoazurin is surrounded by a number of lysine residues. These include Lys10, Lys12, Lys38, Lys57, Lys59, Lys77, Lys109, and Lys110 (see Figure 1). The pH dependence of the electron self-exchange rate constant of pseudoazurin provides compelling evidence that these lysine residues hinder association of two pseudoazurin molecules at neutral pH. It is clearly not possible from our data to identify which of these are more responsible for lowering the electron self-exchange reactivity. Increasing the pH value above 9.0 results in these lysine residues deprotonating, and thus the electrostatic repulsion between two pseudoazurin molecules decreases dramatically. This provides direct proof of the use of the hydrophobic patch of pseudoazurin in the self-exchange reaction. This fact has only previously been directly demonstrated for azurin using variants in which charged amino acids have been introduced, by site-directed mutagenesis, into the hydrophobic patch.[55−58] Furthermore, these studies clearly demonstrate that fast electron self-exchange is not required in the physiological function of pseudoazurin. The lysine residues that are shown here to hinder self-exchange are known to be crucial for the interaction of pseudoazurin with nitrite reductase.[52,53]

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The observation that the high pH form of pseudoazurin has a much larger self-exchange rate constant than the neutral pH form of the protein greatly helps in elucidating the cause of the alkaline transition. The deprotonation of one, or more, surface lysine residues is clearly responsible for the active site transition observed upon increasing the pH value. This is the first direct experimental proof of surface lysine residues being responsible for the alkaline transition of a cupredoxin. Of the lysine residues situated close to the active site (Figure 1) two appear to be the most likely to cause the observed transition. The first is Lys77, which is adjacent to the ligand Cys78, while Lys38, which is situated in the center of the hydrophobic patch, is another possibility.

The alkaline transition of pseudoazurin is slightly different from that observed in the phytocyanin subclass of the cupredoxins. In the case of the phytocyanins, the alkaline form of the proteins has a more rhombic active site.[26] As we have clearly demonstrated here, the alkaline transition of pseudoazurin results in a more axial active site. However, it would appear likely, in light of the results presented, that the deprotonation/protonation of one, or more, surface lysine residues is also responsible for the alkaline transition of the phytocyanins.

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