Spectroscopic and Electrochemical Studies on Active-site Transitions of the Type 1 Copper Protein Pseudoazurin from Achromobacter cycloclastes*

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The single type 1 copper protein pseudoazurin from Achromobacter cycloclastes gives reversible electrochemical behavior at a (4-pyridyl)disulfide-modified gold electrode. Measurements carried out at 25.0°C indicate a midpoint reduction potential of E\text{1/2} = 260 mV versus normal hydrogen electrode at pH 7.0 and a peak-to-peak separation of ΔE\text{p} = 59 mV. The diffusion coefficient and heterogeneous electron transfer rate constant are estimated to be 2.23 × 10\text{-}6 cm\text{s}\text{-}1 and 3.7 × 10\text{-}2 cm\text{s}\text{-}1, respectively. Also, controlled potential electrolysis indicates a 1-electron transfer process and a formal reduction potential of 259 mV versus normal hydrogen electrode for the Cu(II)/Cu(I) couple. The heterogeneous electron transfer rate constant determined at the (4-pyridyl)disulfide-modified gold electrode at pH 4.6 is 6.7 × 10\text{-}7 cm\text{s}\text{-}1, consistent with a slower process at the positively charged electrode surface. At pH 11.3, UV-visible, EPR, and resonance Raman spectra indicate a conversion of the distorted tetrahedral copper geometry to a trigonal structure. The trigonal form has elongated axial bonding and an axial EPR spectrum. At pH 11.3, the reduction potential is further decreased, and Cu–S bands in resonance Raman spectra at 330–460 cm\text{-}1 are shifted to higher energy (−10 cm\text{-}1), consistent with a stronger Cu–S bond.

The type 1 blue copper proteins are a family of electron transfer proteins that have a single copper atom at their active site, an intense absorption band due to the S(Cys) → Cu(II) charge transfer, and a narrow hyperfine coupling constant in the EPR spectra of the oxidized protein (1). The copper atom is located beneath the protein surface at a depth of 5–10 Å (2), with two histidines (imidazole (N)), one cysteine (thiolate (RS\text{-}\text{2})), and one methionine (thioether (S)) as ligands. Recent kinetic (3) and theoretical (4, 5) studies on the blue copper protein plastocyanin have indicated the presence of two distinct electron transfer sites: (i) the adjacent site at the surface-exposed histidine of the hydrophobic path −6 Å from the copper and (ii) the remote site involving acidic patch region −15 Å from the copper with acidic residues on either side of the exposed Tyr-83. Corresponding sites appear to be effective for electron transfer in the plastocyanin-like domain of ascorbate oxidase (and presumably other copper oxidases) and are considered here for the single type 1 copper protein pseudoazurin.

Peroadoxin (−14 kDa) is known to be a component of at least four bacteria, Achromobacter cycloclastes (6), Pseudomonas AM1 (7), Alcaligenes faecalis (8), and Thiophosaera pontotropha (9), where it functions as an electron carrier in the respiratory chain of the microorganism. The A. cycloclastes (10), A. faecalis (8), and T. pontotropha (9) pseudoazurins are electron donors to their respective nitrite reductases. A. cycloclastes pseudoazurin, which has a pl of 8.4 and a charge (from the amino acid composition, with Asp and Glu as 1− and Arg and Lys as 1+) of 1− at pH 7.0, is the subject of this study. The three-dimensional x-ray structure of A. faecalis pseudoazurin has been reported (11, 12), and it has been confirmed that the copper ion is coordinated to two histidines (His-40 and His-81), one cysteine (Cys-78), and one methionine (Met-86) in a distorted tetrahedral configuration. The Cu-S(Met-86) distance (2.76 Å) is shorter than that observed in the plastocyanins from poplar leaves (2.83 Å) (13) and Enteromorpha prolifera (2.93 Å) (14) as well as in azurin from Alcaligenes denitrificans (3.11 Å) (15). Pseudoazurin has a distorted tetrahedral copper center similar to that of plastocyanin, in contrast to azurin, which has a distorted trigonal copper center. More recently, the x-ray structure of pseudoazurin from A. cycloclastes has been reported (Fig. 1) (16), and the structure is almost identical to that of pseudoazurin from A. faecalis (11, 12) and Pseudomonas AM1 (17).

EXPERIMENTAL PROCEDURES

Isolation and Purification of Pseudoazurin—The isolation and purification of pseudoazurin were carried out as described previously (18). Concentrations were determined from the absorbance of the Cu(II) protein at 593 nm (ε = 3700 M\text{-}1 cm\text{-}1) at pH 7 (19).

Spectroscopic Measurements—UV-visible spectra were determined on a Shimadzu UV-2200 recording spectrophotometer. EPR spectra (X-band) on frozen solutions at 77 K were obtained on a JEOL JES- FE-1 EPR spectrometer. All resonance Raman spectra were measured at room temperature with a spinning cell (1800 rpm, diameter = 5 mm).
and Raman shifts were calibrated to an accuracy of 1 cm$^{-1}$ using indene. Resonance Raman scattering was excited at 647.1 nm by a Kr$^+$ ion laser (Spectra-Physics Model 2016) and an Astro-Med CCD 3200 detector attached to a single monochrometer (Ritsu Oyo Kogaku Model DG-100) (20). The laser power at the sampling point was adjusted to 50 milliwatts.

Electrochemical Measurements—Cyclic voltammetry and square-wave voltammetry were carried out using a Bioanalytical Systems Model CV-50W voltammetric analyzer with a Faraday cage. Modification of the gold electrode was carried out as described previously (21).

A single compartment electrochemical cell was used with an Ag/AgCl reference electrode (Bioanalytical Systems, Inc.) and a platinum wire counter electrode separated by a Vicor glass tip from the working solution. Controlled potential electrolysis of pseudoazurin was investigated with a Bioanalytical Systems Model CV-27 voltammograph. Electronic absorption spectra were monitored during the course of electrolysis using a Shimadzu spectroscopic flow cell. Oxygen was removed from the working compartment by passing humidified O$_2$-free argon through the electrochemical cell for 15 min.

**RESULTS**

Cyclic voltammograms were obtained for pseudoazurin in the potential range +600 to 0 mV versus NHE and showed a well defined quasi-reversible faradaic response, with a mid-point potential of $E_{\text{midpoint}} = 260$ mV versus NHE and a peak-to-peak separation of $\Delta E_p = 59$ mV at a 4-pyds/Au electrode at pH 7.0 (Fig. 2). The cathodic peak current ($i_p$) was found to increase linearly with the square root of the potential scan rate ($v^{1/2}$), as expected for a diffusion-controlled process. The diffusion coefficient ($D$) was estimated to be $2.32(4) \times 10^{-6}$ cm$^2$ s$^{-1}$ at pH 7.0 from the slope of the plot $i_p$ against $v^{1/2}$ (Fig. 3A). Fig. 38 shows a linear relationship between the electrochemical kinetic parameter $\Psi$ ($=k_\text{c}/(\pi D n F v/RT)^{1/2}$) and the potential scan rate (22). The heterogeneous electron transfer rate constant ($k_\text{c}$) was estimated to be $3.7 \times 10^{-2}$ cm$^2$ s$^{-1}$ from the linear dependence of the kinetic parameter upon the scan rate.

Controlled potential electrolysis of pseudoazurin was also performed at a 4-pyds/Au mesh electrode, and UV-visible spectra were measured simultaneously at various electrode potentials (Fig. 4A). A decrease in the intense blue absorbance of pseudoazurin at 593 nm indicates reduction of Cu(II) to Cu(I). Reoxidation of electroreduced pseudoazurin could be achieved, confirming that electron exchange of pseudoazurin occurs at the electrode surface without denaturation of the protein. Spectra observed at intermediate potentials are a measure of the ratio of oxidized to reduced forms present. Fig. 4B shows a typical plot of $E_{\text{applied}}$ against log([pseudoazurin$_{\text{red}}$]/[pseudoazurin$_{\text{ox}}$]). The slope of the straight line is 61 mV, which compares favorably with the predicted nernstian value of 59 mV for a 1-electron process. At 25 °C and pH 7.0 (0.10 M phosphate), the reduction potential ($E_{\text{1/2}}$) of pseudoazurin from the potential axis intercept is $259.1 \pm 0.2$ mV versus NHE, which is in good agreement with the value of 260 mV obtained from cyclic and

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$^1$ The abbreviations used are: NHE, normal hydrogen electrode; 4-pyds/Au, (4-pyridyl)disulfide-modified gold.
square-wave voltammetry.

The pH dependence of the reduction potential of the protein displays at least two acid-base equilibria (Fig. 5). At the lower pH values, the behavior is similar to that observed for plastocyanin (23–25) and is attributed to the acid-base properties of an active-site histidine of the Cu(I) protein. From the difference between the \( pK_a \) values for amino acid residues in the two oxidation states of the protein, it is possible to determine, using Equation 1, the effect of protonation or deprotonation of specific amino acid residues on the redox potential of the protein (26):

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E'(\text{pH}) = E'(\text{low pH}) + \left( \frac{RT}{nF} \right) \sum_i \ln \left( \frac{K_i^{\text{red}} + [H^+]}{K_i^{\text{ox}} + [H^+]} \right) \]

(Eq. 1)

where \( K_i^{\text{red}} \) and \( K_i^{\text{ox}} \) are the proton dissociation constants for the residues \( i \) in the reduced and oxidized proteins, respectively. Then the electrochemical data can be fitted to two \( pK_a \) values of 6.6 and 10.4, respectively.

In the case of plastocyanin, it has been demonstrated that protonation results in a loss of redox activity (27). X-ray crystallographic studies on reduced plastocyanin at different pH values (3.8–7.8) have confirmed the effect as an \( H^+ \)-induced dissociation of His-87, resulting in the Cu(I) being coordinated by His-37, Cys-84, and Met-92 in a trigonal arrangement (28).

Similarly, the increase in reduction potential of pseudoazurin at the lower pH values is assigned to the protonation of the corresponding histidine residue (His-81, \( pK_a = 6.6 \)). At pH 11.3, the reduction potential of pseudoazurin is less, \( E_{\text{midpoint}} = 201 \text{ mV versus NHE} \), which is assigned here to a structural transition brought about by the acid dissociation of the -NH\(_3\) e-amino group of Lys-77 (\( pK_a = 10.4 \)).

**Fig. 4.** A, controlled potential electrolysis of pseudoazurin (170 \( \mu \text{M} \)) at 25°C and pH 7.0 (0.1 M phosphate) showing absorption spectra of pseudoazurin during electrolysis at various electrode potentials: 503.8 (spectrum a), 303.8 (spectrum b), 275.3 (spectrum c), 263.1 (spectrum d), 243.8 (spectrum e), 225.2 (spectrum f), 205.3 (spectrum g), and 104.3 (spectrum h) mV versus NHE, respectively. The electrolyzed solution of pseudoazurin at each electrode potential was passed through a spectroscopic flow-cell and recovered into the electrochemical cell. B, the Nernst plot for the controlled potential electrolysis results. pAz, pseudoazurin.

**Fig. 5.** Variation of the reduction potential for the pseudoazurin Cu(II)/Cu(I) couple at different pH values. The data shown by open and closed circles are from cyclic voltammetry and square-wave voltammetry, respectively.

**Active-site Transitions of Pseudoazurin**
A. cycloclastes Cu(II) pseudoazurin exhibits three intense charge transfer bands \((\lambda_{\text{nm}})\) at 452 \((\epsilon = 1400 \text{ M}^{-1} \text{ cm}^{-1})\), 593 \((\epsilon = 3700)\), and 753 \((\epsilon = 1800)\) nm at pH 7.0. Recent works suggest that both the bands at around 600 and 450 nm in the visible absorption spectra of blue copper proteins correspond to \(\text{S(Cys)} \rightarrow \text{Cu(II)}\) electronic transitions (29, 30). The visible spectrum of Cu(II) pseudoazurin is not changed drastically in the pH range 4.6–10.4. At pH 11.3, the intensity of the peak at 593 nm increased \((\epsilon = 3900)\), and that at 452 nm decreased \((\epsilon = 1100)\), indicating a structural transition at the Cu(II) (Fig. 6). Han et al. (29) have pointed out that the sum of the \(\epsilon\) values for these two bands is similar for most type 1 copper proteins. At pH 7.0, the sum of the \(\epsilon\) values at 452 and 593 nm for oxidized pseudoazurin is \(5100 \text{ M}^{-1} \text{ cm}^{-1}\), whereas at pH 11.3, it is \(5000 \text{ M}^{-1} \text{ cm}^{-1}\). This constancy of the sum is in agreement with the behavior of other blue copper proteins and confirms that the changes are not due to denaturation.

The EPR spectra of the type 1 blue copper proteins can be either axial or rhombic. At pH 7.0, the spectrum of pseudoazurin shows a typical rhombic signal with anisotropic spin hamiltonian parameters, \(g_x = 2.01 (A_x = 6.7 \text{ milliteslas})\), \(g_y = 2.09\), and \(g_z = 2.21 (A_z = 5.0 \text{ milliteslas})\) (Fig. 7A). The EPR spectra of pseudoazurin do not change with pH in the range 4.6–10.4, consistent with no active-site effect in the Cu(II) form of the protein. However, at pH 11.3, the rhombic signal becomes almost axial with anisotropic EPR parameters, \(g_x = 2.06\) and \(g_y = 2.20 (A_y = 6.3 \text{ milliteslas})\) (Fig. 7B).

Resonance Raman spectra of blue copper proteins are enhanced by excitation, leading to characteristic spectra with one or two vibrational modes in the 250–280 cm\(^{-1}\) region and as many as nine vibrational modes in the 330–490 cm\(^{-1}\) region. The modes at the lower frequencies are assigned to Cu-His transitions and those at higher frequencies to Cu-Cys transitions (31). A. cycloclastes pseudoazurin has its own unique spectra in the Cu-Cys region, with one strong peak at 392 cm\(^{-1}\) and an additional seven smaller peaks at 454, 439, 411, 383, 368, 353, and 335 cm\(^{-1}\) (Fig. 8A). There is also an isolated Cu-His mode close to 257 cm\(^{-1}\). The multiplicity of vibrational modes between 330 and 460 cm\(^{-1}\) has been ascribed to kinematic coupling of the Cu—S(Cys) stretch with deformations of both the \(\delta(S-\text{C} \rightarrow \text{C} \rightarrow \text{N})\) and \(\delta(\text{C}-\text{C} \rightarrow \text{N})\) modes of the cysteine ligand (32–34). The Raman bands of \(\nu(\text{Cu—S})\) are at 449, 424, 400, 383, 361, and 337 cm\(^{-1}\), and at pH 11.3, an isolated Raman band corresponding to Cu—N(His) is observed at 269 cm\(^{-1}\) (Fig. 8B).

Alternative Raman measurements (31). A. cycloclastes pseudoazurin has its own unique spectra in the Cu-Cys region, with one strong peak at 392 cm\(^{-1}\) and an additional seven smaller peaks at 454, 439, 411, 383, 368, 353, and 335 cm\(^{-1}\) (Fig. 8A). There is also an isolated Cu-His mode close to 257 cm\(^{-1}\). The multiplicity of vibrational modes between 330 and 460 cm\(^{-1}\) has been ascribed to kinematic coupling of the Cu—S(Cys) stretch with deformations of both the \(\delta(S-\text{C} \rightarrow \text{C} \rightarrow \text{N})\) and \(\delta(\text{C}-\text{C} \rightarrow \text{N})\) modes of the cysteine ligand (32–34). The Raman bands of \(\nu(\text{Cu—S})\) are at 449, 424, 400, 383, 361, and 337 cm\(^{-1}\), and at pH 11.3, an isolated Raman band corresponding to Cu—N(His) is observed at 269 cm\(^{-1}\) (Fig. 8B).

DISCUSSION

The transition occurring at pH \(\sim 11.3\) can be monitored by the effect of pH on the visible spectrum of the Cu(II) protein. It is referred to here as a blue copper active-site transition. The ratio \(R\) of the \(\epsilon\) values at 452 and 593 nm is 0.38 at neutral pH and 0.28 at higher pH values (11.3). The value of \(R\) is believed to reflect the strength of the axial coordination (29). The larger \(R\) values indicate stronger axial coordination, leading to a distorted tetrahedral copper environment as in the type 1 copper site of nitrite reductase from A. cycloclastes (29, 30). The smaller value indicates a weaker axial binding as in the case of azurin (\(R = 0.11\)), where the Cu(II) coordination is almost trigonal (15). The EPR spectrum of pseudoazurin at pH 11.3 also suggests that the structural transition at pH 11.3 makes the axial coordination weaker and that the Cu-S(Met-86) bond is elongated compared with the situation at neutral pH.

2 Resonance Raman spectra obtained for Cu(II) pseudoazurin do not show significant changes in going to the more acidic pH values. This is in agreement with the crystallographic studies carried out on poplar Cu(I) plastocyanin at different pH values, which give no evidence for changes in active-site bond lengths.
from the Raman shifts (~10 \text{ cm}^{-1}) of the Cu—S(Cys) and Cu—N(His) vibrational modes to higher energy at pH 11.3. As a result of the axial distortion, the copper atom lies more in the plane of the three equatorial ligands His-40, His-81, and Cys-78 (Fig. 9). It is significant that the trigonal to tetrahedral blue copper transition in the H117G azurin mutant leads to Cu—S(Cys-112) Raman bands coupled with cysteine deformation that are 10–40 cm$^{-1}$ lower in energy (35, 36). A trigonal geometry at the copper, with the S(Cys) lying in the plane formed by His-81, His-40, and Cys-78, is therefore the most reasonable structure for pseudoazurin at high pH. These results are also consistent with the relationship between the blue copper active-site transition and the electron transfer reactivity at an electrode including reduction potential variations brought about by changes in electron density at the copper center.

Pseudoazurin shows a fast electron transfer process at the 4-pyds/Au electrode ($k_s = 3.2 \times 10^{-2}$ cm$^{-1}$ s$^{-1}$) and slower electron transfer at the positively charged 4-pydsh$^+/\text{Au}$ electrode surface ($k_s = 6.7 \times 10^{-3}$ cm$^{-1}$ s$^{-1}$). In the case of electrochemical experiments on cytochrome c, fast electron transfer of the protein is believed to be achieved by hydrogen bonding between the lone-pair electrons on a pyridine moiety at the electrode and positively charged lysine residues on the protein (37). With pseudoazurin, electron transfer is faster at the 4-pyds/Au electrode than at a pyrolytic graphite (38) or glassy carbon (39) electrode. The slower reaction at the pyrolytic graphite and glassy carbon electrodes is probably due to the presence of sites that are less active for electron transfer on a microscopic scale (40). At a 4-pydsH$^+/\text{Au}$ electrode at pH 4.6, the same two determinations were carried out and gave $6.7 \times 10^{-3}$ cm$^{-1}$ s$^{-1}$ and 301 mV versus NHE, respectively. The positively charged electrode surface does not interact as favorably with the positively charged protein, thus impeding electron transfer. In recent studies, the electron self-exchange rate constant for pseudoazurin has been determined by an NMR line-broadening technique and by cross-reaction studies with Pseudomonas aeruginosa azurin, and a value of $2.8 \times 10^{10}$ M$^{-1}$ s$^{-1}$ (average at $I = 0.100$ M and 25°C) was obtained (18). In the case of azurin (P. aeruginosa), rapid self-exchange ($7.0 \times 10^{2}$ M$^{-1}$ s$^{-1}$ at pH 9.0) (41) has been shown to occur via the adjacent hydrophobic patch through which the His-117 ligand is exposed (26, 42). In contrast, pseudoazurin and higher plant plastocyanins have charged residues at or near the adjacent site and subsequently are less efficient at self-exchanging due to electrostatic repulsions. It is interesting to note that the plastocyanin from Anabaena variabilis, which does not possess an acidic patch, has a much larger self-exchange rate constant (44).

In pseudoazurin, the cause of this repulsion is a number of lysine residues, in particular Lys-38, which is located in the middle of what might be described as the adjacent site (at around His-81) and in a similar position to the M44K residue of the P. aeruginosa azurin mutant (26, 42). The latter mutation has a drastic effect on the rate constant for self-exchange of azurin at low pH values. Another positive charge on pseudoazurin comes from Lys-77, which is adjacent to the Cys-78 ligand. This amino acid residue is located in a similar position to Tyr-83 in plastocyanin, which is in the middle of the remote acidic patch of the protein. In studies on cytochrome $c_2$ from Rhodobacter sphaeroides, specific lysine residues have been proposed as a part of the binding site, their role being to create an optional orientation for electron transfer (45), and a similar role for lysine residues in pseudoazurin is also possible.

It has been reported that the negatively charged A. cycloclastes nitrite reductase complexes strongly with positively charged residues on pseudoazurin (21). In the case of the blue copper protein amicyanin, a recent electrochemical study has indicated that the reduction potential of the protein is shifted by 73 mV when complex formation with methyamine dehydrogenase occurs (46). This complex has been confirmed by x-ray structure analysis to have hydrophobic interactions between amicyanin and the enzyme (43). It is possible that complex formation with nitrite reductase through the positive charges on pseudoazurin induces structural changes at the redox center, so that the reduction potential is tuned for a thermodynamically more favorable electron transfer.

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