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Loop-Contraction Mutagenesis of a Type 1 Copper Site

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Engineering studies are constantly highlighting features of proteins which are essential for their structure and functionality. In the case of metalloproteins, various approaches have been utilized to discover the crucial attributes of their active sites, ranging from site-directed mutagenesis of ligating residues to the design of novel metal-binding centers.[1,2] Metal-binding sites in proteins are commonly fabricated from loop regions, and using loop-directed mutagenesis active sites can be grafted onto a particular protein scaffold. The cupredoxins possess a rigid β-barrel structure, which is an ideal scaffold for protein engineering studies, and loop-directed mutagenesis has been used to modify the mononuclear type 1 copper site[3−5] and to introduce a dinuclear Cu_A center.[6,7] The loop mutagenesis studies carried out to date on cupredoxins have all involved lengthening an active-site loop. Herein we report the results of the first loop-contraction mutagenesis experiments on a cupredoxin, in which we have replaced an active-site loop of pseudoazurin (PAz) with the loop from amicyanin which is shorter (see Figure 1).

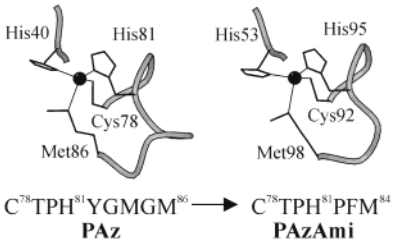

Figure 1. Active-site structures of *A. cycloclastes* pseudoazurin (PAz)[8] and *Paracoccus denitrificans* amicyanin.[9] The sequences of the C-terminal active site loops are shown and indicate the loop-contraction mutation that has been made to PAz to produce PAzAmi.

The type 1 copper site of PAz from *Achromobacter cycloclastes* is shown in Figure 1. The ligands Cys78, His81, and Met86 are connected by the C-terminal active-site loop. We have replaced the native loop of PAz with that of amicyanin (see Figure 1).[10] The PAzAmi protein produced possesses only two intervening residues between the His and Met ligands on this loop. PAzAmi has a cupric site structure very similar to that of PAz as judged by UV/vis[11] and EPR[13] spectroscopy (see Figure 2). The spectral properties of PAzAmi are distinct from those of amicyanin, the protein whose loop has been introduced (see Figure 2). Therefore, the Cu−S(Cys78) interaction, which is largely responsible for the spectroscopic features of a type 1 copper site,[14] is unaltered by the loop contraction. This is consistent with the fact that in PAzAmi the loop between Cys78 and His81 has not been modified. Confirmation that the structure of the cupric site in PAzAmi is very similar to that of PAz is gained from the paramagnetic ^{1}H NMR spectra of the two oxidized proteins which are almost identical.[15]

The loop-contraction mutation lowers the reduction potential (E_m) of PAz.[16] An E_m of 215 mV is obtained at pH 8.0 for PAzAmi compared to 266 mV for PAz (see Figure 3). It is interesting to

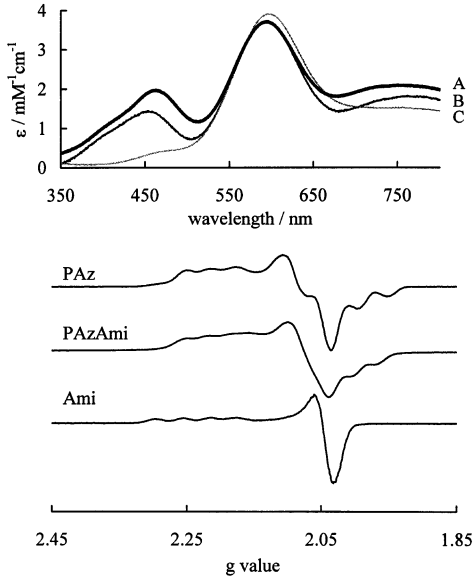

Figure 2. UV/vis (top) and EPR (bottom) spectra of PAz (B), PAzAmi (A), and amicyanin (Ami, C). UV/vis spectra were obtained at 25 °C in 10 mM phosphate pH 8.0, and X-band EPR spectra were obtained at −196 °C in 25 mM Hepes pH 7.6 (40% glycerol).

note that the loop-elongation mutations in amicyanin always result in an increase in E_m.[3−5] The pH dependence of the E_m has also been affected in PAzAmi (see Figure 3). The small influence of pH on the E_m of PAz is due to the protonation of His6, a surface residue some 14 Å from the copper site.[12] The large increase in the E_m of PAzAmi[17] as the pH is lowered from 8 to 5.8 indicates that reduction of the protein is accompanied by the uptake of a proton at the active site in this pH range. A pK_a of 6.6 is obtained from these data[18] which corresponds to the pK_a value determined for His81 in reduced PAzAmi from ^{1}H NMR experiments.[20] The ligand His81 also protonates and dissociates from the cuprous ion in PAz but with a much lower pK_a value of 4.8,[21] and also in a number of other cupredoxins including plastocyanin (pK_a ≈ 5)[19,22−24] and amicyanin (pK_a ≈ 7).[3,23,25] This property of cupredoxins may have a physiological role due to its dramatic influence on the E_m and electron-transfer (ET) capabilities of the type 1 copper site.[3,19,22−27] However, it is currently not clear which features of a cupredoxin's active-site architecture are most significant for determining the pK_a value of the C-terminal His ligand. It is interesting to note that the Pro95Phe mutation in *Paracoccus denitrificans* amicyanin has recently been shown to result in a large decrease in the pK_a of His95.[28] The increase in the pK_a for His81 in PAzAmi, and the similarity of its pK_a to that for the His95 ligand in reduced amicyanin, indicates that the length of the loop between the His and Met ligands can regulate this feature.

The influence of the loop contraction on the ET properties of PAz has been assessed by investigating the electron self-exchange

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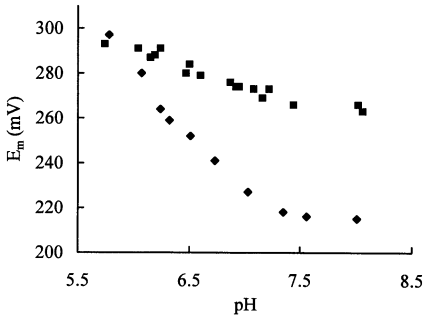

Figure 3. Dependence on pH ($I = 0.10$ M, NaCl) of the reduction potential (E_m) of PAz (■) and PAzAmi (◆). All of the values are referenced to the NHE at 21 °C.

(ESE) reactivity of PAzAmi.[29] The ESE rate constant of PAzAmi is 4.3×10^2 M^{-1} s^{-1} at pH* 7.6 compared to a value of 3.7×10^3 M^{-1} s^{-1} for PAz under identical conditions.[12] The ESE rate constant of PAzAmi decreases to 1.5×10^2 M^{-1} s^{-1} at pH* 6.5 consistent with protonation of the His81 ligand in the reduced protein diminishing the protein's ET capabilities. The PAzAmi variant is still redox active, but its ability to transfer electrons has been diminished by the mutation. A similar influence on ET reactivity has been observed in loop-elongation variants of amicyanin.[3−5]

The properties of the PAzAmi variant are consistent with the loop-contraction mutation not having a major effect on the structure of the copper site. Thus, the β-barrel scaffold of PAz is able to accommodate a shorter C-terminal active-site loop. The decrease in the E_m of PAzAmi indicates that the mutant has an active-site environment more suited to the cupric ion. The increased pK_a value of His81, and the diminished ET reactivity, can be attributed to structural modifications at the cuprous site caused by the loop contraction. This is consistent with observed changes in the ^{1}H NMR spectrum of reduced PAzAmi as compared to that of PAz (see Figure S4). The shortening of the His-to-Met sequence in a cupredoxin facilitates protonation and dissociation of the C-terminal His ligand.

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Supporting Information Available: Figures showing EPR simulation, paramagnetic NMR, CV, NMR titration, and diamagnetic NMR data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) The His81-to-Met86 loop of PAz (*Achromobacter cycloclastes*) was replaced with the sequence HisProPheMet as found in amicyanin using the QuickChange (Stratagene) mutagenesis kit. The mutation was verified by sequencing, and a pTrc99A (Amersham) derivative harboring the PAz construct containing the PAzAmi mutation was utilized to overexpress the protein in *Escherichia coli* BL21. Cell growth, protein isolation and purification were as described elsewhere (Yanagisawa, S.; et al. submitted).
(11) UV/vis spectra (25 °C) were obtained on a λ 35 (Perkin-Elmer) spectrophotometer in 10 mM phosphate at pH 8.0. The spectrum of PAz has bands at 453 nm ($\epsilon = 1600$ M^{-1} cm^{-1}), 594 nm ($\epsilon = 3700$ M^{-1} cm^{-1}), and 758 nm ($\epsilon = 1800$ M^{-1} cm^{-1}), whereas in PAzAmi these absorptions occur at 463 nm ($\epsilon = 1965$ M^{-1} cm^{-1}), 595 nm ($\epsilon = 3700$ M^{-1} cm^{-1}), and 755 nm ($\epsilon = 2080$ M^{-1} cm^{-1}). The UV/vis spectrum of PAzAmi is dependent upon pH in the range 8.0−5.0. The band at 595 nm increases in intensity, whilst that at 463 nm decreases as the pH is lowered (p$K_a = 6.5$) resulting in a A_{463}/A_{595} ratio of 0.48 at pH 5.0. A similar pH-dependence of the UV/vis spectrum has been seen for PAz and assigned to the protonation of the noncoordinated His6.[12]
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(13) The X-band EPR spectra (−196 °C) were obtained on a Bruker EMX spectrometer with the samples in 25 mM Hepes (plus 40% glycerol). Parameters were derived from simulations (see Figure S1) using the program SIMFONIA (Bruker). The spectra of PAz and PAzAmi are both rhombic with the following parameters; PAz: $g_x = 2.015$, $A_x = 7.3$ mT, $g_y = 2.053$, $A_y = 1.8$ mT, $g_z = 2.213$, $A_z = 3.5$ mT, PAzAmi: $g_x = 2.020$, $A_x = 6.0$ mT, $g_y = 2.058$, $A_y = 1.0$ mT, $g_z = 2.205$, $A_z = 4.5$ mT.
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(15) Paramagnetic ^{1}H NMR spectra (see Figure S2) were acquired on a JEOL Lambda 500 spectrometer at 25 °C with the protein in 10 mM phosphate at pH 7.6. The directly observed isotropically shifted resonances are found at 53.5 ppm (His81 $C^{\delta 2}$H), 46.0 ppm (His40 $C^{\delta 2}$H), 33.0 ppm (His40/81 $C^{\epsilon 1}$H), 22.8 ppm (His40 $N^{\epsilon 2}$H), 17.3 ppm (Asn41 C^{α}H), 13.0 ppm (Met86 C^{γ}H), and −9.7 ppm (Cys78 C^{α}H) in PAz, and at 56.4 ppm (His81 $C^{\delta 2}$H), 43.5 ppm (His40 $C^{\delta 2}$H), 30.7 (His40/81 $C^{\epsilon 1}$H), 21.0 ppm (His40 $N^{\epsilon 2}$H), 17.5 ppm (Asn41 C^{α}H), 13.6 ppm (Met84 C^{γ}H), and −10.5 ppm (Cys78 C^{α}H) in PAzAmi.
(16) The reduction potential of PAzAmi was measured using an electrochemical setup described previously[12] with the gold working electrode modified with 4,4-dithiodipyridine. Measurements were made in 20 mM Mes ($I = 0.10$ M, NaCl) in the pH range 5.8−6.7 and for experiments in the range pH 7.0−8.1 20 mM Tris ($I = 0.10$ M, NaCl) was used (see Figure S3 for example cyclic voltammograms).
(17) The reduction potential of PAzAmi is also influenced by the protonation of His6 and pK_a* values of 6.8 and 6.6 have been determined by NMR spectroscopy for this residue in the reduced and oxidized proteins, respectively. In PAz the pK_a* of His6 in the cuprous and cupric forms of the protein are 7.1 and 6.5, respectively.[12]
(18) The pK_a value is obtained by fitting the difference in the E_m values for PAzAmi and PAz as a function of pH to an equation given in ref 19.
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(20) The influence of pH* (pH* indicates a pH meter reading uncorrected for the deuterium isotope effect) on the ^{1}H NMR spectrum of reduced PAzAmi (see Figures S4 and S5) has been studied (25 °C) with the protein in 10 mM phosphate (99.9% D_2O). A pK_a* value of 6.7 is obtained from the influence of pH* on the chemical shift of the His81 $C^{\epsilon 1}$H resonance.
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(29) The self-exchange rate constant of PAzAmi was measured at 25 °C with the protein in 37 mM phosphate at pH* 7.6 and in 60 mM phosphate at pH* 6.5 (99.9% D_2O) as described previously.[12] The influence of oxidized protein concentration on the T_1^{-1} values of ligand His imidazole ring resonances in the reduced protein was measured, and using the relationship: $T_{1,obs}^{-1} = T_{1,red} + k$[Cu(II)] (where $T_{1,obs}^{-1}$ is the observed longitudinal relaxation rate and [Cu(II)] is the concentration of oxidized PAzAmi) which is valid for protons belonging to the slow-exchange regime, the self-exchange rate constant (k) was determined.

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