Crystal Structure Determinations of Oxidized and Reduced Pseudoazurins from Achromobacter cycloclastes

CONCERTED MOVEMENT OF COPPER SITE IN REDOX FORMS WITH THE REARRANGEMENT OF HYDROGEN BOND AT A REMOTE HISTIDINE

(Received for publication, February 3, 1999, and in revised form, April 8, 1999)

Tsuyoshi Inoue, Nobuya Nishio, Shinnichiro Suzuki, Kunishige Kataoka, Takamitsu Kohzuma, and Yasushi Kai

From the Department of Materials Chemistry, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan, the Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, and the Department of Chemistry, Faculty of Science, Ibaraki University, Mito, Ibaraki 310-8512, Japan

The crystal structures of oxidized and reduced pseudoazurins from a denitrifying bacterium, Achromobacter cycloclastes IAM1013, have been determined at 1.35- and 1.6-Å resolutions, respectively. The copper site in the oxidized state exhibits a distorted tetrahedral structure like those of other pseudoazurins. However, not only a small change of the copper geometry, but concerted peptide bond flips are identified. The imidazole ring of remote His6 has a hydrogen bonding distance of 2.73 Å between N-ε1(His6) and O-γ1(Thr30) in the oxidized protein. When the protein is reduced at pH 6.0, the imidazole ring rotates by 30.3° and moves 1.0 Å away from the position of the oxidized state. A new hydrogen bond between N-ε2(His30) and O-ε1(Glu16) is formed with a distance of 3.03 Å, while the hydrogen bond between N-δ1(His30)-O-γ1(Thr30) is maintained with an interatomic distance of 2.81 Å. A concomitant peptide bond flip of main chain between Ile34 and Thr36 occurs.

Pseudoazurin (Mr ~ 14,000) is a type 1 blue copper protein (cupredoxin) found in denitrifying bacteria and methylotrophs. While in methylotrophs pseudoazurins were produced as electron transfer substituted to another blue copper protein named amicyanin at high copper concentration. To explain such difference, the crystal structure analyses of pseudoazurins from four sources, Achromobacter cycloclastes IAM1013, Methyllobacterium extorquens AM1, Alcaligenes faecalis S-6, and Thiosphaera pantotropha (10) have been characterized to date. From their amino acid sequences, it is known that A. cycloclastes pseudoazurin has an extra C-terminal residue giving 124 amino acids in total (11). The greatest degree of similarity among pseudoazurins is 65% conservation of amino acid residues between A. cycloclastes and A. faecalis.

The crystal structure analyses of pseudoazurins from A. faecalis (11–14), T. pantotrophs (15), and M. extorquens (16) revealed that the overall topology of pseudoazurin consists of an eight-stranded β-barrel which resembles the structures of other cupredoxins like plastocyanin and azurin. Differences occur between these proteins regarding the amount and location of helical structure. Pseudoazurin possesses two extra α-helices at the C terminus, whereas azurin has an α-helical flap in the middle of the sequence and plastocyanin generally has a small (1 turn) helix (17). The copper atom is located below the protein surface at a depth of 5–10 Å, with two histidines (imidazole N), a cysteine (thiolate RS-), and methionine (thioether S) as ligands (18). Nine of 13 lysine residues surrounded the hydrophobic face of the molecule through which the histidine ligands protrude slightly. The effect of lysine residues for the electron transportation from pseudoazurin to nitrite reductase was well investigated by using mutant pseudoazurin from A. faecalis S-6 (19). The substitution of these basic lysine residues by amino acids with neutral/acidic side chains decreases the affinity of pseudoazurin for nitrite reductase, which suggests that pseudoazurin may interact with nitrite reductase through its hydrophobic patch. Moreover, the structure of pseudoazurin from T. pantotrophs revealed that the proposed docking motifs based on the positive hydrophobic surface patch for cytochrome cd1 nitrite reductase (15).

Small inorganic complexes such as [Fe(CN)6]3− and [Co(phen)3]3+ (phen = 1,10-phenanthroline) have been used as redox probes to identify potential binding sites on the surface of cupredoxins for electron-transfer partners (20). Recent kinetic and theoretical studies on the blue copper protein plastocyanin have indicated the presence of two distinct electron-transfer sites: (i) the adjacent hydrophobic patch ~6 Å from the copper through which one of the histidine ligands protrudes, and (ii) the remote site involving and acidic patch region ~15 Å from the copper, with acidic residues on either side of the exposed Tyr38 (Tyr33 is next to the copper ligand Cys34). Kinetics studies with small inorganic complexes have been carried out on A. cycloclastes pseudoazurin. The rate constant for the oxidation of the reduced molecule with [Fe(CN)6]3− is 103-fold bigger than that for the [Co(phen)3]3+ oxidation (21), indicating a clear preference of the protein for anionic species. Moreover, the self-exchange rate constant for reduced molecule (about 3 × 106 M−1 s−1) is much smaller than those of most other cupredoxins and is quite similar to that found in higher plant plas-

* This work was supported in part by Grant-in-Aids 09261223, 03241215, 04225216, 05029216, and 08249221 for Scientific Research on Priority Areas, Grants-in-Aid 11750495, 09780632, and 07780572, for Encouragement of Young Scientists from the Ministry of Education, Science, Sports and Culture of Japan, and the Tsukuba Advanced Research Alliance (TARA) Sakabe project. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (codes 1BQK and 1BQR) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

¶ To whom correspondence should be addressed. Tel.: 81-6-6879-7408; Fax: 81-6-6879-7409; E-mail: kai@chem.eng.osaka-u.ac.jp.

This paper is available on line at http://www.jbc.org

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
tocyanins (22). A. cycloclastes nitrite reductase, which possesses many acidic amino acid residues, accepts an electron from a reduced pseudoazurin with a second-order rate constant of $7 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ (5, 23). These findings are all probably due to the effect of a number of conserved lysine residues surround the hydrophobic surface of pseudoazurin.

The effect of pH on the redox reactivity of A. cycloclastes pseudoazurin is well documented (21, 22, 45). The reduced
protein has been shown to participate in an active site protonation/deprotonation equilibrium at His81, which gives an acid dissociation pKₐ of 5.2 from an NMR titration (21) and 4.6 from kinetic studies with inorganic complexes as oxidants (45). The effect of protonation/deprotonation of uncoordinated His6 residue located near to the active site shows a pKₐ value of 7.2 (reduced form) and 6.5 (oxidized form) from NMR titration and 7.3 from kinetic studies of the oxidation of the reduced pseudoazurin with small complexes (21).

The structure of reduced pseudoazurin from A. faecalis at pH 4.4 has been solved and demonstrates that the ligand His81 moves, and the position of solvent molecules changed (24). Preliminary crystallographic studies of A. cycloclastes pseudoazurin were reported by Turley et al. (25), but the crystals obtained were too thin for x-ray diffraction data to be obtained. We have previously reported the crystalization and preliminary x-ray studies on oxidized pseudoazurin from A. cycloclastes at pH 6.0 (26). In this work single crystals of oxidized and reduced pseudoazurin with high resolutional diffraction spots were obtained at pH 6.0. During the last stage of structure refinement, the amino acid sequence was partially corrected (27). We describe here the redox-induced conformational changes at the copper site, and the rearrangement of the hydrogen bonding pattern of the uncoordinated His6, which are quite similar, but not identical, to the pattern of changes found in A. faecalis pseudoazurin reduced at pH 7.0 (24). The peptide flip induced by protonation at His6 is pH-induced conformational transition of His81 in azurin from Pseudomonas aeruginosa (28).

**TABLE II**

<table>
<thead>
<tr>
<th>Distance parameters (Å)</th>
<th>N-δ of His (1)</th>
<th>S-γ of Cys (2)</th>
<th>N-δ of His (3)</th>
<th>S-δ of Met (4)</th>
<th>Oxygen atom</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. cycloclastes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(oxidized, pH 6.0)</td>
<td>1.95 (H40)</td>
<td>2.13 (C78)</td>
<td>1.92 (H81)</td>
<td>2.71 (M86)</td>
<td>3.94 (G39)</td>
</tr>
<tr>
<td>(reduced, pH 6.0)</td>
<td>2.04 (H40)</td>
<td>2.19 (C78)</td>
<td>2.11 (H81)</td>
<td>2.85 (M86)</td>
<td>4.03 (G39)</td>
</tr>
<tr>
<td><strong>Al. faecalis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-6 (oxidized, pH 6.8)</td>
<td>2.20 (H40)</td>
<td>2.14 (C78)</td>
<td>2.27 (H81)</td>
<td>2.67 (M86)</td>
<td>3.99 (G39)</td>
</tr>
<tr>
<td>S-6 (reduced, pH 7.8)</td>
<td>2.16 (H40)</td>
<td>2.17 (C78)</td>
<td>2.29 (H81)</td>
<td>2.91 (M86)</td>
<td>3.83 (G39)</td>
</tr>
<tr>
<td><strong>M. extorquens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM1 (oxidized, pH 8.0)</td>
<td>2.07 (H40)</td>
<td>2.15 (C78)</td>
<td>1.97 (H81)</td>
<td>2.68 (M86)</td>
<td>4.03 (G39)</td>
</tr>
</tbody>
</table>

<FIG. 2. Superimposition of the oxidized (thin lines) and reduced (thick lines) pseudoazurins from A. cycloclastes for whole structures (a) and the copper sites (b). A quite small r.m.s. deviation of 0.14 Å is calculated for whole structures. However, the copper site and the uncoordinated His6 are significantly moved on reduction at pH 6.0.>
highest rotation peak was found at Eulerian angles of $\alpha = 115.40^\circ$, $\beta = 62.23^\circ$, and $\gamma = 302.99^\circ$, and the translation parameters to $x = 0.4537$, $y = 0.1330$, and $z = 0.1387$. The $R$-factor calculated for the model structure was 47.5% for 15.0–3.88-Å resolution data. Refinements for both oxidized and reduced structures have been carried out by using PROLSQ: (33), X-PLOR version 3.0 (34), and TURBO-FRODO (35). The first step of the refinement for both forms was performed using X-PLOR. A simulated annealing calculation at 3000 K, further several steps of positional refinement were performed by X-PLOR using the stepwise increased data. Because of the ambiguity for the energy constraint parameters of metal atom used in the molecular dynamics refinement, somewhat unreliable copper geometries were obtained. In order to obtain exact copper geometry no restraint was imposed on the copper coordination through the final stage of refinement by REFMAC. Every stage includes model improvement by several cycles of refinement. Model improvements were carried out based on the Fourier maps calculated with the coefficients of $(2F_c - F_s)\exp(2\pi i\alpha_{i\alpha})$ and $(F_c - F_s)\exp(2\pi i\alpha_{i\beta})$. The final structure model, further rebuilding, and cycles of refinement for the water structure of oxidized molecule finally decreased the $R$-factor to 17.6% for the data between 8.0- and 1.35-Å resolution with quite reasonable stereochemistry. $R_{free}$ (37) was 18.9% for 5% of total data within the same resolution range. On the other hand, the final $R$- and $R_{free}$-factors for the reduced structure by using the data between 8.0- and 1.6-Å resolution were 17.3 and 21.1%. The results of data collection and refinement are summarized in Table I.

RESULTS

Quality of the Final Model—The final model of oxidized pseudoazurin from A. cycloclastes is made up of one monomer in the asymmetric unit with 124 amino acids, 121 water molecules, and single copper ion. The model remained close to standard geometry throughout refinement. The final model of reduced structure includes 96 water molecules. The mean positional errors of the atoms estimated by Luzzati plots are 0.137 Å for the oxidized protein and 0.153 Å for reduced form, respectively (38). For well defined parts of the structure, especially the $\beta$-strands, the internal side chains and the region around the metal site, the errors are likely to be lower. The quality of the final model is summarized in Table I. The program PROCHECK (39) was used to analyze conformational variations from defined norms. A Ramachandran plot (40) shows that all non-glycine residues have dihedral angles falling in (or near to) energetically preferred regions.

Overall Structure—A ribbon drawing of A. cycloclastes pseudoazurin is presented in Fig. 1. The approximately spher-
ical pseudoazurin molecule has overall dimensions of $38 \times 38 \times 27$ Å. The molecule possesses eight β-strands, forming two β-sheets, and two C-terminal α-helices. β-Sheet I consists of four β-strands: S1, residues 2–8; S2a, 17–19; S3, 30–34; S6, 64–67, and β-sheet II contains five β-strands: S2b, residues 22–25; S4, 42–44; S5, 56–58; S7, 72–77; S8, 87–92. These structural features are very similar to those of the other pseudoazurins (from A. faecalis, M. extorquens, and T. pantoe-
Crystal Structures of Oxidized and Reduced Pseudoazurins

Table III
Change of main chain conformational angles

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>Difference</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe^3</td>
<td>-129</td>
<td>-125</td>
<td>+4</td>
<td>160</td>
<td>163</td>
<td>+3</td>
</tr>
<tr>
<td>Glu^4</td>
<td>-127</td>
<td>-123</td>
<td>+4</td>
<td>137</td>
<td>139</td>
<td>2</td>
</tr>
<tr>
<td>Val^5</td>
<td>-124</td>
<td>-127</td>
<td>-3</td>
<td>128</td>
<td>132</td>
<td>+4</td>
</tr>
<tr>
<td>His^6</td>
<td>-100</td>
<td>-106</td>
<td>-6</td>
<td>138</td>
<td>145</td>
<td>+7</td>
</tr>
<tr>
<td>Met^7</td>
<td>-101</td>
<td>-107</td>
<td>-6</td>
<td>113</td>
<td>119</td>
<td>+6</td>
</tr>
<tr>
<td>Leu^8</td>
<td>-126</td>
<td>-128</td>
<td>-2</td>
<td>148</td>
<td>150</td>
<td>+2</td>
</tr>
<tr>
<td>Asn^9</td>
<td>-69</td>
<td>-69</td>
<td>0</td>
<td>-50</td>
<td>-32</td>
<td>-2</td>
</tr>
<tr>
<td>Thr^10</td>
<td>-115</td>
<td>-116</td>
<td>-1</td>
<td>145</td>
<td>143</td>
<td>-2</td>
</tr>
<tr>
<td>Val^11</td>
<td>-127</td>
<td>-124</td>
<td>+3</td>
<td>126</td>
<td>132</td>
<td>+6</td>
</tr>
<tr>
<td>Thr^12</td>
<td>-102</td>
<td>-105</td>
<td>-3</td>
<td>126</td>
<td>123</td>
<td>-3</td>
</tr>
<tr>
<td>Phe^13</td>
<td>-92</td>
<td>-92</td>
<td>0</td>
<td>114</td>
<td>123</td>
<td>+9</td>
</tr>
<tr>
<td>His^14</td>
<td>-106</td>
<td>-122</td>
<td>-16</td>
<td>128</td>
<td>116</td>
<td>-12</td>
</tr>
<tr>
<td>Pro^15</td>
<td>-79</td>
<td>-74</td>
<td>+5</td>
<td>70</td>
<td>96</td>
<td>+26</td>
</tr>
<tr>
<td>Thr^16</td>
<td>-62</td>
<td>-67</td>
<td>-5</td>
<td>-42</td>
<td>-37</td>
<td>+5</td>
</tr>
<tr>
<td>Asp^17</td>
<td>-107</td>
<td>-103</td>
<td>+4</td>
<td>150</td>
<td>153</td>
<td>+3</td>
</tr>
<tr>
<td>Lys^18</td>
<td>-79</td>
<td>-75</td>
<td>+4</td>
<td>157</td>
<td>152</td>
<td>-5</td>
</tr>
<tr>
<td>Gly^19</td>
<td>97</td>
<td>103</td>
<td>+6</td>
<td>13</td>
<td>10</td>
<td>-3</td>
</tr>
<tr>
<td>His^20</td>
<td>-122</td>
<td>-113</td>
<td>+9</td>
<td>161</td>
<td>162</td>
<td>+1</td>
</tr>
</tbody>
</table>

3.07 Å forming a new hydrogen bond (Fig. 3c). Apparently the hydrogen bonding between His^6 and Thr^36 is weakened. The rearrangement of hydrogen bond of His^6 leads to a concomitant Ile^34-Thr^46 main chain peptide bond flip. The main chain conformational angles are also changed dramatically, particularly around Pro^35 (Table III). The C^α and C atoms of Pro^35 have moved by 0.93 and 2.16 Å, respectively.

Loss of Water Molecules Adjacent to Gly^39—In azurin the backbone carbonyl oxygen of Gly^39 provides a second weakly interacting axial ligand at a distance of 3.1 Å from Cu^2+ in azurin, resulting in a distorted trigonal bypyramidal coordination geometry. In plastocyanins and pseudoazurins the corresponding distance is longer than that in azurin, and the active site geometry is distorted tetrahedral. The O(Gly^39) of A. cycloclastes pseudoazurin, which corresponds to O(Gly^45) in azurin, locates at a distance of 3.94 Å from the copper ion in the oxidized protein, and a water molecule with a temperature factor of 13.9 Å^2 is found at distances of 2.92, 2.83, and 3.04 Å from N(His^40), O(Asp^37), and O(Asn^61), respectively (Fig. 3c).

Because the O(Gly^39) is adjacent atom of N(His^40), the water molecule forms a long range hydrogen-bonding network ranging from O(Gly^39) to O(Asn^61). However, upon reduction the distance between O(Gly^39) and the copper ion is lengthened to 4.04 Å, which shows the extension of ionic radius of the copper center. Moreover, the water molecule is lost providing space for the peptide bond flip of Pro^35. The conformations of the main chain around Gly^39 is changed as shown in Table III. The extended ionic radius of copper and the reduction of the copper charge may influence the water molecule, which may facilitate the peptide bond flip and the rearrangement of the hydrogen bonding of His^6.

Structural Comparison between Pseudoazurins from A. cycloclastes and A. faecalis—The oxidized and reduced pseudoazurins from A. cycloclastes are superimposed on those from A. faecalis, respectively, with r.m.s. deviations for backbone atoms of 0.64 and 0.62 Å. The superimposition reveals the remarkable structural differences at the imidazole ring of His^6 (Fig. 4). The angles between the imidazole rings of His^6 in A. cycloclastes and A. faecalis pseudoazurins are different by more than 70°. The imidazole ring rotates to the perpendicular position relative to that in A. faecalis, which enables it to form the strong hydrogen bond to Thr^36 in the oxidized pseudoazurin from A. cycloclastes. The residue Glu^4 is conserved in both pseudoazurin but the 36th amino acid residue is Val instead of Thr in A. faecalis pseudoazurin. Only one hydrogen bond is therefore possible between His^6 and Glu^4. The N-ε2(His^6)-O-
Crystal Structures of Oxidized and Reduced Pseudoazurins

According to the detailed structural comparison between the oxidized and reduced pseudoazurin from A. cycloclastes, the difference in copper-His\(^6\) distance is bigger between two forms than other copper-ligand distances. The expansion of the ionic radius of the copper, 0.96 Å for Cu\(^{2+}\) and 0.69 Å for Cu\(^{3+}\), and the reduction of copper charge upon reduction results in the loss of a water molecule situated close to the active site. For this reason the peptide bond flip at Pro\(^6\) may occur more easily. The loss of a similar water molecule was found in A. faecalis pseudoazurin at both pH 7.8 and 7.0, while the peptide bond flip was found at only pH 7.0. Because the pK\(_a\)\(_{\text{His}}\) values of the uncoordinated His\(^6\) residue are 7.2 (reduced form) and 6.5 (oxidized form) (21), the protonation at His\(^6\) at pH 7.8 (14) in pseudoazurin, while Val\(^36\) could not fix the nitrogen atom of His\(^6\) and the hydrogen bond between N-e2(His\(^6\)) and O-\(\epsilon1\)(Glu\(^4\)) was already formed in the oxidized state. This is why a small peptide bond flip was observed in A. faecalis pseudoazurin at pH 7.0 (24).

The protonated pseudoazurin at the His\(^6\) position indicated a relatively higher redox potential (42). The protonation at His\(^6\) is important to reduction of the protein, and then, the peptide bond flip occurs in the concomitant region from Ile\(^34\) to Thr\(^36\) upon reduction. Since the His\(^6\) residue is not so far from the copper site (12 Å), this fact might be associated with the electron transfer mechanism.

**Acknowledgments**—We are grateful for helpful and stimulating discussions of Dr. Chris Dennison, University College Dublin. We are also grateful to Professor N. Sakabe, Dr. N. Watanabe, Dr. Suzuki, and Dr. Igarashi for support in data collection at KEK, Japan.

**REFERENCES**

Crystal Structures of Oxidized and Reduced Pseudoazurins

17852

Crystal Structure Determinations of Oxidized and Reduced Pseudoazurins from Achromobacter cycloclastes: CONCERTED MOVEMENT OF COPPER SITE IN REDOX FORMS WITH THE REARRANGEMENT OF HYDROGEN BOND AT A REMOTE HISTIDINE

Tsuyoshi Inoue, Nobuya Nishio, Shinnichiro Suzuki, Kunishige Kataoka, Takamitsu Kohzuma and Yasushi Kāi

doi: 10.1074/jbc.274.25.17845

Access the most updated version of this article at http://www.jbc.org/content/274/25/17845

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 6 of which can be accessed free at http://www.jbc.org/content/274/25/17845.full.html#ref-list-1