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The Crystal Structure of Cobalt-Substituted Pseudoazurin from *Alcaligenes faecalis*

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ABSTRACT:

The Cu(II) center at the active site of the blue copper protein pseudoazurin from Alcaligenes faecalis *has been substituted by Co(II) via denaturing of the protein, chelation and removal of copper by EDTA and refolding of the apo-protein, followed by addition of an aqueous solution of* $CoCl_2$*. Sitting drop vapour diffusion experiments produced green hexagonal crystals, which belong to space group* $P6_5$*, with unit cell dimensions* a = b = *50.03,* c = *98.80 Å. Diffraction data, collected at 291 K on a copper rotating anode X-ray source, were phased by the anomalous signal of the cobalt atom. The structure was built automatically, fitted manually and subsequently refined to 1.86 Å resolution. The Co-substituted protein exhibits similar overall geometry to the native structure with copper. Cobalt binds more strongly to the axial Met86-S*δ *and retains the tetrahedral arrangement with the four ligand atoms, His40-N*δ_1*, Cys78-S*γ*, His81-N*δ_1*, and 86Met-S*δ*, although the structure is less distorted than the native copper protein. The structure reported herein, is the first crystallographic structure of a Co(II)-substituted pseudoazurin.* © *2010 Wiley Periodicals, Inc. Biopolymers 95: 202–207, 2011.*

Keywords: blue-copper protein; pseudoazurin; metal substitution; Co(II)-derivative; crystal structure; Alcaligenes faecalis

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INTRODUCTION

Pseudoazurin is a periplasmic blue-copper protein (123 amino acid residues), a member of the cupredoxin family, participating in the electron transport process leading to the reduction of nitrite to mainly nitric oxide, in denitrifying bacteria.[1] The electron acceptor of pseudoazurin, hereafter called Cu(II)-PA, is the copper enzyme nitrite reductase, the crystal structure of which has been determined,[2] along with its solution structure in complex with Cu(II)-PA.[3] At present there are about 25 deposited Cu(II)-PA structures in the Protein Data Bank, corresponding to proteins from five different bacterial species. About half of the known structures are of the *A. faecalis* protein, reflecting structures of native,[4] various molecular variants containing either Cu(II) or Cu(I),[5] Cu(I)-PA at two pH values,[6] and a metal-free apo-PA.[7] The second structurally most studied pseudoazurin, comes from *Achromobacter cycloclastes*,[8–10] which exhibits 67% sequence identity to the *A. faecalis* protein.

To spectroscopically probe the electronic structure of this redox active site, substitution of Cu(II) by Co(II) or Ni(II) has been carried out successfully in the related blue copper proteins, amicyanin,[11] plastocyanin,[12] and azurin,[13] as well as in Cu(II)-PA from *A. cycloclastes*.[14,15] These and other paramagnetic ^{1}H-NMR and EPR studies have offered a wealth of information for both the "classic" and "perturbed" Type 1 protein copper sites. The latter classification is based on the different spectroscopic fingerprints of these proteins.[16] Moreover, crystal structures have become available for representative examples of metal-substituted "classic" Type 1 copper proteins, namely a cobalt-substituted azurin,[17] a cobalt-substituted amicyanin[18] and a nickel-substituted molecular vari-

ant of azurin,[19] which complemented the above mentioned spectroscopic studies.

To provide further insight into the electronic structure at the active site of Cu(II)-PAs, we substituted the native Cu^{2+} ion of the protein from *A. faecalis* by Co^{2+} and determined its crystal structure at 1.86 Å resolution. The results of the present study will help interpret previous spectroscopy results[15] and facilitate novel spectroscopic studies, which will follow with Co(II)-PA from *A. faecalis*, so as to complete the analysis of this representative member of the "perturbed" blue copper proteins.

RESULTS AND DISCUSSION

Overall Structure

The quality of the final refined structure is reflected in the electron density map shown in Figure 1a. The geometry of the model was analyzed by PROCHECK,[20] from which a G-factor of 0.1 was calculated. Some of these results are shown in Table I, along with statistics of the final cycle of refinement.

The final model assumes the well known β-sandwich fold (Figure 1b) and comprises all but the three last residues (Ser121-Lys123), which could not be fitted in the final electron density maps as a single or a combination of limited conformations. Side chains of four Glu, five Lys and one Ser residues were modeled in two or three different conformations. The estimated standard error for bond distances was 0.11 Å based on the crystallographic reliability factor (R-factor). The overall mean temperature factor was 25.4 $Å^2$ for the entire structure, including the ordered solvent modeled as water.

Compared to the native Cu(II)-PA structure (pdb code, 1paz), Co(II)-PA shows an average rms-displacement σ_{mc} = 0.13 Å (max = 1.1 Å) for its 480 main chain atoms. The corresponding value for the 436 side chain atoms was σ_{sc} = 1.1 Å (max = 7.6 Å). This result shows that PA can refold spontaneously back to its original structure in the absence of its 23-residue leader peptide and any metal ion. This is in agreement with earlier results[7] lending independent evidence to the notion that PA is designed to have a stable metal-binding fold suitable for redox activity. The only significant deviations between the two structures are confined within the main chain atoms of the last three modeled residues (Val118, Ile119, and Ala120) and Glu51 (deviations $>2\sigma_{mc}$, Figure 1b), as well as within the side chains of Lys10, Asp47, Glu54, Lys55, and Lys117 (deviations $>2\sigma_{sc}$). It is worth noting that these side chains are long and flexible and, as such, were assigned high temperature factors (>60 $Å^2$) in the original refined structure (1 paz).

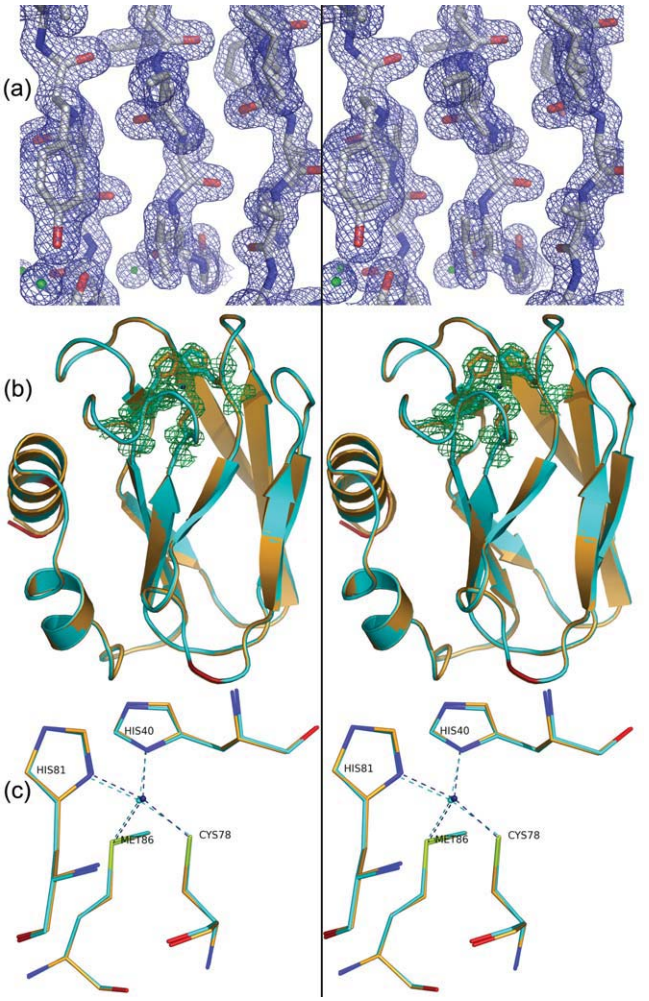

FIGURE 1 Co(II)-PA structure and comparison with native Cu(II)-PA. (a) Stereo pair of a section of the antiparallel β-sheet structure of the final Co(II)-PA refined model. The electron density of the weighted $2F_o$ - F_c map is contoured at 1.5σ (blue). Protein atom coloring, carbon: white, oxygen: red, nitrogen: blue. The green spheres signify ordered water oxygen atoms. (b) Stereo pair of the superposed structures shown as ribbon diagrams. The four ligand side-chains are in stick presentation. Cu(II)-PA: dark yellow, Cu(II): dark blue sphere, Co(II)-PA and Co(II) sphere: cyan, main chain regions deviating $>2\sigma_{mc}$ are in red (see text). The Co(II)-site is shown in electron density of the weighted $2F_o$ - F_c map contoured at 1.8σ (green). (c) Close-up view at the metal sites in PA from *A. faecalis*. The coordinating residues of the least-squares superposed models are shown. Co(II) and its ligands' C-atoms: cyan, Cu(II): dark blue sphere. The ligands of copper are shown in atom colors (carbon: yellow, nitrogen: blue, oxygen: red, sulphur: green).

Cobalt Site

The resulting changes at the copper site of Cu(II)-PA upon substitution with Co(II) are described in Table II and shown in Figure 1c. After superposition, the respective coordinating atoms and the metal ions themselves deviate by 0.0–0.3 Å in the two structures. These distances lie close to the average

Table I Data Collection and Refinement Parameters for Co(II)-PA

Space Group	$P6_5$
Unit-cell parameters (Å)	$a = b = 50.03$, $c = 98.80$
X-ray source[a]/Wavelength (Å)	Rotating anode/1.5418
Resolution range[b] (Å)	43.3–1.86 (1.93-1.86)
No. of measured reflections	120496 (7106)
No. of unique reflections[c]	11710 (1214)
Completeness (%)	99.4 (98.6)
Anomalous completeness (%)	95.4 (84.6)
Mean I/σ(I)	14.5 (8.8)
R_{merge}[d]	0.038 (0.073)
R_{anom}[e]	0.056 (0.098)
Average figure of merit of SAD-phases:	
Starting value/After automated fitting of 77 Ala residues	0.355/0.518
Refinement resolution range (Å)	14.4–1.86 (1.91–1.86)
R_{factor}	0.150 (0.193)
R_{free}[f]	0.178 (0.236)
Average figure of merit of phases from refined structure	0.902
Number of protein atoms	970
Number of ordered waters	109
Number of hetero-atoms (Co^{2+})	1
R.m.s.-deviations from ideal values:	
Bond lengths (Å)	0.015
Bond angles (°)	1.45
Dihedrals (°)	17.6
Chiral center volumes (Å^3)	0.085
Mean atomic B-values (Å^2):	
Main-chain atoms/rms-$B_{main\text{-}chain}$	22.0/0.66
Side-chain atoms/rms-$B_{side\text{-}chain}$	25.2/1.85
Metal ion/Solvent atoms (water oxygens)	21.1/41.7
Ramachandran plot:	
Most favoured region residues (%)	92.2
Allowed region residues (%)	7.8

[a] Rigaku RU-3HR at our laboratory in Heraklion.
[b] Values in parentheses throughout the table refer to the highest resolution shell.
[c] I^+ and I^- were scaled separately.
[d] $R_{merge} = \Sigma|I - <I>|/\Sigma I$, where I is the intensity of reflections.
[e] $R_{anom} = \Sigma|<I^+> - <I^->|/\Sigma I$ is the average anomalous difference.
[f] R_{free} is calculated for a test set of a random 556 reflections. No σ cut-offs were applied to the data during refinement.

coordinate error of 0.11 Å. However, it is noteworthy that the local coordinate error in this well defined region of the molecule ($17 <$ B-factor < 21 Å^2) may be smaller than the one referring to the overall structure. The above results imply that essentially the Co(II)-PA assumes a nearly identical overall structure to the native Cu(II)-PA.

However, there are some prominent differences that have not been observed earlier,[17,18] namely the tighter coordination of Co(II) by the axial 86Sδ (bond distance decreased by 0.3 Å) and the more "canonical" values for the two angles, 40Nδ_1-Co-78Sγ and 40Nδ_1-Co-86Sδ, 126 and 92°, respectively, compared to the ideal tetrahedral value of 109.5°. The corresponding angles in the Cu(II)-PA structure are 136 and 87°, respectively, leading to the highly distorted ("perturbed") Type 1 copper site. Moreover, Co(II) has moved further 0.2 Å out of the equatorial strong ligands' plane (40Nδ_1, 78Sγ, 81Nδ_1) towards the weaker axial ligand 86Sδ and, at the same time, it has moved 0.1 Å towards the planes (40Nδ_1, 81Nδ_1, 86Sδ) and (81Nδ_1, 78Sγ, 86Sδ).

Compared to the known crystal structures of Co(II)-substituted blue copper proteins, azurin from *P. aeruginosa*[17] and amicyanin from *P. denitrificans*,[18] the following noteworthy differences are observed. In the latter structures, contrary to Co(II)-PA, cobalt coordinates significantly less strongly to the axial ligand Sδ. The bond length of Co-Sδ is 3.5 and 3.8 Å, for Co(II)-azurin and Co(II)-amicyanin, respectively. In the latter, the metal ion has moved so much away from 98Sδ, that a water molecule is recruited as its fourth ligand. In Co(II)-azurin, the carbonyl oxygen atom of Gly45 coordinates significantly more strongly (2.2 Å) than in the native Cu(II)-azurin (3.0 Å), thus balancing the weakening of the Co-Sδ interaction. Moreover, in contrast to the Co(II)-PA (Table II), no "relaxation" is observed in Co(II)-amicyanin as regards to its site's tetragonal distortion[21] based on the respective metal-ligand angles.[18]

Finally, the observation of the present study for a stronger interaction with the axial 86Sδ is in agreement with the most prominent paramagnetic effects on the atoms of this coordinated residue, established by the ^{1}H-NMR study of Co(II)-PA from *A. cycloclastes*.[15]

CONCLUSIONS AND PERSPECTIVES

The refined crystal structure of Co(II)-PA from *A. faecalis*, which, to the best of our knowledge, is the first reported structure of a Co(II)-substituted pseudoazurin, shows extensive similarity with the structure of the native Cu(II)-PA. Nevertheless, it is worth noting the more regular tetrahedral geometry of the cobalt site compared with the copper site in the native structure. This implies at least a partial relaxation of the manifested distortion ("perturbation") at this blue copper site.

The expected high-spin, $S = 3/2$, metal center of Co(II)-PA, along with other cobalt-substituted blue copper proteins, could serve as candidate for detailed studies by state-of-the-art spectroscopic methods, such as High-Frequency and Field EPR or ENDOR, and Variable-Temperature Variable-Field MCD spectroscopies, aiming at further elucidating the electronic properties of this biological metal site. Extensive studies by such methods have been recently carried out on mono-

Table II Comparison of the Coordination Geometries in Native and Co(II)-PA

	Distances (Å)			Angles (°)	
	Co^{2+}/Cu^{2+}	Distance of Co^{2+}/Cu^{2+} Ions from Planes			Co^{2+}/Cu^{2+}
$40N\delta_1$	2.0/2.2	Imidazole ring of His40	0.2/0.1	$40N\delta_1$-M[a]-$78S\gamma$	126/136
$78S\gamma$	2.2/2.2	Imidazole ring of His81	0.0/0.0	$40N\delta_1$-M-$81N\delta_1$	101/100
$81N\delta_1$	2.1/2.1	$40N\delta_1$, $78S\gamma$, $81N\delta_1$	0.6/0.4	$40N\delta_1$-M-$86S\delta$	92/87
$86S\delta$	2.5/2.8	$40N\delta_1$, $78S\gamma$, $86S\delta$	0.7/0.7	$78S\gamma$ -M-$81N\delta_1$	113/112
39O[b]	3.8/3.8	$40N\delta_1$, $81N\delta_1$, $86S\gamma$	1.0/1.1	$78S\gamma$-M-$86S\delta$	110/108
$78S\gamma$ -41N[c]	3.6/3.6	$81N\delta_1$, $78S\gamma$, $86S\delta$	0.6/0.7	$81N\delta_1$-M-$86S\delta$	113/112
$40N\varepsilon_2$-$9O\delta_1$[c]	2.8/2.7			M-$78S\gamma$-$78C\beta$[d]	105/105
$81N\varepsilon_2$-O*w*[c]	2.8/2.8				

[a] M denotes either Co^{2+} or Cu^{2+}.
[b] Carbonyl oxygen of Gly39 remains distant to the metal as in the native structure (pdb code 1paz).
[c] These atoms participate in H-bonds with the metal ligand Sγ or with members (Nε_2) of the metal-interacting imidazole rings.
[d] This angle is implicated in the multiple mode of binding of the thiolate S and the copper ion (Holm et al., 1996).[21]

nuclear Co(II)-containing coordination compounds,[22–26] but have been scarce for mononuclear Co(II)-containing biological systems.[27]

MATERIALS AND METHODS

Purification, Substitution of the Metal, and Crystallization Procedures

The protein was purified from transformed *E. coli* C600 cells harbouring the recombinant plasmid pAB301, in which the pseudoazurin gene is under the control of both lac and tac promoters[28] and the ampicillin resistance gene is present. The growth of the bacteria and the protein extraction procedure is described in Vakoufari et al.[6] Q-Sepharose and SP-Sepharose Fast-Flow (GE Healthcare, Piscataway, NJ), replaced the earlier used chromatography materials DEAE Sephacel and CM Sepharose CL 6B, respectively. 22 g of cell pellet yields about 6 mg of essentially pure Cu(II)-PA.

Initial trials to remove the Cu^{2+} ion from Cu(II)-PA by addition of the reducing agent ascorbic acid, the strong metal binding KCN and subsequent addition of aqueous solution of $CoCl_2$ so as to obtain Co(II)-PA, proved fruitless. Therefore, the purified protein was unfolded by incubation for 10 h in a solution containing 50 m*M* Tris-HCl, pH 6.8, 50 m*M* EDTA, and 6*M* guanidinium-chloride. The temperature was kept at 281 K throughout this procedure. The colorless protein was concentrated using an Amicon Ultra 5 kDa device (Millipore) and diluted in 50 m*M* Tris-HCl, pH 6.8, and 6*M* guanidinium-chloride for several times, in order to remove the EDTA and the chelated Cu^{2+} ions. To refold the protein, it was concentrated and diluted in 50 m*M* Tris-HCl pH 6.8. The refolded apoprotein was buffer-exchanged into 50 m*M* Tris-HCl, pH 6.8, and 50 m*M* $CoCl_2$ using repeated concentration/dilution by ultrafiltration. The concentrated protein exhibited a gray-blue color in contrast to the pink filtrate, demonstrating the binding of metal to PA. UV/vis spectra (Figure 2a) taken from protein solutions in water with a nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE) showed the expected electronic absorption pattern for Co^{2+} bound to proteins of this family.[11] Finally, Co(II)-PA was transferred to the crystallization buffer, 50 m*M* Na-citrate pH 5.7, 20 m*M* $CoCl_2$, and concentrated to 5 mg mL^{-1}.

Sitting drop, vapor diffusion experiments against reservoir containing the crystallization buffer in 2.8*M* ammonium sulfate solution produced, within a few days, green bipyramidal crystals of varying size (Figure 2b). The average size of the crystals obtained was 0.5 mm × 0.5 mm × 1.2 mm. Attempts to refold the apo-protein in buffer containing 50 m*M* $CoCl_2$ were also successful, albeit resulting in smaller crystals. The symmetry of the Co(II)-PA crystals was the same and the unit cell parameters similar to the blue crystals of native Cu(II)-PA.[4] The relevant parameters are summarized in Table I.

Data Collection and Processing, Phasing, and Refinement

One single crystal was used for "in house" data collection at room temperature (291 K) using Cu-Kα radiation from a Rigaku RU-3HR rotating-anode generator (Rigaku/MSC, Woodlands TX), coupled with confocal Max-Flux mirrors (Osmic Inc., Troy MI) and a Mar300 imaging plate detector system (MarResearch, Hamburg, Germany). Although the crystal diffracted to a better resolution, it was decided to limit the collection of data due to the potential radiation damage at room temperature. In total, data from two φ-angle sectors were collected and processed with the HKL-package v.1.96.5.[29] Some of the relevant statistics are shown in Table I.

To avoid any possible bias from the native Cu(II)-PA, the structure was solved by the Single-wavelength Anomalous Diffraction (SAD) method, after locating the cobalt sites in the calculated anomalous difference Patterson map (Co K-absorption edge, 1.6083 Å). A typical Harker section of the latter map is shown in Figure 2c. The CCP4 program suite[30] was employed for most of the steps of the present structure solution and refinement. One Co(II) site was selected and used for phasing. Automated model building was carried out by successively using the programs BP3,[31] SOLOMON,[32] BUCANNEER,[33] and REFMAC5,[34] as they are applied by the CRANK pipeline.[35] This resulted in fitting 77 alanine residues in

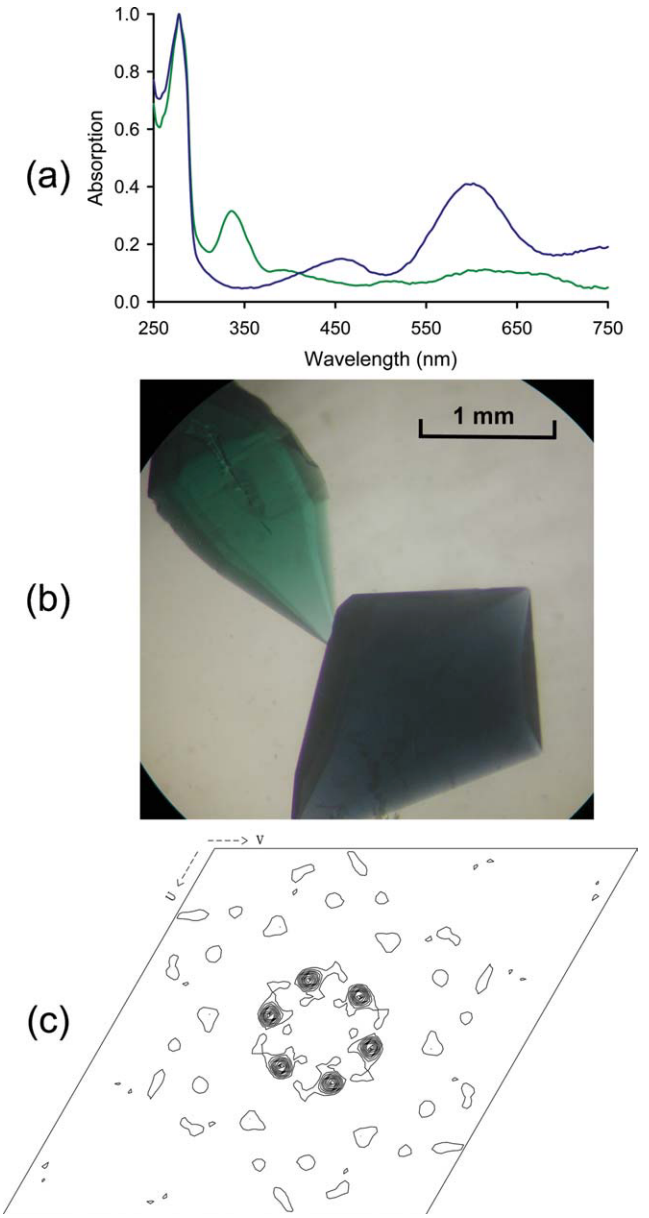

FIGURE 2 UV/vis spectra, crystals and anomalous difference Patterson. (a) Scaled UV/vis spectra of Co(II)-PA (green) and native Cu(II)-PA (blue) in water solutions. Path length = 1 mm. (b) A hexagonal bipyramidal green crystal of Co(II)-PA next to a native blue Cu(II)-PA crystal (lower). (c) A Harker section at $z = 1/3$ of the anomalous difference Patterson map showing peaks of the cobalt self vectors. All but 50 reflections with $|F^{+} - F^{-}| > 5\Delta F_{\text{anom, max}}$ were used for the calculation of the map. The U and V axes are shifted by -0.5. Contouring starts at 1σ level and steps with 1σ.

five different polypeptide chains located in different asymmetric units. These fragments were transformed into segments of the same molecule by the application of the appropriate symmetry operators. The resulting model was completed successfully by ARP/wARP 6.1.[36] The only absent residues were, one at the N-terminus, four at the C-terminus and three in the vicinity of the metal site, where wrong tracing had occurred. Several cycles of manual model building using Xfit[37] were followed by isotropic restrained refinement with REFMAC 5.2.[34] All graphic illustrations of the protein were obtained by PyMOL (http://pymol.sourceforge.net/).

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