Studies of multi-heme cytochromes from *Geobacter sulfurreducens*

P. Raj Pokkuluri, Yuri Y. Londer, Valerie Orshonsky, Lisa Orshonsky, Norma Duke and Marianne Schiffer (PI)

Biosciences Division, Argonne National Laboratory, Argonne, IL 60439

Background

The *Geobacteraceae* family predominates in the reduction of uranium in subsurface environments. We are focusing on the model organism, *Geobacter sulfurreducens;* its genome contains a large number (>100) of cytochromes *c* that function in the metal reduction pathways. Intensive functional genomics and physiological studies are in progress in Prof. Derek Lovley's laboratory, and the complete genome sequence of this organism has been determined by Methé et al. (2003). We are studying cytochromes from the c_7 family that are required for the reduction of Fe(III).

We expressed in *E. coli* (Londer et al., 2002) and determined the three-dimensional structure (Pokkuluri et al., 2004a) of the three-heme cytochrome c_7 PpcA (GSU0612) characterized by Lloyd et al. (2003). Further we identified in the *G. sulfurreducens* genome ORFs for several of its homologs (Pokkuluri et al., 2004a). Four of the ORFs are the same size as PpcA; three other ORFs are polymers of c_7-type domains, two of which consist of four domains and one of nine domains, that contain 12 and 27 hemes, respectively.

By x-ray diffraction we determined structures of all four homologs of PpcA. Though these proteins have highly homologous sequences and three-dimensional structures but their surface characteristics differ from each other. We also found that they have different thermal stabilities and different reduction potentials. Prof. Derek Lovley's group determined that the physiological functions of the above homologs are also different; disabling the individual genes coding for them results in different iron reduction rates (DiDonato et al., 2005, submitted).

The structure of PpcA (Fig. 1), which is most abundant in the periplasm, differs most significantly from the structures of the other homologs. PpcA has a pocket where a guest molecule of deoxycholic acid is located, a feature not observed in the other structures. This pocket might be occupied by a quinone molecule *in vivo.*

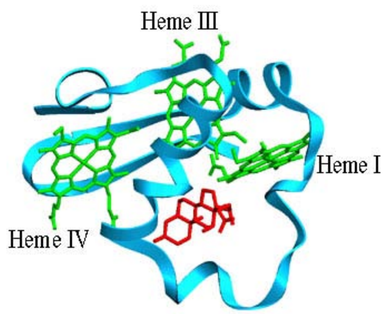

Fig. 1. Structure of PpcA. Protein backbone is in blue, hemes are in green and deoxycholic acid is shown in red.

Thermodynamical characterization of PpcA

The order of heme oxidation was determined for PpcA by NMR methods:

Heme I is the first

Heme IV is the second

Heme III is the last to be oxidized (Pessanha et al., 2004).

This oxidation pattern differs from that of the related cytochrome c_7 from *D. acetoxidans* where heme I and III are oxidized first to a similar extent followed by oxidation of heme IV.

Mutagenesis studies of PpcA

The metal reduction properties of cytochromes are determined by the electrostatic potential of the hemes. The electrostatic potentials of the individual hemes are determined by the ligands of the iron, by their exposure to solvent and by charged residues near them. In a multiheme cytochrome they are also influenced by the neighboring hemes. These factors also determine the E_{app}, the potential where half the hemes are reduced.

To understand the function of individual residues in PpcA and to understand the differences between the c_7 homologs (PpcA, PpcB, PpcC, PpcD, PpcE) we made 25 site-specific mutants of PpcA and determined the structures for 10 of the proteins. The structures were determined at high resolution (1.9 Å) with data from the Advanced Photon Source (APS) at Argonne at the Structural Biology Center (SBC), they were refined to an R-factor of 19%. The thermal stabilities of the mutants were determined by changes in CD spectra as a function of temperature. Their electron transfer properties are being determined by Prof. Carlos Salgueiro (Universidade Nova de Lisboa, Portugal) and the reduction of metals will be determined by Dr. Jon Lloyd (University of Manchester, UK).

Mutants were made in four categories: (structures solved are marked with *)

Conserved hydrophobic residues near the hemes (Val13, Phe15)
replaced by Ala*, Ile, Ser, Thr* and Trp, Tyr, Leu* respectively
Sixth ligand to heme IV (His47)
replaced by Met, Ala, Lys, Arg
Residue that shields heme III from solvent (Met58)
replaced by Ser*, Asn*, Asp*, Lys*
Lysine residues near the hemes
Lys18 near heme I
Lys22 between heme I and III
Lys60 near heme III
Lys9 near heme IV
Lys52 near heme IV (part of chromate ion binding site in c_7 of *D. acetoxidans*, it is Thr in PpcD, where it might interfere with metal binding)
Lys residues were replaced by Glu and Gln; structures for K9E, K22E, K22Q

Detailed results for some of the mutants:

Mutants at residue 15

Phe15 is conserved in all c_7 and c_3 cytochromes, it is located between heme I and III. We exchanged the Phe15 with Trp, Tyr and Leu. The thermostability of all the mutants decreased significantly.

Only the F15L mutant crystallized, though the shapes of the molecules did not change from the wild type as shown by small angle scattering. The relative positions of heme I and heme III were altered by the F15L mutation.

E_{app} of F15L did not change significantly from the wild type value of -136mV, E_{app} of F15W decreased to -152mV, while that of F15Y increased to -122mV at pH 6.9 (Pessanha et al., 2004).

Mutants at residue 13

Val13 is conserved in most c_7 and c_3 cytochromes, it is located between heme III and IV. We exchanged Val with hydrophobic residues Ala and Ile, and polar residues Ser and Thr. The polar residues decreased the thermal stability of the protein.

Ala substitution changed the backbone of residues 13 and 14 as well as the side chain of residue 12.

Thr substitution altered the puckering of heme IV.

Polymers of c_7-type cytochromes

We identified three ORFs coding for proteins that represent multiples of the three-heme proteins (Pokkuluri et al., 2004a). Two of the proteins, GSU0592 and GSU1996, have four repeats (total of 12 hemes) and one, GSU2210, has nine repeats (total of 27 hemes) of the cytochrome c_7-like domain. The repeats within each of the three poly-cytochrome c_7 proteins are highly homologous (Fig. 2). The repeat lengths are longer than that of the cytochrome c_7 molecule; they vary from 73 to 82 residues.

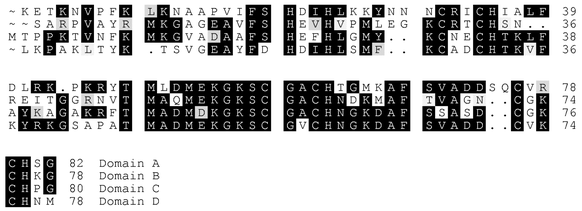

Fig. 2. The alignment of the amino acid sequences of the four c_7-type domains coded by GSU1996

Production and crystallization

A method was developed to express cytochromes *c* containing up to 12 hemes in *E. coli* strain JCB7123; we produced the complete four domain protein coded by GSU1996. In the initial purification attempts large fraction of the protein was insoluble probably due to interaction with the cell membranes. However, we were able to selectively solubilize the protein using 0.5 M sodium chloride. In experiments with detergents, such as HEGA-10 or β-octylglucoside, significant amounts of other cellular proteins were also extracted together with the cytochrome. The protein solubilized in the presence of NaCl was dialyzed and purified following our standard approach (cation exchange followed by gel-filtration). The purified protein was analyzed by mass-spectrometry and confirmed to have 12 covalently attached hemes. The yield of pure cytochrome was 0.5 mg per liter of culture. The protein was crystallized from 0.5 M ammonium sulfate + 2.5 M sodium chloride (Fig. 3). The same approach was used to produce the other 12 heme protein GSU0592. This protein was crystallized from 3.5 M sodium formate + 0.1 M sodium acetate, pH 4.6. X-ray diffraction data have been collected on both four domain cytochromes at the SBC beamline at APS.

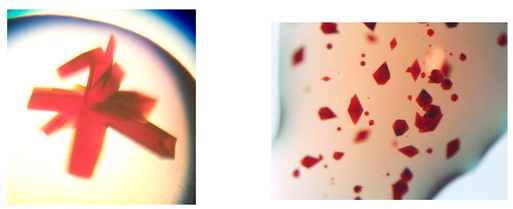

Fig. 3. Crystals of four domain cytochromes GSU1996 (left) and GSU0592 (right)

Discussion

The structures of the two domain proteins show that the domains could form a chain, a protein wire, as we have predicted based on the packing of the molecules in the crystals formed by PpcA, though the chain probably will not be completely linear. Based on the geometries of the two-domain proteins and on the amino acid sequences of the individual domains we can suggest that an electron in the polymer will travel from its C-terminus (domain D) to the N-terminus (domain A). The Fe-Fe distance between neighboring hemes of domains A and B is 9.7 Å while it is 14.7 Å between domains C and D; this gives a kinetic advantage for the electrons transferred between A and B. Further the excess of positively charged residues increases from domain D to domain A. The measured E_{app} of domain D is more negative than that of domain C. We speculate that the protein might interact with the cell membrane or a protein imbedded in the membrane by a large patch of four positively charged Arg and Lys residues.

Structural characterization

The x-ray structure of the third domain, domain C (Fig. 4), and the structure of the two domain protein – domains CD (Fig.5), third and fourth domains of the four domain protein GSU1996, were determined (Pokkuluri et al., 2004b; Londer et al., 2005). The c_7-type domains that form the polymers represent a new family of cytochromes *c* that has not been previously described. While two of the hemes in each domain are bis-histidine coordinated as found in cytochromes c_7 and c_3 the third one is coordinated by a histidine and a methionine, which is expected to make its redox potential more positive than those of the other two. Indeed, the midpoint reduction potential of domain C is -105mV, 50mV more positive than that of PpcA (Pokkuluri et al., 2004b).

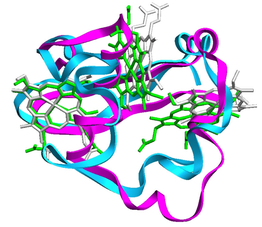

Fig. 4. The structures of PpcA and domain C overlapped using only heme IV atoms. Domain C: blue; PpcA: magenta; hemes in domain C: green; hemes in PpcA: grey

Recently we produced the first two domains (AB) of protein GSU1996 by methods developed for the complete protein described above. To get well diffracting crystals a reductive methylation procedure was applied. Mass spectrometry on the resulting protein sample has confirmed that all 18 lysine residues and the amino terminus were methylated. The methylated protein has produced crystals suitable for x-ray diffraction data collection from 2 M ammonium sulfate and 0.1 M sodium acetate, pH 4.6. A preliminary structure of the AB domain shows (Fig. 6) that this fragment is different from the CD fragment (Fig. 5). The hemes of neighboring domains are much closer in AB than in CD. It is also expected that the redox properties of the two fragments will be different.

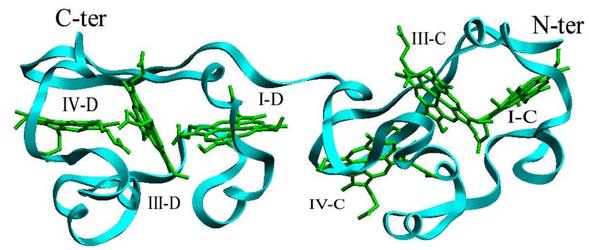

Fig. 5. The refined structure of the two-domain unit CD. The Fe atoms of heme IV-C and heme I-D are 14.7 Å apart.

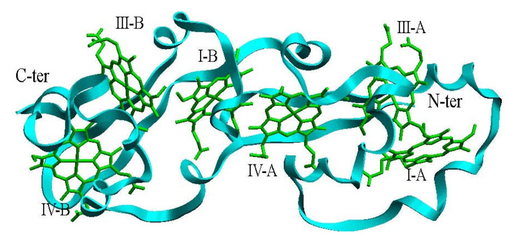

Fig. 6. The structure of the two-domain unit AB. The Fe atoms of heme IV-A and heme I-B are 9.7 Å apart, 5 Å closer than in CD. The hemes between the A and B domains are closer to each other than the hemes within the individual domains; they are in van der Waals contact.

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Acknowledgments

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