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Desulfovibrio desulfuricans G20 Tetraheme Cytochrome Structure at 1.5 Å and Cytochrome Interaction with Metal Complexes

M. V. Pattarkine[1], J. J. Tanner[1,2], C. A. Bottoms[3], Y.-H. Lee[4] and J. D. Wall[1]*

[1]*Biochemistry Department University of Missouri-Columbia Columbia, MO 65211, USA*

[2]*Chemistry Department University of Missouri-Columbia, Columbia, MO 65211 USA*

[3]*Health Management & Informatics, University of Missouri-Columbia, Columbia MO 65211, USA*

[4]*Structural Biology Core University of Missouri-Columbia, Columbia, MO 65211 USA*

**Corresponding author*

The structure of the type I tetraheme cytochrome c_3 from *Desulfovibrio desulfuricans* G20 was determined to 1.5 Å by X-ray crystallography. In addition to the oxidized form, the structure of the molybdate-bound form of the protein was determined from oxidized crystals soaked in sodium molybdate. Only small structural shifts were obtained with metal binding, consistent with the remarkable structural stability of this protein. *In vitro* experiments with pure cytochrome showed that molybdate could oxidize the reduced cytochrome, although not as rapidly as U(VI) present as uranyl acetate. Alterations in the overall conformation and thermostability of the metal-oxidized protein were investigated by circular dichroism studies. Again, only small changes in protein structure were documented. The location of the molybdate ion near heme IV in the crystal structure suggested heme IV as the site of electron exit from the reduced cytochrome and implicated Lys14 and Lys56 in binding. Analysis of structurally conserved water molecules in type I cytochrome c_3 crystal structures identified interactions predicted to be important for protein stability and possibly for intramolecular electron transfer among heme molecules.

Keywords: *Desulfovibrio*; tetraheme cytochrome c_3; metal reduction; X-ray crystallography; bioremediation

Introduction

Sulfate-reducing bacteria of the genus *Desulfovibrio* are anaerobes that derive energy from the dissimilatory reduction of sulfate with the oxidation of dihydrogen or organic substrates. Additionally, these bacteria enzymatically reduce toxic metals such as chromium (VI), manganese (IV), iron (III),[1] technetium (VII),[2] and uranium (VI).[3] However, metal reduction has not been demonstrated to support growth with any consistency. When uranium is reduced, the soluble U(VI) is converted to U(IV), which precipitates as the insoluble mineral uraninite, UO_2. Currently, this metabolism is being explored for its potential application to bioremediation of groundwater contaminated with uranium.[4]

The tetraheme cytochrome c_3 is a periplasmic redox protein from Gram-negative sulfate-reducing bacteria.[5] As their most abundant *c*-type cytochrome, it has been implicated in the transfer of electrons and protons from periplasmic hydrogenases[6,7] and other periplasmic dehydrogenases to the electron transfer chain for sulfate respiration and ATP synthesis, respectively. The role of the *Desulfovibrio* cytochrome c_3 in uranium reduction has been investigated *in vitro* through spectroscopic studies and by pathway reconstitution experiments.[8] The results of these studies supported a model of electron flow from hydrogen through hydrogenase to cytochrome c_3 to U(VI). While recent evidence from mutant analyses confirmed that cytochrome c_3 was a part of the *in vivo* electron transfer pathway to U(VI) from hydrogen, evidence was found for alternative pathways from organic donors that could bypass this tetraheme cytochrome.[9]

This small (~13,000 Da) soluble *c*-type cytochrome has four heme molecules with bis-histidinyl axial

Present address: Y.-H. Lee, Department of Biological Sciences, Louisiana State University, 202 Life Sciences Bldg, Baton Rouge, LA 70803, USA.

Abbreviations used: CD, circular dichroism; RMSD, root-mean-square difference.

E-mail address of the corresponding author: wallj@missouri.edu

ligation.[5] Because it is encoded by a monocistronic gene, has CXXCH and CXXXXCH heme-binding motifs, and has the characteristic conserved lysine residues, it is classified as type I cytochrome c_3 (TpI-c_3).[10–12] The adjacent pairs of heme planes are almost perpendicular to each other and are oriented by a minimum of protein matrix.[5] The different redox potentials of the four heme molecules have been shown to be modulated by several factors, such as the nature of the axial ligands, solvent accessibility and interaction of the porphyrin ring substituents with the neighboring amino acid residues.[5,13–15] To reach an understanding of the mechanism of electron transfer by cytochrome c_3 at the molecular level, knowledge of the architecture of the heme pockets is a critical requirement. High-resolution X-ray structure data for the oxidized tetraheme cytochrome c_3 from five different species of *Desulfovibrio* and one from *Desulfomicrobium*[16–21] have revealed key amino acid residues that are likely to be critical for electron transfer. NMR studies support the correctness of these structures and have been used to elucidate the solution structure of the reduced protein.[22–25] The three-dimensional structures of cytochromes c_3 from these species show highly conserved architecture for the heme core, despite very low levels of sequence homology.[5,20] This diversity in sequence generates different environmental conditions around each heme, resulting in a range of redox potentials, pH-dependence and rates of electron transfer.[5,22]

Positive cooperativity during reduction of cytochromes c_3 has been demonstrated and suggested to be a mechanism for simultaneous multi-electron capture.[7,12] In addition, heterotropic cooperativity or redox Bohr effects have been documented that show thermodynamic linkage between reduction and protonation.[12,23] It has been suggested that this linkage allows charge separation in the absence of a membrane.[7] Because of its dual activities and the limited occurrence of this cytochrome in prokaryotes outside of the δ-Proteobacteria, it may play a critical role in the metabolism of these bacteria.

The specificity of intermolecular electron transfer is provided by molecular recognition. Cytochrome c_3 interacts effectively with a large array of electron-transfer proteins, including hydrogenases,[6,26] a unique high molecular mass cytochrome *c* (Hmc),[27] ferredoxins,[28] flavodoxins,[29] rubredoxin,[29] and a large inorganic polyanion.[30] Several of these successful interactions are unlikely to have physiological meaning, since the proteins occur in different cellular compartments; however, information about the sites of potential electron transfer into and out of cytochrome c_3 has been gained from these studies. In most cases, *in silico* docking of crystal structures of proteins and NMR chemical shift perturbations point to heme IV (the heme numbering convention used is as the binding site occurs in the amino acid sequence) as the site of protein–protein interactions for electron transfer.[26,31] The broad range of substrates would suggest that specificity of the interactions is low and that general features of the protein, such as surface charges allowing electrostatic interactions, may dominate the docking that facilitates electron transfer.

In order to understand the role of cytochrome c_3 in energy transduction as well as enzymatic metal reduction, the interplay of various molecular influences on redox state and heme–heme interactions involved in the process must be elucidated. High-resolution structures of cytochrome c_3 coupled with spectral studies of this redox active protein may provide information about the molecular mechanism involved. Here, we describe the crystal structure of the oxidized cytochrome c_3 from *Desulfovibrio desulfuricans* strain G20 (G20c_3) at 1.5 Å resolution. The structure of the oxidized protein bound to the molybdate ion revealed a binding site near heme IV, in agreement with the inference that this site effectively interacts with substrates.[26,31] Binding of molybdate caused no large protein rearrangement. The crystal structures were complemented with functional studies of Mo(VI) and U(VI) oxidation of reduced G20c_3. These studies provide the foundation for the elucidation of the specific amino acid residues of G20c_3 essential for metal interaction and for normal physiological function of cytochrome c_3 in these bacteria. Finally, we examined the conservation of protein-bound water in available crystal structures of the TpI-c_3 family. Several highly conserved water molecules are likely important for overall protein structure and stability, while a pocket of water molecules at the intersection of heme I, heme II, and heme III may be involved in electron transfer among the heme molecules.

Results and Discussion

UV-Vis spectroscopy measurements

To determine whether efficient electron transfer could occur from reduced purified G20c_3 to the uranyl ion, the changes in UV-visible light absorption by G20c_3 following reduction and metal exposure to the reduced G20c_3 were monitored. The absorption spectrum of as-isolated oxidized G20c_3, dithionite-reduced and G20c_3 oxidized following addition of uranyl acetate are shown in Figure 1. The addition of U(VI) as the uranyl ion clearly oxidized the reduced G20c_3 and the spectrum of this metal-oxidized protein resembled that obtained for the as-isolated air-oxidized protein (spectrum 4 *versus* 1). The amount of U(VI) necessary for apparent complete protein oxidation was near the theoretical stoichiometry of 2:1.

The Soret peak of the U(VI)-oxidized protein at 408 nm was consistently decreased about 5–8% in absorbance with respect to that expected for the amount of G20c_3 present. The cause of this decrease was not apparent, although G20c_3 binding to insoluble metal oxides has been documented.[32] When subjected to three sequential cycles of reduction and metal addition, the shapes of the spectra were identical, although the absorbance at