Preliminary X-ray studies of the tetra-heme cytochrome c_3 and the octa-heme cytochrome c_3 from *Desulfovibrio gigas*

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A tetra-heme and an octa-heme cytochrome c_3 from the sulfate bacterium *Desulfovibrio gigas* have been crystallized. Diffraction quality crystals of the tetra-heme cytochrome are obtained from solution by the addition of polyethylene glycol at pH 6.5. The crystals are orthorhombic, space group $P2_12_12$ with unit cell parameters $a=42.27$ Å, $b=52.54$ Å and $c=52.83$ Å. The octa-heme cytochrome crystals develop from low ionic strength solutions of phosphate or Tris-Cl in the pH range 6.2–7.6. The crystals belong to the trigonal system, space group $P3_1$ or the enantiomorph $P3_2$, with unit cell parameters $a=b=57.4$ Å, $c=97.3$ Å, $\gamma=120°$. Single crystal diffraction studies of the structures of these two low-potential cytochromes are in progress.

Multiheme cytochrome *Cytochrome* *Low-potential cytochrome* *Crystallography* *Electron transport-* (Desulfovibrio)

1. INTRODUCTION

The multiheme cytochromes of *Desulfovibrio gigas* are electron transport proteins involved in sulfur metabolism at low redox potentials in the approximate range -200 to -300 mV. The tetra-heme cytochrome c_3 (cyto 4Hc3) has four hemes covalently bound to a single polypeptide chain of 111 amino acid residues [1] and a mass of ~14400 Da, whereas the octa-heme cytochrome c_3 (cyto 8Hc3) has eight hemes and a mass ~26000 Da. Cyto 8Hc3 is not a dimer of cyto 4Hc3, however, but differs in amino acid composition, solubility and redox potential [2]. Due to the extremely low solubility of the 8Hc3 protein, this molecule has not been as well studied as the 4Hc3 cytochrome.

The above cytochromes differ from the mitochondrial cytochromes of eukaryotes which characteristically have one covalently bound heme with fifth and sixth axial ligands of histidine and methionine, respectively, and operate at redox potentials of ~250 mV. The multiheme cyto 4Hc3s have histidine residues at both the fifth and sixth axial ligand positions and have heme midpoint potentials ranging from ~ -30 mV to -400 mV, depending upon the particular cytochrome and individual heme environment [3–5]. Although not firmly established, the cyto 8Hc3s are presumed to contain histidine at these positions [6]. Neither the cyto 4Hc3 nor the cyto 8Hc3 molecule can replace the other in their respective reactions. (See LeGall and Fauque [7] for a review of the involvement of these cytochromes in sulfate-reducing bacteria.)

2. TETRA-HEME CYTOCHROME

Studies by Moura et al. [8] have indicated that *D. gigas* cyto 4Hc3 is reduced by ferredoxin I in the

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production of hydrogen and, in turn, reduces ferredoxin II (FdII) in the formation of sulfide. An interpretation of these observations has cyto 4Hc3 playing a strategic role in the production of hydrogen and sulfur by *D. gigas*. The interaction of *D. gigas* cyto 4Hc3 with ferredoxin has been corroborated by Xavier et al. [9] through redox titrations coupled to EPR spectroscopy. A similar interaction has been observed between cyto 4Hc3 and the four iron ferredoxin I from the Norway 4 strain of *D. desulfuricans* [10].

Several cyto 4Hc3 proteins have been isolated from different sulfate-reducing bacteria and most show distinct differences in the midpoint redox potentials of their heme groups, probably a consequence of the differing amino acid sequences and the resulting variation in arrangement of residues associated with each heme (see [4,7,11]). Evidence exists for modulation of the redox potentials of each heme resulting from heme-heme interaction and pH effects [1,12].

Two cyto 4Hc3 structures have been elucidated by X-ray diffraction analysis, and the structures show very similar polypeptide chain folding and heme arrangement [13–17] despite significant differences in amino acid sequences and heme potentials. Although the 3-D structures of cyto 4Hc3 from *D. vulgaris* (Miazaki) and *D. desulfuricans* (Norway 4) are known in considerable detail, there is no clear understanding of the effects produced by the differing amino acid residues in the structures. Solving the crystal structure of a third cyto 4Hc3 will provide valuable information for broadening the base of knowledge in the investigation of these cytochromes.

Proton NMR investigations of several cyto 4Hc3 have been reported, indicating different intra- and intermolecular electron exchange [8,10–12]. The *D. gigas* cyto 4Hc3 has been studied extensively by NMR because of the slow electron exchange in this protein relative to the NMR time scale [11,18,19]. The detailed 3-D structure will be extremely useful in correlating and explaining the NMR observations.

The 3-D structure analysis of the three iron FdII of *D. gigas* is in progress [20], and since the *D. gigas* cyto 4Hc3 is known to interact specifically with FdII [8,9], knowledge of the structure of both should aid in the interpretation of the interaction of these two by other techniques.

3. OCTA-HEME CYTOCHROME

Cyto 8Hc3 has been shown to stimulate thiosulfate reduction in *D. gigas* cell extracts but little is known beyond this observation [2,7,23]. The low solubility of the protein has hindered the investigation of this unusual cytochrome by spectroscopic techniques. A recent report by Guerlesquin et al. indicates that the cyto 8Hc3 from *D. desulfuricans* (Norway 4) is a dimeric molecule [24].

The multiheme cytochromes provide an excellent opportunity for investigating the differing heme environments in order to increase our understanding of structure and function of the cytochrome family.

4. CRYSTALLIZATION AND DIFFRACTION EXPERIMENTS

Crystallographic studies have been in progress for some time on the cyto 4Hc3 and cyto 8Hc3 from *D. gigas*, and the preliminary results are presented here.

Cyto 4Hc3 crystallizes from a 0.5–1.0% protein solution, buffered with 0.03 M Tris-maleate at pH 6.5, which has been adjusted to 35–40% (g/100 ml) polyethylene glycol (PEG, M_r = 4000–6000). Crystals can be produced by the hanging drop method, the free interface technique or the batch method at either 4°C or room temperature. Once the material has crystallized, the crystals can be stabilized at 4°C. The crystals grow as extremely long needles which tend to develop inclusions if the time of crystal growth is extended. 2-Methyl-2,4-pentanediol (MPD) can be used as a precipitant, but the crystalline material tends to polymerize, changing the crystals into ill-defined waxy threads after a period of several weeks. The crystals grown from solutions containing PEG also polymerize, but more slowly and to a lesser extent.

Purified *D. gigas* cyto 4Hc3 tends to polymerize if kept at temperatures in the range 0–23°C. Storing at −70°C avoids the polymerization problem. It is likely that the tendency to polymerize causes the problem of crystal stability. Crystallization attempts with polymerized protein reveal that the higher molecular mass material is much more soluble but will not crystallize. The use of ammonium

sulfate as a precipitant resulted in no useful crystals from either form of cyto 4Hc3.

Preliminary X-ray diffraction studies of cyto 4Hc3 grown from MPD and PEG show symmetry of both crystal forms consistent with space group $P2_12_12$. Crystals grown from solutions of 60% MPD have cell parameters $a = 40.8$ Å, $b = 51.7$ Å, and $c = 52.5$ Å as determined from precession films. Crystals grown from solutions of 40% PEG have the following cell parameters as determined by diffractometer measurement: $a = 42.27$ Å, $b = 52.54$ Å and $c = 52.83$ Å with only small variations being observed among different crystal preparations. The cell differences may be due to the precipitants or be caused by impurities in the reagents used. Distinct differences are observed in the intensities on precession photos of the *h*01 nets of crystals grown in MPD and those grown in PEG.

Determinations of the crystal structure are being attempted by the use of anomalous scattering of the four iron atoms and by the molecular replacement method [25] based on the structure of cyto 4Hc3 of *D. vulgaris* (Miazaki).

The octa-heme cytochrome 8Hc3 of *D. gigas* is very insoluble, but the material can be dissolved in 1 M phosphate or 4 M Tris-Cl at pH 7.6 and the protein solution concentrated by micropore pressure techniques. Crystals can be grown over the pH range 6.2–7.6 by a controlled reduction of the ionic strength of the solution. The crystals appear as deep red acute rhombohedra or as elongated trigonal spikes, depending on the concentration of protein and type of buffer in solution. Dialysis of the protein solution against buffered 35% MPD also produces the acute rhombohedral form. Both crystal forms have the same space group, $P3_1$ ($P3_2$) and the same cell parameters: $a = b = 57.4$ Å, $c = 97.3$ Å, $\gamma = 120°$. The unit cell volume is 277600 Å^3. For two molecules of 26 kDa per asymmetric unit, the volume per Da (V_m) is 1.77; for one molecule per asymmetric unit, $V_m = 3.54$. These values fall at the extremes of the normal range found for protein crystals [26]. Since this protein has not been fully characterized, the molecule may be larger than the initial studies indicate and thus give a V_m closer to the normal value of 2.4. This protein is very stable and can be recycled numerous times for crystal growth experiments, in contrast to the cyto 4Hc3 material.

Attempts to find useful heavy atom derivatives necessitated use of a 35% MPD solution to stabilize the crystals grown from low ionic strength solutions. The increased strength from the added heavy atom reagent caused dissolution of the crystal. Diffraction intensity changes have been observed from soaking the crystals in millimolar solutions of the reagents: dimercury acetate, samarium nitrate, potassium uranyl pentafluoride, *p*-chloromercury benzenesulfonate and sodium mersalyl. Thus far, the heavy atom sites in the derivatives have not been located.

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