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Crystallization of cytochrome b_{562} from *Erwinia chrysanthemi*

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Abstract

Cytochrome b_{562} from *Erwinia chrysanthemi* has been crystallized using the hanging-drop vapour-diffusion method with ammonium sulfate as the precipitant. X-ray precession photographs show that the crystals formed belong to either of the enantiomorphic space groups $P4_12_12$ or $P4_32_12$ with the cell parameters $a = b = 98.6$ and $c = 62.7$ Å. Estimation of the crystal density and consideration of the possible values for V_m indicate that there is either a dimer or trimer in the asymmetric unit. Experiments using the synchrotron radiation source at the CCLRC Daresbury Laboratory have shown that the crystals diffract to at least 2.7 Å resolution. An analysis of the N-terminal sequence indicates that this cytochrome shows limited homology to the cytochrome b_{562} from *E. coli*. Determination of the structure will therefore allow analysis of the relationship between these two proteins.

1. Introduction

Cytochromes are a group of iron-containing electron-transfer proteins that contain iron-porphyrin prosthetic groups which are attached *via* covalent [*e.g.* cytochrome *c'* (Ren, Meyer & McRee, 1993)] or non-covalent interactions (*e.g.* cytochrome b_{562}) and undergo reversible Fe^{II}–Fe^{III} oxidation/reduction changes during their catalytic cycle. In nearly all cytochromes the fifth and sixth axial ligand positions of the haem Fe atom are occupied by side chains of specific amino-acid residues of the protein, commonly histidine [Hamada, Bethge & Mathews, 1995; Carter *et al.*, 1985; Xia & Mathews, 1990; Frolow, Kalb(Gilboa) & Yariv, 1994; Martinez, Huang, Szczepaniak, Cramer & Smith, 1994]. Cytochromes fall into four groups differing in the nature of the haem prosthetic group. Cytochromes *b* and *c* contain a protohaem IX prosthetic group which is bound to the protein non-covalently or covalently through thioester linkages, respectively. Type *a* cytochromes contain haem groups which are decorated with a formyl group and may also contain a long-chain alkyl group. Finally, the *d*-type cytochromes contain haem groups which contain dihydroporphyrin. All cytochromes in their reduced state have three distinctive absorption bands α, β and γ. The wavelength of the α band of the particular reduced cytochrome species, which is absent in oxidized cytochromes, is thus useful in differentiating the various cytochromes. Specific cytochromes are then classified by the position of their α band and this is then used as a number subscript *e.g.* cytochrome b_{562}.

The structure of cytochrome b_{562} from *E. coli*, a soluble, 106-residue, monomeric protein, has been determined to a resolution of 1.4 Å and it has been shown that this protein is made up of four α-helices connected by two short loops, between helices *A* and *B*, *C* and *D*, and one longer loop between helices *B* and *C* (Nikkila, Gennis & Sligar, 1987; Hamada *et al.*, 1995). The haem group is found at the divergent end of the molecule between the N and C-terminal helices with the haem Fe atom coordinated through histidine and methionine side chains (His102, Met7). The structure of the cytochrome b_{562} from *E. coli* has been compared with several cytochromes *c* from *R. molichianum* (Finzel, Weber, Hardman & Salemme, 1985), from *C. vinosum* and from *R. rubrum* (Yasui *et al.*, 1992) which have a similar four-helix bundle structure. However, in contrast to the non-covalent linkage between the haem group and the protein in cytochrome b_{562} from *E. coli*, the *c'* cytochromes bind the haem group covalently *via* a *CXX*CH sequence region with the two cysteine residues forming covalent bonds with the haem vinyl groups and the histidine residue acting as a fifth haem Fe-atom ligand. Interestingly in these cytochromes the haem Fe atom has only five ligands rather than the six present in cytochrome b_{562} from *E. coli*.

Cells of *E. chrysanthemi* contain high levels of a cytochrome with an α band of 562 nm. This protein can be readily purified (Goward, Stevens, Tattersall & Atkinson, 1992) and analysis using electrospray mass spectrometry indicates that it has a molecular weight of 10.6 kDa (excluding the haem group) suggesting that this cytochrome is somewhat shorter than the equivalent *E. coli* protein. The sequence of the first 47 N-terminal residues has been identified, using the Applied Biosystems model 476A protein sequencer, and shown to be 25% identical to the *E. coli* cytochrome b_{562} (Fig. 1). In order to compare the structure of *E. chrysanthemi* cytochrome b_{562} with that of *E. coli* and the cytochromes *c* from *R. molichianum*, *C. vinosum* and *R. rubrum* we have initiated a structural study of this protein.

2. Cell culture and protein purification

Cytochrome b_{562} was purified from a crude extract of *E. chrysanthemi* cell paste prepared by thawing cells in 1 m*M* EDTA at a final pH of 5.6–5.8 (Goward, Stevens, Collins, Wilkinson & Scawen, 1989). The pH of the suspension was adjusted to 11.5 by addition of 0.5 *M* NaOH and the suspension was stirred for 30 min to extract protein from the cells. The pH of the cell extract was adjusted to 6.5 with 25%(*v*/*v*) acetic acid and the precipitated extraneous protein removed by centrifugation at 13 000*g*. The supernatant was adjusted to pH 4.8 by further addition of 25%(*v*/*v*) acetic acid and the precipitate removed by centrifugation as before. The supernatant was diluted with deionized water to a conductivity of less than 2.5 mS and loaded onto a column of S-Sepharose FF equilibrated with 40 m*M* sodium acetate buffer, pH 4.8 (Goward *et al.*, 1992). The column was washed with 40 m*M* sodium

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acetate buffer, pH 4.8, to remove proteins not bound to the S-Sepharose FF and cytochrome b_{562} was eluted from the column in an extended wash step with 40 m*M* sodium phosphate buffer, pH 6.0. Fractions containing cytochrome b_{562} and the protein solution were dialysed against 10 m*M* potassium phosphate buffer, pH 6.0, and the resulting solution was applied to a column of CM-Sepharose FF. The protein was eluted with a gradient of 10–100 m*M* potassium phosphate buffer at pH 6.0 and the fractions containing cytochrome b_{562} which were easily identifiable from their red colour were pooled. The final purity of the protein as estimated by sodium dodecyl sulfate gel electrophoresis was 80%.

3. Crystallization

For initial crystallization trials the protein was dialysed against 0.1 *M* Tris buffer, pH 7.0 at 277 K. The resulting protein solution was then used for vapour-diffusion crystallization trials, using the hanging-drop method, by mixing 5 µl of the protein solution with 5 µl of the precipitant and equilibrating over the precipitant at 290 K. Trials with ammonium sulfate in the concentration range 3.0–3.5 *M* saturated in 0.1 *M* sodium phosphate buffer, pH 7.0 and with a protein concentration of 2–5 mg ml^{-1} resulted in the formation of small bipyramidal crystals. It was found that larger crystals could be grown using large sitting-drop crystallization trials rather than hanging-drop trials. For these trials the protein was dialysed against 2.0 *M* ammonium sulfate in 100 m*M* sodium phosphate buffer at pH 7.0 and 150 µl samples were then set down in each of three wells of a Corning depression slide. These were sealed in small plastic boxes and surrounded by a reservoir containing the precipitant (3.0–3.5 *M* ammonium sulfate in 100 m*M* sodium phosphate buffer at pH 7.0). Using this method generally larger crystals could be grown with a maximum dimension up to 1.0 mm. These crystals could be stabilized in 0.1 *M* sodium phosphate buffer, pH 7.0, with 3.5 *M* ammonium sulfate. X-ray precession photographs of the cytochrome b_{562} crystals (Figs. 2*a* and 2*b*) show that the crystals belong to the tetragonal system, point group 422. Reflections have been shown to be present only when $l = 4n$ and h or $k = 2n$ identifying the space group as either one of the enantiomorphic pair $P4_12_12$ or $P4_32_12$ with the cell parameters $a = b = 98.6$ and $c = 62.7$ Å. If the asymmetric unit contains a dimer or a trimer then the V_m is 3.3 or 2.2 Å^3 Da^{-1}, respectively, which are both within the range given by Matthews (1977) whereas the value for a monomer falls outside the range.

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                1               10              20
                * *               *   *           **  *  *
E.chrysanthemi  -AVKDEMGAM      AKSYKGASSA      TDAATLKADL
E.coli          ADLEDNMETL      NDNLKVIEKA      DNAAQVKDAL

                30                  40          44
                * *  *               *
E.chrysanthemi  LNIKAHATKA      ------KADP      EFNN
E.coli          TKMRAAALDA      QKATPPKLED      KSPD
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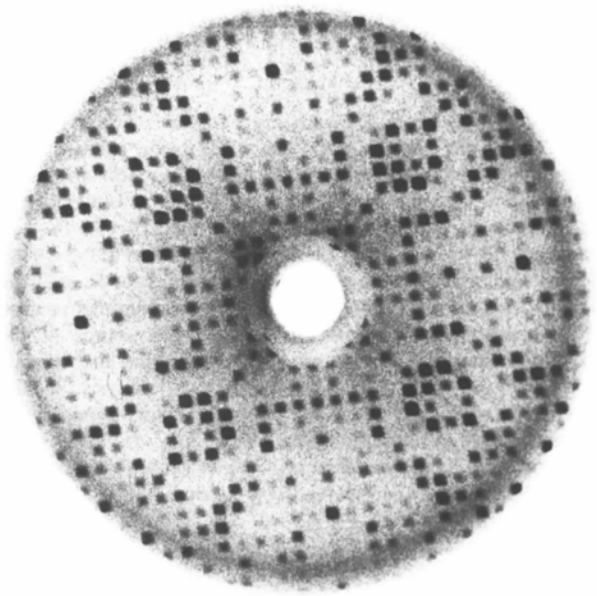
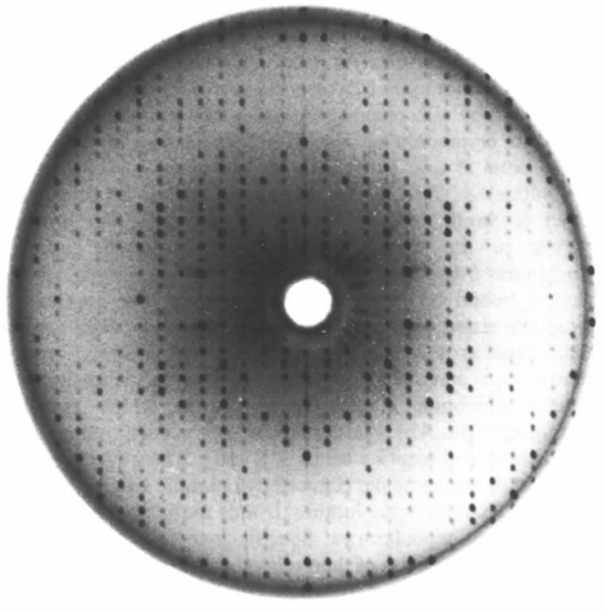
Fig. 1. Sequence alignment of the first 47 N-terminal residues of the cytochromes b_{562} from *E. chrysanthemi* and *E. coli*. Numbers refer to the sequence from *E. chrysanthemi*. Residues indicated by * are identical in both sequences.



(*a*)



(*b*)

Fig. 2. (*a*) A 7° precession photograph of the *hk*0 zone of the *E. chrysanthemi* cytochrome b_{562} crystals (crystal-to-film distance = 75 mm). (*b*) A 10° precession photograph of the *h*0*l* zone of the *E. chrysanthemi* cytochrome b_{562} crystals (crystal-to-film distance = 75 mm) with the *c* axis vertical.

4. Crystal density measurement

The Ficoll density-gradient method (Westbrook, 1976; Bode & Schirmer, 1985) was used to determine the density of the cytochrome b_{562} crystals and to thus obtain an estimate for the number of subunits in the asymmetric unit. The density gradient was constructed from 1.00 to 1.25 g cm^{-3} and calibrated using mixtures of water saturated xylene and bromobenzene of known density. Since the crystals were both large and dark red in colour they were easily visible within the gradient. Duplicate experiments were carried out which produced consistent results giving a density of 1.176 ± 0.004 g cm^{-3} for the cytochrome b_{562} crystals, averaged over a total of four crystals. The estimated density indicated that the mass of protein per asymmetric unit in the cytochrome b_{562} crystals is 31.8 kDa. Since the subunit molecular weight for cytochrome b_{562} is approximately 11.6 kDa (including the haem group) this again suggests that the asymmetric unit contains either a dimer or a trimer but at this stage a resolution of this ambiguity is not possible.

The crystals produced to date are suitable for structural studies and a native data set has been collected at the CCLRC Daresbury Laboratory to a resolution of 2.7 Å. Elucidation of the three-dimensional structure of this enzyme will enable a detailed comparison to be made with that of *E. coli* cytochrome b_{562} in order to examine the nature of the haem binding site. Such a comparison may also help to lead to an understanding of the electron-transfer mechanism for the b_{562} cytochromes whilst the molecular evolution relating the cytochromes b_{562} and c' may be examined.

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