Acta Cryst. (1997). D**53**, 461–463

Crystallization and preliminary X-ray crystallographic analysis of the electron-transferring flavoprotein from *Megasphaera elsdenii*

CAROLINE T. SHARKEY,[a] MARTIN A. WALSH,[b] STEPHEN G. MAYHEW[c] AND TIMOTHY M. HIGGINS[a]* at [a]*Department of Chemistry, University College, Galway, Ireland,* [b]*European Molecular Biology Laboratory (EMBL), c/o DESY, Notkestrasse 85, 2000 Hamburg 52, Germany, and* [c]*Department of Biochemistry, University College Dublin, Dublin 4, Ireland.*
E-mail: tim.higgins@ucg.ie

(*Received 30 July 1996; accepted 2 January 1997*)

Abstract

Electron-transferring flavoprotein from the rumen bacterium *Megasphaera elsdenii* is a heterodimer ($M_r = 75$ kDa) containing FAD as cofactor and functioning solely to mediate electron transfer between the prosthetic groups of other proteins. The enzyme was crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 4000 as precipitant. The crystals obtained belong to the space group $P2_12_12_1$ with unit-cell dimensions of $a = 58.75$, $b = 61.77$ and $c = 122.27$ Å. Interestingly the crystals exhibit a low solvent content. Crystals diffracted to beyond 2.5 Å using synchrotron radiation.

1. Introduction

Electron-transferring flavoprotein (ETF) is a heterodimer that contains non-covalently bound FAD, and that functions as an electron carrier between other flavoenzymes (Thorpe, 1991). First isolated from mammalian mitochondria (Crane & Beinert, 1956), it was subsequently found in a variety of organisms. Mitochondrial ETF couples the oxidation of reduced acyl-CoA, glutaryl-CoA, sarcosine and dimethyl glycine dehydrogenases to the reduction of ETF-ubiquinone oxidoreductase, an enzyme that in turn transfers electrons to ubiquinone in the inner mitochondrial membrane. Defects in mammalian ETF can result in glutaric aciduria type II, a metabolic disease that is often fatal (Ikeda, Keese & Tanaka, 1986).

The ETF's isolated from bacterial sources have functions analogous to those of mitochondrial ETF. The enzyme from *M. elsdenii* is of particular interest because its catalytic and physico-chemical properties differ in several respects from those of ETF preparations from other sources. *M. elsdenii* is a rumen bacterium that ferments lactate to molecular hydrogen, CO_2 and short-chain fatty acids which are excreted into the growth medium (Elsden, Volcani, Gilchrist & Lewis, 1956). ETF in this organism transfers electrons from the flavoenzyme D-lactate dehydrogenase to butyryl-CoA dehydrogenase, an enzyme which operates physiologically to reduce crotonyl-CoA. In contrast to ETF from other sources which transfer reducing equivalents only between other redox enzymes, *M. elsdenii* ETF also catalyzes the oxidation of NADH, which can, therefore, serve to reduce the enoyl-CoA.

In common with other ETF's, the *M. elsdenii* enzyme is a dimer of different subunits, the flavin in the enzyme is at the level of FAD and the protein-bound flavin is unusually fluorescent. Also the form of the flavin semiquinone stabilized by the protein is red and anionic. The smaller β-subunit has been cloned recently together with a part of the α-subunit (O'Neill, Butler & Mayhew, 1995), and while very little overall sequence homology was found with the β-subunits of ETF from human liver (Finocchiaro *et al.*, 1993) and *Paracoccus denitrificans* (Bedzyk, Escudero, Gill, Griffin & Frerman, 1993), several regions of conserved sequence were identified. In marked contrast to other ETF's which contain only one molecule of FAD, the *M. elsdenii* enzyme has two molecules of flavin. A fraction of the flavin in some preparations consists of 6-OH-FAD and 8-OH-FAD (Ghisla & Mayhew, 1976), but since the content of hydroxy-flavins varies with the methods used to isolate the enzyme, it is unlikely that the modified flavins occur naturally. Binding experiments with these flavins, and also with the flavin derivatives 8-Cl-FAD and 8-F-FAD which react covalently with the enzyme (O'Nuallain & Mayhew, 1987), have suggested that the two flavin-binding sites are not equivalent. It is possible that redox reactions with NAD(H) occur at one flavin site while reactions with redox enzymes occur at the other. Analysis of the products of reaction with 8-F-FAD showed that only the α-subunit is modified, suggesting that both FAD sites may be on this subunit.

The three-dimensional structure of any ETF has not yet been published, though ETF from human and *P. denitrificans* (Roberts, Herrick, Frerman & Kim, 1995), as well as *Methylophilus* W3A1 (White, Matthew, Rohlfs & Hille, 1994) have been crystallized. The low sequence homology between ETF from *M. elsdenii* and other ETF's, the presence of two, possibly non-equivalent, FAD binding sites and the diaphorase activity prompted us to crystallize ETF from *M. elsdenii* for structure determination. The three-dimensional structure coupled with the availability of the cloned gene for the enzyme will allow the rational design of site-directed mutants.

2. Results

Cells of *M. elsdenii*, strain LC1, grown in iron-poor media were prepared as published (Mayhew & Massey, 1969). ETF was purified essentially as already published (Whitfield & Mayhew, 1974). On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), ETF showed two major bands corresponding to two non-identical subunits, 41 and 34 kDa. The concentrated protein was stored in 20 m*M* potassium phosphate buffer pH 6, 0.3 m*M* EDTA at 253 K. For crystallization, the stock solution of ETF was concentrated and desalted using an Amicon centricon-10 concentrator. Protein concentrations were calculated using the extinction coefficient (450 nm) for ETF + FAD (Whitfield & Mayhew, 1974).

Crystals were obtained at 291 K using the hanging-drop vapour-diffusion technique after an initial screening of conditions with a sparse-matrix search (Jancarik & Kim, 1991). Further optimization of conditions eventually improved the crystals from thin plates to ones of X-ray diffraction quality.

The hanging drops were prepared by mixing 1 μl of the enzyme solution (25 mg ml^{-1}) with 1 μl of the reservoir solution. The reservoir solution contained 17% polyethylene glycol 4000 and 22% isopropanol in 100 m*M* sodium citrate buffer pH 5.6. Crystals were yellow–green in colour.

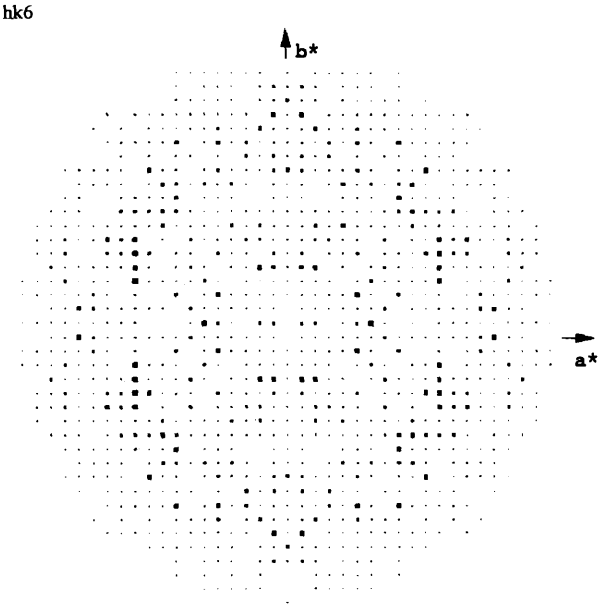

Fig. 1. The pseudoprecession plot of the *hk*6 zone to 3 Å resolution calculated by the *HKLVIEW* program (Collaborative Computational Project, Number 4, 1994).

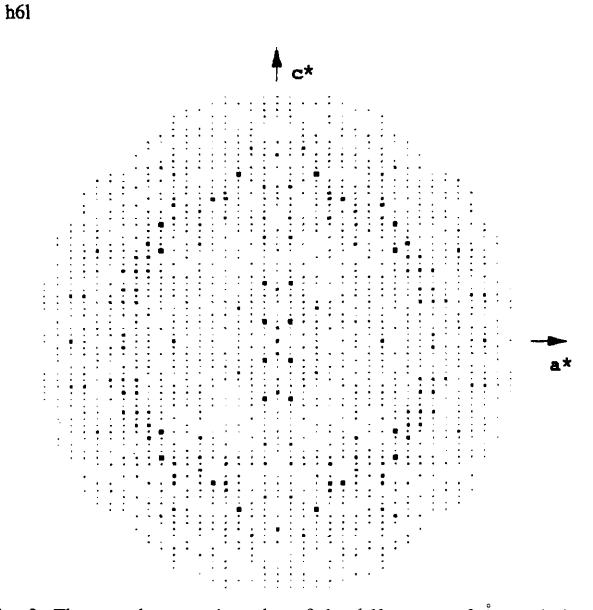

Fig. 2. The pseudoprecession plot of the *h*6*l* zone to 3 Å resolution calculated by the *HKLVIEW* program (Collaborative Computational Project, Number 4, 1994).

Diffraction data were collected at room temperature, to 2.5 Å, with synchrotron radiation ($\lambda = 0.99$ Å), from the EMBL X31 beamline at the DORIS storage ring, DESY, Hamburg. One tiny crystal (0.05 × 0.05 × 0.2 mm) provided 180° of data. However, these data were only 85% complete because of loss of high-order diffraction in the later stages of data collection. There were no visible signs of twinning either under the polarizing microscope or in the diffraction images. The reflection data were integrated and scaled using the programs *DENZO* and *SCALEPACK*, respectively (Otwinowski, 1993). The data were initially processed in the orthorhombic space group $P2_12_12_1$. Indexation proceeded smoothly giving an excellent fit to all data. The unit-cell dimensions are $a = 58.75$, $b = 61.77$, $c = 122.27$ Å, $\alpha = \beta = \gamma = 90°$. The data to 3 Å resolution was 98.7% complete and contained a total of 64 989 observations, which were reduced and merged to 9 117 unique reflections with an R_{merge} of 9.6% (based on intensities).

PAGE gels from dissolved crystals were identical to those from ETF prior to crystallization and so assuming that the asymmetric unit contains one ETF molecule with a molecular mass of 75 kDa (Whitfield & Mayhew, 1974), the V_m value is calculated as 1.5 Å^3 Da^{-1}, equivalent to a solvent content of 20%(*v*/*v*). Matthews coefficients as low as this, the lowest extreme end of the range observed for most protein crystals (Matthews, 1968), are not unprecedented (Nagendra, Sudarsanakumar & Vijayan, 1995; Gallagher, Alexander, Bryan & Gilliland, 1994). This suggests a very efficient packing and possibly extensive intermolecular interaction.

The possibility that the data belong to a lower Laue symmetry space group was investigated by reprocessing as triclinic. The 64 989 observations, to 3 Å resolution, now merged to 29 702 unique reflections with an R_{merge} of 7.8% (intensities). Plots of the reciprocal lattice planes clearly showed *mm* symmetry (see Figs. 1 and 2). Examination of the systematic absences clearly showed the presence of twofold screws on all three axes.

The search for heavy-atom derivatives for phase determination by multiple isomorphous replacement is in progress.

We would like to thank Eleanor and Guy Dodson for their continued support. This work was funded in part by a grant awarded by Forbairt, the Irish science and technology agency.

References

Bedzyk, L. A., Escudero, K. W., Gill, R. E., Griffin, K. J. & Frerman, F. E. (1993). *J. Biol. Chem.* **269**, 20211–20217.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Crane, F. L. & Beinert, H. (1956). *J. Biol. Chem.* **218**, 717–731.
Elsden, S. R., Volcani, B. E., Gilchrist, F. M. C. & Lewis, D. (1956). *J. Bacteriol.* **72**, 681–689.
Finocchiaro, G., Colombo, I., Garavaglia, B., Gellera, C., Valdoameri, G., Garbuglio, N. & Didonato, S. (1993). *Eur. J. Biochem.* **213**, 1003–1008.
Gallagher, T., Alexander, P., Bryan, P. & Gilliland, G. (1994). *Biochemistry*, **33**, 4721–4729.
Ghisla, S. & Mayhew, S. G. (1976). *Eur. J. Biochem.* **63**, 373–390.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Ikeda, Y., Keese, S. M. & Tanaka, K. (1986). *J. Clin. Invest.* **78**, 997–1002.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mayhew, S. G. & Massey, V. (1969). *J. Biol. Chem.* **244**, 794–802.
Nagendra, H. G., Sudarsanakumar, C. & Vijayan, M. (1995). *Acta. Cryst.* D**51**, 390–392.

O'Neill, H. M., Butler, G. & Mayhew, S. G. (1995). *Biochem. Soc. Trans.* **23**, 379S.

O'Nuallain, E. M. & Mayhew, S. G. (1987). In *Flavins and Flavoproteins*, edited by D. B. McCormick & D. E. Edmondson, pp. 361–364. Berlin: Walter de Gruyter.

Otwinowski, Z. (1993). *Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 560–562. Warrington, England: Daresbury Laboratory.

Roberts, D. L., Herrick, K. R., Frerman, F. E. & Kim, J. P. (1995). *Protein Sci.* **4**, 1654–1657.

Thorpe, C. (1991). In *Chemistry and Biochemistry of Flavoenzymes*, edited by F. Mller, pp. 471–476. Boca Raton: CRC Press.

White, S. A., Matthews, F. S., Rohlfs, R. J. & Hille, R. (1994). *J. Mol. Biol.* **240**, 265–266.

Whitfield, C. D. & Mayhew, S. G. (1974). *J. Biol. Chem.* **249**, 2801–2810.