Acta Cryst. (1995). D51, 402–403

Crystallization of a calcium-binding EGF-like domain. By Z. RAO, *Laboratory of Molecular Biophysics and Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QT, England,* P. A. HANDFORD, *Sir William Dunn School of Pathology, and Oxford Centre for Molecular Sciences, South Parks Road, Oxford, OX1 3QT, England,* V. KNOTT and M. MAYHEW, *Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, England,* G. G. BROWNLEE, *Sir William Dunn School of Pathology, and Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QT, England,* and D. STUART, *Laboratory of Molecular Biophysics, and Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QT, England*

(*Received* 20 *May* 1994; *accepted* 26 *August* 1994)

Abstract

Crystals of a calcium-binding epidermal growth factor (EGF)-like domain of human clotting factor IX suitable for X-ray diffraction analysis have been obtained by vapour diffusion (sitting drop) against 48% PEG 400. The crystals belong to the tetragonal space group $P4_32_12$, with unit-cell dimensions $a = b = 40.3$, $c = 98.2$ Å. The crystals diffract beyond 1.5 Å resolution and are relatively stable in the X-ray beam. This is the first reported crystallization of a calcium-binding EGF-like domain.

Introduction

EGF-like domains are found in a variety of extracellular and membrane proteins (Doolittle, Feng & Johnson, 1984; Campbell & Bork, 1993). The precise function of the EGF-like domains in the majority of these proteins remains unknown (Stenflo, 1991), however, a significant number bind calcium and are thought to be involved in modulating protein–protein interactions (Stenflo, 1991; Campbell & Bork, 1993). The calcium-binding domains contain a consensus sequence Asp, Asp/Asn, Glu/Gln, Asp*/Asn*, Tyr/Phe (where * indicates a β-hydroxylated residue) (Rees *et al.*, 1988; Campbell & Bork, 1993). The biological importance of these residues has been demonstrated by the identification of mutations in them in haemophilia B patients (defective in human clotting factor IX) (Gianelli *et al.*, 1993) and in patients with Marfan's syndrome (defective in the connective tissue protein fibrillin) (Dietz *et al.*, 1993).

An investigation of the calcium-binding properties of the first EGF-like domain from human factor IX and the structure of the calcium-free form, determined by NMR, has been reported (Handford *et al.*, 1990; Handford *et al.*, 1991; Mayhew *et al.*, 1992; Baron *et al.*, 1992). However, the precise structural basis of calcium binding was not determined. Here we report the crystallization and preliminary X-ray analysis of this domain.

Experimental

The calcium-binding EGF-like domain (residues 46–84 of human clotting factor IX, either recombinant or synthetic) was purified as described (Handford *et al.*, 1990; Mayhew *et al.*, 1992). Briefly, the lyophilized peptide was dissolved in 0.1% trifluoroacetic acid in H_2O and filtered through a low protein-binding 0.45 µm filter (Millipore) to remove particulate matter. High-pressure liquid chromatography (HPLC) purification of the peptide was performed using an Ultrapore C8 reversed-phase semi-preparative column (Beckman). The protein was eluted using a linear gradient of 20–80% of a solution containing 80% acetonitrile, 20% H_2O and 0.1% trifluoroacetic acid.

Table 1. *Crystallization conditions*

Method	Sitting drop (vapour diffusion)
Drop	20 mg ml^{-1} of protein 50 m*M* Tris–HCl 20 m*M* calcium chloride
Reservoir	48% polyethylene glycol (PEG) 400
Drop size	1 µl
Temperature	289 K
pH	7.3
Time	One week
Crystals	Largest size 1.0 × 0.4 × 0.25 mm
Note	No reservoir solution was added to the drop!

For crystallization the protein was dissolved in Tris chloride buffer, pH 7.3, containing 20 m*M* calcium chloride and filtered through a low protein-binding 0.45 µm filter (Millipore). It was then concentrated in a centricon tube (Amicon corporation) to a protein concentration of 20 mg ml^{-1}. The peptide purity was assessed by HPLC. In all cases the purity exceeded 99%.

Crystallization conditions were explored using the hanging- and sitting-drop vapour-diffusion methods at room temperature and at 277 K with reservoirs containing varying precipitants, solvents and pH. The first crystal was found in an experiment in which, by an oversight, no solution was transferred from reservoir to the protein drop. This unusual aspect of the crystallization protocol proved to be essential and is preserved in the final conditions, detailed in Table 1.

Results and discussion

The most commonly observed form of the crystals was single transparent and brick-like. The largest crystals span the drops (Fig. 1). The approximate size of the best crystal was 1.0 × 0.4 × 0.25 mm. Unfortunately, the crystals adhered strongly to the surface of the sitting-drop bridge and it was almost impossible to remove them without damage. In an attempt to avoid this problem, crystallization experiments were set up using nylon, propylene, polyester, polystyrene and glass supports. Non-adhesive propylene microbridges (Harlos, 1992) gave the best results, and provided all the crystals for the structure analysis. These bridges may well prove useful in other cases where manipulation of the crystal is otherwise difficult. Siliconization appeared to have little effect.

For X-ray diffraction studies, crystals were mounted in thin-walled quartz capillaries. A set of native data (92% complete to 2.5 Å) was collected on an imaging plate (MAR research) using 0.90 Å monochromatic radiation at a temperature of between

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ISSN 0907-4449 © 1995

273 and 277 K at station 9.5 of the SRS, Daresbury. A total of 30 frames were collected with 2° rotation range per frame. Only one crystal was used during the data collection and there was no obvious radiation damage. Examination of somewhat large crystals on the F1 line at CHESS demonstrated that some diffracted to Bragg spacings of better than 1.5 Å (Fig. 2).

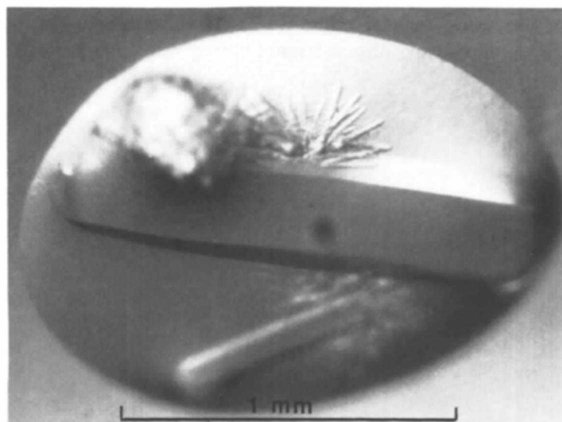

Fig. 1. A crystal of the calcium-binding EGF-like domain. The crystals are single transparent rectangular prisms. The largest crystal spans the drop. The approximate size of the best crystals was 1.0 × 0.4 × 0.25 mm.

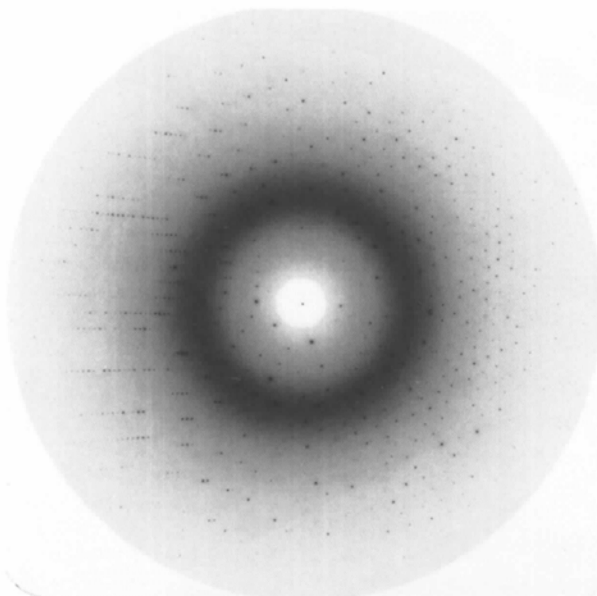

Fig. 2. An X-ray diffraction photograph from an EGF-like domain crystal recorded on the F1 line at CHESS. This demonstrated that the crystal diffracted to a Bragg spacing of better than 1.5 Å. Oscilation range 1.0°, $\lambda = 0.91$ Å, exposure time = 10 s. Crystal-to-film distance = 175 mm.

Analysis of the diffraction data indicated that the crystals belong to the tetragonal space group, $P4_12_12$ (or $P4_32_12$), with unit-cell dimensions $a = b = 40.3$, $c = 98.3$ Å. If it is assumed that there are two EGF-like domains in each asymmetric unit, the solvent content of these crystals is approximately 30%.

Analysis using a proton microprobe (Grime, Dawson, Marsh, McArthur & Watt, 1991) was performed to estimate the calcium content of the crystals, and the results corresponded to 1.7 (±0.2) calcium ions per protein domain (E. F. Garman, unpublished results).

The structure has now been solved using isomorphous-replacement and anomalous-scattering methods and refined at 1.5 Å resolution to an R factor of 15.7% for all measurements in the range 30–1.5 Å (manuscript in preparation). This confirmed the space group as $P4_32_12$ and also confirmed that there are two protein molecules and three calcium ions in the crystallographic asymmetric unit, in agreement with the microprobe results.

It is possible that aspects of the method described here may prove useful for the crystallization of other small proteins and peptides, which have often proved difficult to crystallize.

The Oxford Centre for Molecular Sciences is supported by the SERC and MRC. PH is supported by a 1992 Royal Society University Research Fellowship. Thanks to Drs E. Fry, E. Y. Jones, K. Harlos and E. F. Garman and other colleagues in our laboratory for assistance with data collection and to the staff at Daresbury and CHESS for their cooperation with the project.

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