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A new crystal form of carboxypeptidase G_2 from *Pseudomonas* sp. strain RS-16 which is more amenable to structure determination

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Abstract

Carboxypeptidase G_2 is a zinc-dependent exopeptidase which has applications in cancer therapy. Crystallization of carboxypeptidase G_2, first achieved more than a decade ago, yields large crystals; however, problems with non-isomorphism between native crystals as well as failure to obtain any useful heavy-atom derivatives have precluded structure solution. A modification of the crystallization protocol leading to a promising new crystal form which diffracts beyond 3.0 Å resolution on a rotating-anode source is now reported. These crystals are readily indexed on an apparent *C*-centred orthorhombic lattice with $a = 81.35$, $b = 230.9$ and $c = 105.5$ Å, but the correct crystal system is monoclinic. The crystals have space group $P2_1$, with $a = 81.35$, $b = 105.5$, $c = 122.4$ Å and $\beta = 109.3°$. There are two possible non-equivalent monoclinic indexings with these lattice constants. A partial native data set collected at the SRS, Daresbury, indicates that 1.9 Å diffraction is attainable. Structure determination using MIR methods is in progress.

1. Introduction

The carboxypeptidase G class of enzymes hydrolyses the C-terminal glutamate moiety from folic acid and analogues such as methotrexate, polyglutamate derivatives of folic acid, subfragments of folic acid such as *p*-aminobenzoyl glutamate and specific small peptides with C-terminal glutamate residues (Kalghatgi & Bertino, 1981). Carboxypeptidase G_2 (CPG_2) is a zinc-dependent metalloenzyme localized in the periplasmic space of *Pseudomonas* sp. strain RS-16. It exists as a homodimer of molecular weight $2 \times 41\,800$ Da, and atomic absorption analysis indicates binding of four zinc ions per dimeric molecule (Sherwood, Melton, Alwan & Hughes, 1985). There is no amino-acid sequence homology with other carboxypeptidases for which structural information is available.

The key role of reduced folates as co-enzymes in many biochemical pathways, including those leading to DNA synthesis *via* the pyrimidines and purines, has made folic acid a target molecule for chemotherapy. Depletion of folic acid can inhibit the growth of tumours with high folic acid requirements (Rosen & Nichol, 1962). Depletion of the reduced folate acid pool has been most commonly achieved by the use of folic acid antagonists, notably methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid), which inhibit the enzyme dihydrofolate reductase (Osborn, Freeman & Huennekens, 1958). Folate depletion can also be achieved by enzymic cleavage of reduced folates by the carboxypeptidase G group of enzymes (Kalghatgi & Bertino, 1981). Carboxypeptidase G enzymes can also be used in rescue therapy following high-dose methotrexate regimes in patients in whom prolonged treatment with the drug leads to toxicity. Provided that CPG_2 administration follows after about 24 h, higher doses of methotrexate can be used in therapy, and patients who have accidentally received methotrexate overdoses can be treated (Widemann, Hetherington, Murphy, Balis & Adamson, 1995). CPG_2 can be targeted to tumour sites by conjugation to antibodies directed at tumour-associated antigens. In a two- or three-stage process, after clearing residual enzyme activity from the blood, a relatively non-toxic prodrug which is a substrate for the tumour-located enzyme is given, resulting in the generation of the cytotoxic drug selectively at tumour sites (Bagshawe, 1987; Bagshawe *et al.*, 1988; Sharma *et al.*, 1991). Several enzyme-prodrug systems are being evaluated but only the carboxypeptidase G_2 system has entered clinical trials (Knox & Connors, 1995).

The yield of protein from *Pseudomonas* sp. strain RS-16 is very low (< 0.1% of cell soluble protein). In order to increase yields of CPG_2, so as to provide sufficient material for a full assessment in clinical trials, the gene coding for CPG_2 has been cloned in *Escherichia coli* (Minton, Atkinson & Sherwood, 1983), where it is expressed at 3–5% of the soluble protein. The samples used in this project contained an N-terminal glutamine to threonine mutation (Werlen *et al.*, 1994), and were expressed in *E. coli* and purified at the Division of Biotechnology, CAMR, Porton Down, England, following a previously described protocol (Sherwood, Melton, Alwan & Hughes, 1985). In order to identify the active site of the enzyme and to allow modification of the protein and/or substrate with a view to improving the therapeutic properties of the enzyme, the three-dimensional structure determination by X-ray crystallography was undertaken. CPG_2 crystals of space group *C*2 have been grown by hanging-drop vapour diffusion (Lloyd, Collyer & Sherwood, 1991), however, there was variation in the cell dimensions between different native crystals (non-isomorphism) and despite extensive searching no heavy-atom derivatives were found that would allow structure determination. We now report a modification of the crystallization protocol which yields isomorphous crystals and has allowed heavy-atom derivatization which we believe will lead to structure determination.

2. Crystallization

On occasion, it was noted that a second distinct crystal form would appear in the same hanging drop as the original *C*2 crystal form, leading to the belief that a slight change in crystallization conditions would possibly provide a more amenable crystal form for structural analysis. The *C*2 crystal

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form is grown in the presence of 200 m*M* zinc acetate. Addition of 200 m*M* zinc acetate as an ingredient in the crystallization mixture reduces the pH of the system to about pH 5.9, even though 100 m*M* Tris–HCl buffer is also present. Therefore, the concentration of the buffer component of the system was raised, resulting in a pronounced shift from the *C*2 morphology to the new form. The diffraction from the new crystal form was rather weak at this stage, but the addition of 10% glycerol improved the resultant crystals dramatically, perhaps by stabilizing the protein conformation (Sousa & Lafer, 1990). The final crystallization protocol involves mixing 4 µl of reservoir solution [200 m*M* Tris–HCl pH 7.2; 12%(*w*/*v*) polyethylene glycol 4000; 200 m*M* zinc acetate; 10%(*v*/*v*) glycerol] with 4 µl of 20.0 mg ml^{-1} protein solution to form a hanging drop which was allowed to equilibrate with the reservoir solution at room temperature. Crystals grew to their final size of 0.4 × 0.4 × 0.4 mm within 6–8 d.

3. Crystal characterization

Native data were collected on a 30 cm diameter MAR Research image plate mounted on an Enraf–Nonius FR571 rotating-anode generator equipped with a Cu target and a graphite monochromator and operated at 40 kV and 90 mA, producing Cu $K\alpha$ X-radiation which was collimated through horizontal and vertical slits set at 0.5 mm. A single crystal was mounted in a glass capillary at room temperature and exposed for 30 min per 1° of crystal rotation. The detector was positioned 200 mm from the crystal and data were collected to 3.0 Å resolution. The data could be indexed using the auto-indexing routines available in a version of the program *XDS* (Kabsch, 1988) adapted for use with image plates. Misleadingly, the crystals can be indexed on a *C*-centred orthorhombic lattice (space group $C222_1$) with $a = 81.35$, $b = 230.9$, $c = 105.5$ Å and angles of 90.0°. However, examination of the intensity-weighted reciprocal lattice clearly indicates the presence of only one mirror plane (perpendicular to the 105 Å axis), thus the correct crystal system is monoclinic. The true space group is $P2_1$, with cell dimensions $a = 81.35$, $b = 105.5$, $c = 122.4$ Å and $\beta = 109.3°$ which would accommodate two dimers in the asymmetric unit giving a volume to mass ratio $V_m = 2.93$ Å^3 Da^{-1}, corresponding to a solvent content of 58% (Matthews, 1968). Self-rotation studies (data not shown) using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) showed the presence of three mutually perpendicular non-crystallographic twofold axes. No special relationship between the new and the old crystal forms could be detected. The relationship between the indexings of the new crystal form is shown by a schematic representation of the lattice in Fig. 1. Also clear from this figure is that there are two non-equivalent monoclinic indexings with identical unit-cell parameters: data indexed with one indexing need to be reindexed before they can be compared with data indexed with the other indexing. Since the indexing selected is not known *a priori*, care must be taken when comparing data sets during, for example, the search for heavy-atom derivatives. As expected, data merged using the incorrect orthorhombic indexing showed relatively poor statistics. For the correct indexing, R_{sym} for all data to 3.0 Å is 6.2% (38 093 unique data derived from 119 191 measurements; 96.9% complete; 93.4% complete in the highest resolution shell 3.5–3.0 Å; $R_{sym} = 15.9\%$ in the highest resolution shell). A partial synchrotron data set collected on a 30 cm MAR Research image plate at station 9.6 at the SRS, Daresbury, England, showed that diffraction to 1.9 Å (Fig. 2) was attainable, however this resolution limit decayed to worse than 2.5 Å within 4° of crystal rotation for all crystals examined, indicating that crystal freezing at 100 K would be desirable for high-resolution data collection. Significant intensity changes were found in data collected from crystals soaked in 5 m*M* K_2PtCl_4 for 1 d and from crystals soaked in 5 m*M* $K_2Pt(NO_2)_4$ for 2 d, and structure determination using MIR methods is in progress using the 3 Å rotating-anode data.

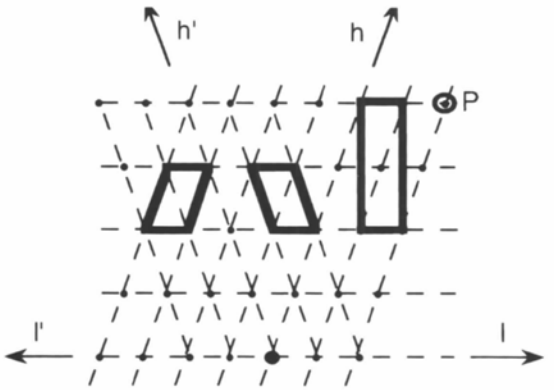

Fig. 1. Schematic diagram of one lattice plane, highlighting the misleading *C*-centred orthorhombic unit cell and two correct non-equivalent monoclinic unit cells with identical cell parameters. Data need to be reindexed to the same monoclinic indexing if they are to be compared. The reindexing transformation is $h_{new} = h$, $k_{new} = -k$, $l_{new} = -h - l$. Thus, the indices of point **P** would change from 4,*k*,2 to 4,– *k*,– 6.

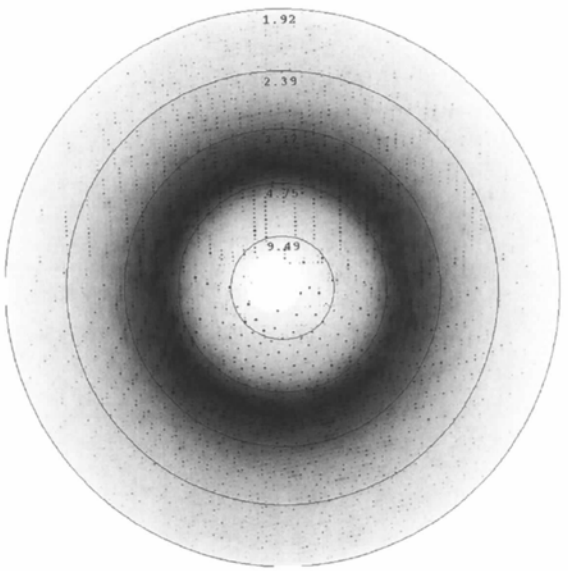

Fig. 2. A digitized image showing 1.9 Å diffraction obtained at station 9.6 at the SRS, Daresbury, using a 30 cm MAR Research image plate positioned 300 mm from the crystal and a wavelength of 0.88 Å.

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