Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary characterization of crystals of the C-terminal half fragment of tropomodulin

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Received 4 December 2000
Accepted 1 March 2001

Tropomodulin (40 kDa) stabilizes the actin–tropomyosin filament by capping the P end (slow-growing end). The C-terminal half (C20, 20 kDa), an independently folded domain that is believed to be responsible for the P-end capping, has been crystallized. Crystals grew in the presence of Zn^{2+} as the solution pH was increased from 3 towards the pI of the protein. The crystals belong to the trigonal space group $R3$. They have unit-cell parameters $a = b = 69.6$, $c = 101.3$ Å (mean values, with a estimated standard deviation of 0.009 Å) and diffract to 1.9 Å resolution when the frozen crystals were measured at 120 K on a rotating-anode X-ray source at 120 K.

1. Introduction

Tropomodulin (40 kDa) is the unique P-end (slow-growing end) capping protein of the actin–tropomyosin filament. Tropomodulin isoforms have been found in striated muscle (Almenar-Queralt *et al.*, 1999), in the cytoskeleton of erythrocyte membranes (Fowler, 1996), in eye-lens cells (Fischer *et al.*, 2000) and in the brain (Watakabe *et al.*, 1996). The N-terminal region of tropomodulin interacts with the N-terminus of tropomyosin (Sung & Lin, 1994; Vera *et al.*, 2000). The tropomodulin–actin interaction has been implicated by the fact that tropomodulin alone, without tropomyosin, inhibits the elongation of actin filaments (Weber *et al.*, 1994). Moreover, the C-terminal region of tropomodulin is believed to be responsible for the capping activity; elongation of actin filaments resumed upon injection into cells of monoclonal antibodies against the C-terminal region (Gregorio *et al.*, 1995). Nevertheless, no binding site has been identified on the C-terminal region for interaction with actin.

We have elucidated the following structural properties of tropomodulin, which led us to develop our successful crystallization strategy. By use of an improved *Escherichia coli* tropomodulin expression system, large fragments have been obtained: a fragment of the C-terminal half (C20) and a fragment that covers the N-terminal quarter (N11) (Kostyukova *et al.*, 2000). Small-angle X-ray scattering (Fujisawa *et al.*, unpublished results) and differential scanning calorimetry studies (Kostyukova *et al.*, unpublished results) have revealed that the C-terminal half of tropomodulin is compactly folded and globular, whereas the N-terminal half is elongated and has no tertiary structure. Because of its compact conformation and its important role in actin-filament capping, we have chosen C20 as the first target for crystallization. No atomic structure is so far known of this protein or of any B-end capping proteins except gelsolin. Here, we report the first crystallization and preliminary X-ray crystallographic analysis of the C20 tropomodulin fragment. The crystals diffract X-rays to 1.9 Å resolution.

The atomic structure of the tropomodulin C20 fragment would enable us to determine whether C20 can interact with any other protein, especially with actin, by comparing it with a number of known structures that interact with actin. This information would shed light on the thin-filament P-end capping mechanism and the regulation of actin dynamics.

2. Methods and results

2.1. Protein preparation

E-tropomodulin with a His tag (six His residues) at the N-terminus and lacking 15 C-terminal residues, denoted Tmod (N39), was expressed in *BL21(DE3)plysE* cells and purified as previously described using methods described in Kostyukova *et al.* (2000). A fragment (C20) containing the C-terminal half, with a weight of 20 kDa, was obtained by limited proteolysis of Tmod (N39) with *Streptococcus aureus* V8 protease and was purified on a MonoQ column as described by Kostyukova *et al.* (2000).

It was found that at neutral to basic pH conditions, Tmod (N39) as well as C20 tends to form aggregates in the presence of salts, even with 200 m*M* NaCl. This was confirmed on

native PAGE gels and by dynamic light scattering (data not shown). Moreover, differential scanning calorimetry indicated that at neutral to basic pH, thermal denaturation of Tmod (N39) and C20 was irreversible owing to aggregation in the presence of salt, even after dialysis of samples into buffer without salts. However, at a pH of around 3 (below the calculated pI of tropomodulin of 5.2; Appel *et al.*, 1994) the denaturation was totally reversible (Kostyukova *et al.*, unpublished results). Based on these observations, a solution of C20 in 25 m*M* glycine buffer pH 3.0 was used as the initial protein solution for crystallization trials.

2.2. Crystallization

Crystals were grown by the hanging-drop vapour-diffusion method. Crystals could be obtained over a wide range of protein concentrations, 5–12 mg ml^{-1}, while PEG at a concentration in the range 15–25% was used in the reservoir as a precipitant. The molecular weight of PEG is not critical for crystallization, but PEGs of smaller molecular weights are preferable as they give nucleation rates that give rise to crystals that are larger in size and are better shaped. $ZnSO_4$ is required; crystals could only be obtained in the presence of $ZnSO_4$ at a concentration of between 2 and 50 m*M* in the reservoir.

The best crystals, used for data collection, were grown under the following conditions. A hanging drop was made up by mixing 4 µl protein solution (10 mg ml^{-1} of C20 in 25 m*M* glycine buffer pH 3.0) with 2 µl precipitant solution composed of 0.1 *M* MES–NaOH buffer pH 6.5, 24%(*v*/*v*) PEG 400 and 10 m*M* $ZnSO_4$. The reservoir solution contained 0.1 *M* MES–NaOH buffer pH 6.5, 15%(*v*/*v*) PEG 400 and 6 m*M* $ZnSO_4$. Crystals grew in 3–7 d to a size suitable for data collection and grew further up to 1 mm in length. All crystallization trials were undertaken at room temperature (295 K) in order to avoid possible pH fluctuation arising from temperature change associated with handling of crystals during inspection and mounting.

2.3. X-ray diffraction analysis

For X-ray analysis, the crystals were soaked in a cryoprotectant solution (25% glycerol in the mother liquor), mounted in nylon cryoloops (Hampton Research, CA, USA) and flash-cooled in liquid nitrogen. From the frozen crystals kept at 120 K, data were collected using a R-AXIS IV image-plate detector (Rigaku, Tokyo) on a Rigaku FRC rotating-anode X-ray generator (λ = 1.54 Å). The data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). From the apparent unit-cell parameters obtained in this way, the estimated standard deviation (e.s.d.) of the unit-cell parameters was calculated. The data-collection parameters, crystallographic parameters and data-processing results are summarized in Table 1.

Table 1
Data collection, crystallographic parameters and data-processing statistics.

Values in parentheses refer to the highest resolution shell.

Crystal dimensions (mm)	0.3 × 0.2 × 0.8
Crystal-to-detector distance (mm)	120
Maximum data resolution (Å)	1.9
Oscillation range for each exposure (°)	1
Temperature (K)	120
Exposure time (min)	10
Total crystal rotation (°)	180
Unit-cell parameters (Å)	$a = b = 69.6$, $c = 101.3$
E.s.d. of unit-cell parameters (Å)	0.009
Space group	*R*3
Molecules per asymmetric unit	1
Resolution range (Å)	20–1.89
Completeness (%)	95.0 (84.8)
χ^2	1.141 (0.735)
$R_{\rm merge}$	0.067 (0.264)

3. Results and discussion

The key point in the crystallization of C20 was pH in combination with Zn^{2+}. The pH of the protein solution was first adjusted to pH 3.0; for crystallization the pH was then increased by adding MES–NaOH buffer pH 6.5 in the presence of Zn^{2+}. We suppose that at neutral to basic pH tropomodulin binds cations that may change its surface properties and cause aggregation. On the other hand, at pH values below the calculated pI of 6.8 for C20 (Appel *et al.*, 1994), the surface properties may be different, helping to avoid aggregation. Crystals grow as the pH is raised from 3 towards 6.5, which is close to the pI, in the presence of Zn^{2+}. We suppose that this change of pH in solution allows C20 to bind only Zn^{2+} ions to critical sites on the protein surface, which appears to be essential for crystallization.

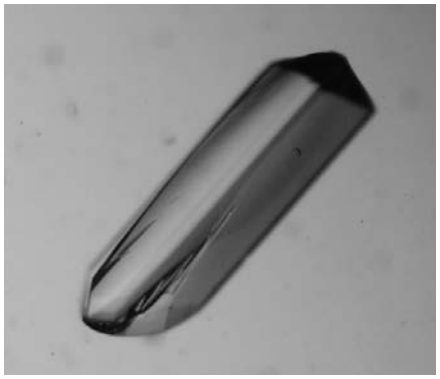

Figure 1
Crystal of the C-terminal tropomodulin fragment (C20). The crystal shown has approximate dimensions 0.25 × 0.2 × 1 mm. The crystal belongs to space group *R*3, with unit-cell parameters $a = b = 69.6$, $c = 101.3$ Å.

Crystals of C20 (Fig. 1) diffract to at least 1.9 Å on a rotating-anode X-ray source. The reflections were indexed by an *R*3 trigonal lattice with unit-cell parameters $a = b = 69.6$, $c = 101.3$ Å; the mean e.s.d. was 0.009 Å. Assuming one molecule per asymmetric unit, the Matthews coefficient (Matthews, 1968) was calculated to be 2.3 Å^3 Da^{-1}, which corresponds to a solvent content of 47.1%. A search for heavy-atom derivatives is under way.

The authors are grateful to Dr Atsuko Yamashita for advice on X-ray data acquisition and to Dr Fadel Samatey (Protonic NanoMashine Project, ERATO) for help with data analysis. This work was partly supported by the Special Coordination Funds for Promoting Science and Technology of the Science and Technology Agency of the Japanese Government.

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