Acta Cryst. (1998). D**54**, 1401–1404

Crystallization and preliminary X-ray analysis of the unliganded recombinant catalytic subunit of cAMP-dependent protein kinase

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(*Received 21 August 1997; accepted 20 April 1998*)

Abstract

X-ray diffraction-quality crystals of the unliganded mouse recombinant catalytic subunit of cAMP-dependent protein kinase were grown by the hanging-drop vapour-diffusion technique using 2-methyl-2,4-pentanediol as precipitant. The crystals belong to the monoclinic space group $P2_1$ with unit-cell parameters a = 48.9, b = 147.4, c = 54.2 Å, β = 110.2°. A data set to 3.0 Å resolution with 92% completeness has been collected using synchrotron radiation. The unit cell contains four molecules of molecular weight 40 kDa with a corresponding volume solvent content of 45%.

1. Introduction

The protein kinases are a large and diverse family of enzymes that play an important role in many signal-transduction pathways in eukaryotic cells (Hanks & Quinn, 1991). cAMP-dependent protein kinase (cAPK), one of the smallest and simplest members of the protein kinase family, serves as a template for the entire family because all share a conserved catalytic core (Knighton, Zheng *et al.*, 1991). The enzyme in its native form exists as an inactive tetramer containing two regulatory (R) and two catalytic (C) subunits. When intracellular levels of cAMP are elevated, cAMP binds with a high affinity to the R subunit (Granot *et al.*, 1980), resulting in dissociation of the complex as indicated:

$$R_2C_2 + 4\text{cAMP} = R_2 - (\text{cAMP})_4 + 2C.$$

It is the dissociated monomeric form of the C subunit ($M_r \simeq 40000$) that is active as an ATP–protein phosphotransferase. It catalyzes the transfer of the γ-phosphate from adenosine triphosphate (ATP) to serine or threonine residues on the substrate peptides or proteins preceded in the sequence by two basic residues, usually arginines (Kemp *et al.*, 1976).

The structure of the catalytic subunit of cAPK was the first to be determined in the protein kinase family and continues to serve as a biochemical and structural prototype for this class of enzymes (Knighton, Zheng *et al.*, 1991). The mouse recombinant C subunit (rC) of cAPK has a bi-lobal structure with ATP binding at the base of the cleft between the two lobes and peptide binding at the edge of the cleft (Knighton, Zheng *et al.*, 1991; Zheng, Trafny *et al.*, 1993). Subsequently, several binary and ternary complexes of both the mouse recombinant (rC) and the porcine heart C subunit (mC) have been solved, providing evidence for 'open', 'intermediate' and 'closed' conformations (Karlsson *et al.*, 1993; Zheng, Knighton *et al.*, 1993; Madhusudan *et al.*, 1994; Bossemeyer *et al.*, 1993; Narayana *et al.*, 1997). The binding of substrates induces a conformational change that leads to the closing of the active-site cleft. To further understand the conformational changes that occur upon binding of substrates or inhibitors, the mouse rC subunit was crystallized in an unliganded form (apo-rC). In this article we report the crystallization and preliminary X-ray analysis at 3.0 Å resolution of the mouse apo-rC subunit.

2. Materials and methods

The mouse rC was expressed in *Escherichia coli* and purified as described previously (Herberg *et al.*, 1993). The purified enzyme was concentrated to 10 mg ml^{-1} in 150 m*M* ammonium acetate, 10 m*M* 2-mercaptoethanol and 50 m*M* Bicine buffer, pH 8.0. The active isozyme of rC is phosphorylated at three sites: Ser10, Thr197 and Ser338 (Herberg *et al.*, 1993). Single crystals of rC were grown by the hanging-drop vapour-diffusion method using Linbro multi-well tissue plates. The mother liquor (15–20 µl) contained 0.6 m*M* protein in 100 m*M* Bicine buffer, pH 8.0, and 3% 2-methyl-2,4-pentanediol (MPD). The reservoir solution (1 ml) was made up of 15% MPD in 100 m*M* Tris–HCl buffer (pH 7.5). Plate-like or rod-shaped clusters of crystals with approximate dimensions of 0.05 × (0.2–1.0) × (0.3–1.0) mm grow at 277 K, generally in about 2–3 months (Fig. 1). These crystals diffracted to only about 4 Å resolution when exposed to a graphite monochromated Cu $K\alpha$ beam from a Rigaku RU-200 rotating-anode X-ray generator operating at 5 kW. The unit-cell dimensions were a = 49.8, b = 145.6, c = 63.5 Å, β = 105.0° and the crystals belonged to the monoclinic system with space group $P2_1$. The crystals exhibited a high level of decay on exposure to X-rays at 277 K and lost their power of diffraction after a few hours of X-ray data collection using the multi-wire area detector (Hamlin, 1985); it was not possible to collect a complete set of data from a crystal belonging to such a low-symmetry space group as $P2_1$. Also, collecting a complete data set using multiple crystals was difficult because crystals suitable for X-ray work were seldom obtained. Attempts to improve the quality and extent of diffraction by the addition of detergents such as octyl-β-D-glucopyranoside (β-OG), octanoyl-*N*-methylglucamide (MEGA-8) and decanoyl-*N*-methylglucamide (MEGA-10) were not successful.

To reduce X-ray radiation damage, common cryo-protectants such as MPD, polyethylene glycol (PEG 400), glycerol and additives such as dioxane, dimethyl sulfoxide (DMSO) and ethylene glycol, were used to flash-freeze the crystals (Hope, 1990). Among the cryo-protectants used, MPD resulted in

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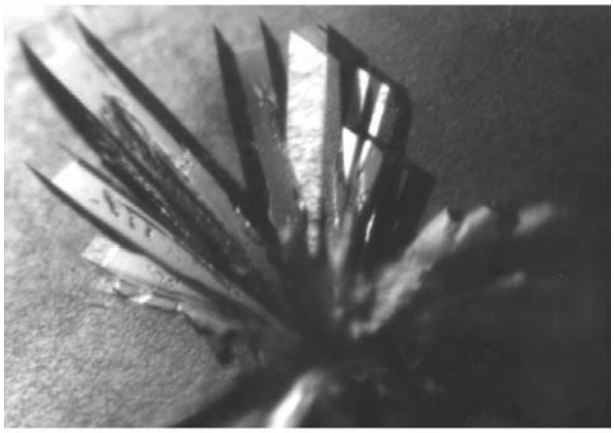
least damage to the crystals. However, direct transfer of crystals from the mother liquor to a solution containing 0.6 m*M* protein and 25% MPD in 100 m*M* Bicine buffer, pH 8.0, caused mechanical shock in the crystals resulting in a large mosaic spread and overlapping reflections. To overcome this problem, a good-quality crystal grown using the above-mentioned protocol was scooped out of the mother liquor using a rayon loop mounted on a stainless-steel tube and plunged into a 20 µl drop containing 0.6 m*M* protein and 10% MPD in 100 m*M* Bicine buffer at pH 8.0. This crystal was then successively transferred to other 20 µl drops containing 0.6 m*M* protein and 15, 20 and 25% MPD in 100 m*M* Bicine buffer at pH 8.0. The crystals tended to either dissolve or lose crystallinity if they were left in the cryo-protectant drops for more than a minute. Therefore, the entire process of transferring the crystal from the mother liquor to the final step in the above-mentioned protocol was completed in about 3 min. The cryo-protected crystal was then placed in a cold stream at 170 K. The flash-frozen crystal, when irradiated with X-rays, gave a good diffraction pattern on a MAR Research image plate. The unit-cell dimensions were the same as those obtained at 277 K. However, the crystals diffracted to only about 4 Å resolution. After the determination of a suitable cryo-condition the stage was set to use a synchrotron source to extend the resolution of diffraction.

Table 1. *Crystal and data-collection parameters*

Crystal size (mm)	0.05 × 0.2 × 0.5
Temperature (K)	100
Oscillation range (°)	1
Exposure time (s frame^{-1})	30
Unit-cell parameters	a = 48.9, b = 147.4, c = 54.2 Å; β = 110.2°
Space group	$P2_1$
Packing density (Å^3 Da^{-1})	V_m = 2.2; two molecules per asymmetric unit
Volume solvent content (%)	45
Resolution (Å)	3.0
Total observations	103346
Unique reflections	13296
Redundancy for all data	8
R_{sym} for all data†	0.14
Completeness of data (%)	92.0
I/σ for all reflections	6.0

† The R factor is defined as $\sum |I_i - \langle I \rangle| / \sum \langle I \rangle$, where I_i is the intensity of the ith observation, $\langle I \rangle$ is the mean intensity value of the reflection and the summations are over all observed reflections.

(*a*)



(*b*)

Fig. 1. Crystals of apo-rC. (*a*) Plate-like crystals in most drops had layered structures where thin crystals stack one on top of another. Crystal dimensions are about 0.05 × 0.4 × 1.0 mm. (*b*) Droplet containing good-quality rod-shaped crystals suitable for X-ray diffraction. Crystal dimensions are about 0.1 × (0.2–1.0) × (0.5–1.0) mm.

The unliganded rC-subunit crystal was flash-frozen at 100 K using the protocol described above, and exposed to X-rays on beamline 7-1 at the Stanford synchrotron radiation laboratory (SSRL). The X-ray intensity data were collected on a single crystal with an exposure time of 30 s per 1° oscillation. A total of 180 frames were collected using large (30 cm radius) and small (18 cm radius) MAR imaging plates positioned at a distance of 250 cm from the crystal. Owing to lack of allocated time, the last 80 frames were collected using the small plate. X-ray intensity data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski, 1993).

3. Conclusions

The crystal diffracted to 3 Å resolution (Fig. 2) and belongs to space group $P2_1$ with unit-cell dimensions a = 48.9, b = 147.4, c = 54.2 Å and β = 110.2°. Based on the assumption of two molecules per asymmetric unit, V_m was calculated to be 2.2 Å^3 Da^{-1} and the solvent content was 45%, which is well within the range expected for protein crystals (Matthews, 1968). Data reduction yielded a 92% complete data set up to 3 Å resolution with an R_{sym} value of 0.11. R_{sym} for the last three resolution shells, 3.38–3.23, 3.23–3.11 and 3.11–3.00 Å, is 0.40, 0.51 and 0.67, respectively. Some details of crystal and data-collection parameters are given in Table 1.

The present unit-cell dimensions are different from those seen at 277 K and for crystals flash-frozen at 170 K in our laboratory (a = 49.8, b = 145.6, c = 63.5 Å, β = 105.0°). Surprisingly, this was the first observation of a significant change in cell dimensions for the apo-rC enzyme still maintaining the space group $P2_1$. This may be due to differences in the cooling temperatures, which were 100 K at SSRL and 170 and 277 K at our laboratory, implying loose crystal packing, particularly along the unit-cell c axis which has shrunk by about 15%. The present unit-cell dimensions correspond to a

solvent content 10% lower than the usually observed value of 55%. To our knowledge this is the largest shrinkage of a cell length upon flash-freezing a crystal. Alternatively, the change in unit-cell dimensions may signify that this particular crystal indeed belongs to a new crystal form preserving the space group and is not a consequence of the difference in cooling temperatures of the crystals. Although the reason for the observed variation in the unit-cell dimensions is unknown, it appears to reflect the flexible nature of the enzyme structure as well as weak intermolecular interactions in the crystals.

The apo-enzyme of the catalytic subunit from porcine heart was crystallized previously in two crystal systems: monoclinic and cubic (Sowadski *et al.*, 1984; Knighton, Xuong *et al.*, 1991; Zheng, Knighton *et al.*, 1993). The monoclinic crystals belong to space group $P2_1$ with unit-cell dimensions (a = 64.24, b = 143.58, c = 48.4 Å, β = 106.9°) close to the values seen in the present apo-rC enzyme, but where the unit-cell a and c axes are swapped. The cubic crystals belong to space group $P4_132$. These two crystal forms of the enzyme purified from porcine heart diffracted poorly, with useful data not exceeding 4 Å resolution (Zheng *et al.*, 1991). A similar trend in diffraction capability was observed for the present apo-rC crystals with a laboratory X-ray source. The apo-rC enzyme was grown using novel crystallization conditions, and unlike the mammalian enzyme (2–3 days) it takes a longer period of time to crystallize using the above-mentioned protocol (2–3 months) and has poor reproducibility. It may be noted that the apo C-subunit crystals from porcine heart were grown previously (Sowadski *et al.*, 1984) using PEG 6000 as a precipitant and not MPD as in the present apo-rC crystals.

Structure solution was initiated using the molecular-replacement technique. Several search models were constructed from different conformational states of the enzyme whose structures have been solved previously: the open mammalian C-subunit purified from porcine heart complexed with the peptide inhibitor, PKI(5–24) (Karlsson *et al.*, 1993; Zheng, Knighton *et al.*, 1993), the intermediate rC complexed with balanol (Narayana *et al.*, private communication) and the closed rC complexed with ATP and PKI(5–24) (Zheng, Trafny *et al.*, 1993). Rotation and translation searches were performed using the *X-PLOR* program (Brünger, 1990, 1993). Further analysis is in progress.

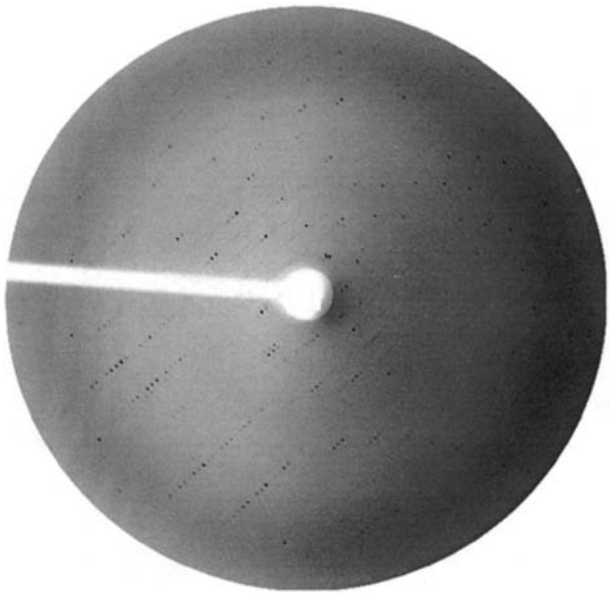


Fig. 2. X-ray diffraction pattern. The 1° oscillation image was obtained at SSRL using an MAR Research image plate of radius 18 cm positioned at a distance of 250 cm from the crystal. The X-ray wavelength used was 1.08 Å and the crystal diffracted to 3.0 Å resolution.

A complete data set to at least 3.0 Å or better for the unliganded enzyme, either for the enzyme from porcine heart or the recombinant protein, was elusive for a number of years owing to a variety of difficulties, including crystal production, reproducibility, crystal decay, high mosaicity, poor diffraction and difficulties in flash-freezing the crystals. The present complete data set to 3 Å resolution will reveal the overall conformation of the enzyme in an unliganded form for the first time at medium resolution. The structural comparison between the apo-rC enzyme and many rC complexes whose structures have been determined previously will yield insights into the conformational changes that occur upon binding of ligands in the active-site cleft. The structural comparison can also be extended to related protein kinases in order to enhance our understanding of the conformational changes brought about by the binding of substrates or inhibitors. The apo-rC enzyme structure may be used as another model in ligand-docking experiments using computers.

We thank Dr Victor Ashford, Chris Nielsen, Don Sullivan and Nick Nguyen for support at the NIH National Research Resource at UCSD multiwire-area-detector facility during many of our initial trials. We thank Nick Nguyen for assistance while using the MAR image plate with cryosystem at UCSD. NN thanks Raja Elhayek for technical support in growing crystals. We thank Dr Xiaoping Dai for his timely help in successful processing of the data. We thank Dr Peter Kuhn for help at the Stanford Synchrotron Radiation Laboratory, which is supported by the DOE and NIH Biomedical Resource Technology Program. This work was supported by the Lucille P. Markey Charitable Trust, by NIH grants GM19301 (SST), RR01644 and RR10748 (NX).

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