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Crystallization and preliminary X-ray analysis of UMP/CMP-kinase from *Dictyostelium discoideum* with the specific bisubstrate inhibitor P^1-(adenosine 5′)-P^5-(uridine 5′)-pentaphosphate (UP_5A)

Lisa Wiesmüller[a,d], Klaus Scheffzek[a], Werner Kliche[a], Roger S. Goody[c], Alfred Wittinghofer[b], Jochen Reinstein[a,c,*]

[a]*Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, 69120 Heidelberg, Germany*
[b]*Max-Planck-Institut für molekulare Physiologie, Abteilung strukturelle Biologie, Rheinlanddamm 201, 44139 Dortmund, Germany*
[c]*Max-Planck-Institut für molekulare Physiology, Abteilung physikalische Biochemie, Rheinlanddamm 201, 44139 Dortmund, Germany*
[d]*Max-Planck-Institut für Biochemie, Abteilung Zellbiologie, am Klopfspitz, 82152 Martinsried, Germany*

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Abstract UMP/CMP-kinase (UK) from the slime mold *Dictyostelium discoideum* has been purified to high homogeneity and co-crystallized with the bisubstrate inhibitor P^1-(adenosine 5′)-P^5-(uridine 5′)-pentaphosphate (UP_5A). UP_5A binds to UK with a dissociation constant (K_d) of 3 ± 0.5 nM at 25°C and pH 7.5. This is some 50-fold tighter than the binding of P^1,P^5-(diadenosine 5′)-pentaphosphate (AP_5A, K_d = 160 ± 15 nM). AP_5A is a bisubstrate inhibitor that is specific for adenylate kinase. The crystals have the symmetry of the tetragonal space group $P4_12_12$ or its enantiomorph $P4_32_12$. The unit cell dimensions are $a = b = 78.5$ Å and $c = 101.4$ Å. The crystals diffract to a Bragg spacing of 2.1 Å.

Key words: UMP/CMP-kinase; Substrate specificity; Phosphoryl transfer; Bisubstrate inhibitor; Crystallization

1. Introduction

Nucleoside monophosphate kinases (ATP:NMP phosphotransferases) catalyze the reversible transphosphorylation between nucleoside triphosphates and nucleoside monophosphates. The best studied enzyme of this class is adenylate kinase (EC 2.7.4.3; AK) which catalyzes the phosphoryl transfer from the donor ATP to the acceptor molecule AMP. Whereas AK is highly specific for AMP as acceptor molecule, UMP/CMP-kinase from the slime mold *Dictyostelium discoideum* (EC 2.7.4.14; UK_{dicty}) appears to accept both UMP and CMP as acceptors [1] and thus belongs to the group of pyrimidine monophosphate kinases.

Pyrimidine kinases occupy a strategic position in the biosynthesis of pyrimidine nucleotides, since their phosphate acceptor substrates are products of both the de novo and the salvage pathways. We are interested in the specificity of UK_{dicty} in relation to other monophosphate kinases such as adenylate kinase or guanylate kinase and therefore need to investigate the architecture of the active site, in particular the monophosphate binding site. The structure of UMP-kinase from yeast was solved recently [2]. However, comparative complementation analysis of enzymatic activity indicated that UK_{yeast} has different properties than UK_{dicty} since it accepts AMP almost as well as UMP or CMP as the phosphate acceptor. It therefore has to be considered to be not specific for pyrimidine nucleotides [3]. Secondly, the crystallized protein contained two adenosine nucleotide molecules and not just one ATP or ADP at the donor site, which would be expected for a kinase that is highly specific for pyrimidines at the acceptor site. It thus appears that the enzyme referred to as UMP-kinase from yeast should be called an unspecific NMP-kinase.

We report here the crystallization of UK_{dicty} with the bisubstrate inhibitor UP_5A at its active site. We show by fluorescence measurements that UK_{dicty} binds UP_5A much tighter than AP_5A, the bisubstrate inhibitor that is specific for adenylate kinases [4,5]. Determination of the crystal structure of this complex should enable us to answer the question of how UMP/CMP kinase achieves its specificity for pyrimidine nucleotides.

2. Materials and methods

2.1. Preparation of the bisubstrate inhibitor UP_5A

The bisubstrate inhibitor UP_5A was prepared from AP_4 and UMP according to the method of Feldhaus et al. [5].

2.2. Protein expression and purification

UMP/CMP kinase from *Dictyostelium discoideum* was expressed in *E. coli* as described [1]. 50 g cells were lysed with 300 mg lysozyme (Serva) in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 mM DTE and 0.04% (v/v) sodium azide. Subsequently, cell debris was removed as described [6]. The crude extract was applied to a blue Sepharose pseudo affinity column of 7 × 18 cm (bed volume of 700 ml). The column was equilibrated with the same buffer as described above. The flow rate was 200–450 ml/h. The column was washed with 2 vol. of the buffer. Most kinases from *E. coli*, such as adenylate kinase, were removed with a washing step of 1.3 l buffer that contained 0.25 M NaCl.

UMP/CMP kinase was eluted with 1 M NaCl (1 l) at a flow rate of 100 ml/h. The eluate (1 g protein total) was dialyzed three times against 10 l of 25 mM Tris-acetate, pH 8.3, 1 mM EDTA, 20 mM DTE and 0.04% (v/v) sodium azide (chromatofocusing buffer). The sample at a concentration of at most 10 mg/ml was applied at 30 ml/min to a chromatofocusing column of 1.7 × 60 cm (bed volume 140 ml) filled

*Corresponding author. Max-Planck-Institut für molekulare Physiologie, Abteilung physikalische Biochemie, Rheinlanddamm 201, 44139 Dortmund, Germany. Fax: (49) (231) 120 6229.
E-mail: joachim.reinstein@mpi-dortmund.mpg.de

Abbreviations: UK, UMP/CMP kinase in general (EC 2.7.4.14); UK_{dicty}, UMP/CMP kinase from *Dictyostelium discoideum*. UK_{yeast}, UMP/CMP kinase from *Saccharomyces cerevisiae*. AK, adenylate kinase (EC 2.7.4.3); AP_5A, P1,P5-(diadenosine-5′)-pentaphosphate; UP_5A, P1-(adenosine 5′)-P5-(uridine 5′)-penta phosphate; mAP_5Am, α,ω-[di(-(3′ or 2′)-*O*-(*N*-methyl-anthraniloyl)adenosine-5′-*O*-)]pentaphosphate; EDTA, ehtylenediamine-tetraacetate; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol.

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with polybuffer exchanger 94 (Pharmacia). The column was equilibrated with 10 vol. of chromatofocusing buffer prior to the application of the sample. After washing the column with 600 ml of chromatofocusing buffer, UK was eluted at pH 6.5 with a gradient from pH 8.3 to 5.0 using 1.4 l of polybuffer. Adenylate kinase from *E. coli* elutes at pH 5.2 and therefore this step is efficient in removing residual amounts of this enzyme. After dialysis against concentrated ammonium sulfate solution the precipitate was redissolved in 6 ml s-buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 20 mM DTE and 0.04% sodium azide). Aliquots of 2 ml of this concentrated protein solution were applied to a Superdex 75 size exclusion column (Pharmacia) at 1 ml/min with s-buffer (see above). The protein concentration was determined according to Bradford [7] with bovine serum albumin as the standard protein.

2.3. Protein crystallization

A 1 : 1 complex of UK_{dicty} and UP_5A was prepared by mixing UK_{dicty} and UP_5A at a 1 : 1 ratio. The protein was concentrated to 20 mg/ml in a centricon 10 centrifugation chamber (Amicon). Crystals of the UK : UP_5A complex were grown at room temperature with the hanging drop method [8]. Equal volumes of concentrated protein–nucleotide solution and a solution of 28% (w/v) polyethylene glycol 3350 (Sigma), 100 mM Tris-HCl, pH 8.8, 200 mM $MgCl_2$, 0.04% sodium azide, 20 mM DTE were mixed and equilibrated against a reservoir of the same composition but without protein or nucleotide. DTE is not essential for crystallization.

3. Results and discussion

3.1. Binding measurements

To learn how specific UK_{dicty} is for UP_5A, we measured the dissociation constant of UP_5A and compared it to that of AP_5A which is the bisubstrate inhibitor specific for adenylate kinases [4,5]. Binding of the fluorescent bisubstrate inhibitor mAP_5Am [9] to UK_{dicty} resulted in an increase in fluorescence intensity of 200% at 360 nm excitation wavelength and 440 nm emission wavelength. The dissociation constant for mAP_5Am was determined as described [9] and is 154 nM. The dissociation constants of AP_5Am and UP_5A were determined in a competition experiment with mAP_5Am [9] to be 160 ± 15 nM for AP_5A and 3 ± 0.5 nM for UP_5A. All equilibrium experiments were performed at 25°C, 50 mM Tris-HCl, pH 7.5, 1.6 mM $MgCl_2$, 100 mM KCl and 20 mM DTE. It has been noted that the catalytic activity of UMP-kinases is highly dependent on the presence of a reducing agent such as DTE [10] and we also found the presence of 20 mM DTE to be necessary to gain full catalytic activity. In conclusion, UK_{dicty} is specific for UP_5A.

3.2. Protein crystallization

Crystals grew within 3–5 days to a typical size of $500 \times 600 \times 600\,\mu m^3$. For biochemical analysis the crystals were washed several times with artificial motherliquor which did not contain protein or nucleotide. Then the crystals were dissolved and subjected to SDS-PAGE [11]. The crystals contained UK with the expected apparent mass of 25 kDa, showing that degradation had not occurred. Crystals treated the same way as described above but analyzed with a C_{18} reverse-phase column were found to contain the nucleotide UP_5A.

3.3. Collection of X-ray data

Crystals were mounted in thin-walled glass capillaries. Two native datasets were collected using an Elliot GX13 rotating anode (CuK_α) as the source of X-rays, a modified rotation camera and a Siemens/Nicolet area detector. The crystals diffract to 2.1 Å resolution and are very stable in the X-ray beam. The determination of the space group symmetry and of the unit cell parameters was performed with an extended version of the computer program XDS [12]. The crystals belong to the tetragonal space group $P4_12_12$ or the enantiomorphous space group $P4_32_12$ with unit cell dimensions of $a = b = 78.5$ Å and $c = 100.4$ Å.

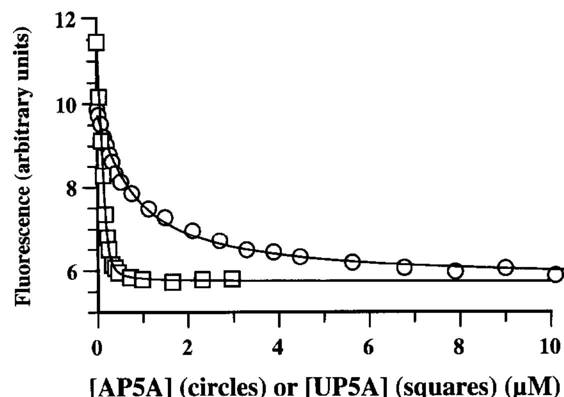

Fig. 1. The displacement of the fluorescently labeled bisubstrate inhibitor α,ω-[di(-(3′ or 2′)-*O*-(*N*-methyl-anthraniloyl)adenosine-5′-*O*-)]pentaphosphate (mAP_5Am) by the bisubstrate inhibitors UP_5A (□) or AP_5A (○). The dissociation constant of UP_5A was calculated as 3 ± 0.5 nM and that of AP_5A as 160 ± 15 nM (see text). 600 µl of titration buffer contained 0.4 µM of the fluorescent bisubstrate inhibitor analog mAP_5Am, 0.4 µM UK, 1.6 mM $MgCl_2$, 50 mM Tris, pH 7.5, 100 mM KCl, 10 mM DTE at 25°C. Signal was the decrease in the intensity of the emission of mAP_5Am at λ = 440 nm. The excitation wavelength was 360 nm.

Although density measurements of UK : UP_5A crystals in a CCl_4/Xylol density gradient suggest two molecules per asymmetric unit (the density is 1.27 g/cm³), corresponding to a V_M of 1.8 Å³/Da (28% solvent content) non-crystallographic symmetry could not be detected unequivocally by self rotation studies. Structure determination will have to reveal the actual number of UK : UP_5A molecules per asymmetric unit.

Initial attempts to solve the structure by molecular replacement with known structures of adenylate kinase from *E. coli* or pig muscle (AK1) failed. We are currently collecting datasets with heavy atom derivatives.

In conclusion, determination of the structure of the UK_{dicty} : UP_5A complex should reveal how this type of NMP kinase gains specificity for the pyrimidine phosphate acceptor. Specifically, the comparison with the structure of the UK : AP_5A complex is very promising, since it crystallizes in the same space group and with virtually identical cell dimensions, so that any differences in the mode of binding of AP_5A should be attributable to the specificity of the nucleotide binding pocket.

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