Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, purification and crystallization of human 3′-phosphoadenosine-5′-phosphosulfate synthetase 1

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Received 1 September 2003
Accepted 2 December 2003

3′-Phosphoadenosine-5′-phosphosulfate (PAPS) is used to incorporate sulfate into biomolecules. The human PAPS synthetase 1 catalyzes two steps leading from adenosine triphosphate (ATP) and sulfate to PAPS. The ATP sulfurylase domain catalyzes the formation of the intermediate adenosine-5′-phosphosulfate (APS). The APS kinase domain then adds a phosphate group to the 3′-ribose and releases PAPS. In this article, the recombinant expression, purification and crystallization of the full-length protein is described. In *Escherichia coli* the protein is only partly soluble and copurifies with GroEL. The pure protein migrates as a dimer in gel-filtration chromatography. It is moderately active, forming 25 nmol PAPS per minute per milligram. Crystals grow to 100 × 100 × 300 µm and diffract to 1.75 Å.

1. Introduction

Sulfation is a common modification of biomolecules. The extracellular matrix of skin, cartilage and hair contains sulfated sugars. Extracellular proteins have been found to be tyrosine-sulfated. In addition to these modifications, which take place in the Golgi apparatus, sulfation of steroids is important for detoxification and transport. Sulfated hormones, bile salts and neurotransmitters have been reported (Negishi *et al.*, 2001; Weinshilboum *et al.*, 1997).

To attach sulfate to biomolecules, organisms synthesize the high-energy sulfate ester 3′-phosphoadenosine-5′-phosphosulfate (PAPS). In humans, this reaction is catalyzed by the bifunctional PAPS synthetases 1 and 2. In the first step, ATP sulfurylase replaces the β-phosphate of ATP by sulfate and releases pyrophosphate. In the second step, the newly formed adenosine-phosphosulfate (APS) is phosphorylated by APS kinase at the 3′-position of the ribose, yielding the final product PAPS. To date, bacterial, fungal and yeast ATP sulfurylases and a fungal APS kinase have been characterized by crystallography (MacRae *et al.*, 2000, 2001; Beynon *et al.*, 2001; Ullrich *et al.*, 2001). In contrast to the human bifunctional protein, these enzymes exist as separate polypeptides. The fungal ATP sulfurylase contains a C-terminal domain which resembles the APS kinase. This catalytically inactive domain has been shown to inhibit ATP sulfurylase allosterically (MacRae *et al.*, 2002). In order to analyze the structural and functional consequences of the bifunctional domain linkage, we decided to determine the crystal structure of human PAPS synthetase 1 (hPAPSS1).

2. Methods

2.1. Cloning and expression

The cDNA of hPAPSS1 cloned into the vector pGEX-6p3 (Amersham) was a kind gift from H. Fuda and C. A. Strott (Fuda *et al.*, 2002). Six histidines were attached to the C-terminus *via* PCR and the full-length construct (AS 1–624-His_6) was inserted into a *Nco*I/*Sac*I-prepared modified pET27b vector (Klostermeier *et al.*., 1998). *Escherichia coli* BL21 DE3 Codon plus cells (Stratagene) were transfected and grown in a 30 l fermenter (B. Braun Biotech). Expression was induced at OD_{600} = 1.0 with 1 m*M* IPTG, the temperature was lowered to 293 K and after 16 h the cells were washed [25 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 6.9, 140 m*M* sodium chloride, 10 m*M* trimethylamine and 10 m*M* trimethylamine oxide], harvested and frozen.

2.2. Purification

50 g bacteria were resuspended in 120 ml buffer {50 m*M* 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.2, 300 m*M* potassium chloride, 10 m*M* potassium acetate, 10 m*M* guanidinum hydrochloride, 10 m*M* trimethylamine (TMA), 10 m*M* trimethylaminoxide (TMAO), 0.01% Triton X-100, 1 m*M* 2-mercaptoethanolate, 20 m*M* imidazole and 0.5 m*M* phenylmethanesulfonyl fluoride (PMSF)} and lysed using a microfluidizer (Microfluidics Corp.). The lysate was cleared by ultracentrifugation (Sorvall AH-629, 29 000 rev min^{-1}) and subjected to metal-affinity chromatography (Ni–NTA, Qiagen or Novagen, Waters HPLC system). A HR 10/10 column (Amersham) was chosen so

that it would be slightly overloaded. The lysate was applied at a flow rate of 1 ml min^{-1} and the column was washed with the above affinity buffer (without TMA, TMAO and PMSF). After the optical density returned to the base level, 1 ml of 10 m*M* ATP with 10 m*M* magnesium sulfate in the above buffer was injected. The protein was washed and eluted with a step gradient (100 min of 40, 70 and 250 m*M* imidazole in the above buffer). The protein fraction was dialyzed six times against 500 ml of 25 m*M* 2-amino-2-hydroxymethylpropane-1,3-diol adjusted to pH 7.8 with hydrochloric acid (Tris–HCl), 2 m*M* 2-mercaptoethanolate and 200 µ*M* ethylenediaminotetraacetic acid (EDTA, complexon-II). Using centrifugal filter devices with 30 kDa molecular-weight cutoff (Millipore or Vivascience), hPAPSS1 was concentrated to 30 mg ml^{-1} and frozen in small aliquots at 193 K.

Gel filtration was performed on Superdex 200 columns (26/60 at 2.5 ml min^{-1} and 10/30 at 0.5 ml min^{-1}) in 25 m*M* Tris–HCl pH 7.8, 2 m*M* 2-mercaptoethanolate and 200 µ*M* EDTA. The columns were calibrated with high- and low-molecular-weight standards (Amersham).

2.3. Enzymatic activity

The activity of the enzyme was determined with a coupled enzymatic assay (Seubert *et al.*, 1983). Here, the amount of adenosine diphosphate (ADP) generated during the formation of PAPS is measured. Briefly, pyruvate kinase (PK) catalyzes the regeneration of ATP using phosphoenolpyruvate; lactate dehydrogenase (LDH) converts pyruvate to lactate, oxidizing β-nicotinamide adenine dinucleotide (NADH), which displays a strong absorption at 340 nm; the consumption of NADH followed photometrically is proportional to the production of ADP. The following conditions were used: 6 m*M* ATP, 75 m*M* ammonium sulfate, 100 m*M* Tris–HCl pH 7.6, 5 m*M* magnesium chloride, 0.1% Triton X-100, 4 m*M* 1,4-dimercaptobutane-2,3-diol (DTT), 400 µ*M* phosphoenolpyruvate, 342 µ*M* NADH, 9.5 U LDH, 5.4 U PK and 2.5 U pyrophosphatase.

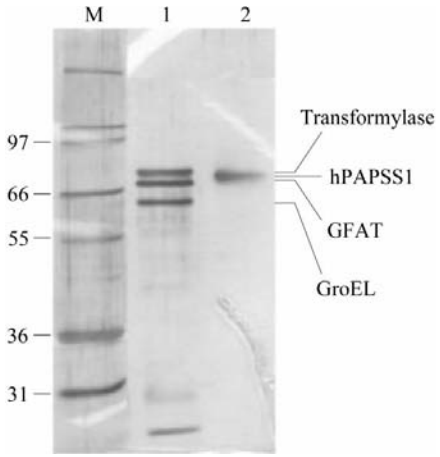

Figure 1
Silver-stained SDS–PAGE of protein fractions eluted from a Ni–NTA column during purification of hPAPSS1. Lane M, molecular-weight markers (kDa); lane 1, low-affinity (70 m*M* imidazole); lane 2, high-affinity (250 m*M* imidazole).

2.4. Protein analysis

Protein concentration was determined by standard assay (Bradford, 1976), which was reproduced gravimetrically. 10% polyacrylamide gels containing 0.1% SDS were used for protein analysis. Gels were either silver- or Coomassie-stained (Sambrook & Russell, 2001).

Bands to be identified were cut from the gel, washed with 100 m*M* ammonium bicarbonate and processed as described in Shevchenko *et al.* (1996). After overnight digestion with trypsin, soluble fragments were diluted 1:10 with matrix [saturated 2-cyano-3-(4-hydroxyphenyl)acrylic acid (α-cyano-4-hydroxycinnamic acid) in 0.1% trifluoracetic acid, 50% acetonitrile] and dried on a steel plate. MALDI–TOF spectra were recorded using a Voyager DE (Perseptive Biosystems) in reflector mode. Spectra were calibrated using matrix peaks.

For mass-spectrometric determination of molecular weight, purified protein was diluted 1:10 in matrix [saturated 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylic acid (sinapinic acid) in 0.1% trifluoracetic acid, 50% acetonitrile]. The mixture was dried on a steel plate and linear spectra were recorded.

For dynamic light scattering, the protein and molecular-weight standard (Amersham) solutions at 2 mg ml^{-1} were centrifuged (358 000*g*, 25 min, 293 K). The clarified solution was inserted into a 12 µl cuvette which was then centrifuged to remove air. Measurements were performed with a DynaPro device (Protein Solutions).

2.5. Crystallization

Hanging-drop vapour-diffusion crystallization was performed in 24-well plates (Linbro, ICN) using glass cover slides of appropriate size (treated with dichlorodimethylsilane). The thawed protein solution was diluted to 10 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.8 and 2 m*M* tri(2-carboxyethyl)phosphine hydrochloride (TCEP). 2.5 µl of protein solution was placed on a glass cover slide and the same amount of crystallization solution was added. The glass plate was placed on top of a well containing 0.5 ml crystallization solution and sealed with vacuum grease (medium viscosity, Bayer).

2.6. Data collection

In-house data were collected using an Enraf–Nonius rotating-anode generator FR591 equipped with MaxFlux mirror optics (Osmic Inc.). Diffraction images from a frozen crystal mounted in a Cryostream (Oxford Cryosystems) were collected using a MAR 345 image plate. CCD detectors were used at the ESRF (ADSC Q4R) and a MAR CCD detector was used at the Swiss Light Source (SLS).

3. Results and discussion

Metal-affinity chromatography of histidine-tagged recombinant proteins is a quick purification procedure. Under conditions of high stringency, most of the copurifiing host proteins elute at 70 m*M* imidazole and can be separated. In the present case, four proteins were found in the range 50–80 kDa (Fig. 1). The bands were cut out of a polyacrylamide gel, digested with trypsin and subjected to MALDI–TOF mass spectrometry (Shevchenko *et al.*, 1996). The resulting fragments were compared with a protein database (Clauser *et al.*, 1999). The protein eluting at 250 m*M* imidazole is of course hPAPSS1 (Fig. 1). Two of the other three proteins eluting at 70 m*M* imidazole are always present in preparations using this *E. coli* strain. They were identified as transformylase (74 kDa) and glucosamine-fructose-6-phosphate-aminotransferase (67 kDa). The third protein at 54 kDa molecular weight could be identified as the chaperone component GroEl (Fig. 1). This protein copurifies when proteins with folding problems are expressed (Landry, 1994). To stabilize hPAPSS1 and to utilize the refolding activity of GroEL, the column-bound protein was washed with ATP before elution.

In total, only one third of the expressed hPAPSS1 could be recovered. The purified protein showed a high degree of aggregation. Addition of aggregation inhibitors to the affinity buffer and pressure protectants (Yancey *et al.*, 2002) to the wash and lysis buffer improved purification. Under these conditions, the protein eluted as a single peak on a gel-filtration column. From the retention time on two different columns, a molecular weight of 162 $\pm$ 4 kDa could be estimated. A monomer molecular weight of 71.6 kDa was determined by MALDI–TOF.

Table 1
Data collection of human PAPS synthetase 1 crystals in the $P2_1$ monoclinic unit cell: a = 782, b = 825, c = 133 Å, $\alpha = \gamma = 90$, $\beta = 105^\circ$.

Processing was performed with the program *XDS* (Kabsch, 1993). Values in parentheses refer to the highest resolution shell.

Crystal	Nat2	Nat3	Ectoine
Beamline	SLS X06SA-PX	ESRF ID14-4	ESRF ID14-1
Detector	MAR CCD	Q4R ADSC-CCD	Q4R ADSC-CCD
Wavelength (Å)	0.980	0.920	0.934
Resolution (Å)	30–2.0 (2.1–2.0)	30–2.0 (2.1–2.0)	30–1.75 (1.85–1.75)
Completeness (%)	82 (71)	97 (93)	96 (88)
$\langle I/\sigma(I)\rangle$	4.9 (2.0)	13 (2.9)	11 (2.1)
No. unique observations	88947 (10357)	108980 (14100)	159267 (22268)
Redundancy	3.5 (3.5)	6.4 (6.3)	3.6 (3.2)
R_{sym} (%)	17 (64)	6.9 (58)	6.9 (58)

The recombinant APS kinase domain of hPAPSS1 (AS 1–236-His_6) also migrates at the size of a dimer. These observations have been reproduced using dynamic light scattering. As the closely related APS kinase of *Penicillum chrysogenum* is also a dimer (MacRae *et al.*, 2000), there is evidence that hPAPSS1 itself might be a dimer in solution. It is likely that the APS kinase domain of hPAPSS1 contains a putative dimer interface.

The purified human PAPS synthetase 1 is moderately active, producing 25 nmol PAPS per minute per milligram of protein at 303 K. This activity is similar to a previous report (Fuda *et al.*, 2002), but considerably slower than the monofunctional fungal enzymes ATP sulfurylase or APS kinase (Seubert *et al.*, 1983; Renosto *et al.*, 1984). Initial crystallization trials at 293 K using the Hampton Research Crystal Screen Lite revealed four different conditions for crystal formation. The pH, the salt concentration and the polyethylene glycol (PEG) concentration of the most regular crystals were fine-tuned. The optimal crystallization solution consists of 91 m*M* sodium citrate pH 5.6, 270 m*M* ammonium acetate and 12% PEG 4000. At 285 K, crystals appeared after 1 d and continued to grow for several days. The crystals were transferred to cryosolution (the above crystallization solution containing 30% glycerol) in five steps and frozen in liquid nitrogen. A data set to 2.0 Å resolution was collected at Swiss Light Source (SLS) beamline X06SA-PX (Table 1).

The crystal quality was further increased. To remove precipitate and dust particles, protein and crystallization solutions were mixed and cleared by centrifugation (17 500*g*, 15 min, 277 K). This led to an increase in crystal size and in turn to a diffraction data set with a higher completeness (Table 1). When the crystallization solution additionally contained 100 m*M* 2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid (Ectoine), crystals of a more regular shape and up to five times larger were obtained. The X-ray diffraction resolution increased to 1.75 Å (Table 1). The monoclinic crystals grew to dimensions of around 100 × 100 × 300 µm (Fig. 2*a*). Based on the Matthews parameter (Matthews, 1968; Collaborative Computational Project, Number 4, 1994), we estimated there to be one dimer in the asymmetric unit (Matthews coefficient 2.9 $Å^3$ Da^{-1}; 57.3% solvent). We are currently solving the structure of human PAPS synthetase 1 using a combination of isomorphous and molecular replacement.

Six weeks after having grown the crystals shown in Fig. 2(*b*), they were washed and subjected to SDS–gel electrophoresis. The migration of the protein of the dissolved crystal is the same as a freshly purified fraction. However, the non-crystallizing protein which remained in the mother liquor of the crystallization drop indicates degradation.

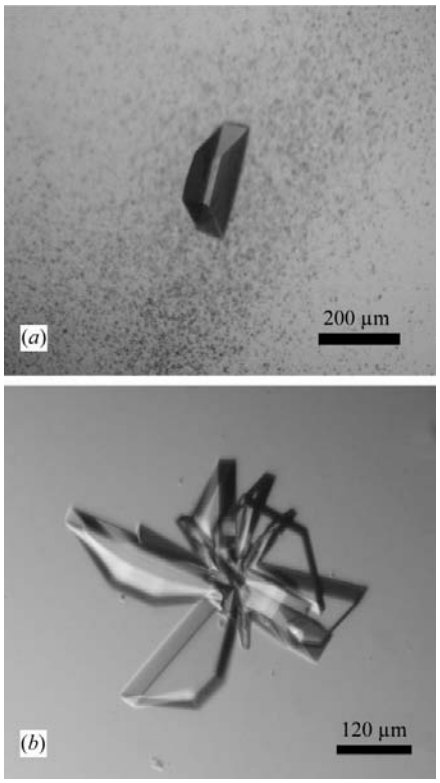

Figure 2
Crystals of human PAPS synthetase 1.

We thank P. Janning, H. Prinz and K. Reinecke for help with MS, D. Fiegen for explaining DLS and W. Blankenfeld, T. Dambe, B. Klink and O. Yildiz for help with data collection. We acknowledge the European Synchrotron Radiation Facility for provision of synchrotron-radiation facilities and we would like to thank E. Mitchell, R. Ravelli and A. McCarthy for assistance in using beamlines ID29, ID14-1 and ID14-4. Part of this work was performed at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland. We would like to thank C. Schulze-Briese and T. Tomizaki for assistance. We gratefully thank the DFG project 1624/4-1 (SH, PB) and the Fonds der chemischen Industrie Deutschlands eV (PB) for financial support.

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