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Expression, purification, crystallization and preliminary X-ray analysis of *Escherichia coli* 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase

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A recombinant form of *Escherichia coli* 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (E.C. 3.2.2.9) has been purified to homogeneity and crystallized using the hanging-drop vapour-diffusion technique. While several different crystallization conditions were obtained, only one set of conditions yielded crystals suitable for X-ray diffraction analysis. These crystals grow as diamond-shaped wedges, with unit-cell parameters $a = 50.92$, $b = 133.99$, $c = 70.88$ Å, $\alpha = \beta = \gamma = 90°$. The crystals belong to space group $P2_12_12$ and diffract to a minimum d spacing of 2.3 Å on a MAR345 image plate with a Rigaku RU-200 rotating-anode X-ray generator. On the basis of density calculations, two monomers are predicted per asymmetric unit (Matthews coefficient, $V_M = 2.37$ Å^3 Da^{-1}), with a solvent content of 48%.

1. Introduction

E. coli 5′-methylthioadenosine/*S*-adenosyl-homocysteine (MTA/AdoHcy) nucleosidase (E.C. 3.2.2.9) is a 232 amino-acid enzyme (25.4 kDa) which is involved in two catabolic reactions. MTA/AdoHcy nucleosidase is involved primarily in the irreversible cleavage of the glycosidic bond of 5′-methylthioadenosine (MTA) to yield 5′-methylthioribose (MTR) and adenine (Duerre, 1962). With a reduced enzyme reactivity (35–42% of maximal cleavage; Cornell *et al.*, 1996), this nucleosidase also cleaves the glycosidic bond of *S*-adenosylhomocysteine (AdoHcy) to produce adenine and *S*-ribosylhomocysteine (Della Ragione *et al.*, 1985; Shimizu *et al.*, 1988; Zappia *et al.*, 1985).

MTA/AdoHcy nucleosidase represents an ideal target for the design of antimicrobial drugs. An exploitable metabolic difference exists in which mammalian and prokaryotic cells catabolize MTA (Riscoe *et al.*, 1989). In mammalian cells, MTA is reversibly catabolized to 5′-methylthioribose-1-phosphate (MTR-1-P) and adenine by a specific MTA phosphorylase (Pegg & Williams-Ashman, 1969). Adenine enters the purine-salvage pathway (Kamatani & Carson, 1981; Kamatani *et al.*, 1984), while MTR-1-P is recycled in a series of enyzmatic steps to methionine (Backlund & Smith, 1981, 1982). In contrast, many pathogenic microbes do not have an MTA phosphorylase. Instead, MTA is first cleaved by MTA/AdoHcy nucleosidase to adenine and MTR. MTR is then phosphory-lated to MTR-1-P by MTR kinase. By designing potential therapeutic agents to the nucleosidase, invading microbes should be selectively killed owing to accumulation of cytotoxic MTA.

Computer-assisted database searches have failed to locate any sequence homology between MTA/AdoHcy nucleosidase and any other known proteins. However, functional similarities with other enzymes have been reported. MTA/AdoHcy nucleosidase shares a similar substrate and function with AdoHcy hydrolase (Della Ragione *et al.*, 1985; Walker & Duerre, 1975) and inosine-uridine nucleoside *N*-ribohydrolase (IUNH), respectively. AdoHcy hydrolase breaks down AdoHcy to adenosine and homocysteine, while IUNH catalyzes the hydrolysis of the glycosidic bond of purine ribosides to form the purine base and ribose. The three-dimensional crystal structure of IUNH (Degano *et al.*, 1996) shows striking structural similarity to the catalytic domain of AdoHcy hydrolase (Turner *et al.*, 1998). The catalytic domain of AdoHcy hydrolase and IUNH both exhibit a core of parallel α/β structures (Degano *et al.*, 1996; Turner *et al.*, 1998). Structural superimposition of AdoHcy hydrolase with IUNH shows an r.m.s. difference for 94 Cα positions of 2.14 Å (Turner *et al.*, 1998). Although no primary sequence homology has been detected between the three enzymes, there may be structural similarity based on the common substrate and reaction catalyzed. The crystallization and preliminary X-ray analysis of recombinant *E. coli* MTA/AdoHcy nucleosidase reported here represents the first steps towards determining the enzyme's structure and catalytic mechanism.

2. Expression and purification

An *Eco*RI/*Not*I fragment from p5Xmtan (Cornell & Riscoe, 1998) containing the complete *E. coli* MTA/AdoHcy nucleosidase gene (accession No. U24438) was ligated into *Eco*RI/*Not*I-digested pPROEX HTa expression vector (Gibco BRL) and transformed into *E. coli* strain TOP10F′. The expressed enzyme contains a 31-residue N-terminal tag consisting of a six-histidine tag, a spacer sequence and an rTEV protease cleavage site prior to the native initiating methionine of the nucleosidase. A starter culture of 10 ml LB with 100 µg ml⁻¹ ampicillin was inoculated with a single transformed colony and grown overnight at 310 K in a water-bath shaker (200 rev min⁻¹). This overnight culture was added to 1 l of LB media containing 100 µg ml⁻¹ ampicillin and incubated at 310 K in a water-bath shaker
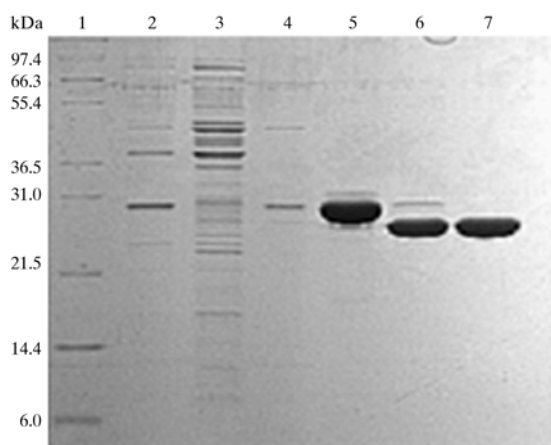
(200 rev min⁻¹) until an OD_{600} reading of 0.7. At this point, protein expression was induced by isopropyl-β-D-thiogalacto-pyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested 3 h post-induction by centrifugation (5000 rev min⁻¹, Beckman JA-10 rotor, 277 K, 10 min) and resuspended in 40 ml B-PER (Pierce) containing a protease-inhibitor cocktail tablet (Boehringer-Mannheim). The cells were lysed by gentle vortexing at room temperature (295 K) for 10 min. The cell debris was removed by centrifugation at 12 000 rev min⁻¹ for 20 min in a Beckman JA-20 rotor. The supernatant was directly applied to a 5 ml Ni-NTA (Qiagen) column pre-equilibrated in buffer A (50 mM sodium phosphate pH 7.5) with 20 mM imidazole. The column was subsequently washed with 25 ml of buffer A plus 20 mM imidazole and the protein eluted from the column in a single 15 ml fraction of buffer A with 250 mM imidazole. The protein was subsequently dialyzed against 1 l of buffer A overnight at 277 K.

Since attempts to crystallize the N-terminally His-tagged protein failed and cleaving the His-tag using rTEV resulted in the protein precipitating, limited proteolysis was used to find a smaller protein fragment that was more suitable for crystallographic study. MTA/AdoHcy nucleosidase was incubated with chymotrypsin (1:1000 molar ratio of chymotrypsin to protein) at room temperature (295 K). After 1 h, the reaction was stopped with the addition of phenylmethyl-sulfonyl fluoride (PMSF) to a final concentration of 1 mM. The resulting reaction mixture (15 ml) was reapplied to a 5 ml

Ni-NTA column pre-equilibrated in buffer A to remove the N-terminal fragment and any unproteolyzed protein. The flowthrough was collected and EDTA was immediately added to a final concentration of 1 mM to bind any leached nickel. The protein was concentrated to approximately 2 ml using an Ultrafree-15 BioMax-10K (Millipore) centrifuge concentrator prior to application to a gel-filtration column. MTA/AdoHcy nucleosidase was applied in 0.5 ml fractions to a Superdex-75HR FPLC column pre-equilibrated with 50 mM sodium HEPES pH 7.5 and isocratically eluted at a flow rate of 0.5 ml min⁻¹. Fractions were pooled according to the chromatogram and concentrated to 15 mg ml⁻¹ in an Ultrafree-0.5 BioMax-10K microconcentrator. This preparation of enzyme was then subsequently used in crystallization trials.

Protein purity was assessed using SDS–PAGE stained with Coomassie blue (Fig. 1). From 1 l of bacterial culture, approximately 30 mg of ~99% pure soluble protein was obtained. The concentrations of MTA/AdoHcy nucleosidase were measured using the Coomassie Plus (Pierce) protein-determination method (Bradford, 1976). Analysis of the proteolytic fragment using N-terminal amino-acid sequencing revealed that in addition to the six-histidine tag, 11 amino acids located in the N-terminal spacer region were cleaved (Fig. 2). A total of 21 residues were proteolyzed from the N-terminal region. The molecular weight of the protein, determined by electrospray mass spectrometry (25 464 Da), confirmed that no other residues had been excised.

Figure 1
Coomassie-stained 15% SDS–PAGE gel showing MTA/AdoHcy nucleosidase purification. Lane 1, molecular-weight markers; lane 2, soluble lysate; lane 3, Ni-NTA column flowthrough; lane 4, Ni-NTA column wash; lane 5, Ni-NTA column elution with 250 mM imidazole; lane 6, chymotrypsin-cleaved MTA/AdoHcy nucleosidase; lane 7, MTA/AdoHcy nucleosidase after FPLC gel filtration (Superdex-75HR).
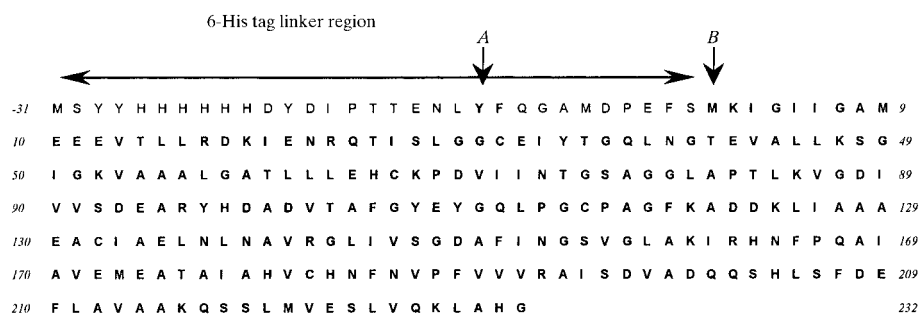
3. Crystallization

Initial screening for crystallization conditions was performed using commercially purchased sparse-matrix screens (Jancarik & Kim, 1991) from Hampton Research (Crystal Screens I and II) and Emerald Biostructures (Wizard I and II). Two different crystallization conditions of *E. coli* MTA/AdoHcy nucleosidase were obtained. At present, only one of these conditions (condition 2) has yielded crystals suitable for X-ray diffraction studies. All crystals were grown using the hanging-drop vapour-diffusion technique by mixing 2 µl of protein (15 mg ml⁻¹) in 50 mM sodium HEPES pH 7.5 with 1 µl of precipitating solution on a siliconized coverslide and equilibrating against 1.0 ml of the same precipitant solution. Crystals were grown in an incubator maintained at 293 K.

Figure 2
Amino-acid sequence of the expressed MTA/AdoHcy nucleosidase enzyme. Arrows A and B indicates the site of cleavage by chymotrypsin and the start site of the MTA/AdoHcy nucleosidase enzyme, respectively. The numbering of the protein starts at the initiating methionine; residues in the fusion are numbered from −31 to −1.

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3.1. Condition 1

Microcrystals were obtained overnight
from 4.0 M sodium formate. Larger hexa-
gonal disc-like crystals (0.4 × 0.4 × 0.1 mm)
were grown by lowering the precipitant
concentration to 3.2 M sodium formate and
adding 50 mM guanidine hydrochloride.
When irradiated with X-rays, these crystals
diffracted to 6 Å.

3.2. Condition 2

Small rod-like microcrystals were
obtained within 2 d from 1.0 M sodium
citrate, 100 mM 2-(N-cyclohexamino)-

Figure 3
Crystals of *E. coli* MTA/AdoHcy nucleosidase
(condition 2). The crystals have approximate dimen-
sions 0.6 × 0.2 × 0.1 mm.

ethanesulfonic acid (CHES) pH 9.5. Opti-
mization of this condition {0.72–0.77 M
sodium citrate, 100 mM CHES pH 8.5,
0.8 mM {3-[(3-cholamidopropyl)dimethyl-
ammonio]-1-propanesulfonate (CHAPS)}
produced diamond-shaped crystals (0.6 ×
0.2 × 0.1 mm) within 5–7 d (Fig. 3).

4. X-ray data collection and analysis

Prior to data collection, a crystal (0.4 × 0.2
× 0.1 mm) was transferred to a cryoprotec-
tant solution containing 15%(w/v) glucose,
0.9 M sodium citrate, 100 mM CHES pH 8.5
for 2 min. The crystal was subsequently
transferred to a 30%(w/v) glucose, 0.9 M
sodium citrate, 100 mM CHES pH 8.5 solu-
tion for an additional 2 min prior to being
cooled in a stream of nitrogen gas (100 K).
Data were collected using a MAR345 image
plate with a Rigaku RU-200 rotating-anode
X-ray generator. A total of 244 frames of 1°
$\Delta\varphi$ oscillations were collected. The crystals
diffracted to a minimum d spacing of 2.3 Å.

Preliminary autoindexing, refinement of
the cell and setting parameters and data
processing were performed using the *HKL*
data-processing suite (Otwinowski & Minor,
1997). The unit-cell parameters were found
to be $a = 50.92$, $b = 133.99$, $c = 70.88$ Å,
$\alpha = \beta = \gamma = 90°$. The full data-reduction
statistics are presented in Table 1. Exam-
ination of the systematic absences uniquely
determined the space group to be $P2_12_12$.
On the basis of density calculations
($V_M = 2.37$ Å^3 Da^{-1}; Matthews, 1968), we
estimate that two monomers are present in
the asymmetric unit. The structure deter-
mination of this protein is currently in
progress.

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