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Crystallization of butyrate kinase 2 from *Thermotoga maritima* mediated by vapor diffusion of acetic acid

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The sitting-drop method of crystallization uses the evaporation of water to increase the concentration of the protein and precipitant in the drop. The presence of other volatile components, such as acetic acid, can have a marked impact on crystallization. A member of the ASKHA (acetate and sugar kinases/Hsc70/actin) superfamily of proteins, isobutyrate kinase (Buk2) from *Thermotoga maritima*, was expressed in *Escherichia coli* with six histidine residues added to the C-terminus. The purified protein was crystallized in a sitting drop with a well solution consisting of 1.7–3.0 *M* sodium formate, with the pH of the well solution alone adjusted to 4.5 with acetic acid. Diffraction data collected at 100 K show that the crystals diffract to 3.1 Å and belong to space group *I*422, with unit-cell parameters $a = b = 198.12$, $c = 58.93$ Å. Both the crystal form and the results of dynamic light-scattering studies suggest that Buk2 is an octomer, the first to be identified in the ASKHA superfamily.

1. Introduction

Butyrate kinase 2 (Buk2; EC 2.7.2.7), encoded by TM1756 or *buk*2 from *Thermotoga maritima*, catalyzes the phosphorylation of isobutyrate to isobutyryl phosphate, which is then converted to isobutyryl-CoA. Such isobutyrate kinases exist in many species of eubacterial and archaeal taxa. In the well characterized species *Clostridium acetobutylicum*, a predominant biochemical pathway is the fermentation of butyric acid to butanol, in which butyryl-CoA is converted to butyric acid through the reactions running in the reverse direction (Davies & Stephenson, 1941). In contrast, the butyrate biochemical pathways from *Bacillus subtilis* (Debarbouille *et al.*, 1999; Kaneda, 1977), *Enterococcus faecalis* (Ward *et al.*, 1999) and *T. maritima* (Nelson *et al.*, 1999) may be involved in the catabolism of branched-chain amino acids or the reversible conversion of branched-chain amino acids into the corresponding fatty acids. Therefore, we refer to the *buk*2 gene product as isobutyrate kinase and have crystallized the protein in the presence of isobutyrate. The *buk*2 gene from *T. maritima* encodes a protein consisting of 375 amino acids, with a predicted molecular weight of 42 kDa. The structure of acetate kinase from *Methanosarcina thermophila*, a protein with 24% sequence identity to *buk*2, has been determined to 2.5 Å (Buss *et al.*, 2001). Isobutyrate kinase belongs to the ASKHA superfamily, a diverse collection of enzymes including acetate and sugar kinases, Hsp70 and actin. Comparison of the structures and activities of isobutyrate kinase and acetate kinase will help identify the properties of the two enzymes that allow them to distinguish between the similar compounds acetate and isobutyrate.

Acetic acid is a common buffer used at pH 4.5 in protein crystallization. This compound is volatile and in this way is different from other buffers frequently used in crystallization, such as citrate (pH 5.5), MES (pH 6.5), HEPES (pH 7.5) and Tris (pH 8.5), but similar to ammonium sulfate (Mikol *et al.*, 1989). Normally, buffers in crystallization are part of the reservoir solution and a small amount is mixed with the protein solution in the crystallization drop. In this case, adding acetate buffer directly to the drop appears to alter the pH too rapidly. Adding the acetate buffer only to the reservoir causes a slow lowering of pH in the drops through evaporation of acetic acid and produces larger more regular crystals.

2. Materials and methods

2.1. Protein expression and purification

The *buk*2 gene was PCR-amplified using *T. maritima* genomic DNA as a template and primers specific to butyrate kinase 2 (GGTCGTGACATATGTTCAGAATCCTCACCATAAATCCCG for the 5′ end and CCTCAGCACTCGAGTAAGATCCCATCAAGATACGAATC for the 3′ end). These primers contained *Nde*I and *Xho*I restriction enzyme sites, respectively, which allowed the gene to be inserted into the pET-30a(+) vector (Novagen). The gene is immediately followed by six CAC codons, which encode a C-terminal hexahistidine tag. We will refer to the protein

produced by the plasmid as hBuk2 to denote the hexahistidine tag.

The *buk*2 gene contains 16 AGA arginine codons that are rarely used in *E. coli*. Therefore, hBuk2 was expressed in BL21 codon-plus (DE3)-RIL strain (http://www.stratagene.com), which produces higher levels of the tRNA required to translate AGA codons. Cells were grown at 310 K in 2×YT culture (16 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl pH 7.0), induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside at an OD_{600} of 0.6–0.8 and harvested after an additional 3–4 h of growth. The cell pellet from 6 l of culture was resuspended in 90 ml buffer *A* [300 m*M* NaCl, 10%(*v*/*v*) glycerol, 50 m*M* NaH_2PO_4 pH 8.0] with 20 m*M* imidazole, frozen in liquid N_2, thawed at room temperature and lysed with a French press (SLM-Aminco) after addition of protease inhibitor cocktail (P8849, Sigma) at 1 ml per 20 g of cells. The lysate was spun in an ultracentrifuge at 55 000 rev min^{-1} in a Ti 70 rotor for 2 h and was loaded onto an Ni–NTA column (Qiagen) in buffer *A* with 20 m*M* imidazole. The column was washed with 60 m*M* imidazole and hBuk2 was eluted with a gradient of 60–500 m*M* imidazole, all in buffer *A*. EDTA and DTT, both at pH 8.5, were added to the eluate to 5 m*M* and the eluate was then desalted on a Sephadex G25 column in buffer *B* (50 m*M* NaH_2PO_4 pH 8.0, 5 m*M* DTT) and loaded onto a Hitrap blue column (Amersham Biosciences) with an equilibration buffer of buffer *B* and an elution buffer of buffer *B* containing 1.5 *M* KCl. A gel-filtration column (XK16/60 Hiload Superdex 200, Amersham Biosciences) equilibrated with 25 m*M* Tris–HCl pH 8.5, 150 m*M* NaCl, 5 m*M* DTT, 10%(*w*/*v*) glycerol was then used to further isolate hBuk2. After addition of 1 m*M* ADP, $MgCl_2$ and isobutyrate, hBuk2 was concentrated to 25–30 mg ml^{-1} in an Amicon ultrafiltration cell (Amicon, Beverly, MA), frozen as aliquots in liquid N_2 and stored at 203 K.

2.2. Enzyme assay

The activity of hBuk2 was measured with an adapted hydroxamate assay (Nishimura & Griffith, 1981). HBuk2 (2.3 µg) was added to 0.5 ml assay solution (50 m*M* sodium isobutyrate, 50 m*M* disodium succinate, 5 m*M* $MgCl_2$, 5 m*M* ATP, 500 m*M* hydroxylamine hydrochloride neutralized with NaOH, 50 m*M* triethanolamine pH 8.3) and incubated at 323 K. The reaction was stopped by the addition of 0.75 ml of stop solution (370 m*M* $FeCl_3$, 200 m*M* trichloroacetic acid, 2.68 *M* HCl) to aliquots every 5 min for 25 min and absorbance was measured at OD_{535}.

2.3. Size determination

The molecular weight of hBuk2 in solution (0.5 mg ml^{-1} in 25 m*M* Tris–HCl, 200 m*M* NaCl, 5 m*M* DTT) was determined by dynamic light scattering using a DynaPro-Ms/X (Protein Solutions). The equation $MW = (1.68R)^{2.3398}$ was used to calculate the molecular weight of a globular protein, where MW (in kDa) is the molecular weight of the particle and *R* (in nm) is the radius of the particle (see Kratochvil, 1987).

2.4. Diffraction data collection

Diffraction data from hBuk2 crystals were collected to 3.1 Å resolution at the Advanced Photon Source BioCARS beamlines at Argonne National Laboratory. A total of 180° of data were collected with an oscillation range of 1° at a wavelength of 1.0 Å and a detector distance of 225 mm. The programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) were used to process the diffraction data and *CCP*4 (Collaborative Computational Project, Number 4, 1994) was used for further analysis.

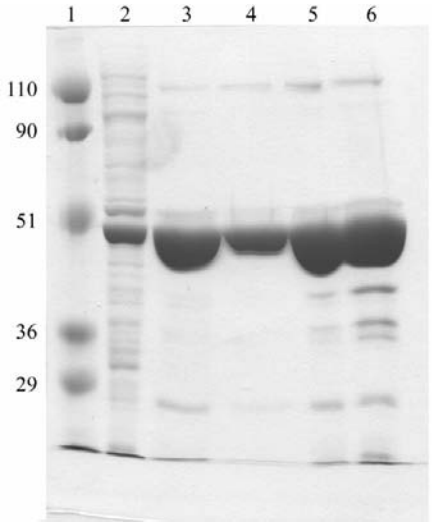

Figure 1
Analysis of the purification of hBuk2 by SDS–PAGE. Lane 1, molecular-weight standards; lane 2, supernatant; lane 3, Ni–NTA column; lane 4, Hitrap blue column; lane 5, gel-filtration column; lane 6, final purified hBuk2. The amount of sample loaded was not normalized. Numbers at the left indicate the molecular mass of the standards in kilodaltons.

Table 1
Purification of hBuk2.

	Volume (ml)	Protein (mg)	Activity (units†)	Specific activity (units mg^{-1})
Supernatant	35	475	3653 ± 527	7.7 ± 1.1
Ni–NTA	120	173	2596 ± 251	15.0 ± 1.5
Hitrap blue	130	55	815 ± 52	14.9 ± 1.0
Gel filtration	17	38	453 ± 10	12.0 ± 0.3

† A unit is defined as micromoles of product produced per minute. The error is the standard deviation between the results of two assays.

3. Results and discussion

3.1. Purification

The first step of purification of hBuk2 (Buk2 with a hexahistidine tag at the C-terminus), ultracentrifugation at 55 000 rev min^{-1} for 2 h, ensures that only one peak of hBuk2 will elute from the final gel-filtration column. The next step in the purification of hBuk2, which possesses six histidines at the C-terminal end, is a nickel–NTA column (Table 1, Fig. 1). Two other columns, Hitrap Blue and gel filtration, were added to the purification to ensure the removal of aggregates. During the purification, the pH is kept between 8.0 (with phosphate as buffer) and 8.5 (with Tris–HCl as buffer), as changing buffer to Na HEPES at pH 7.5 precipitates hBuk2. Glycerol is used in the purification to reduce possible hydrophobic interactions between proteins and to increase the capacity of the nickel–NTA column (Qiagen, 1998).

3.2. Crystallization

Optimization of the crystallization conditions originally determined from Crystal Screen kits (Hampton Research) required the addition of acetic acid to the wells alone to produce crystals that diffract to about 3 Å. In the current crystallization conditions, 2–4 µl of protein solution containing 25–30 mg ml^{-1} hBuk2, 0.55 m*M* *n*-octyl-β-D-glucoside, 25 m*M* Tris–HCl pH 8.5, 150 m*M* NaCl, 5 m*M* DTT, 10%(*w*/*v*) glycerol, 1 m*M* ADP, 1 m*M* $MgCl_2$ and 1 m*M* isobutyrate was mixed with an equal volume of the initial reservoir solution, 1 ml of 1.7–3.0 *M* sodium formate. Sodium acetate (0.1–0.2 ml, 1 *M*, pH 4.5) was only added to the wells. The sitting-drop vapor-diffusion method was employed to prevent the spread of the crystallization solution over cover slips. Crystals appeared in several days (Fig. 2) and were flash-cooled with 4 *M* sodium formate pH 4.5, 5% glycerol in liquid nitrogen for data collection. Mixing protein solution directly with reservoir solution of 0.1 *M* sodium acetate pH 4.6 and 2 *M* sodium formate results in precipitation of

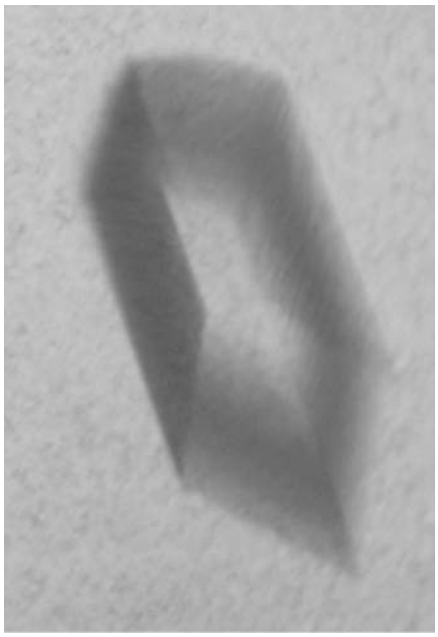

Figure 2
A crystal of hBuk2 formed in a drop of 12.5 m*M* Tris–HCl pH 8.5, 75 m*M* NaCl, 2.5 m*M* DTT, 5%(*v*/*v*) glycerol, 0.5 m*M* ADP, 0.5 m*M* $MgCl_2$, 0.5 m*M* isobutyrate, 0.27 m*M* *n*-octyl-β-D-glucoside, 1.4 *M* sodium formate, with a reservoir solution of 2.8 *M* sodium formate and 0.1 *M* sodium acetate pH 4.5. Crystal dimensions are 0.6 × 0.15 × 0.15 mm.

hBuk2 and often produces no crystals. Using vapor diffusion, acetic acid from the reservoir redistributes slowly into the sitting drop and thereby lowers the pH in the sitting drop gradually, allowing large crystals to grow reproducibly. Lower concentrations (<0.17 *M*) of the buffer sodium acetate pH 4.5 in the wells result in slow growth and well packed cubic crystals, while higher concentrations result in fast-growing poorly diffracting rod-like crystals.

3.3. hBuk2 is likely to be an octomer

Dynamic light-scattering data suggests a radius of 7.5 nm for purified hBuk2, corresponding to a molecular weight of approximately 380 kDa. This measurement is consistent with the formation of octomers or nonamers by the 43 kDa hBuk2 monomer. As many members of the ASKHA superfamily form dimers, including the closely related proteins *M. thermophila* acetate kinase (Buss *et al.*, 2001) and *Clostridium acetobutylicum* butyrate kinase (Huang *et al.*, 2000), it is likely that hBuk2 is an octomer formed from dimers. In fact, hBuk2 crystallizes in space group *I*422, with unit-cell parameters $a = b = 198.12$, $c = 58.93$ Å (Table 2). Solvent-fraction calculations on hBuk2 crystals support the presence of one protein molecule in the asymmetric unit, with a solvent fraction of 62% (Matthews, 1968). These data suggest crystallization of an octomer at a special position of 422 symmetry. Work is under way to solve the structure of hBuk2 by the multiple-wavelength anomalous dispersion method.

Table 2
Crystallographic data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Space group	*I*422
Unit-cell parameters	
$a = b$ (Å)	198.12
c (Å)	58.93
Resolution (Å)	33.0–3.1 (3.21–3.1)
Observed reflections	67355 (4985)
Unique reflections	10317 (1010)
Average redundancy	6.5 (4.9)
$R_{\rm merge}$† (%)	4.4 (16.5)
Completeness (%)	93.1 (93.9)
$I/\sigma(I)$	22.7 (8.3)

† $R_{\rm merge} = \sum |I_i - \langle I \rangle| / I_i$, where I_i is an individual intensity observation of a reflection, $\langle I \rangle$ is the mean intensity for that reflection and the summation is over all reflections.

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