crystallization papers

N-Acetyl-L-glutamate kinase from *Escherichia coli*: cloning of the gene, purification and crystallization of the recombinant enzyme and preliminary X-ray analysis of the free and ligand-bound forms

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The gene for *Escherichia coli* N-acetyl-L-glutamate kinase (NAGK) was cloned in a plasmid and expressed in *E. coli*, allowing enzyme purification in three steps. NAGK exhibits high specific activity ($1.1 \ \mu mol \ s^{-1} \ mg^{-1}$), lacks Met1 and forms dimers (shown by cross-linking). Crystals of unliganded NAGK diffract to 2 Å and belong to space group $P6_122$ or its enantiomorph $P6_522$ (unit-cell parameters $a = b = 78.6, c = 278.0$ Å) with two monomers in the asymmetric unit. Crystals of NAGK with acetylglutamate and the ATP analogue AMPPNP diffract to 1.8 Å and belong to space group $C222_1$ (unit-cell parameters $a = 60.0, b = 71.9, c = 107.4$ Å), with one monomer in the asymmetric unit. NAGK crystallization will allow the determination of proposed structural similarities to carbamate kinase.

1. Introduction

N-Acetyl-L-glutamate kinase (NAGK; E.C. 2.7.2.8) phosphorylates the γ-COOH group of N-acetyl-L-glutamate (NAG) in the second step of microbial arginine biosynthesis (Cunin *et al.*, 1986). In many microorganisms NAGK is a crucial control point, being feedback-inhibited by the final product arginine (Cunin *et al.*, 1986). Little is known about the structure and catalytic mechanism of NAGK. The *P. aeruginosa* enzyme is a homodimer of 29 kDa subunits and appears to also form higher oligomers (Haas & Leisinger, 1975). The gene-deduced amino-acid sequences indicate that bacterial and chloroplast NAGK polypeptides (Marina *et al.*, 1998) are composed of 258–304 amino-acid residues and exhibit considerable sequence identity. Substantial sequence similarity (approximately 20% identity and 40% conservative replacement) was also found with carbamate kinase (Marina *et al.*, 1998), the final enzyme of the arginine deiminase pathway of arginine catabolism and an enzyme whose three-dimensional structure we have recently determined (Marina *et al.*, 1999). Carbamate kinase is a homodimer of polypeptides of approximately 30 kDa and, similarly to NAGK, catalyzes the transfer of the γ-phosphoryl group of ATP to a COO^- group. These similarities suggest that carbamate kinase and NAGK are structurally and functionally similar (Marina *et al.*, 1999). Since carbamate kinase presents a new α/β fold, NAGK and carbamate kinase might be members of a new structural family which may also include, given the sequence similarities (data not shown), γ-glutamyl kinase and long-chain fatty acyl CoA synthetase. The present report is a necessary preliminary step in testing this possibility.

2. Experimental

The *NAGK* gene was PCR-amplified from *E. coli* DNA, using Deep Vent DNA polymerase (from New England Biolabs) and the primers 5′-CTTATTTACTAGTGTCATGATGAATC-CATTAATTATCAAACTGGGC-3′ and 5′-GCTGCGCCGCTCAGCAACAAAACTTA-AGCTAAAATCCGC-3′, which introduce *Bsp*HI and *Blp*I sites at the initiator ATG and downstream of the stop codon, respectively. The *Bsp*HI- and *Blp*I-digested amplified fragment was ligated using T4 ligase into the *Nco*I and *Blp*I sites of plasmid pET-15b (Novagen) and *E. coli* DH5α cells (from Clontech) were transformed. Plasmid pNAGK24 was isolated and was shown by restriction analysis and automated DNA sequencing to harbour the full *NAGK* gene. *E. coli* BL21(DE3) cells (from Novagen) transformed with pNAGK24 and grown to $A_{600} = 0.6$ at 310 K in 1.5 l LB broth with 75 µg ml^{-1} ampicillin were induced for 3 h with 1 m*M* isopropyl-β-D-thiogalactoside and were harvested by centrifugation. Subsequent steps were carried out at 277 K. The cells, suspended as 10 ml g^{-1} cells in 0.1 *M* sodium phosphate pH 7.0, 0.2 m*M* dithioerythritol, were disrupted by sonication. After centrifugation (30 min, 35000*g*), the protein in the supernatant which precipitated between 40 and 60% saturation (at 273 K) of ammonium sulfate was dissolved in 20 ml of buffer *A* (10 m*M* sodium phosphate pH 7.0, 0.2 m*M* dithioerythritol) and dialyzed against the same buffer before application to a Q-Sepharose

Fast Flow (Pharmacia Biotech) column (1 × 18 cm) equilibrated with buffer A. After washing the column with 75 ml of buffer A, a 350 ml linear gradient of 0–0.5 M NaCl in the same buffer was applied. NAGK was eluted as a single peak at approximately 0.12 M NaCl. Fractions rich in enzyme were mixed, dialyzed against 20 mM Tris–HCl pH

(a)

(b)

Figure 1
Expression, purification and cross-linking of NAGK. (*a*) SDS–PAGE of cell extracts of *E. coli* BL21(DE) cells transformed with plasmids pET-15b (lane 1) or pNAGK24 (lane 2). Lane 3, purified enzyme. (*b*) Cross-linking with dimethylsuberimidate (DMS), using the method of Davies & Stark (1970), of 4 mg ml^{-1} NAGK or aldolase (from rabbit muscle; monomer mass 40 kDa). The minus sign indicates omission of DMS. Arrows mark the migration positions of protein standards of the masses indicated.

7.5, 20 mM MgCl$_2$, 0.2 mM dithioerythritol and applied to an Affigel Blue (Bio-Rad) column (2 × 20 cm), which was equilibrated and washed with three column volumes of the dialysis buffer. Pure NAGK was eluted with two column volumes of this buffer supplemented with 2.5 mM ATP and 20 mM NAG and was precipitated with ammonium sulfate (70% saturation) and stored as a slurry at 277 K. For crystallization, the enzyme was placed in the appropriate solution by repeated centrifugal ultrafiltration (Microsep 10K, Pall Filtron).
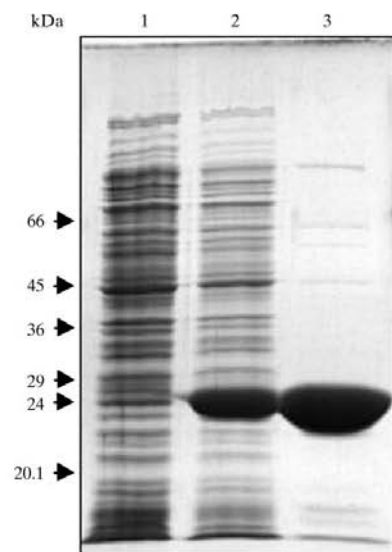
Initial crystallization conditions using the hanging-drop vapour-diffusion method were tested with the sparse-matrix sampling procedure (Jancarik & Kim, 1991), mixing 1.5 µl of reservoir fluid and 1.5 µl of 10 mg ml^{-1} NAGK in 10 mM sodium/potassium phosphate pH 7.0, 1 mM dithioerythritol, either alone or supplemented with 24 mM NAG, 30 mM MgCl$_2$ and 6 mM of the inert ATP analogue AMPPNP ('substrates'). The best crystals, reaching about 0.6 mm in the largest dimension, were produced in about a week at 295 K. In the absence of substrates, crys-

(a)

(b)

Figure 2
Typical crystals of *E. coli* NAGK grown at 295 K (*a*) in the absence of ligands or (*b*) in the presence of 12 mM NAG, 15 mM MgCl$_2$ and 3 mM AMPPNP. In both cases, the largest dimension was approximately 0.6 mm.

tals were obtained with 0.1 M sodium citrate pH 5.6, 26–32% PEG 4K (Sigma) and 0.1–0.3 M ammonium acetate; in the presence of substrates, crystals were obtained with 0.1 M sodium acetate pH 4.6, 27–32% PEG monomethylether 2K (Hampton) and 0.1–0.3 M ammonium sulfate. Harvesting solutions were 36% PEG 4K, 0.3 M ammonium acetate and 10% ethylene glycol in 0.1 M sodium citrate pH 5.6 for crystals grown without substrates and 36% PEG monomethylether 2K, 0.24 M ammonium sulfate and 5% ethylene glycol in sodium acetate pH 4.6 for crystals grown with substrates. Crystals of about 0.3 mm in the longest dimension were examined with a MAR Research image-plate area detector mounted on a Rigaku rotating copper-anode X-ray source operating at 40 kV and 100 mA. Data were collected at 100 K from crystals flash-cooled using an Oxford cryosystem. The data set for crystals grown with substrates was processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski, 1993), whereas for crystals grown without substrates *MOSFLM* (Leslie, 1990) and *SCALA* (Evans, 1997) were used.
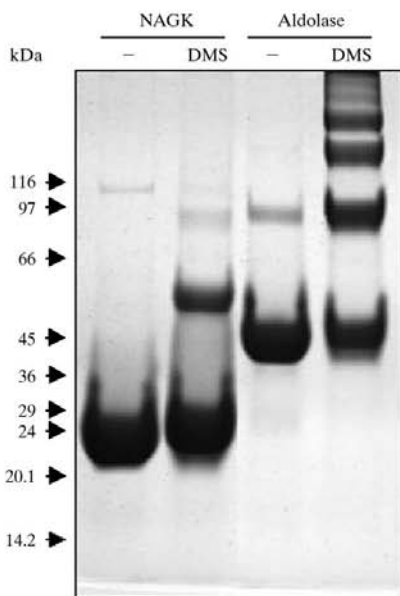
NAGK activity was assayed using the method of Haas & Leisinger (1975). One enzyme unit (16.7 × 10^{-9} mol s^{-1}) yields 1 µmol min^{-1} hydroxamate at 310 K. Protein was assayed using the method of Bradford (1976) with bovine serum albumin as standard. SDS–PAGE was carried out using the method of Laemmli (1970). Cross-linking with dimethyl suberimidate (DMS; Pierce) and SDS–PAGE of the covalent adducts was performed using the method of Davies & Stark (1970).

3. Results and discussion

The product generated by PCR amplification of *E. coli* DNA was identical in size and DNA sequence to that expected from the *NAGK* gene (Parsot *et al.*, 1988). Extracts of *E. coli* BL21(DE3) transformed with an expression plasmid (pNAGK24) which contains as an insert the amplified DNA (see §2), but not extracts of cells transformed with the parental vector without the insert, exhibited NAGK activity (data not shown) and showed a prominent band on SDS–PAGE (Fig. 1*a*) migrating with the expected mass for NAGK (mass estimate from semi-logarithmic plots of protein standards, 27 kDa; mass expected from the sequence, 27.15 kDa). The enzyme, purified in three steps from pNAGK24-transformed cells (Fig. 1*a*, lane 3), exhibited similar activity (1.06 µmol s^{-1} mg^{-1} protein) to the purest bacterial preparation reported thus far

(Haas & Leisinger, 1975). Automated Edman degradation yielded the expected N-terminal sequence (MNPLIIK), except for the lack of Met1, which is removed post-translationally. Upon cross-linking with dimethylsuberimidate (DMS), a new band with the mass of a dimer was observed by SDS–PAGE (Fig. 1b) indicating that, as with *P. aeruginosa* NAGK (Haas & Leisinger, 1975), *E. coli* NAGK forms dimers. With the tetrameric enzyme aldolase, used as a control, the four expected bands were observed (Fig. 1b; Davies & Stark, 1970).

NAGK crystals obtained in the absence of substrates had a fronded appearance (Fig. 2a), diffracting as monocrystals to at least 2 Å resolution with a conventional X-ray source. For practical reasons, a complete data set was only collected at 2.95 Å resolution (93.4% completeness; R_{merge} = 12.1). The space group was hexagonal $P6_122$ or its enantiomorph $P6_522$, with unit-cell parameters $a = b = 78.6$, $c = 278.0$ Å. Packing-density considerations (Matthews, 1968) indicate that, for a monomer mass of 27 kDa, the unit cell could contain 24 monomers ($V_m = 2.6$ Å^3 Da^{-1}; solvent content 53%), corresponding to two monomers per asymmetric unit.

In the presence of substrates, crystals with a rhomboidal shape (Fig. 2b) grew from an amorphous precipitate and diffracted to at least 1.8 Å, although a complete data set was collected at 2.3 Å (90.4% completeness, R_{merge} = 11.5). The space group was $C222_1$, with unit-cell parameters $a = 60.0$, $b = 71.9$, $c = 107.4$ Å and an estimated eight monomers per unit cell ($V_m = 2.1$ Å^3 Da^{-1}; solvent content, 42%) or one monomer per asymmetric unit. If the molecule is organized as a dimer, then the molecular twofold symmetry axis should be coincident with a crystallographic dyad axis. In fact, crystal soaking in solutions of K_2PtCl_6 yielded a Pt derivative with a single location of the metal on the crystallographic dyad X axis, suggesting that this axis could coincide with the molecular twofold axis.

Molecular replacement with the program *AMoRe* (Navaza, 1994) using a polyalanine model of residues 2–105 and 160–310 of carbamate kinase (residues 106–159 form a flexible subdomain and were omitted; Marina *et al.*, 1999) has thus far yielded suggestive but not conclusive solutions. Further heavy-atom derivatives and the selenomethionine-substituted protein are presently being prepared.

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