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# Towards structural understanding of feedback control of arginine biosynthesis: cloning and expression of the gene for the arginine-inhibited *N*-acetyl-L-glutamate kinase from *Pseudomonas aeruginosa*, purification and crystallization of the recombinant enzyme and preliminary X-ray studies

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*N*-Acetyl-L-glutamate kinase (NAGK) catalyzes the second step in the pathway of arginine biosynthesis in microorganisms and plants. In many species, it is the pathway-controlling enzyme and is subject to feedback inhibition by arginine. The gene for the best characterized arginine-inhibitable NAGK, that from *Pseudomonas aeruginosa*, has been cloned in a pET22 plasmid and overexpressed in *Escherichia coli*. The enzyme was purified in three steps to 95% purity and was shown by cross-linking to form dimers. It was crystallized by the hanging-drop vapour-diffusion method at 277 K in the presence of ADP, Mg and *N*-acetyl-L-glutamate. The crystallization solution contained 0.1 *M* sodium cacodylate pH 6.5, 150–170 m*M* magnesium acetate and 13% polyethylene glycol 8000. Prismatic crystals of maximum dimension approximately 0.5 mm diffract to 2.75 Å resolution and belong to space group *P*1 (unit-cell parameters $a = 71.86$, $b = 98.78$, $c = 162.9$ Å, $\alpha = 91.49$, $\beta = 92.03$, $\gamma = 107.56^\circ$). Packing density considerations agree with 6–18 NAGK monomers in the asymmetric unit, with a corresponding solvent content of 79–36%. Self-rotation function calculations confirm the space group and suggest the presence of 3–7 dimers in the unit cell.

## 1. Introduction

Microorganisms and plants synthesize arginine from glutamate. Glutamate is first N-acetylated and then $\gamma$-phosphorylated by *N*-acetyl-L-glutamate kinase (NAGK) to give $\gamma$-phosphoryl *N*-acetyl-L-glutamate, which is converted in two steps to *N*-acetyl-L-ornithine (Cunin *et al.*, 1986; Shargool *et al.*, 1988). After deacetylation, the ornithine is finally converted to arginine. There are two variants of this pathway (Cunin *et al.*, 1986). In one, typified by the pathway in *Escherichia coli*, glutamate is acetylated by acetyl-CoA and acetylornithine is deacylated hydrolytically. In the other, typified by the pathway in *Pseudomonas aeruginosa*, the acetyl group is recycled by reversible transacetylation from acetylornithine to glutamate. Both of these pathways are subject to feedback inhibition by arginine. In the *E. coli* pathway, inhibition occurs at the initial acetylation of glutamate. In the *P. aeruginosa* pathway, phosphorylation of *N*-acetylglutamate (NAG) by NAGK is inhibited by arginine (Cunin *et al.*, 1986; Haas & Leisinger, 1975*b*).

In our laboratory, we have recently determined the three-dimensional structure of *E. coli* NAGK bound to NAG and to the ATP analogue AMPPNP, having thus determined the mechanism of catalysis by this enzyme (Ramón-Maiques *et al.*, 2002). However, since *E. coli* NAGK is not a controlling enzyme and is not inhibited by arginine (Cunin *et al.*, 1986), the mechanism of allosteric regulation of NAGK by arginine is an important issue that remains to be clarified. The determination of the regulatory mechanism may also be relevant for understanding the inhibition by arginine of NAG synthetase in those organisms that do not recycle the acetyl group (Leisinger & Haas, 1975; Cunin *et al.*, 1986). A protein module conferring sensitivity to arginine might be incorporated either in NAG synthetase or in NAGK, depending on the organism and the metabolic organization of the route. To understand in physical terms the mechanism of inhibition of NAGK by arginine, structural studies have been initiated with the NAGK from *P. aeruginosa*. This enzyme is well characterized biochemically and functionally and is strongly inhibited by arginine (Haas & Leisinger, 1975*a*,*b*). The crystallization and initial X-ray diffraction studies of this enzyme, overexpressed in *E. coli* from the cloned gene, are reported here. The crystals pose an interesting and difficult crystallographic problem, given their triclinic character (*P*1 group) and the large number of enzyme monomers in the unit cell.

## 2. Experimental

*P. aeruginosa* PAO1 (donated by Dr Dieter Haas, University of Lausanne), the strain originally used to purify NAGK (Haas & Leisinger, 1975*a*), was the source of the genomic DNA. Using this DNA as template, the putative *P. aeruginosa* NAGK gene (*argB*; PA5323, *Pseudomonas* genome project, http://www.pseudomonas.com) was amplified by PCR utilizing a high-fidelity proofreading thermostable DNA polymerase (Deep Vent, from New England Biolabs) and the direct and reverse primers 5′-TCGGAGCTCC*AT***ATGACCCTGAGTCGCGATGACG**-3′ and 5′-GCGCGCCG*AA*GC*TT*GCGA**TCAGTGACGCTTGCGGTTGCTGATCA**-3′, respectively. These primers (derived from nucleotides 5993778–5993811 and the complementary sequence 5994670–5994713 of the *P. aeruginosa* genome) encompass the beginning and the end of the ORF (the coding sequences are in bold) and short flanking genomic *P. aeruginosa* sequences. They include mutations (shown in italic) to introduce *Nde*I (direct primer) and *Hin*dIII (reverse primer) sites after the initiator and stop codons.

The amplified fragment, digested with *Nde*I and *Hin*dIII and ligated using T4 ligase into the same sites of plasmid pET-22b (Novagen), was used to transform *E. coli* DH5$\alpha$ cells (from Clontech), allowing the isolation of plasmid pNAGK-PA25, which carries in its insert the NAGK gene (as shown by DNA sequencing). To overexpress the gene, *E. coli* BL21 (DE3) cells (from Novagen) were transformed with pNAGK-PA25 and were grown at 310 K with aeration to a cell density of $A_{600}$ = 0.6 in 1.5 l LB broth containing 0.1 mg ml$^{-1}$ ampicillin. 1 m*M* isopropyl-$\beta$-D-thiogalactoside (IPTG) was then added, the incubation was continued for 3 h and the cells were collected by centrifugation. Subsequent steps were carried out at 277 K. The pellet, suspended in 15 ml 0.1 *M* sodium phosphate pH 7.0 per gram of cells, 0.2 m*M* dithioerythritol, was sonicated and after centrifugation (30 min, 35 000*g*) the supernatant was precipitated sequentially with $(NH_4)_2SO_4$ at 30 and 65% saturation at 273 K. The 65% saturation precipitate was dissolved in 20 ml of buffer *A* (20 m*M* sodium phosphate pH 8.0, 1 m*M* dithioerythritol) and dialysed against the same buffer before application to a 1 × 8 cm column of Q-Sepharose Fast Flow (Pharmacia Biotech) that had been equilibrated with buffer *A*. After a 100 ml wash with buffer *A*, a 400 ml linear gradient of 0–0.5 *M* NaCl in this buffer was applied. Enzyme-rich fractions (monitored by SDS–PAGE) eluted at approximately 0.12 *M* NaCl. They were pooled, dialysed against 20 m*M* sodium phosphate buffer pH 7.0 containing 20 m*M* $MgCl_2$ and 1 m*M* dithioerythritol and were applied to a 2 × 20 cm column of Affigel Blue (Bio-Rad) that had been equilibrated with the same solution. After washing with 180 ml of the solution, a 500 ml linear gradient of 0–1.0 *M* NaCl in this solution, devoid of $MgCl_2$, was applied to the column and NAGK eluted at approximately 0.75 *M* NaCl. The enzyme was concentrated by ultrafiltration to 5 mg ml$^{-1}$, supplemented with 10% glycerol and stored at 253 K.

The sparse-matrix sampling vapour-diffusion method (Jancarik & Kim, 1991) was used for crystallization tests carried out at 277 and 294 K in hanging drops in multiwell plates utilizing commercial kits (Crystal Screen I and II, from Hampton Research). The drops contained 1.5 µl reservoir solution and 1.5 µl of 10 mg ml$^{-1}$ NAGK solution prepared by repeated centrifugal ultrafiltration (Microsep 10K, Pall Filtron) of the enzyme in 20 m*M* HEPES pH 7.5, 1 m*M* dithioerythritol, 10% glycerol and 0.02% sodium azide. The best crystals were grown in the presence of 30 m*M* $MgCl_2$, 20 m*M* *N*-acetyl-L-glutamate and 10 m*M* ADP at 277 K in about two weeks using as reservoir solution 0.1 *M* sodium cacodylate pH 6.5, 150–170 m*M* magnesium acetate and 13% polyethylene glycol 8000 (Hampton Research). Crystals were flash-cooled using an Oxford cryosystem and diffraction was carried out at 100 K using synchrotron radiation (ESRF, Grenoble; beamline ID14-4) and a Quantum ADSC Q4R CCD detector. The data set was processed and scaled with *MOSFLM* (Leslie, 1990) and *SCALA* (Evans, 1997). The self-rotation functions (Rossmann & Blow, 1962) were calculated with *MOLREP* (Vagin & Teplyakov, 1997).

Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard. Densitometry of digitized gel images, carried out with the program *SIGMAGEL* (Jandel Scientific) was used for quantification of proteins in Coomassie-stained electrophoretic gels. For all other experimental techniques, see Gil *et al.* (1999).

## 3. Results and discussion

SDS–PAGE (Fig. 1*a*) of *E. coli* BL21 (DE3) transformed with plasmid pNAGK-PA25 revealed a prominent band, representing 18% (densitometric estimate) of the bacterial protein, which was not observed when the cells were transformed with pET-22b not carrying the insert. The electrophoretic mass estimate for this band (30 kDa) agrees, within experimental error, with a previous electrophoretic estimate of the mass of NAGK purified from *P. aeruginosa* (29 kDa; Haas & Leisinger, 1975*a*) and with the sequence-deduced mass of the enzyme polypeptide (31.85 kDa). Furthermore, the extracts of the *E. coli* cells trans-

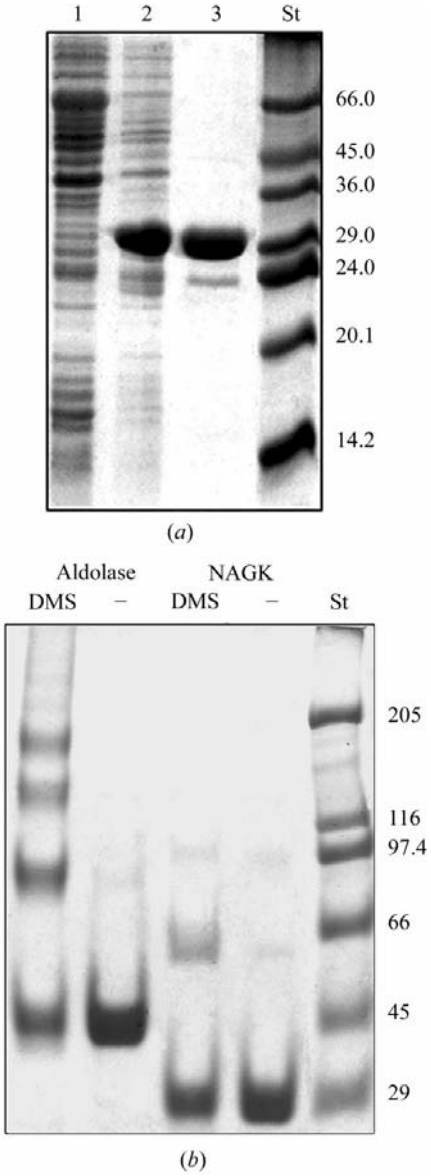


**Figure 1**
Expression, purification and cross-linking of NAGK. St, protein standards of the masses indicated (kDa). (*a*) SDS–PAGE of the sonicates of *E. coli* BL21 (DE3) cells transformed with either plasmid pET-22b (lane 1) or pNAGK-PA25 (lane 2), or of the purified enzyme (lane 3). (*b*) Cross-linking with dimethylsuberimidate (DMS; Pierce) using the method of Davies & Stark (1970) of 5 mg ml$^{-1}$ NAGK or aldolase (used as a cross-linking control; from rabbit muscle, monomer mass 40 kDa). The minus sign indicates omission of DMS.

**Figure 2**
Typical crystal of *P. aeruginosa* NAGK grown at 277 K in the presence of 30 m*M* $MgCl_2$, 20 m*M* NAG and 10 m*M* ADP.

formed with plasmid pNAGK-PA25 but not those of the cells transformed with pET22b exhibited important NAGK activity (4.01 µmol $min^{-1}$ $mg^{-1}$) that was essentially abolished by the addition to the assay of 1 m*M* arginine, again as expected for *P. aeruginosa* NAGK (Haas & Leisinger, 1975*b*). In contrast, pure *E. coli* NAGK (Gil *et al.*, 1999) was not inhibited significantly even by 10 m*M* arginine.

A three-step purification protocol (see §2) lasting 2–3 d yielded 95% pure enzyme (purity monitored by SDS–PAGE using densitometry; Fig. 1*a,* lane 3). The specific activity of the purified enzyme ($88 \pm 7$ µmol $min^{-1}$ $mg^{-1}$) was comparable, although somewhat higher, than that reported for the purest preparation from *P. aeruginosa* (68.8 µmol $min^{-1}$ $mg^{-1}$; Haas & Leisinger, 1975*a*). The enzyme activity was inhibited 13, 54 and 96% by 0.1, 0.5 and 1 m*M* arginine, respectively. Automated Edman sequencing (performed by the sequencing service, Institute de Biomedicina de Valencia) yielded the expected N-terminal sequence TLSRDDAAQVAKVLSEA, except for the lack of the initial methionine, which must therefore be removed post-translationally. MALDI-TOF mass spectrometry (kindly performed by Dr J. J. Calvete, Institute de Biomedicina de Valencia, with the Voyager System 6221; Applied Biosystems) of the pure protein yielded a molecular weight of 31 711 Da, to be compared with 31 718 Da deduced from the gene sequence after removal of Met1. In summary, all the properties indicate that the recombinant enzyme corresponds to the genuine *P. aeruginosa* NAGK. In addition, similar to *E. coli* NAGK (Gil *et al.*, 1999; Ramon-Maiques *et al.*, 2002), the enzyme from *P. aeruginosa* seems to be dimeric, as a band corresponding to the dimer was observed on SDS–PAGE (Fig. 1*b*) in addition to the band arising from the monomer upon cross-linking of the enzyme with dimethylsuberimidate.

Prismatic crystals of approximately 0.5 mm maximal dimension were obtained in the presence of 30 m*M* $MgCl_2$, 20 m*M* NAG and 10 m*M* ADP at 277 K (Fig. 2). Upon diffraction with synchrotron radiation, data were collected to a resolution of 2.75 Å (91.4% completeness, $R_{\rm merge}$ = 5.7). The space group was triclinic (*P*1), with unit-cell parameters $a$ = 71.86, $b$ = 98.78, $c$ = 162.9 Å, $\alpha$ = 91.49, $\beta$ = 92.03, $\gamma$ = 107.56°. Packing-density considerations (Matthews, 1968) for a monomer mass of 31 714 Da suggest 6–18 monomers ($V_{\rm M}$ = 5.79–1.93 $Å^3$ $Da^{-1}$, corresponding to a solvent-content range of 79–36%). To cross-check the space group, the self-rotation function (Rossmann & Blow, 1962) was calculated with *MOLREP* (Vagin & Teplyakov, 1997), yielding peaks that were always weaker than 25% of the origin peak, supporting the absence of crystallographic axes with rotational symmetry, which is in agreement with a triclinic space group *P*1. For $\chi$ = 180°, the self-rotation gives a number of well defined peaks (Fig. 3). The sharpest 3–7 peaks, all situated with orientations almost perpendicular to $a^*$, could correspond to molecular dyad axes representing 3–7 dimers (6–14 subunits) in the crystal unit cell. The broad peak at an angle of about 15° from the $a^*$ axis could correspond to a relationship close to 180° between the orientation of different dimers (Fig. 3). Attempts at finding an initial solution by molecular replacement (Navaza, 1994) using a polyalanine model of residues 1–258 of NAGK from *E. coli* (Ramón-Maiques *et al.*, 2002) are under way, but have thus far given no conclusive solutions.


**Figure 3**
Representation of the $\chi$ = 180° section of the self-rotation function from *P. aeruginosa* NAGK crystals. Start level is 1$\sigma$ and step size is 0.75$\sigma$. The orthogonalization code follows the PDB convention. See text for interpretation.

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## References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Cunin, R., Glansdorff, N., Piérard, A. & Stalon, V. (1986). *Microbiol. Rev.* **50**, 314–352.
Davies, G. E. & Stark, G. R. (1970). *Proc. Natl Acad. Sci. USA*, **66**, 651–656.
Evans, P. R. (1997). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **33**, 22–24.
Gil, F., Ramón-Maiques, S., Marina, A., Fita, I. & Rubio, V. (1999). *Acta Cryst.* D**55**, 1350–1352.
Haas, D. & Leisinger, T. (1975*a*). *Eur. J. Biochem.* **52**, 365–375.
Haas, D. & Leisinger, T. (1975*b*). *Eur. J. Biochem.* **52**, 377–383.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24,** 409–411.
Leisinger, T. & Haas, D. (1975). *J. Biol. Chem.* **250**, 1690–1693.
Leslie, A. G. W. (1990). *Crystallographic Computing.* Oxford University Press.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Ramón Maiques, S., Marina, A., Gil, F., Fita, I. & Rubio, V. (2002). *Structure*, **10**, 329–340.
Rossmann, M. G. & Blow, D. M. (1962). *Acta Cryst.* **15**, 24–31.
Shargool, P. D., Jain, J. C. & McKay, G. (1988). *Phytochemistry*, **6**, 1571–1574.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.