Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cobalt-, zinc- and iron-bound forms of adenylate kinase (AK) from the sulfate-reducing bacterium *Desulfovibrio gigas*: purification, crystallization and preliminary X-ray diffraction analysis

A. V. Kladova,[a] O. Yu. Gavel,[a,b] A. Mukhopaadhyay,[a] D. R. Boer,[a] S. Teixeira,[a] V. L. Shnyrov,[b] I. Moura,[a] J. J. G. Moura,[a] M. J. Romão,[a] J. Trincão[a]* and S. A. Bursakov[a,c]*

[a]REQUIMTE, Departamento de Química, Centro de Química Fina e Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal, [b]Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, 37007 Salamanca, Spain, and [c]Departamento de Protección Ambiental, Estación Experimental del Zaidín, CSIC, C/Profesor Albareda 1, 18008 Granada, Spain

Correspondence e-mail: trincao@dq.fct.unl.pt, sergey@dq.fct.unl.pt

Received 19 May 2009
Accepted 22 July 2009

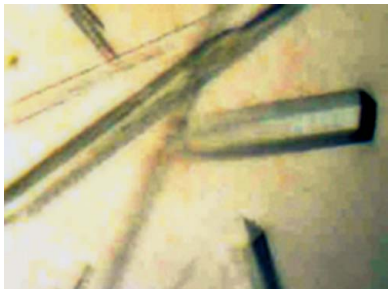
Adenylate kinase (AK; ATP:AMP phosphotransferase; EC 2.7.4.3) is involved in the reversible transfer of the terminal phosphate group from ATP to AMP. AKs contribute to the maintenance of a constant level of cellular adenine nucleotides, which is necessary for the energetic metabolism of the cell. Three metal ions, cobalt, zinc and iron(II), have been reported to be present in AKs from some Gram-negative bacteria. Native zinc-containing AK from *Desulfovibrio gigas* was purified to homogeneity and crystallized. The crystals diffracted to beyond 1.8 Å resolution. Furthermore, cobalt- and iron-containing crystal forms of recombinant AK were also obtained and diffracted to 2.0 and 3.0 Å resolution, respectively. Zn^{2+}–AK and Fe^{2+}–AK crystallized in space group *I*222 with similar unit-cell parameters, whereas Co^{2+}–AK crystallized in space group *C*2; a monomer was present in the asymmetric unit for both the Zn^{2+}–AK and Fe^{2+}–AK forms and a dimer was present for the Co^{2+}–AK form. The structures of the three metal-bound forms of AK will provide new insights into the role and selectivity of the metal in these enzymes.

1. Introduction

Adenylate kinases (AKs; ATP:AMP phosphotransferases; EC 2.7.4.3) catalyze the reaction Mg^{2+}ATP + AMP ↔ Mg^{2+}ADP + ADP. Like most nucleotide-binding proteins, AKs belong to the α/β class of proteins (a five-stranded β-sheet surrounded by several α-helices; Schulz, 1992). AK comprises three domains: the CORE, which contains the P-loop that plays an important role in binding the triphosphate group of ATP, the AMP-binding domain and the LID (Yan & Tsai, 1999). The AMP-binding domain contributes to the binding of AMP, closing over bound AMP. The LID domain is exposed to the solvent and undergoes a large shift during catalysis and upon substrate binding, closing over the active site and protecting the MgATP/AMP ternary complex from bulk water, thus facilitating phosphoryl transfer and preventing hydrolysis (Müller *et al.*, 1996). Motions can also be observed for substrate-free AK and follow the direction of the catalytically competent conformation (Henzler-Wildman *et al.*, 2007).

AKs from Gram-positive bacteria usually contain a Cys-X_2-Cys-X_{16}-Cys-X_2-Cys/Asp structural motif in the LID domain that is responsible for zinc ion binding (Gilles *et al.*, 1994; Berry & Phillips, 1998; Bae & Phillips, 2004).

Gram-negative bacteria are usually devoid of metal ions as the cysteine residues are substituted by other highly conserved amino acids: His, Ser, Asp and Thr. Nevertheless, the following exceptions have been reported: AKs from *Desulfovibrio gigas* (metal-chelating motif ^{129}Cys-X_5-His-X_{15}-Cys-X_2-Cys) and *D. desulfuricans* ATCC 27774 that contain either cobalt or zinc (Gavel *et al.*, 2004), recombinant AK from *Paracoccus denitrificans* overproduced in *Escherichia coli* (^{126}Cys-X_2-Cys-X_{16}-Cys-X_2-Cys; Vielle *et al.*, 2003), which binds either zinc or iron, and zinc-containing AKs from *Thermotoga neapolitana* (^{134}Cys-X_2-Cys-X_{16}-Cys-X_2-Cys) and *Chlamidia pneumoniae* (^{133}Cys-X_2-Cys-X_{12}-Cys-X_2-Cys) (Perrier *et al.*, 1998; Fenton *et al.*, 1978). The motif Cys-X_5-His-$X_{14–19}$-Cys-X_2-Cys is also present in AKs from other *Desulfovibrio* and *Streptomyces* species and may also capture metal ions (Gavel *et al.*, 2008).



Thus, three different metal atoms, zinc, cobalt and iron, have been found in AKs from Gram-negative bacteria.

In this work, we report the purification, crystallization and preliminary X-ray characterization of the native zinc-containing and overexpressed cobalt-containing and iron-containing forms of AK from *D. gigas*. This enzyme consists of 223 amino acids, with a calculated MW of 24 512 Da. Sequence alignment of AK from *D. gigas* with structurally characterized AKs from Gram-negative bacteria revealed 23% sequence identity with AK from *Sulfolobus acidocaldarius* (Vornhein *et al.*, 1998), 35% sequence identity with AK from *Aquifex aeolicus* (Henzler-Wildman *et al.*, 2007) and 38% sequence identity with AK from *M. voltae* (Criswell *et al.*, 2003).

Diffraction data were collected for Co^{2+}–AK, Zn^{2+}–AK and Fe^{2+}–AK to 2.0, 1.8 and 3.0 Å resolution, respectively. These data will allow the structure determination of all three holo-AKs, which will help in elucidation of the metal-coordination sphere and of its role in the folding of the LID domain and may shed some light on the thermodynamic properties of AK (Gavel *et al.*, 2004).

2. Materials and methods

2.1. Purification

D. gigas cells were grown in a 400 l fermenter (Dr René Toci, Bacterial Chemistry Laboratory, CNRS, Marseille, France).

D. gigas cells were grown under anaerobic conditions in a basal medium as described by LeGall *et al.* (1965) using a lactate/sulfate medium. Cells were harvested at the beginning of the stationary phase, resuspended in 10 m*M* Tris–HCl buffer pH 7.6 in a 1:4(*w*:*v*) ratio and passed through an EmulsiFlex C5 homogenizer at 75 kPa. 1 m*M* phenylmethylsulfonyl fluoride (PMSF) was added to the homogenate as an inhibitor of serine proteases. The extract was centrifuged at 15 000*g* for 65 min, after which the pellet was discarded. The supernatant was subjected to further centrifugation at 180 000*g* for 90 min at 277 K in order to eliminate the membrane fraction. A clear supernatant containing the soluble fraction was then used for the purification of AK, which was processed immediately.

The gene coding for the AK of *D. gigas* (670 bp DNA fragment; EMBL accession No. FN424087) was amplified (annealing temperature 337 K) from *D. gigas* genomic DNA by polymerase chain reaction (PCR) with the appropriate upstream primer 5′-GGGGC-TCGAG**CA/TATG**AACATCCTGATCTTCGGTCCGAACGGC-3′ and downstream primer 5′-CCCCGGATCC**A/AGCTT**TTAGGCA-AGCTGGGCCAG-3′. The PCR fragments containing upstream *Nde*I and downstream *Hin*dIII restriction sites were cloned into the pMOS*Blue* vector (GE Healthcare) and the nucleotide sequence of the PCR product was verified by DNA sequencing. The *Nde*I and *Hin*dIII digestion product of the DNA fragment was then cloned into the expression vector pET-22b(+) (Novagen). The resulting plasmid pET-22b(+)/AK with the gene inserted was used for protein expression in *E. coli* strain BL21 (DE3) (Stratagene).

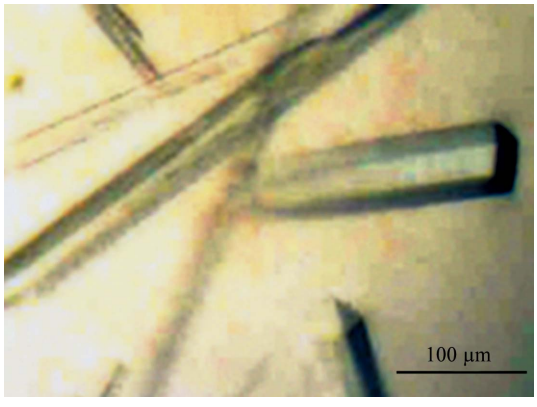


Figure 1
Crystals of native AK from *D. gigas* (approximate dimensions 0.30 × 0.07 × 0.07 mm).

The *E. coli* cells were grown at 310 K in minimal medium M63B1 (0.1 *M* KH_2PO_4, 15 m*M* ammonium sulfate, 0.8 m*M* $MgSO_4$, 3 µ*M* vitamin B_1, pH 7.4) supplemented with 0.4% glucose (Perrier *et al.*, 1998). The time of incubation and the metal concentration in the medium were optimized for the purpose of homogeneous protein production (with a metal:protein ratio of 1:1). Expression was induced with isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 m*M* and an appropriate concentration of metal ions when the culture reached an OD_{600} of 0.6. For Co^{2+}–AK the optimal conditions were 160 µ*M* $CoCl_2$ and 6 h incubation, for Zn^{2+}–AK they were 250 µ*M* $ZnCl_2$ and 4 h incubation, and for Fe^{2+}–AK they were 130 µ*M* $FeCl_2$ and 4 h incubation.

Purification of the native and recombinant AK was performed as described previously (Gavel *et al.*, 2004) by a two-step procedure involving FPLC chromatography (Pharmacia) on a Blue Sepharose Fast Flow column followed by gel filtration on a Superdex 75 column. Both purification steps were performed aerobically at 277 K. The specific activities of the enzymes were determined at each step in the purification process (Gavel *et al.*, 2008).

The purity of the protein was determined by SDS–PAGE at 12.5%(*w*/*v*), as described by Laemmli (1970), at each purification step.

The quantification of metals was performed by ICP-AES analysis (inductively coupled plasma atomic emission spectroscopy) in an Ultima model apparatus (Horiba Jobin-Yvon) with standard solutions (Aldrich) containing cobalt, iron and zinc ions.

2.2. Crystallization, data collection and processing

After purification, the buffer was exchanged to 20 m*M* Tris–HCl pH 7.6 and the protein was concentrated to ∼10 mg ml^{-1}. Crystallization trials of the AK were performed using the hanging-drop vapour-diffusion method using 2 µl drops (with protein:well solution ratios of 1:1, 1:2 and 1:3) over 700 µl well solution. Initial crystallization conditions were screened using an in-house modified version of the sparse-matrix method of Jancarik & Kim (1991) in combination with the commercial screens Crystal Screen and Crystal Screen 2 from Hampton Research (California, USA) at 277 and 293 K. Crystallization conditions were improved by screening additives and varying the protein and precipitant concentrations.

Multiple data sets were collected either on an in-house Cu *Kα* rotating-anode generator or at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Crystals were flash-cooled directly in liquid nitrogen and stored or transferred to a gaseous nitrogen stream (100 K) using Paratone oil as a cryoprotectant. The best data set was collected on beamline ID14-3 at the ERSF using an ADSC Quantum-4R detector. The data were processed using *MOSFLM* v.7.0.1 and *SCALA* from the *CCP*4 package v.6.0.2 (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Native zinc/cobalt-containing AK from *D. gigas* was purified to homogeneity and crystallized. Metal analysis of AK using atomic absorption spectroscopy indicated the presence of cobalt and zinc.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Zn^{2+}–AK				
Data set	Peak	Inflection	Remote	Co^{2+}–AK	Fe^{2+}–AK
X-ray source	ID14-3			ID29	ID29
Crystal data					
Crystal system	Orthorhombic			Monoclinic	Orthorhombic
Unit-cell parameters					
a (Å)	39.39			131.53	38.80
b (Å)	119.44			39.51	119.16
c (Å)	149.59			94.30	146.71
α (°)	90.0			90.0	90.0
β (°)	90.0			109.43	90.0
γ (°)	90.0			90.0	90.0
Max. resolution (Å)	1.8			2.0	3.0
Mosaicity	0.73			0.63	0.73
Molecules per ASU	1			2	1
Matthews coefficient (Å^3 Da^{-1})	3.59			2.31	3.46
Solvent content (%)	65.5			48.3	64.3
Data collection and processing					
Space group	*I*222			*C*2	*I*222
Resolution limits (Å)	25.0–1.8	25.0–1.8	25.0–1.8	44.5–2.0	59.5–3.0
Wavelength (Å)	1.2852	1.2855	1.2825	1.6064	1.7266
No. of observed reflections	202932 (17350)	184103 (14708)	195516 (17373)	109104 (16014)	25156 (3637)
No. of unique reflections	33146 (4618)	33841 (4649)	33272 (4723)	31103 (4417)	6444 (918)
Redundancy	6.1	5.4	5.9	3.5	3.9
$R_{\rm p.i.m.}$†	0.063 (0.447)	0.069 (0.539)	0.049 (0.414)	0.058 (0.327)	0.078 (0.246)
Completeness (%)	99.6 (97.3)	99.3 (95.5)	99.8 (99.1)	97.7 (94.4)	91.3 (92.0)
Anomalous completeness (%)	97.8 (87.6)	95.6 (78.9)	97.6 (89.2)	—	—
$\langle I/\sigma(I)\rangle$	11.0 (1.7)	9.4 (1.4)	12.4 (2.0)	9.0 (2.1)	15.0 (5.1)

† Precision-indicating merging R factor: $R_{\rm p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, with N being the number of times a given reflection hkl was observed; $I_i(hkl)$ is the ith observation of reflection hkl (Weiss, 2001).

The cobalt and zinc contents were 0.4 ± 0.02 mol cobalt and 0.3 ± 0.03 mol zinc per mole of protein for the *D. gigas* enzyme. AKs from *Desulfovibrio* strains were the first AKs found to contain cobalt (Gavel *et al.*, 2004).

The best crystallization conditions were 0.2 *M* sodium/potassium tartrate, 0.1 *M* MES pH 6.5 and 20%(*w*/*v*) PEG 8K (the protein:well solution ratio in the drop was 1:1, 1:2 or 1:3, with a final drop volume of 4, 6 or 8 µl) using a protein stock concentration of ∼10 mg ml^{-1} at 277 K. Crystals grew to dimensions of about 0.30 × 0.07 × 0.07 mm (Fig. 1).

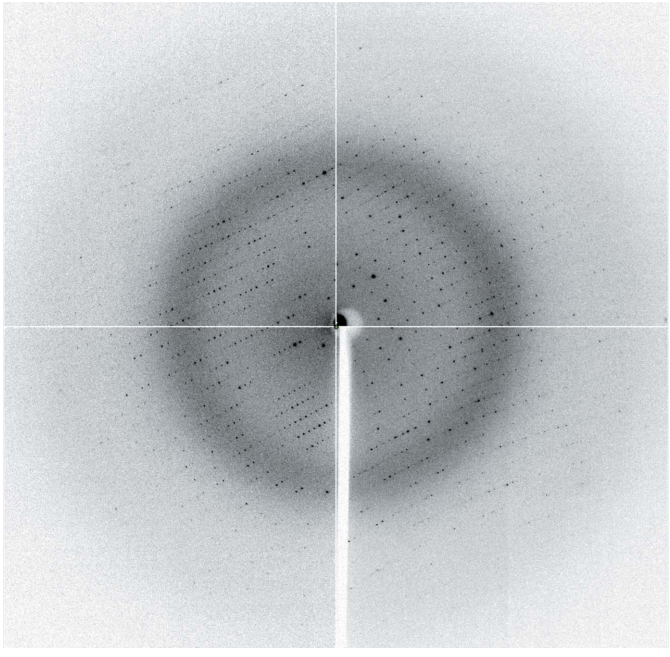


Figure 2
Diffraction pattern of the *D. gigas* AK crystal (the resolution at the edge is 1.5 Å) obtained on beamline ID14-3 (ESRF).

Cobalt and iron-containing AKs were overexpressed in *E. coli* cells grown in controlled media in order to ensure full metal occupancy. The 1:1 ratio of metal to protein was assayed by metal analysis. Recombinant AKs were crystallized using similar conditions as for the native protein. The Co^{2+}–AK, Zn^{2+}–AK and Fe^{2+}–AK crystals diffracted to beyond 1.8, 2.0 and 3.0 Å resolution, respectively (Fig. 2). Zn^{2+}–AK and Fe^{2+}–AK crystallized in space group *I*222 with similar unit-cell parameters, whereas Co^{2+}–AK crystallized in space group *C*2 (Table 1). A full 180° data set was collected for each crystal with 1° oscillation per image. The calculated Matthews coefficients (Matthews, 1968) for Zn^{2+}–AK, Co^{2+}–AK and Fe^{2+}–AK crystals were 3.59, 2.31 and 3.46 Å^3 Da^{-1}, respectively, assuming the presence of a monomer in the asymmetric unit for both the Zn^{2+}–AK and Fe^{2+}–AK forms and of a dimer for the Co^{2+}–AK form. Data-collection and processing statistics are shown in Table 1.

X-ray fluorescence scans were performed for each crystal form, which allowed confirmation of the nature of the anomalous scatterer present. MAD data sets were collected for each crystal at three wavelengths corresponding to the peak, the inflection point and a remote wavelength for each atom. These data sets are now being used to obtain the structures of each form of the enzyme. Comparison of these structures will help in understanding the available biophysical and biochemical data as well as in elucidating the role of the metal present in these enzymes.

This work was supported in part by Fundacão para a Ciência e Tecnologia POCI/QUI/59119/2004, by CRUP–Acções Integradas Luso-Esp. E-62/06 projects and FCT grants SFRH/BD/24744/2005

(AVK), SFRH/BPD/28380/2006 (OYuG) and SFRH/BPD/30142/2006 (AM).

References

Bae, E. & Phillips, G. N. Jr (2004). *J. Biol. Chem.* **279**, 28202–28208.
Berry, M. B. & Phillips, G. N. Jr (1998). *Proteins*, **32**, 276–288.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Criswell, A. R., Bae, E., Stec, B., Konisky, J. & Philips, G. N. Jr (2003). *J. Mol. Biol.* **330**, 1087–1099.
Fenton, D. E., Schroeder, R. R. & Lintvedt, R. L. (1978). *J. Am. Chem. Soc.* **100**, 1932–1934.
Gavel, O. Yu., Bursakov, S. A., Di Rocco, G., Trincão, J., Pickering, I. J., George, G. N., Calvete, J. J., Shnyrov, V. L., Brondino, C. D., Pereira, A. S., Lampreia, J., Tavares, P., Moura, J. J. G. & Moura, I. (2008). *J. Inorg. Biochem.* **102**,1380–1395.
Gavel, O. Yu., Bursakov, S. A., Pina, D. G., Zhadan, G. G., Moura, J. J. G., Moura, I. & Shnyrov, V. L. (2004). *Biophys. Chem.* **110**, 83–92.
Gilles, A. M., Glaser, P., Perrier, V., Meier, A., Longin, R., Sebald, M., Maignan, L., Pistotnik, E. & Barzu, O. (1994). *J. Bacteriol.* **176**, 520–523.
Henzler-Wildman, K. A., Thai, V., Lei, M., Ott, M., Wolf-Watz, M., Fenn, T., Pozharski, E., Wilson, M. A., Petsko, G. A., Karplus, M., Hübner, C. G. & Kern, D. (2007). *Nature (London)*, **450**, 838–844.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
LeGall, J., Mazza, G. & Dragoni, N. (1965). *Biochim. Biophys. Acta*, **99**, 385–387.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Müller, C. W., Schlauderer, G. J., Reinstein, J. & Schulz, G. E. (1996). *Structure*, **4**, 147–156.
Perrier, V., Barlacu-Miron, S., Boussac, A., Meier, A. & Gilles, A. M. (1998). *Protein Eng.* **11**, 917–923.
Schulz, G. E. (1992). *Curr. Opin. Struct. Biol.* **2**, 61–67.
Vielle, C., Krishnamurthy, H., Hyun, H.-H., Savchenko, A., Yan, H. & Zeikus, G. (2003). *Biochem. J.* **372**, 577–585.
Vornhein, C., Bönisch, H., Schäfer, G. & Schulz, G. E. (1998). *J. Mol. Biol.* **282**, 167–179.
Weiss, M. S. (2001). *J. Appl. Cryst.* **34**, 130–135.
Yan, H. & Tsai, M. D. (1999). *Adv. Enzymol. Relat. Areas Mol. Biol.* **73**, 103–134.