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Zinc-, cobalt- and iron-chelated forms of adenylate kinase from the Gram-negative bacterium *Desulfovibrio gigas*

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ABSTRACT

Adenylate kinase (AK) from the sulphate-reducing bacterium *Desulfovibrio gigas* (AK) has been characterized earlier as a Co^{2+}/Zn^{2+}-containing enzyme, which is an unusual characteristic for adenylate kinases from Gram-negative bacteria, in which these enzymes are normally devoid of metal ions. AK was overexpressed in *E. coli* and homogeneous Co^{2+}-, Zn^{2+}- and Fe^{2+}-forms of the enzyme were obtained under *in vivo* conditions. Their structural stability and spectroscopic and kinetic properties were compared. The thermal denaturation of Co^{2+}- and Zn^{2+}-forms of AK was studied as a cooperative two-state process, sufficiently reversible at pH 10, which can be correctly interpreted in terms of a simple two-state thermodynamic model. In contrast, the thermally induced denaturation of Fe^{2+}-AK is irreversible and strongly dependent upon the scan rate, suggesting that this process is under kinetic control. Practically identical contents of secondary-structure elements were found for all the metal-chelated-forms of AK upon analysis of circular dichroism data, while their tertiary structures were significantly different. The peculiar tertiary structure of Fe^{2+}-AK, in contrast to Co^{2+}- and Zn^{2+}-AK, and the consequent changes in the physico-chemical and enzymatic properties of the enzyme are discussed.

1. Introduction

Adenylate kinase (AK, ATP:AMP phosphotransferase, EC 2.7.4.3), a member of the nucleoside monophosphate kinase family, is a small monomeric protein that mediates the reversible transfer of phosphate groups between adenine nucleotides, which are the main substrates, co-factors, or allosteric effectors in a series of key metabolic reactions [1].

Like many nucleotide-binding proteins, AK belongs to the α/β class, with a five-stranded β-sheet surrounded by several α-helices [2]. The formation of the ternary complex stabilizes the enzyme in a form where the mobile small LID and AMP-binding sub-domains close over the remaining CORE region. This rearrangement of the two mobile sub-domains is necessary for the accommodation of the nucleotides in an optimal catalytic geometry, and the resulting closed enzyme conformation provides a solvent-free environment for phosphoryl transfer [3].

Generally, the AK from Gram-positive bacteria contain a Cys-X_2-Cys-X_{16}-Cys-X_2-Cys/Asp structural motif in the LID domain that is responsible for the binding of zinc ion [4–6], whereas the AK from Gram-negative bacteria are usually devoid of metal ions, since their Cys residues are substituted by another four highly conserved amino acids – His, Ser, Asp and Thr, respectively [7]. Nevertheless, exceptions are the AK from *Desulfovibrio gigas* and *Desulfovibrio desulfuricans* ATCC 24774, which contain either cobalt or zinc [8], the AK from *Paracoccus denitrificans*, overproduced in *E. coli* [9], which binds either zinc or iron, and the AK from *Thermotoga neapolitana* and *Chlamydia pneumoniae*, which contain zinc [10,11]. Thus, to date three different metal ions – zinc, cobalt and iron-have been found to be present in the AK from a few Gram-negative bacteria.

Besides the somewhat rare presence of metal ions in the AK of several Gram-negative bacteria, attention has focused the varieties of the metal-binding centers (Cys-X_{1-5}-Cys/His-X_{14-19}-

Abbreviations: AK, adenylate kinase(s); Da, Dalton; *D. desulfovibrio*, *Desulfovibrio desulfuricans* ATCC 27774; *D. gigas*, *Desulfovibrio gigas* NCIB 9332; FPLC, fast performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; U, units.

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Cys-X_2-Cys/Asp) involved in different organisms [8], whose specific properties may be involved in metal selection.

Metal ions play a variety of roles in natural proteins, including nucleophilic catalysis, electron transfer, and the stabilization of protein structure. They also play a structural role in AK, as confirmed in several crystallographic studies [6,12,13]. Mutations in the LID domain lead to considerable differences in the overall stability of AK [7,14,15]. Zinc binding at the genetically engineered zinc-chelating site of *E. coli* AK affords the microorganism considerably higher thermal stability, with a T_m value of 61.6 °C *versus* 52.5 °C for the AK from the wild-type, with hydrogen bonding in the LID domain [7,15]. Previous studies have also shown that zinc depletion reduces the T_m value of *D. gigas* by 5.3 °C [16], that of *T. neapolitana* by 6.3 °C, and that of *Bacillus* (*Geobacillus*) *stearothermophilus* by 7.5 °C [10,17].

A method for the production of chimeras by exchange of certain parts of thermophilic and mesophilic organisms has been applied to confirm their main responsibilities as regards the activity and stability of AK [18]. This, together with mutagenesis studies, has shown that the CORE of AK is responsible for the structural stabilities and dynamics of the LID domain, and AMP_{bind} domains have been suggested to be related to catalysis [3,13,18,19]. Differential scanning calorimetry (DSC) results show that the stabilities of the mesophilic and thermophilic AMP_{bind} and LID domains are similar, and hence their overall stabilities are limited by the stabilities of their CORE domains. Additionally, the results of activity assays clearly show that the two mobile domains themselves (the AMP_{bind} and LID domains) may control their own functional dynamics and may cause differences in activity. Bae and Phillips confirmed their suggestion that AK catalysis is regulated by the intrinsic properties of the moving domains [18].

Knowledge of structural stability and functional activity is important for understanding the binding mode and the putative role of different metal ions in AK. Thus, in the present work we describe a detailed investigation of the thermal stability of homogeneous Co^{2+}-, Fe^{2+}- and Zn^{2+}-forms of AK from *D. gigas*, using different independent methods such as DSC, UV–Vis-spectroscopy, circular dichroism (CD) and kinetic assays.

2. Materials and methods

2.1. Materials

The reagents hexokinase, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, $NADP^+$, NADH, AMP, ADP, MgATP were from Sigma. Glucose-6-phosphate dehydrogenase was from Merck.

2.2. Gene cloning and expression screening

The gene coding for the AK of *D. gigas* (EMBL Nucleotide Sequence Database accession number FN424087) was cloned and resulting plasmid pET-22b(+)/AK with the gene inserted was used for protein expression in *E. coli* strain BL-21(DE3) (Stratagene) as described earlier [8].

2.3. Protein production

Translation of the insert gene induced in mid-log phase ($A_{660nm} \approx 0.6$–0.7) by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) to minimal M63B1 medium (0.1 M KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 3 μM vitamin B_1, pH 7.4) supplemented with 0.4% glucose [20] at 37 °C with vigorous shaking for 4–6 h depends on the type of AK to be expressed. The incubation temperature and the metal concentration in the medium were optimized in order to obtain homogeneous protein with a single metal. Typically, cells were grown in 1 L of M63B1 medium containing 100 μg/ml of ampicillin and the expression conditions were as follows: for Co^{2+}-AK, 6 h and minimal medium supplemented with 160 μM $CoCl_2$; for Zn^{2+}-AK, 4 h and 250 μM $ZnCl_2$; for Fe^{2+}-AK, 4 h and 130 μM $FeCl_2$ at 37 °C.

After these incubation periods, cells were harvested by centrifugation at 5000 rpm for 30 min at 4 °C. Then, cells were resuspended in 20 mM Tris–HCl, pH 7.6, at a ratio of 1:4 (w/v), and disrupted by passing them through an EmusTex-C5 Homogenizer at 9000 psi. Phenylmethylsulphonyl fluoride (PMSF) at 1 mM concentration was added to the broken cells fraction as an inhibitor of serine proteases. The extract was centrifuged at 125,000 × *g* for 90 min at 4 °C and the pellet was discarded. A clear supernatant containing the soluble fraction was then used for the purification of AK; it was processed immediately to avoid protein degradation.

2.4. Purification of recombinant AK from E. coli

The AK of *D. gigas* overexpressed in *E. coli* was purified as described earlier [8,21] in a two-step FPLC (Pharmacia) procedure on Blue Sepharose (Cibacron Blue 3G-A Sepharose CL-6B) fast flow and Superdex-75 gel filtration columns at 4 °C. After the second column, the protein was eluted in 50 mM Tris–HCl buffer, pH 7.6, and 250 mM NaCl, after which it was concentrated, dialyzed, and kept frozen at −20 °C. The protein concentration was determined with the Bicinchoninic Acid Protein Assay Kit from Sigma.

2.5. Determination of protein purity and metal analysis

Protein purity was determined by SDS-PAGE at 12.5 % (w/v), as described by Laemmli [22], in each purification step. The molecular mass standards from Bio-Rad were myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Protein staining was performed using R-250 Coomassie blue.

The quantification of metals was performed by ICP-AES (inductively coupled plasma-atomic emission spectroscopy) analysis on a Horiba Jobin–Yvon apparatus, model Ultima. Standard solutions containing cobalt, iron and zinc were supplied by Aldrich.

2.6. Measurement of the velocity of the forward reaction (AMP and MgATP as the substrates)

Measurement of the velocity of the forward reaction was performed by monitoring the oxidation of NADH at 340 nm by coupling with pyruvate kinase (PK) and lactate dehydrogenase (LDH). The reaction mixture contained 50 mM Tris–HCl, pH 7.6, 100 mM KCl, 0.25 mM $MgCl_2$, 0.2 mM NADH, 1 mM PEP, 15.5 U/ml of LDH and 25 U/ml of PK. The concentrations of AMP and MgATP were varied for the specific requirements of each experiment. The reaction was started by adding 30–50 ng of AK. One unit (U) is defined as 1 μmol of ADP (MgADP) generated per minute.

2.7. The rate of the backward reaction (MgADP and ADP as substrates)

The rate of the backward reaction was measured by following the reduction of $NADP^+$ at 340 nm in an enzyme solution coupled with hexokinase and glucose-6-phosphate dehydrogenase. The reaction mixture contained 50 mM Tris–HCl, pH 7.6, 100 mM KCl, 1 mM glucose, 8 mM $NADP^+$, 5 U of hexokinase, and 5 U of glucose-6-phosphate dehydrogenase. The concentrations of ADP and $MgCl_2$ were varied for the individual needs of each experiment. The reaction was started by adding 30–50 ng of AK. One unit is defined as 1 μmol of ATP generated per minute.