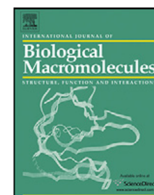
ARTICLE IN PRESS

International Journal of Biological Macromolecules xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Characterization and origin of bacterial arginine kinases

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ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 27 February 2013
Accepted 27 February 2013
Available online xxx

Keywords:
Q3 Bacterial arginine kinase
Phosphagen kinase

ABSTRACT

Arginine kinase (AK) plays a key role in ATP buffering systems of tissues and nerves that display high and variable rates of ATP turnover and is widely distributed in invertebrate animals. The enzyme is also found in unicellular organisms, protists and bacteria, but its occurrence is intermittent among species. The AK sequence is structurally divided into two domains, N- and C-terminal domains. The purpose of this study is to clarify the origin of bacterial AK.

A search of over 1700 bacterial genomic sequences revealed eight species from Deinococcus–Thermus (*Oceanithermus profundus*) and Proteobacteria (*Ahrensia* sp., *Nitratifractor salsuginis*, *Desulfobacterium autotrophicum*, *Desulfotalea psychrophila*, *Myxococcus xanthus*, *Moritella* sp. and *Sulfurovum* sp.) possessing a complete AK sequence homologue. In addition, we searched another key protein that is homologous with that of the C-terminal domain of AK (mcsB). The mcsB is more widely distributed in about 150 species across at least nine bacterial genera. In agreement with the report by other authors, a phylogenetic tree of AK homologues shows that the eight species are separated into two clusters: cluster-A with AKs from ciliates Tetrahymena and Sterkiella and a porifera and the larger cluster-B, including most of the invertebrate AKs. We cloned and expressed the AK from *Sulfurovum lithotrophicum* in cluster-A and determined its enzymatic properties. Bacterial AKs were characterized as having the highest catalytic efficiency among known AKs, although there was a marked difference in k_{cat} values for cluster-A and -B bacterial AKs. These observations suggest that bacterial AKs in cluster-B may be the prototype of invertebrate AKs. On the other hand, it appears that bacterial AKs in cluster-A diverged at an early stage of bacterial evolution after the appearance of AK, or introduced by horizontal gene transfer.

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1. Introduction

Arginine kinases (AKs) are enzymes that catalyze the reversible transfer of the γ-phosphoryl group of ATP to arginine yielding ADP and a phosphorylated arginine, playing a key role in ATP buffering systems in animal cells that have high and variable rates of ATP turnover [1,2]. AKs have a molecular mass of 40 kDa, consist of the N- (∼100 amino acids) and C-terminal (∼250 amino acids) structural domains and typically function as monomers [3]. A wide range of invertebrates, including arthropods, molluscs and cnidarians, universally express at least one AK gene, and some species of unicellular phyla, Protista and Bacteria, have an AK gene, suggesting its ancient origin [4–7].

Andrews et al. [6] were the first to report the presence of AK homologue genes in several proteobacteria, *Desulfotalea psychrophila*, *Myxococcus xanthus*, *Moritella sp.* and *Sulfurovum sp.* They cloned the AK gene from *D. psychrophila*, determined the kinetic constants using recombinant enzyme and showed that the enzyme has minimal, but significant AK activity in both of forward and reverse reactions [6]. In addition, they proposed that bacterial AKs had been introduced by horizontal gene transfer, based mainly on the observation that species with AK homologues are not closely related and that several closely allied species lacked an AK gene [6,7]. Bacterial AK in *Myxococcus xanthus* has been proposed to play a role in fruiting body formation and cell differentiation [7].

The purpose of this study is to clarify the origin of bacterial AK. We assembled a database of over 1700 bacterial species in order to search for AK gene homologues and confirmed that only eight species contain a complete AK gene homologue but that about 150 species have a sequence homologous with that of the C-terminal domain of AK, known as mcsB [17,25,26]. A Bayesian phylogenetic tree showed that eight bacterial AK homologues were separated into two distinct clusters named A and B of which the latter had a close affinity with mcsB.

We cloned the putative AK gene from *Sulfurovum lithotrophicum*, expressed in *E. coli* and determined the kinetic parameters. The k_{cat} (or V_{max}) of the enzyme was 30-fold larger compared with those of *D. psychrophila* [6] and *Myxococcus xanthus* [7]. Based on phylogenetic and enzymatic data, we discussed the two distinct clusters of bacterial AK homologues.

Abbreviation: AK, arginine kinase.

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http://dx.doi.org/10.1016/j.ijbiomac.2013.02.023

2. Materials and methods

2.1. Search for bacterial AK homologues and related proteins (mcsB), alignment of amino acid sequences and construction of phylogenetic tree

Genomic sequences of bacterial AK homologues and mcsBs were identified from searches of the available databases using tBLASTn.

Multiple sequence alignment of 200 AK sequences was done with the ClustalW program available on the DDBJ homepage (http://ddbj.nig.ac.jp/) using the default settings, except that the PAM model was used to construct the distance matrix. The NJ tree was made using the program on the DDBJ homepage, and the Bayesian tree was constructed with the MrBayes 3.1.2 program [24].

2.2. Cloning and expression of *S. lithotrophicum* AK in *E. coli*

S. lithotrophicum is a sulfur-oxidizing chemolithoautotroph belonging to the epsilon-Proteobacteria isolated from Okinawa Trough hydrothermal sediments [8]. *S. lithotrophicum* was obtained from the RIKEN BioResource Center, Japan.

The open reading frame of the *S. lithotrophicum* AK homologue was amplified using primers designed from flanking sequences of AK homologue from *Sulfurovum* sp. (YP_001359116.1): a forward primer with an *Nde* I site and a reverse primer with 6× His-tag encoding sequence and a stop codon. The fragments were subcloned into the pGEM-T Easy Vector and sequenced. Of the 343 deduced amino acids of *S. lithotrophicum* AK, differences from *Sulfurovum* sp. were found at 29 positions. The plasmid vectors were digested with *Nde* I and *Eco* RI and the *S. lithotrophicum* AK DNA was cloned into the *Nde* I/*Eco* RI site of the pET30b vector (Novagen, WI, USA). The pET30b plasmids were sequenced and confirmed to be free of intended mutations in the coding region.

The fusion protein with a hexameric His tag at the C-terminal end was expressed in *E. coli* BL21 (DE3) cells (Novagen) by induction with 1.0 mM IPTG at 20 °C for 24 h. The cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), sonicated and the resulting soluble recombinant protein was purified by affinity chromatography using a Ni-NTA Superflow column (QIAGEN, CA, USA). The purity of the expressed enzyme was verified by SDS-PAGE. The enzymes were placed on ice until assay of enzymatic activity within 12 h.

2.3. Enzyme assay

Enzyme activity was measured by NADH-linked spectrophotometric assay at 25 °C [9] for the forward reaction (phosphagen synthesis). The basic reaction mixture (total volume, 1.0 ml) was 0.65 ml of 100 mM Tris/HCl (pH 8), 0.05 ml of 750 mM KCl, 0.05 ml of 250 mM Mg-acetate, 0.05 ml of 25 mM phosphoenolpyruvate made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of 5 mM NADH made up in 100 mM Tris/HCl (pH 8), 0.05 ml of pyruvate kinase/lactate dehydrogenase mixture made up in 100 mM imidazole/HCl (pH 7), 0.05 ml of 100 mM ATP made up in 100 mM imidazole/HCl (pH 7) and 0.05 ml of recombinant enzyme. The reaction was started by adding 0.05 ml of arginine of the appropriate concentration made up in 100 mM Tris/HCl (pH 8). Initial velocity values were obtained by varying the concentration of one substrate, arginine, while holding the concentration of the second substrate, ATP, constant. The concentration of recombinant enzyme was estimated by the absorbance at 280 nm using the extinction coefficient (units, $M^{-1}\,cm^{-1}$) obtained with the computer program ProtParam (http://ca.expasy.org/tools/protparam.html).

2.4. Determination of kinetic constants

Since the kinetics of AK can be explained as a random-order, rapid-equilibrium kinetic mechanism [10], the initial value of the velocity (v) is given by the following equation.

$$v = \frac{V_{\max}}{K_a^{\mathrm{ATP}} \cdot K_{ia}^{\mathrm{Arg}}/[\mathrm{Arginine}][\mathrm{ATP}] + K_a^{\mathrm{ATP}}/[\mathrm{ATP}] + K_a^{\mathrm{Arg}}/[\mathrm{Arginine}] + 1} \quad (1)$$

$$K_a^{\mathrm{Arg}} \cdot K_{ia}^{\mathrm{ATP}} = K_a^{\mathrm{ATP}} \cdot K_{ia}^{\mathrm{Arg}} = KT = \frac{[\mathrm{E}][\mathrm{Arg}][\mathrm{ATP}]}{[\mathrm{E}-\mathrm{Arg}-\mathrm{ATP}]} \quad (2)$$

and

$$V_{\max} = k_{\mathrm{cat}}[\mathrm{E_0}] = k_{\mathrm{cat}}([\mathrm{E}] + [\mathrm{E}-\mathrm{Arg}] + [\mathrm{E}-\mathrm{ATP}] + [\mathrm{E}-\mathrm{Arg}-\mathrm{ATP}]) \quad (3)$$

where K_a is the ternary dissociation constant in the presence of the second substrate, K_{ia} is the binary dissociation constant in the absence of the second substrate, and K_T is the dissociation constant of the [E – Arg – ATP] complex (see Eq. (2)). To determine the kinetic parameters, data were fitted directly to Eq. (1) according to the method of Cleland [11] using software written by Dr. R. Viola (Enzyme kinetics Programs, ver. 2.0) or SigmaPlot 12 (Systat Software, Inc.).

3. Results and discussion

3.1. Search of database for bacterial AK homologues and its related protein (mcsB)

tBLASTn searches of bacterial genomic DNA from over 1700 bacterial species identified 8 complete AK homologues in which key residues necessary for AK function were conserved from newly identified *Ahrensia* sp. and seven species reported by Fraga's group [6,7]: *Nitratifractor salsuginis Desulfobacterium autotrophicum*, *D. psychrophila*, *Myxococcus xanthus*, *Moritella* sp., *Sulfurovum* sp. and *Oceanithermus profundus*. All species belong to Proteobacteria, except for *Oceanithermus profundus* (Deinococcus–Thermus), based on the bacterial taxonomy of the NCBI taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/).

We previously reported another bacterial protein family that was homologous with that of the C-terminal domain of AK, having a well-conserved C-terminal domain of about 250 residues but completely lacking the N-terminal domain of about 100 residues necessary for guanidine substrate recognition, was distributed among bacteria [12]. Now this protein is identified as mcsB protein [13,25] and known to function as a protein arginine kinase [26]. In this study, we extracted a total of 151 mcsBs from 1710 bacteria from the following phyla: 100 from Firmicutes, 12 from Chlamydiales, 15 from Spirochaetales, 8 from Planctomycetes, 3 from Synergistetes, 9 from Verrucomicrobia, 1 from Lentisphaerae, 2 from Dictyoglomi and 1 from Gemmatimonadetes. It is interesting to note that 713 species in Proteobacteria and 14 species in Deinococcus–Thermus, which were found to contain 8 complete AK homologues, possessed no mcsB. Presence of mcsB in at least 9 bacterial phyla might suggest that it has a bacterial origin but other possibilities have not been ruled out.

3.2. Characterization of amino acid sequences of bacterial AKs and AK-related protein (mcsB)

Amino acid AK sequences of 8 bacteria identified by tBLASTn and *S. lithotrophicum* (this work) were aligned (data not shown) with that of *Limulus* AK, whose TSAC (transition state analog complex) structure is known [3,14].

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Please cite this article in press as: T. Suzuki, et al., Int. J. Biol. Macromol. (2013), http://dx.doi.org/10.1016/j.ijbiomac.2013.02.023

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