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Identification and characterization of two arginine kinases from the parasitic insect *Ctenocephalides felis*

Margaret Werr, Jörg Cramer, Thomas Ilg*

Intervet Innovation GmbH, Zur Propstei, 55270 Schwabenheim, Germany

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ABSTRACT

Arginine kinase (ATP:L-arginine ω-N-phosphotransferase, EC2.7.3.3.; AK) is an enzyme crucial for the energy metabolism of insects and other invertebrates, that has known allergenic potential in humans and that has been proposed as a pesticidal drug target. Here we report the identification, cDNA cloning, genomic gene structure and functional expression of AK genes from *Ctenocephalides* (*C.*) *felis* (cat flea). In contrast to other insect species investigated so far, *C. felis* possesses two AK genes, *cfak1* and *cfak2*, encoding the functional enzymes CfAK1 and CfAK2 that can be distinguished by their guanidino substrate specificity and the kinetic parameters for their natural substrates. Molecular modelling on CfAK1 and CfAK2 based on the *Limulus polyphemus* AK X-ray structure (Zhou et al., 1998) and substrate docking studies provide a potential rational for the observed specificities. Evidence is provided that adult fleas express predominantly CfAK1 as an abundant soluble protein, and that *in vivo* in *C. felis*, the AK metabolites are present in concentration ranges relevant for this enzyme.

1. Introduction

Phosphagen kinases catalyze the reversible phosphorylation by adenosine triphosphate (ATP) of guanidino groups on compounds such as creatine, glycocyamine, taurocyamine, opheline, lombricine or arginine, whose phosphorylated forms are collectively called phosphagens (Watts and Watts, 1968; Ellington, 1989). Phosphagen kinases play a key role in cellular energy metabolism as ATP buffers. In addition, functions of these enzymes in intracellular energy transport from mitochondria to sites of ATP consumption, in regulation of inorganic phosphate concentrations, and in proton buffering are also discussed. The exclusive phosphagen system in vertebrates consists of creatine and its corresponding creatine kinase, while in protochordates, invertebrates and some protozoa, seven distinct phosphagen/phosphagen kinase combinations have been identified in addition to the phospho-creatine/creatine kinase system (Ennor and Morrison, 1958; Watts and Watts, 1968; Wallimann et al., 1998; Pereira et al., 2000; Wyss and Kaddurah-Daouk, 2000; Ellington, 2001; Uda et al., 2006; Suzuki et al., 2009). In arthropods, phospho-L-arginine appears to serve as the predominant phosphagen, that is a substrate of the corresponding arginine kinases (Lohmann, 1935; Lewis and Fowler, 1962; Virden et al., 1965; Blethen, 1972; Strong and Ellington, 1995). Arginine kinase has been studied in a number of insect groups, including muscid flies (Lewis and Fowler, 1962; Sacktor and Hurlbut, 1966; Rockstein and Kumar 1972; Wallimann and Eppenberger, 1973; Wyss et al., 1995), locusts (Newsholme et al., 1978; Schneider et al., 1989; Li et al., 2006), lepidopterans (Rosenthal et al., 1977; Chamberlin, 1997; Binder et al., 2001), hymenopterans (Kucharski and Maleszka, 1998; Wang et al., 2009), cockroaches (Brown and Grossman, 2004; Brown et al., 2004) and beetles (Tanaka et al., 2007). The majority of the studies concluded the presence of a single type of enzyme with approximately 40 000 Da molecular mass, that is presumably encoded by a single copy gene. It has been demonstrated that insect arginine kinase has allergenic potential contributing to allergies against Indianmeal moth (Binder et al., 2001) and cockroaches (Sookrung et al., 2006). There is even evidence that this enzyme may be a cross-reactive invertebrate pan-allergen (Binder et al., 2001; Yu et al., 2003).

The cat flea *Ctenocephalides* (*C.*) *felis* is the commercially most important parasitic insect of companion animals and occurs worldwide as frequent ectoparasite of cats and dogs. Besides causing blood loss in affected animals and being a nuisance to both pets and pet owners, *C. felis* elicits flea allergy dermatitis and is also the transmitting intermediate host of the intestinal cestode *Dipylidium caninum* (Rust and Dryden, 1997; Rust, 2005). Treatment and control of *C. felis* infections rely on chemotherapy with insecticides and insect growth regulators, such as pyrethroids, neonicotinoids, organophosphates and carbamates, macrocyclic lactones, phenylpyrazoles, benzoylphenyl ureas or juvenile hormone analogs.

* Corresponding author. Tel.: +49 6130 948315.
E-mail address: thomas.ilg@sp.intervet.com (T. Ilg).

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However, resistance development threatens the efficacy of many of these compound classes and new chemotherapeutic treatment options will be needed in the future (Rust, 2005). Due to its assumed prominent role in energy metabolism and its specific occurrence in invertebrates, arginine kinase has been proposed as a novel target structure for innovative insecticides to combat insect pest species (Brown and Grossman, 2004; Wang et al., 2009; Wu et al., 2009), and this enzyme has also attracted our interest as a potential chemotherapeutic target for treatment of ectoparasite infection in veterinary animal species.

In this study we describe the identification, molecular cloning, genomic structure and heterologous functional expression of two arginine kinase genes from *C. felis*. We report the kinetic properties of the two enzymes as well as their guanidino substrate specificities. Molecular modelling and substrate docking experiments on the two enzymes outline a potential rational for the observed specificities. Furthermore, we provide information on the *in vivo* concentrations of arginine kinase metabolites and the arginine kinase expression in different flea life stages.

2. Materials and methods

2.1. Bacterial strains, plasmids, chemicals, column materials and insects

Bacterial cultures were grown in Luria–Bertani (LB) medium modified with supplements as specified. The *Drosophila* (*D.*) *melanogaster* expressed sequence tag clone GH11670 was purchased from Invitrogen. Polymerase chain reaction (PCR) products were cloned into pCR2.1-Topo and introduced into *Escherichia* (*E*). *coli* Top 10 cells (Invitrogen). The hexa-His tag expression vector pQE30 and its *E. coli* host strain M15 as well as Ni^{2+}–NTA agarose were from Qiagen. L-Arginine, L-homo-arginine, L-canavanine, L-arginine-O-ethyl ester, L-Nα-acetyl-arginine, L-creatine, L-Nω-methyl-arginine, L-Nω-nitro-arginine, agmatine, 4-guanidino butyric acid, phospho-L-arginine, dihydronicotinamide adenine dinucleotide (NADH), phosphoenolpyruvate, adenosine triphosphate (ATP), pyruvate kinase and lactate dehydrogenase were from Sigma. *C. felis* larvae, pupae and adult fleas (adapted to artificial feeding, Wade and Georgi, 1988) used in this study were collected from long term cultures maintained at Intervet Innovation GmbH, Schwabenheim/FRG, Germany.

2.2. Cloning and expression of the D. melanogaster arginine kinase gene and antibody generation against the recombinant product

The *D. melanogaster* expressed sequence tag clone GH11670 was used as a template for polymerase chain reaction (PCR) with the primer TCCGGATCCGCATGCATGGTTGATGCCGCTGTTCTCGCT and TAAGCTTCCCGGGTTAGCTCTTCTCGAGCTTGATCAGCTCGGTGAT, which introduce flanking restriction enzyme sites (underlined). PCR products were cloned into pCR2.1-Topo and introduced into *E. coli* Top 10 cells (Invitrogen). Cloned PCR product inserts were sequenced, the translation products compared with the database sequence NM_079264 and matching clones were selected. The open reading frame was excised by Bam HI and Sma I digests and subcloned into Bam HI/Sma I-cut pQE30 (pQE30-*dmak*). After introduction of pQE30-*dmak* into *E. coli M15*, protein expression experiments were performed at room temperature. The soluble recombinant *D. melanogaster* arginine kinase (DmAK) was purified under native conditions by Ni^{2+}–NTA agarose chromatography as outlined by the manufacturer (Qiagen, Hilden, FRG). The purified His_6-DmAK was used for immunization of rabbits by a standard protocol involving a primary immunization and three booster immunizations with 200 μg antigen each (SEQLAB, Goettingen/FRG). Blood samples were taken before primary immunization and 2 weeks after each booster immunization. The sera were stored at −20 °C. Specific DmAK antibodies were isolated by affinity purification of the rabbit anti-His_6-DmAK antiserum on electroblotted recombinant protein on nitrocellulose slices as outlined earlier (Ilg et al., 1999).

2.3. Identification, isolation and phylogenetic analysis of the C. felis arginine kinase genes

Total RNA was extracted from adult *C. felis* fleas by a modification of the guanidinium thiocyanate/phenol extraction method (Chomczynski and Sacchi, 1987; Trizol, Sigma). Reverse transcription (RT-) PCR was performed using the Titan one tube RT-PCR system (Roche) with total RNA (0.5–1 μg/50 μl) as template. In some cases, reverse transcription of total RNA was performed in a separate step followed by PCR under various conditions. For the identification of arginine kinase genes of *C. felis*, degenerate primer pairs were constructed from the peptide sequences DDHFLFKEG and EHT(E/K)(A/S)EGG, which are conserved in arthropod species as distant as *Limulus polyphemus* and *D. melanogaster* (Fig. 1). Degenerate primer RT-PCR was performed using total RNA isolated from *C. felis* imagines. RT-PCR products of the expected size (~416 bp) were cloned and sequenced. The deduced protein sequence of the degenerate primer RT-PCR products showed 87% sequence identity to the *D. melanogaster* arginine kinase gene (NM_079264). The missing cDNA sequences of the *C. felis* arginine kinase gene were then obtained by 5′-rapid amplification of cDNA ends (RACE; specific primer: CAACTTCTTCCAACTTGGCGCGGTT AGCAG) and 3′-RACE (specific primer: TGCTAATGCTTGCCGTT TCTGGCCAACTGG) using total RNA from flea imagines as templates. 5′-RACE- and 3′-RACE-PCR was performed using the SMART RACE cDNA Amplification Kit (Clontech) and total RNA as template, *C. felis* arginine kinase specific 5′-RACE- and 3′-RACE primers derived from specific degenerate primer RT-PCR products and generic flanking 5′- and 3′-RACE primer as outlined by the manufacturer. Based on the deduced start and stop codon positions in the 5′- and 3′-RACE product sequences, PCR primers (CTGGATCCAGCATGGTTGACGC AGCCGTCTTGGAC and GACTGCAGTTACATTTCTTTCTCCATCTTAATG AG) were then designed for the RT-PCR amplification of the full-length genes from adult flea total RNA. The restriction enzyme sites introduced by the primer are underlined. The PCR product was cloned into pCR2.1-Topo and sequenced. Thereby a 1087 bp PCR product with a 1068 bp open reading frame (*cfak1*) encoding *C. felis* arginine kinase 1 (CfAK1) was identified. A consensus sequence devoid of PCR errors was identified by sequencing of 3 independent plasmid-cloned PCR fragments and performing ClustalW alignments with the translated insert DNA sequences. The *cfak1* open reading frame was excised by Bam HI/Sal I digests and ligated into Bam HI/Sal I-cut pQE30 (pQE30-*cfak1*). For overexpression experiments pQE30-*cfak1* was transformed into *E. coli M15*. In a separate 5′-RACE experiment with adult flea total RNA and the primer GCAAGGGTTGAATGGATAGCCTTCCATGG, a PCR product was identified that encoded a C-terminally incomplete protein homologous, but not identical, to CfAK1. The C-terminus of this second arginine kinase-like protein was identified by a nested 3′-RACE using the primer CTGATGAAATTGATGGAGGTGATTTAAGTGAGC followed by ATCCTGCCAAAAACTGGGGTGATGTGAACTCCC. From these 5′- and 3′-RACE product sequences, the start and stop codons of the encoded gene were deduced and PCR primers (CAGGATCCACC ATGCCTGTTGAAATTGTTGCCGG and CTCTGCAGTTACATTTCTTTTT CAATTCTTATC) were then designed for the RT-PCR amplification of the full-length genes from flea total RNA. The 1130 bp PCR product was cloned into pCR2.1-Topo and sequenced, a consensus sequence was selected, and a 1119 bp open reading frame (*cfak2*) encoding