

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

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ARTICLE INFO

Article history:

Received 27 May 2009
Received in revised form
30 June 2009
Accepted 6 July 2009

Keywords:

Arginine kinase
Insect energy metabolism
Cat flea
Molecular modelling

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.

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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 Malleszka, 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.

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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 rationale 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 Ni²⁺-NTA agarose were from Qiagen. L-Arginine, L-homo-arginine, L-canavanine, L-arginine-O-ethyl ester, L-N α -acetyl-arginine, L-creatine, L-N ω -methyl-arginine, L-N ω -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, Schwanheim/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 TCCGGATCCGCATGCATGGTTGATGCCGCTGTCTCGCT and TAAGCTTCCCGGTTAGCTCTTCTCGAGCTTGATCAGCTCGGTGAT, 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 Ni²⁺-NTA agarose chromatography as outlined by the manufacturer (Qiagen, Hilden, FRG). The purified His₆-Dmak was used for immunization of rabbits by a standard protocol involving a primary immunization and three booster immunizations with 200 μ g 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-His₆-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 μ g/50 μ l) 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: CAACTCTCTCCAACCTGGCGCGGTTAGCAG) and 3'-RACE (specific primer: TGCTAATGCTTGCCGTTTCTGGCCAACCTGG) 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 (CTGGATCCAGCATGGTTGACGCAGCCGCTCTGGAC and GACTGCAGTTACATTTCTTCTCCATCTTAATGAG) 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 ATCCTGCCAAAACTGGGGTGATGTGAAGTCCC. From these 5'- and 3'-RACE product sequences, the start and stop codons of the encoded gene were deduced and PCR primers (CAGGATCCACCATGCCTGTTGAAATTGTTGCCGG and CTCTGCAGTTACATTTCTTTTCAATCTTATC) 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

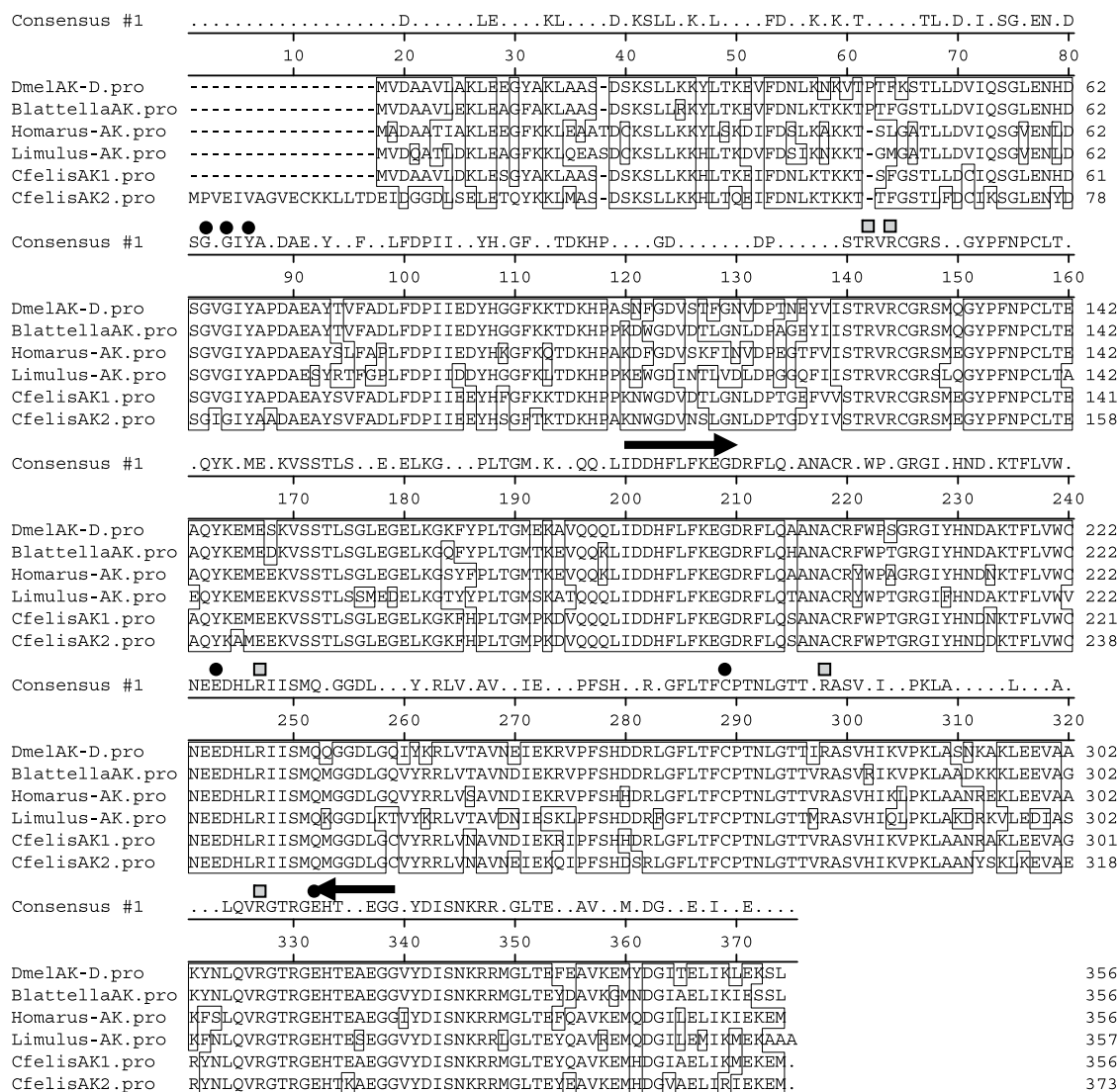


Fig. 1. Alignment of AK protein sequences of *C. felis* and other arthropods. *C. felis* AK1 and AK2 identified in this study were aligned with *Drosophila melanogaster* AK1 (NM_079264), *Blattella germanica* AK (DQ358231), *Homarus vulgaris* AK (AM940133) and *Limulus polyphemus* AK (U09809). The peptide sequences used for degenerate primer design are marked with horizontal arrows. The conserved residues implicated in substrate guanidinium binding are marked with filled circles; the five conserved arginine residues implicated in ADP binding are highlighted by grey boxes (Zhou et al., 1998). The alignment was carried out using ClustalW/DNASTar.

C. felis arginine kinase 2 (CfAK2) was identified. For overexpression, the *cfak2* open reading frame was subcloned into pQE30 and transformed into *E. coli* M15. ClustalW multiple sequence alignments of *C. felis* arginine kinases and other arthropod enzymes, the generation of phylogenetic trees and bootstrap analyses were done within the DNASTar Lasergene software package.

2.4. Protein expression in *E. coli* and protein purification

Overnight cultures of *E. coli* M15 carrying pQE-*cfak1* or pQE-*cfak2* in LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (37 °C) were diluted 1:20 in fresh antibiotics-containing medium and cultured for 3 h at room temperature. CfAK1 or CfAK2 expression was induced by the addition of 1 mM isopropyl thio-β-D-galactoside (IPTG). After a further 3 h of room temperature culture, the cells were harvested and frozen at -80 °C. Purification of the hexa-His-tagged CfAK1 or CfAK2 (=His₆-CfAK1; His₆-CfAK2) was performed by *E. coli* extraction and native Ni²⁺-NTA agarose chromatography as outlined by the manufacturer (Qiagen), except for supplementing the elution buffer with 1 mM dithiothreitol

(DTT). Enzyme-containing 250 mM imidazole eluates were stored at 4 °C, because freezing led to precipitate formation and loss of enzymatic activity.

2.5. Analytical methods

Protein concentration in column eluates was determined using bovine serum albumin as a standard by the Coomassie blue dye binding method (Bradford, 1976). A standard arginine kinase assay was performed in the direction of phospho-L-arginine production at 22 °C in 0.5 ml or 1 ml volumes of 50 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 0.9 mM ATP, 0.2 mM NADH, 2.5 mM phosphoenolpyruvate, 4 mM DTT, 2 units/ml pyruvate kinase and 1 unit/ml lactate dehydrogenase. After addition of the arginine kinase sample, the reaction was started with 5 mM L-arginine (final concentration). For assays using *C. felis* extracts, background rates without specific substrates were determined and subtracted from the extract reaction rates to obtain the specific rates. For the determination of the Michaelis-Menten constant (*K_M*) for L-arginine, the concentrations of this amino acid were varied between 0.2 mM and 10 mM, and the

initial reaction velocity was recorded. The accurate determination of the K_M for ATP was not possible due to the marked and increasing inhibition of the pyruvate kinase indicator enzyme at concentrations above 1 mM ATP. To determine the K_M values for adenosine diphosphate (ADP) and for phospho-L-arginine, the assay was performed into the direction of ATP production. In this case 50 mM Tris–HCl pH 7.5, 20 mM MgCl₂, 10 mM glucose, 2 mM nicotinamide adenine dinucleotide phosphate (NADP⁺), 4 mM DTT, 2 units/ml hexokinase and 1 unit/ml glucose-6-phosphate dehydrogenase were used as basic buffer. For the determination of phospho-L-arginine K_M values, this solution was supplemented with 5 mM ADP, and the phospho-L-arginine was varied between 0.05 mM and 5 mM. For the determination of ADP K_M values, the phospho-L-arginine concentration was held at 5 mM, and ADP was varied between 0.1 mM and 5 mM. SDS-polyacrylamide gel electrophoresis of purified proteins or extracts was performed using 10% acrylamide/bis-acrylamide (29:1) gels, that were subsequently either stained with Coomassie Blue or blotted onto nitrocellulose membranes for immunodetection of arginine kinase. The analysis of *in vivo* substrate concentrations in adult fleas was performed by perchloric acid extraction of the entire organisms essentially as described by Bergmeyer (1985) followed by determination of ATP, ADP and phospho-L-arginine in the extract as outlined by others (Sacktor and Hurlbut, 1966), and by the determination of L-arginine using the standard arginine kinase assay described above.

2.6. Isolation of *C. felis* arginine kinase genomic gene copies and Southern blot analysis

Genomic DNA from adult *C. felis* was isolated as outlined in standard protocols (Sambrook and Russell, 2001). PCR (Expand High Fidelity PCR system, Roche) was performed to amplify genomic gene copies using genomic DNA (0.5 µg/50 µl) as template with the same primer combinations as for *cfak1* and *cfak2* full-length cDNA amplification (Section 2.3). Southern blot analysis of restriction enzyme-digested *C. felis* genomic DNA was performed by capillary blotting using cationized nylon membranes (Roche) as described in standard protocols (Sambrook and Russell, 2001). Digoxigenin (DIG) labeled probes were generated by PCR with the PCR DIG-probe synthesis kit (Roche) using the appropriate *cfak1* cDNA or genomic DNA templates and the primer combinations AGTATGTTGACGACGCCGCTCTGGAC and TTACATTCTTTCTCCATCTTAATGAGTTCAG for AK1-orf-DIG, CTGGTAAGATCTAGGAAAC TAAC and CTAATAAATACAAAATGAGACATGAGC for AK1-intron-DIG, GGCAAGATTCTATCAGTATTC and CTAGAACAATTGTAATGA ATTAC for AK1-3-UTR-DIG, and using the appropriate *cfak2* genomic gene copy as template the primer pair ATGCCTGTTGA AATTGTTGCCGGTG and CCATCAATTTCATCAGTTCTAAAGCAG for AK2-intron1-DIG. The DIG-labeled probes were designed based on sequence comparisons of the genomic gene versions of *cfak1* and *cfak2* to be nondiscriminating (AK1-orf-DIG), specific for *cfak1* (AK1-intron-DIG and AK1-3-UTR-DIG), or specific for *cfak2* (AK2-intron1-DIG). Hybridization and detection of membrane-bound DNA probes was done according to the instructions of the DIG hybridization and detection kit (Roche) using CDP-Star (Roche) as chemiluminescence substrate and corresponding films (Hyperfilm ECL; Amersham/GE) for exposure and development.

2.7. Analysis of arginine kinase in *C. felis* extracts by enzyme assays and immunoblot studies

For the analysis of arginine kinase solubility, 30 *C. felis* imagines were homogenized in 500 µl homogenization buffer (50 mM Tris–HCl pH 7.5, 5 mM EDTA, 4% SDS, 10 µM leupeptin, 1 µM pepstatin and 0.5 mM phenylmethanesulfonyl fluoride) by using ceramic

beads (diameter 1.4 mm) in a ball mill. After centrifugation (13 266 g at 4 °C for 15 min) the supernatant (SN1) was stored and the homogenization and centrifugation steps were repeated three times with the pellet (SN2–SN4). A fifth extraction was performed using homogenization buffer containing 300 mM KCl and 0.5% Triton-X-100 (SN5). The samples SN1–SN5 were then analyzed by immunoblotting and standard arginine kinase assays. For comparative immunoblots of *C. felis* larvae, pupae and imagines, lysates were generated by homogenization in homogenization buffer with mortar and pestle. The homogenates were then centrifuged at 13 266 g at 4 °C for 15 min, the supernatants recovered and stored at –70 °C. The protein concentrations in the homogenate supernatants were determined by the bicinchoninic acid method as outlined by the manufacturer (Pierce) using bovine serum albumin as a standard. For immunoblot analysis of the *C. felis* imagines extracts SN1–SN5, 16 µl samples were loaded onto a 12% SDS-polyacrylamide gel. Furthermore, the equivalent of 100 µg of homogenate protein of the larvae, pupae and imagines samples was loaded onto 10% SDS-polyacrylamide gels together with 20 ng standards of His₆-CfAK1 and His₆-CfAK2. The electrophoretically separated proteins were in both cases electro-transferred (Trans-blot, BioRad) to a nitrocellulose membrane (Protean, Whatman). After blocking of non-specific binding sites with 5% skim milk powder in phosphate-buffered saline (PBS, 10 mM Na₂HPO₄/NaH₂PO₄ pH 7.4, 137 mM NaCl, 2.7 mM KCl) for 1 h at room temperature, the membrane was incubated overnight at 4 °C in 5% skim milk powder/PBS containing affinity-purified anti-DmAK antibodies corresponding to an approximately 1:20 dilution of the initial antiserum. The membrane was washed four times with PBS, 0.1% Tween-20 followed by a 30 min incubation in a 1:20 000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG (Jackson ImmunoResearch) in 5% skim milk powder/PBS. After 5 washes with PBS, 0.1% Tween-20, bound antibody conjugates were detected using a peroxidase chemiluminescence substrate (ECL, Amersham) using films (Hyperfilm ECL, Amersham/GE) as outlined by the manufacturer.

2.8. Generation of structure models and molecular docking studies

The modelling software package Molecular Operating Environment (MOE) 2008.10 (Chemical Computing Group, Inc., Montreal, Quebec, Canada) was used for homology modelling studies. The homology models of CfAK1 and CfAK2 were generated using the PDB entry 1M15 as template sequence and structure. The sequence alignment was calculated with MOE's implemented BLOSUM 65 algorithm under default conditions. The Charmm 27 forcefield (Beglov and Roux, 1994; MacKerell et al., 2004) was applied with a born shell solvation approximation for the homology model calculation. The best structure was defined by the lowest number of violations for side chain torsion angles and van der Waals clashes. The best model structure passing the criteria mentioned above was used for our docking studies. Docking experiments were performed using Gold 4.0 as docking software (Jones et al., 1995, 1997). The substrate cavity was defined by a 5 angstrom radius around the X-ray coordinates of the substrate L-arginine of PDB template 1M15. As the focus of the study was on marginal guanidino substrates, all water molecules, ADP, the nitro-molecules as well as the Mg atom of template structure were kept for the docking study. H-bond interactions of native substrate were set as constraints under default conditions. In case of CfAK1 the side chains of residues Ser 55, Ser 62, Val 64, Tyr 67, Glu 224, Cys 270 and Glu 313 were treated flexible using Gold's protein rotamer database, whereas the flexible side chains of CfAK2 were Ser 72, Ile 81, Ile 83, Glu 241, Glu 330 and His 331. The selection of the flexible side chains relied on the visual inspection of directed

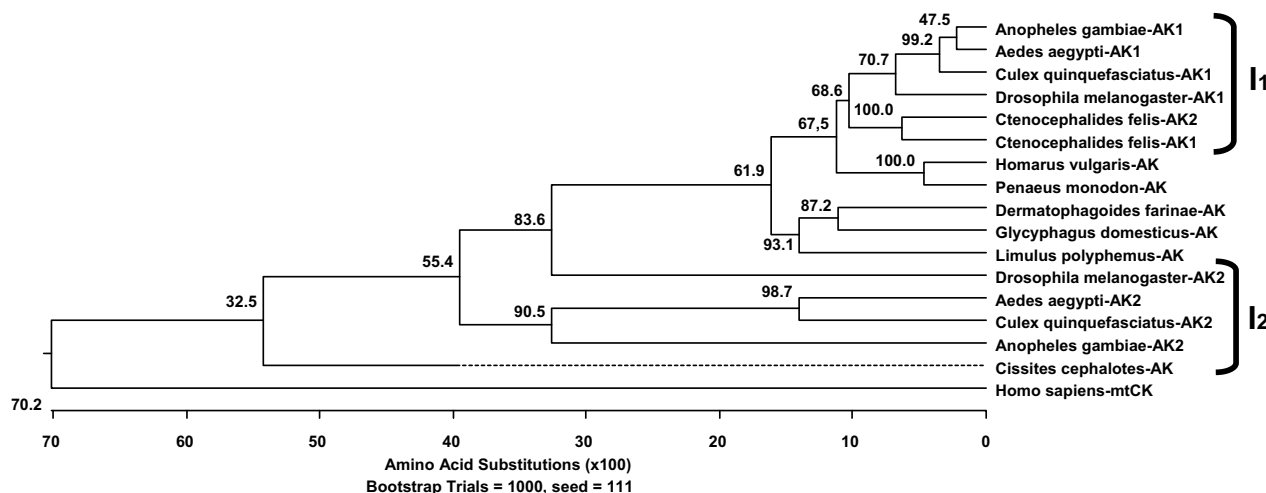


Fig. 2. Phylogenetic dendrogram of 16 arthropod AKs and one creatine kinase. The dendrogram (DNASTar) was derived from ClustalW-aligned protein sequences of *C. felis* AK1 and AK2 identified in this study together with 14 other arthropod AKs and one creatine kinase (CK) with the following Genbank accession numbers: *D. melanogaster* AK1 (NM_079264), *D. melanogaster* AK2 (BT012434), *Anopheles gambiae* AK1 (XM_001688645), *Anopheles gambiae* AK2 (XM_321020), *Aedes aegypti* AK1 (XM_001653673), *Aedes aegypti* AK2 (XM_001657339), *Culex quinquefasciatus* AK1 (XM_001849602), *Culex quinquefasciatus* AK2 (XM_001843516), *H. vulgaris* AK (AM940133), *Penaeus monodon* AK (AF479772), *Dermatophagoides farinae* AK (AY211951), *Glycyphagus domesticus* AK (EU106620), *L. polyphemus* AK (U09809), *Cissites cephalotes* AK (AB275000) and *Homo sapiens* mitochondrial CK (J04469). Bootstrap values (1000 replications) are shown at the branching points. The two insect AK groups proposed by Tanaka et al. (2007) are indicated by the parentheses marked I1 (group 1 insect AKs) and I2 (group 2 insect AKs).

interactions for the docking results of the original substrate docked against the model structures. The docking algorithms were run under default conditions. The start conformations of all docked ligands were minimized by MOE (MMFF94 forcefield MOE2008.10) to 3d coordinates also under default conditions and were provided in sdf file format (Symix MDL).

3. Results

3.1. Identification and isolation of the *C. felis* arginine kinase genes *cfak1* and *cfak2*

For the identification of the arginine kinase gene 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 *D. melanogaster*, *L. polyphemus* and *Homarus (H.) vulgaris* (Fig. 1). Degenerate primer RT-PCR was performed using total RNA isolated from *C. felis* imagines. An RT-PCR product of the expected size (~416 bp) was cloned and sequenced (accession No. FN421424). The deduced protein sequence of the degenerate primer RT-PCR product showed 87% identity to the *D. melanogaster* arginine kinase gene (NM_079264). The missing cDNA sequences of the *C. felis* arginine kinase gene was then obtained by 5'- and 3'-RACE using total RNA as template (FN421425, FN421428, FN421430). Based on the deduced start and stop codon positions in the 5'- and 3'-RACE product sequences, PCR primers were then designed for the RT-PCR amplification of the full-length genes from total RNA. An RT-PCR product with a 1068 bp open reading frame (termed *C. felis* arginine kinase 1, *cfak1*) was identified (FN421431). Consensus sequences devoid of PCR errors were identified by sequencing of 6 independent plasmid-cloned PCR fragments. The deduced polypeptide sequence of this new *C. felis* gene, *cfak1*, showed extensive sequence identity to arginine kinases from other species, particularly to *D. melanogaster* arginine kinase (86.2%), but also to *L. polyphemus* arginine kinase (75%) and *H. vulgaris* arginine kinase (83.4%) (Fig. 1). However, closer inspection of 16 distinct 5'-RACE products showed that 15 sequences were derived from the *cfak1* gene, but a single PCR product was identified that differed in its DNA sequence from

cfak1, although it showed considerable homology to this gene (FN421432). Specific primers were designed for a 3'-RACE experiment, which yielded a DNA fragment again homologous yet not identical to *cfak1* (FN421426). The start and stop codons of this second putative *C. felis* arginine kinase gene were identified by 5'- and 3'-RACE, and based on this information PCR primers were designed for RT-PCR amplification of the full-length gene, which yielded a DNA fragment containing an open reading frame of 1119 bp (*cfak2*, FN421427). The translation product showed 88.5% sequence identity to CfAK1 and was consequently designated *C. felis* arginine kinase 2 (CfAK2; Fig. 1). CfAK2 showed also protein sequence identities to *D. melanogaster* arginine kinase (80.3%), *L. polyphemus* arginine kinase (69.4%) and *H. vulgaris* arginine kinase (76.9%), that were somewhat lower than those observed for CfAK1, but still, the observed homologies were pronounced (Fig. 1). A major difference between CfAK2 and the other AK proteins was the 17 amino acids N-terminal extension (Fig. 1). For both CfAK1 and CfAK2, six out of seven amino acid residues that were reported to interact with the substrate arginine in *L. polyphemus* AK (G64, G66, Y68, C271, E314, E225; Zhou et al., 1998) and all five arginines reported to interact with the ADP phosphates (R124, R126, R229, R280, R309; Zhou et al., 1998) were conserved (Fig. 1). Extended polypeptide sequence comparisons, as illustrated in the DNASTar/ClustalW-based molecular tree (Fig. 2) showed that both *cfak1* and *cfak2* genes identified and cloned in this study belong to the arginine kinase family and both *C. felis* genes cluster within the insect group 1 arginine kinases (I1, Fig. 2) and not within the insect group 2 branch (I2, Fig. 2) that has recently been described (Tanaka et al., 2007).

3.2. Genomic organization and copy number of the *C. felis* arginine kinase genes *cfak1* and *cfak2*

To investigate the genomic structures of the *C. felis* *cfak1* and *cfak2* genes, PCR was performed using the respective full-length primer and *C. felis* genomic DNA as templates. PCR products were readily obtained for both genes and subsequently cloned and sequenced.

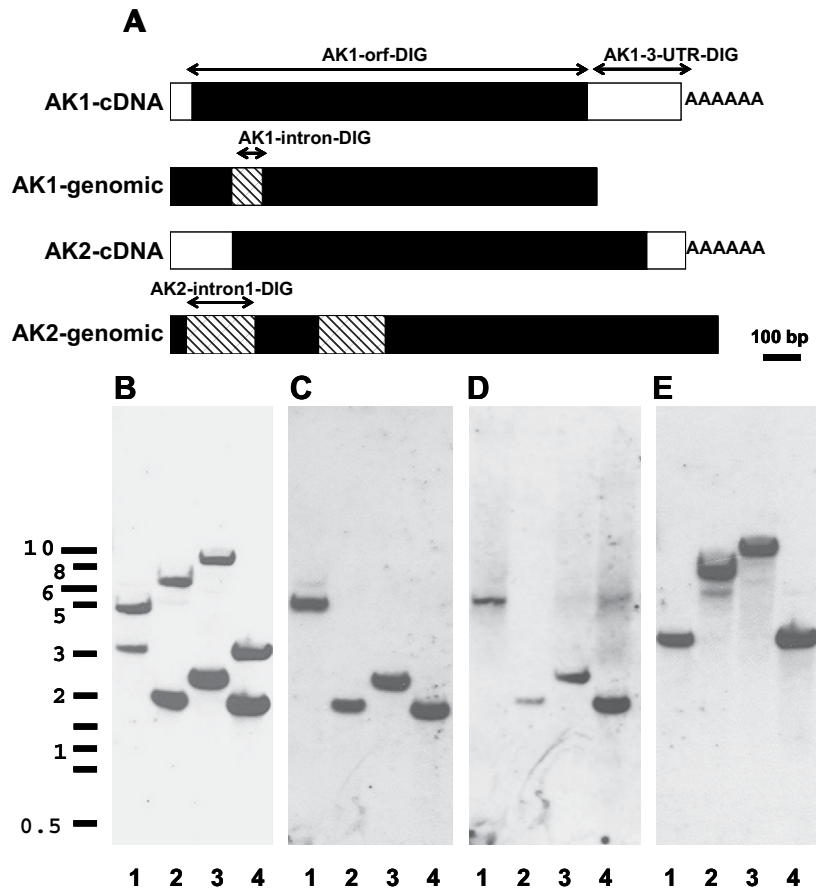


Fig. 3. Gene structure and Southern Blot analysis of *C. felis* arginine kinase genes *cfak1* and *cfak2*. (A) 1) Organization of *cfak1* and *cfak2* mRNA-derived cDNA compiled from 5'-RACE products, 3'-RACE products and the full-length cDNA open reading frame (black areas), the 5'- and 3'-untranslated regions (UTRs) are in white, the 3'-polyA tail is indicated. 2) Organization of the genomic copies of *cfak1* and *cfak2*. The exonic regions are in black, the introns are highlighted as hatched areas. The sequences encompassing the DIG-labeled Southern blot probes AK1-orf-DIG, AK1-3'-UTR-DIG, AK1-intron-DIG and AK2-intron1-DIG are indicated by the double arrows. (B–E): Southern blot membranes probed with the DIG-labeled probes: (B) AK1-orf-DIG; (C) AK1-3'-UTR-DIG; (D) AK1-intron-DIG; (E) AK2-intron1-DIG. The individual lanes were loaded with 10 µg of *C. felis* genomic DNA digested with the following restriction enzymes: lanes 1, Cla I; lanes 2, Mfe I; lanes 3, Xba I/Spe I double digest; lanes 4, Cla I/Mfe I double digest. The positions of DNA fragments from a 1 kb ladder (NEB) are indicated in kb.

In the case of *cfak1*, the genomic PCR product (1150 bp) (FN421429) was longer than the RNA-derived cDNA gene version (1068 bp). The difference was accounted for by one 82 bp intron in the genomic gene version not present in the cDNA (Fig. 3A). For *cfak2*, the genomic PCR product was 1478 bp long (FN421433) compared to 1119 bp for the corresponding cDNA. The larger size of the genomic *cfak2* copy is caused by the presence of two introns encompassing 182 bp and 177 bp, respectively. To investigate the copy number of *cfak1* and *cfak2* in the *C. felis* genome, Southern blot analysis was performed. Restriction enzymes were chosen that do not cut within the sequence of the genomic versions of *cfak1* and *cfak2* and the known 5'- and 3' flanking regions. The hybridization signal pattern was investigated for one digoxigenin-(DIG-) labeled probe not expected to distinguish between *cfak1* and *cfak2* (AK1-orf-DIG), two DIG-probes designed to be specific for *cfak1* (AK1-3'-UTR-DIG and AK1-intron1-DIG) and one DIG-probe expected to be specific for *cfak2* (AK2-intron1-DIG) (Fig. 3A). The promiscuous probe AK1-orf-DIG recognized 2 bands in each restriction enzyme digest (Fig. 3B), which were interpreted as corresponding to the *cfak1* and *cfak2* genes, respectively. This notion was confirmed by the use of *cfak1*- and *cfak2*-specific probes, which yielded single bands in each restriction enzyme digest with positions on the blots that when combined gave the pattern observed for the promiscuous probe (Fig. 3C–E, compare with Fig. 3A). Taken together the

Southern blot analysis described here suggests that the *cfak1* and *cfak2* genes are each present in single copies in the *C. felis* genome.

3.3. Heterologous expression of the *C. felis* arginine kinase genes *cfak1* and *cfak2*, and characterization of the purified enzymes

The PCR primers-introduced flanking Bam HI and Sal I restriction enzyme sites were used to subclone the full-length *cfak1* and *cfak2* genes of *C. felis* into the *E. coli* expression vector pQE30. Protein expression was performed for both genes in *E. coli* M15 at room temperature, which yielded largely soluble expression products that were purified by Ni²⁺-NTA agarose chromatography via their N-terminal His₆-tag. The purified gene products of *cfak1* and *cfak2* showed the expected size on Coomassie Blue-stained SDS-polyacrylamide gels (41.5 and 43.4 kDa, respectively; Fig. 4A) and the purity as judged by overloaded gels (not shown) was greater than 90%. The yields were in the range of 5 mg/100 ml *E. coli* culture for CfAK1 and 3 mg/100 ml *E. coli* culture for CfAK2. Both protein preparations showed enzymatic activity in a standard arginine kinase assay (phospho-L-arginine production), and the specific activities of the purified recombinant proteins were 75 units/mg for CfAK1 and 5 units/mg for CfAK2. The determination of the Michaelis–Menten (K_M) constants for the arginine kinase substrates L-arginine, phospho-L-arginine and ADP gave distinct

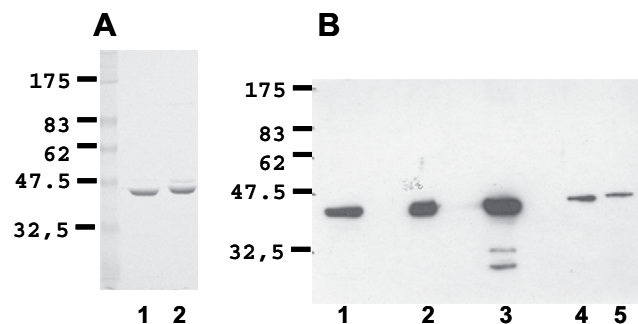


Fig. 4. 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant His₆-CfAK1 and His₆-CfAK2 preparations and immunoblot of *C. felis* extracts from different life stages. (A) 10% SDS-PAGE stained with Coomassie Blue; lane 1, 2 μ g His₆-CfAK1; lane 2, 2 μ g His₆-CfAK2. (B) 10% SDS-PAGE/immunoblot: 1: *C. felis* larvae lysate; 25 μ g protein; 2: *C. felis* pupae lysate; 25 μ g protein; 3: *C. felis* adult flea lysate; 25 μ g protein; 4: 20 ng recombinant His₆-CfAK1 5: 20 ng recombinant His₆-CfAK2. The immunoblot was incubated with affinity-purified rabbit *D. melanogaster* AK antibodies. (A and B) The relative position of protein size markers are indicated in kDa.

values for CfAK1 and CfAK2 in the high μ M/low mM range, that are expected for this type of enzyme (Table 1). The investigation of substrate specificity with potential arginine substrate analogs showed a high specificity of CfAK1 for L-arginine. Among the potential guanidino substrates investigated, only L-canavanine showed an appreciable rate ($\sim 10\%$ of that obtained with L-arginine), while L-arginine-O-ethyl ester and L-homo-arginine were extremely poor substrates with only 3.5% and 2% of the L-arginine reaction rate. L-N α -acetyl-arginine, agmatine, creatine, 4-guanidino butyric acid, N ω -methyl-arginine and N ω -nitro-arginine were no substrates for CfAK1 (Fig. 5A,D). The substrate specificity of CfAK2 was not as strict as that of CfAK1. L-homo-arginine proved to be a fair substrate for CfAK2 with reaction rates approaching 37%, while L-arginine-O-ethyl ester showed $\sim 16\%$ and L-canavanine only 9.5% of the L-arginine reaction rate. Minor substrates for CfAK2 were L-N α -acetyl-arginine and 4-guanidino butyric acid with 1.5% and 1.9% of the L-arginine reaction rate, respectively. Agmatine, creatine, L-N ω -methyl-arginine and L-N ω -nitro-arginine were no substrates for CfAK2 (Fig. 5B,D).

3.4. Molecular modelling and docking studies on CfAK1 and CfAK2

Based on the *L. polyphemus* arginine kinase crystal structure as a template (Zhou et al., 1998, PDB No. 1M15) structural homology models were generated for CfAK1 and CfAK2. Both models did not contain ϕ, ψ – torsion angles outside the allowed regions of the Ramachandran plot. The root mean square deviation was on average below 0.5 Å for C α atoms as well as for the heavy atoms. The overall folding of both models was identical to the template structure. We performed docking experiments for both CfAK1 and CfAK2 with the natural substrate L-arginine and the two artificial substrates L-homo-arginine and L-arginine-O-ethyl ester that are phosphorylated by CfAK2, but are marginal substrates for CfAK1

(Fig. 5A and B). In both CfAK1 and CfAK2, the position of L-arginine as seen in the *L. polyphemus* arginine kinase template was reproduced by the docking algorithm (Fig. 6A,D). Docking of the artificial substrates into the CfAK1 substrate binding pocket led in the case of L-homo-arginine to van der Waals violations at the carboxylate group with the substrate cavity residue Phe 193 CfAK1 (Fig. 6B) while in the case of L-arginine-O-ethyl ester the additional ethyl group could not be properly accommodated at the arginine binding site showing close contacts with either backbone atoms or side chains of Ser 62 and Phe 193 of CfAK1 (Fig. 6C). By contrast the docking algorithm predicted for the artificial substrates a fit into the arginine binding pocket of CfAK2 that was close to the arrangement of the natural substrate and without steric clashes with the exception of L-arginine-O-ethyl ester showing for the best pose one potential steric interaction with the flexible site chain of His 314 (Fig. 6E and F). Taken together the results of the CfAK1 and CfAK2 modelling and docking experiments (Fig. 6A–F) are consistent with the observations in enzyme assays (Fig. 5A and B). Referring to the structure template we do not observe the active configuration of the mechanism. However the model implies that the possible structural changes of the substrate cavity cannot compensate the steric needs of the substrate analogs in case of the CfAK1 and a steric hindrance of CfAK2 for the L-arginine-O-ethyl ester.

3.5. Characterization of arginine kinase from *C. felis* extracts and determination of in vivo arginine kinase substrate concentrations

To determine whether arginine kinase is a soluble enzyme, we performed four sequential extractions of *C. felis* imagines with detergent-free aqueous low salt buffers and a final extraction with Triton-X-100 and high salt-containing buffer. The extracts were subjected to an SDS-PAGE/immunoblot analysis using antibodies raised against recombinant *D. melanogaster* arginine kinase that were affinity-purified. These antibodies recognize both recombinant CfAK1 and CfAK2 on immunoblots (Fig. 4B, lanes 4 and 5). The bulk of immunoreactive material was detected in extract 1, with some also present in extract 2, while only traces could be detected in extract 3 and none in extract 4 (Fig. 7A, lanes 2–5). Application of detergent and high salt failed to extract any further immunoreactive material (Fig. 7A, lane 6). This finding is exactly mirrored by arginine kinase enzyme assays on these extracts. 84.5% of the activity is detected in extract 1, a further 12.4% in extract 2, 2.4% in extract 3, while both extract 4 and 5 contain less than 0.4% of the total arginine kinase activity (Fig. 7B). Taken together these results suggest that in *C. felis* imagines, $>99\%$ of arginine kinase activity is soluble and not bound to cellular structures by either hydrophobic or ionic interactions. The specific arginine kinase activity of the combined *C. felis* adult flea extracts was 2.76 units/mg protein. We investigated also whether arginine kinase is expressed in other *C. felis* life stages. To this end whole organism extracts were generated from *C. felis* larvae, pupae and imagines, normalized to protein content and subjected to SDS-PAGE/immunoblotting. The

Table 1
 K_M values determined for arginine kinase substrates.

	<i>C. felis</i> arginine kinase 1 (CfAK1)	<i>C. felis</i> arginine kinase 2 (CfAK2)	<i>Manduca sexta</i> arginine kinase ^b	<i>Periplaneta americana</i> arginine kinase ^c
L-arginine	1.27 $\pm$ 0.032 mM ($n = 3$) ^a	0.21 $\pm$ 0.026 mM ($n = 3$) ^a	0.5 mM	0.49 mM
Phospho-L-arginine	2.31 $\pm$ 0.05 mM ($n = 3$)	0.74 $\pm$ 0.042 mM ($n = 3$)	0.7 mM	0.94 mM
ADP	0.7 $\pm$ 0.029 mM ($n = 2$)	0.284 $\pm$ 0.015 mM ($n = 3$)	0.45 mM	0.09 mM

^a Determined at 0.9 mM ATP. We refrained from determining the arginine kinase K_M for ATP via the coupled pyruvate kinase/lactate dehydrogenase assay, because ATP inhibits pyruvate kinase in the test concentration range leading to erroneous readings.

^b Taken from Rosenthal et al. (1977).

^c Taken from Brown et al. (2004).

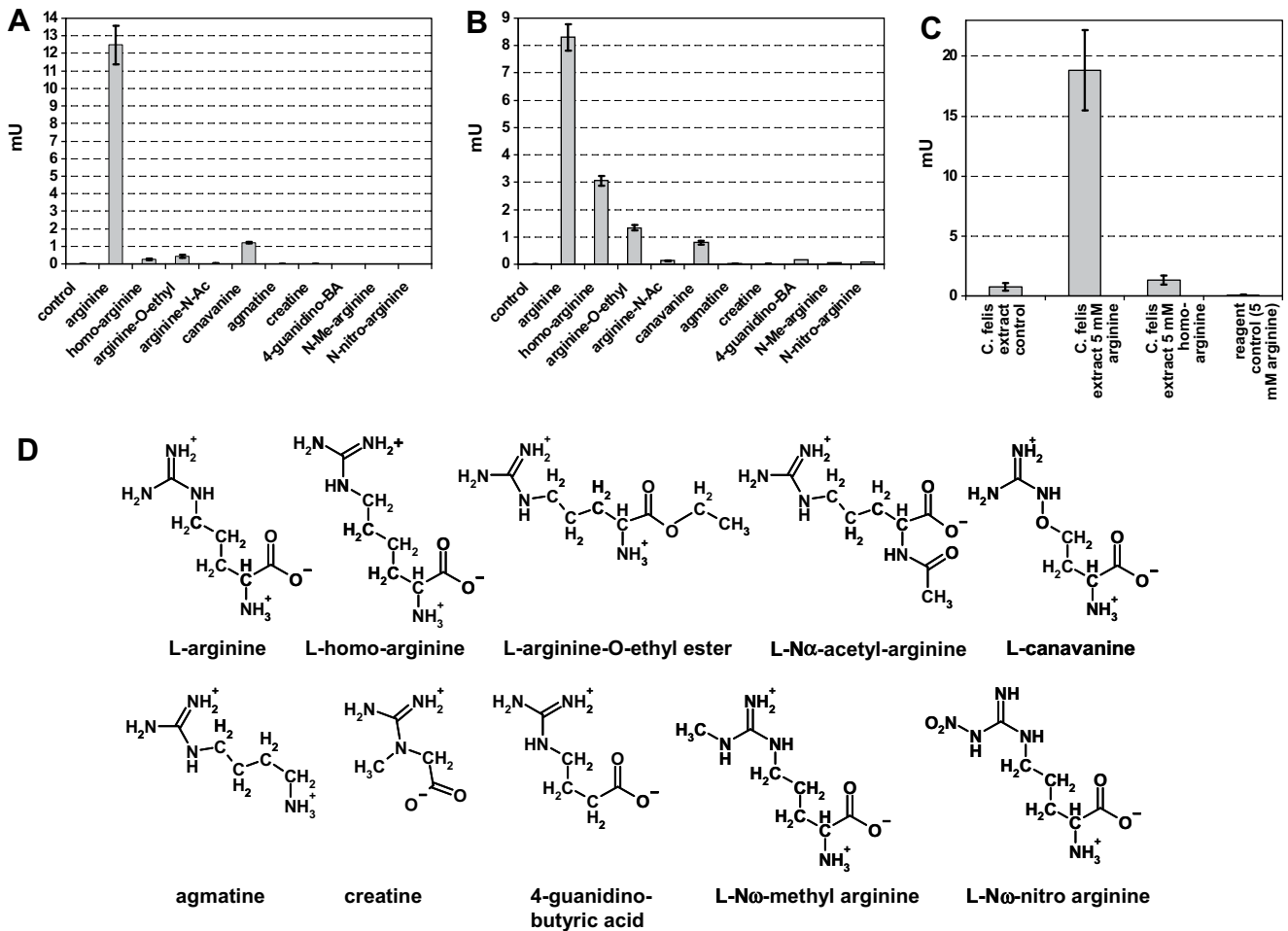


Fig. 5. Guanidino substrate specificity of His₆-CfAK1, His₆-CfAK2 and AK in *C. felis* imago extract. Standard arginine kinase assay conditions were applied, where L-arginine was replaced by 5 mM concentrations of other guanidino compounds. The NADH production rates were determined photometrically and arginine kinase units were calculated. (A) Purified recombinant His₆-CfAK1. (B) Purified recombinant His₆-CfAK2. (C) *C. felis* imago soluble extract. (D) Chemical structures of guanidino compounds used in arginine kinase assays.

result of this study suggested that arginine kinase is expressed in all *C. felis* life stages, and, although not rigorously quantified, the relative signal strength in immunoblots suggested an increase of expression from the larval stage over the pupae to the imago (Fig. 4B). Since the immunoblot studies do not allow to distinguish between CfAK1 and CfAK2, we employed enzyme assays using the diagnostic substrate pair L-arginine/L-homo-arginine (Fig. 5A,B) to gain information on their presence in a soluble *C. felis* imago extract. Standard enzyme assays were performed using L-arginine and L-homo-arginine as substrates, and the ratio of their reaction rates (L-arginine versus L-homo-arginine) was compared with the ratio determined for the purified recombinant enzymes: the *C. felis* imago extract showed a substrate ratio of 64.1, while recombinant CfAK1 had a substrate ratio of 48.5 and recombinant CfAK2 a substrate ratio of 2.7. This result suggested that CfAK1, and not CfAK2, is the dominant arginine kinase activity in *C. felis* imago extract. To investigate the *in vivo* concentrations of arginine kinase substrates, perchloric acid extracts (Bergmeyer, 1985) were generated from whole adult fleas, and the amounts of arginine, phospho-arginine, ATP and ADP in the extract were determined (Sacktor and Hurlbut, 1966). The most abundant substrate was phospho-L-arginine with >3 $\mu\text{mol/g}$ followed by ATP with nearly 3 $\mu\text{mol/g}$, L-arginine with >2 $\mu\text{mol/g}$ and ADP with approximately 0.4 $\mu\text{mol/g}$ fleas (Table 2).

4. Discussion

Arginine kinase (AK) is the only member of the phosphagen kinase family that has so far been identified in insects. It was commonly assumed that insect AK is derived from single genes in each species (group 1 insect AKs, see Fig. 2), until Suzuki and coworkers pointed out that based on gene predictions from genome sequences a second divergent family of insect AKs can be postulated (group 2 insect AKs, see Fig. 2; Uda et al., 2006; Tanaka et al., 2007). In this study, we report the identification and molecular cloning of two arginine kinase genes from the cat flea *C. felis*, *cfak1* and *cfak2*. Phylogenetic analysis suggests that, unexpectedly, while *cfak1* clearly falls into the group 1 insect AK family, *cfak2* is not clustering into group 2, but is also a member of group 1 (Fig. 2), which is so far unique in insects. Neither CfAK1 nor CfAK2 possess an N-terminal mitochondrial import sequence predictable by Bioinformatics tools. Both genes *cfak1* and *cfak2* encode functional arginine kinases, as shown by heterologous expression of the gene products and enzyme assays. Southern blot analyses suggest that both the *cfak1* and the *cfak2* gene are present as single copies in the *C. felis* genome. The genomic version of *cfak1* contains one intron, while genomic *cfak2* possesses two intronic regions. The position and phase of the single *cfak1* intron and intron 2 of *cfak2* correspond to intron A identified in *Anopheles gambiae* AK1,

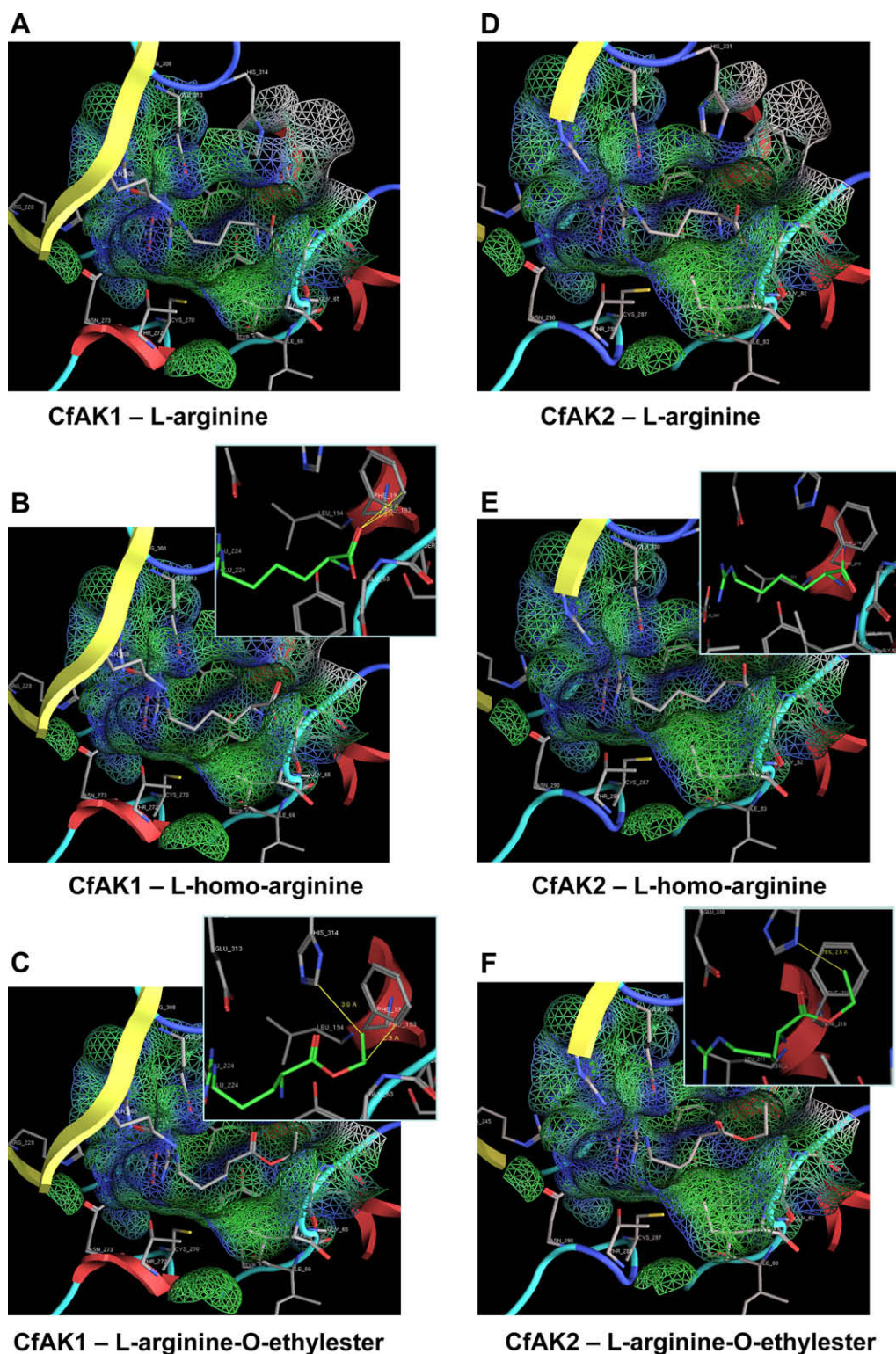


Fig. 6. Molecular models of the CfAK1 and CfAK2 L-arginine binding pocket and docking of guanidino substrates. The X-ray position of the L-arginine (A,D) and the best poses of docking experiments of the artificial substrates L-homo-arginine (B,E) and L-arginine-O-ethyl (C,F) ester are shown for substrate binding pockets of the CfAK1 and CfAK2 homology models. (A–C) show the substrate binding site of CfAK1, while in (D–F) the corresponding cavity of CfAK2 are shown. Green areas indicate lipophilic protein areas whereas blue stays for hydrophilic protein spheres. Exposed protein surface areas are coloured in grey. Predicted van-der-Waals violations for substrate homologues are illustrated for the docking results of CfAK1 as well as CfAK2 using a stick representation of the molecules and shown in the smaller graphs in the upper left (B,C,E,F). The key interactions between the ligand and the enzyme site chains are marked by yellow lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

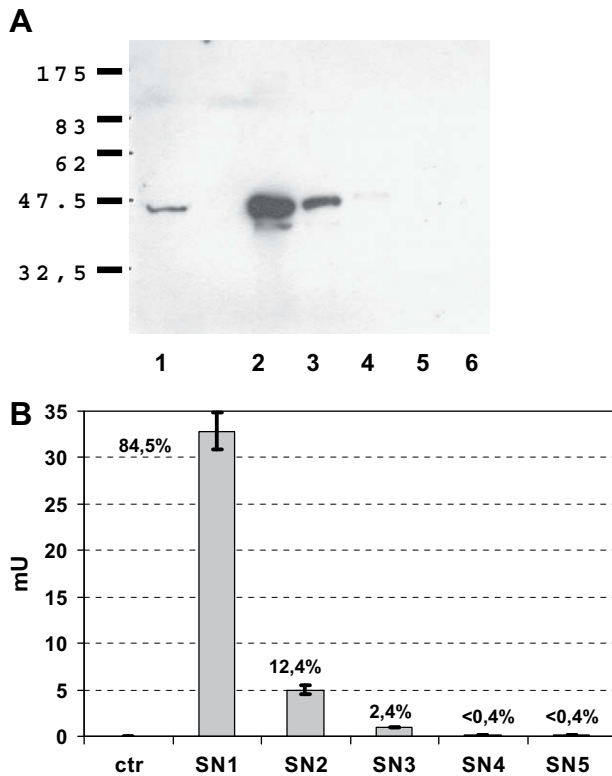


Fig. 7. 10% SDS-PAGE/immunoblot and arginine kinase assays of sequential extracts from *C. felis* imagines. (A) 10% SDS-PAGE/immunoblot: 1: 20 ng recombinant His₆-CfAK1; 2: *C. felis* imago lysate, soluble supernatant 1 (SN1); 3: SN2; 4: SN3; 5: SN4; 6: SN5 (detergent/high KCl). The loading of the lanes was normalized to the extraction volumes. The immunoblot was incubated with affinity-purified rabbit *D. melanogaster* AK antibodies. The relative position of protein size markers is indicated in kDa. (B) Standard arginine kinase assay using aliquots of SN1–SN5 normalized to the extraction volumes. The assay mixture without extracts served as background control (ctr). The NADH production rates were determined photometrically and arginine kinase units were calculated.

Bombyx mori AK and several platyhelminth and molluscan AKs (Uda et al., 2006), while the arrangement of intron1 from *cfak2* is unique. The Michaelis–Menten constants (K_M) of the two gene products CfAK1 and CfAK2 determined for the AK substrates phospho-L-arginine, L-arginine and ADP (Table 1) as well as their specific activities are for both enzymes within the range observed for other arginine kinases (e.g. Uhr et al., 1966; Blethen and Kaplan, 1968; Rosenthal et al., 1977; Brown et al., 2004). The investigation of guanidino substrate specificity revealed that CfAK1 and CfAK2, although highly homologous in their primary structures, show subtle differences: CfAK1 is highly specific for L-arginine and, amongst the guanidino compounds tested, phosphorylates only the toxic analog L-canavanine with appreciable rate. By contrast CfAK2 accepts not only L-canavanine in addition to L-arginine, but also phosphorylates L-homo-arginine and L-arginine-O-ethyl ester (Fig. 5). This is an indication that the architecture of the active site in CfAK1 and CfAK2 is different. Indeed, when structure models of the two *C. felis* arginine kinases were generated based on the *L. polyphemus* AK crystal structure (Zhou et al., 1998), and the substrates or substrate analogs L-arginine, L-homo-arginine and L-arginine-O-ethyl ester were docked into the respective arginine binding pockets, differences between the two enzymes could be demonstrated. As expected, the specific substrate L-arginine showed a perfect fit in both enzyme models. By contrast, the latter two compounds led to steric clashes and van der Waals radius violations in the CfAK1 active site, while the equivalent site in

Table 2

Arginine kinase *in vivo* substrate concentrations in *C. felis* imagines.

	μmol/g fresh weight
L-arginine	2.18 ± 0.06 (n = 4)
Phospho-L-arginine	3.25 ± 0.34 (n = 4)
ADP	0.38 ± 0.06 (n = 2)
ATP	2.93 ± 0.4 (n = 3)

CfAK2 accommodates the two substrate analogs in arrangements close to that of the natural ligand L-arginine. These substrate analog acceptance differences between CfAK1 and CfAK2 can be exploited to investigate their expression *in vivo*. AK extracted from adult *C. felis* is unable to phosphorylate L-homo-arginine at an appreciable rate, which suggests that CfAK2 is present at very low levels or not at all, and that CfAK1 is most likely the dominant enzyme form in this life stage. The results of consecutive extractions also suggest that flea AK is largely soluble and not strongly associated with cellular structures. In adult fleas, CfAK1 is a highly abundant protein: based on its specific activity as purified recombinant enzyme and the specific activity of extract material, it can be calculated that CfAK1 represent >3.5% (w/w) of the soluble protein of adult fleas. Immunoblot studies with *C. felis* larvae and pupae suggest that AK is present in these life stages too. However, due to the lack of specific antibodies, it remains unclear whether this larval and pupal AK is also CfAK1, like in the adult stage, or whether CfAK2 is specifically expressed in these earlier life stages. We have investigated whether in adults fleas the *in vivo* metabolite levels relevant to arginine kinase are in a range suitable for CfAK1 function (Table 2). Given that >50% of the adult flea fresh weight is recovered as insoluble cuticle residue after perchloric acid extraction, it can be deduced that the cellular concentrations of phospho-L-arginine, L-arginine, ATP and ADP exceed 6 mM, 4 mM, 6 mM and 0.8 mM, respectively, which compares well with the values observed earlier for flight muscle extracts of the blowfly *Phormia regina* (Sacktor and Hurlbut, 1966) or of locusts and beetles (Newsholme et al., 1978), and which are in line with the measured K_M values for the recombinant CfAK1 product.

In the case of the vertebrate phospho-creatine/creatine (PCr/CK) system, several roles in cellular energy metabolism are discussed. These include temporal energy buffering, where the PCr/CK system stabilizes ATP levels under conditions where ATP consumption exceeds ATP production by glycolysis and/or mitochondrial respiration, and spatial energy buffering, where intermitochondrial space and cytosolic creatine kinase interact via the highly diffusible PCr, thereby optimizing ATP transport from the production to the consumption sites (Wallimann et al., 1992). The precise function of the phospho-L-arginine/AK system in insects in this context is still unresolved and a matter of debate. Newsholme et al. (1978) argue that in flight muscles of four insect species a transfer function (spatial energy buffering) for phospho-L-arginine from the mitochondria to the myofibrils is not plausible due to the unfavourable ratio of maximal AK activity and the maximal rate of ATP turnover. They consider a temporal energy buffer function for this metabolite as more likely. By contrast, Schneider et al. (1989) conclude that in *Locusta migratoria* flight muscles, a temporal energy buffer function is inconsistent with their results and contend that a spatial energy buffering function for the phospho-L-arginine/AK system is a more plausible scenario. However, for spatial energy buffering, a mitochondrial pool of arginine kinase is expected to be present, but in *D. melanogaster* (Wyss et al., 1995) and *Manduca sexta* flight muscles (Ellington and Hines, 1991) none can be detected. Confusingly, in other insect organs such as the *M. sexta* midgut, the presence of mitochondrial AK has been demonstrated (Chamberlin, 1997). It appears that further work is needed to resolve the functions of

phospho-L-arginine/AK in insect flight muscles. Different from the situation in flight muscles from the same organism, for femoral muscles of *L. migratoria* responsible for the intermittent jumping movements of the animal Schneider et al. (1989) propose a temporal energy buffer function for the phospho-L-arginine/AK system, that is convincingly supported by their data. The movement of fleas has similarities to locust jumping and it is conceivable that the phospho-L-arginine/AK system of this organism may also serve a temporal energy buffer function.

It should be pointed out that the role of the phospho-L-arginine/AK system is not limited to insect muscle tissue. Arginine kinase is highly expressed in growth cones of the developing central nervous system in *Schistocerca americana* (Wang et al., 1998) and the brain of *Calliphora erythrocephala* (Rivera and Langer, 1983). Furthermore, high activities of the enzyme has been found in the compound eyes of *Apis mellifera* (Kucharski and Maleszka, 1998) and *C. erythrocephala* (Rivera and Langer, 1983). Ion-transporting epithelia of the lepidopteran midgut (Chamberlin, 1997) and the locust hindgut (Chamberlin and Phillips, 1983) are also rich sources for AK. It will be of interest to explore whether the so far elusive CFAK2 has possibly a specialized function in the flea in one of these organs or in earlier developmental phases of this insect.

Arginine kinase has been proposed as a target for innovative pesticidal compounds (Brown and Grossman, 2004; Wang et al., 2009; Wu et al., 2009) due to its assumed prominent role in energy metabolism and its absence in vertebrates. However, there are several challenges ahead: the role of AK in insects has not yet been clearly defined and is likely to be distinct in different insects, organs and developmental stages. While it has been shown that mutations in the AK1 gene of *D. melanogaster* can result in lethal phenotypes (Vernon et al., 1998; Buszczak et al., 2007), this may be due to developmental defects and is no proof that pharmacological inhibition of AK in larval or adult insects will lead to the death of the organisms. Furthermore, *in vivo* expression levels of AK are extraordinarily high, most likely reaching concentrations of tens or even hundreds of μM in enzyme-expressing insect cells. Therefore, pesticidal/parasitocidal compounds acting on AK as their mode of action will have to be potent and to be present in relatively high concentrations.

This report has outlined the investigation of the phospho-L-arginine/AK system in the cat flea *C. felis* by gene identification, cloning, enzymology, molecular modelling and docking, *in vivo* protein expression and metabolite analyses. Future studies should focus on the definition of the role of CFAK1 in different flea organs, the elucidation of the role of CFAK2, and the identification of potent AK inhibitors to allow a pharmacological validation of this enzyme as a pesticidal/parasitocidal drug target.

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