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Interaction of nanoparticles with arginine kinase from *Trypanosoma brucei*: Kinetic and mechanistic evaluation

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ABSTRACT

Arginine kinase is not only absent from mammalian hosts but is critical to the survival of trypanosomes under stressful conditions and consequently its inhibition may lead to an effective treatment for trypanosomiasis. The His-tagged enzyme was cloned from *Trypanosoma brucei* genomic DNA, expressed in *Escherichia coli* BL21 DE3 cells and purified on a Ni-affinity column and by FPLC on a Superdex 200 HR. The enzyme had a specific activity of 2.92 μmol min^{-1} mg $protein^{-1}$, molecular mass of 40 kDa, temperature and pH optima of 30 °C and 7.8, and K_m and V_{max} as 2.94 mM and 0.161 μmol ml^{-1} min^{-1} (arginine substrate). The interaction of the enzyme with silver and gold nanoparticles showed a non-competitive inhibition with, respectively, 75% and 62% decrease in activity; K_i values ranged from 1.5 nM (Ag) to 3.1 nM (Au). A mechanism for this inhibition was by interaction with Cys^{271} positioned 3.3 Å from the reactive NH^1 of substrate arginine. This cysteine controls electrophilic and nucleophilic character of the guanidinium group that is crucial for enzymatic phosphoryl transfer between ADP and ATP.

1. Introduction

Trypanosomes are responsible for the economically important veterinary infections and severe human diseases. In Africa, *Trypanosoma brucei* causes sleeping sickness also known as the African trypanosomiasis, while in America, *Trypanosoma cruzi* causes Chagas disease [1–3]. Infection by the *T. brucei* is fatal if untreated. It is however unfortunate that the current treatments are beset with several shortcomings including limited efficacy, unwanted toxicity and emergence of resistant strains of trypanosomes. These and other factors highlight the need for innovative strategies to combat the disease. The absence of effective anti-trypanosomal therapies coupled with unsuccessful efforts at vaccine development occasioned by the phenomenon of antigenic variation has stimulated the numerous research investigations directed at understanding the molecular biology and biochemistry of trypanosomes. Among the biomedical target molecules which have been identified as possible drug targets in trypanosomatids is arginine kinase (AK), a phosphotransferase enzyme responsible for the reversible formation of phosphoarginine using L-arginine and ATP as substrates (Fig. 1) [4]. Phospho-arginine can act as an emergency reservoir, not only of ATP but also for inorganic phosphate [5–7].

The enzyme AK is absent in humans [8], a fact that makes it an attractive target choice for trypanocide development. These researchers demonstrated an increased activity of AK in trypanosomes when linked to oxidative stress and its inhibition, in any way possible, would be lethal to the parasite supporting the importance of AK to the survival of the trypanosomes. During the parasite life-cycle the trypanosomes are constantly exposed to the pro-oxidants in the blood of mammalian host, and consequently either the absence of AK or its decrease in activity would present serious consequences to the growth and survival of the parasites. In light of this, compounds that selectively inhibit AK are desirable and should become candidates for early development of trypanocides.

Metal nanoparticles, especially silver and gold, with their increased solubility, have been reported to possess interesting properties including anti-microbial activity [9,10], and to cause changes in enzyme activities [11–13].

Engineering nanoparticles that are capable of selective binding to enzymes, is a new research direction with exciting prospects in biomedical applications and there are two fundamental approaches used – either a direct covalent or non-covalent link [14,15]. In the former, there is multiple non-specific binding of the nanoparticle to cysteine side chains leading to a loss of enzyme activity, usually with conformational changes to protein structure. With the second, non-covalent method, either 'naked' or fully functionalised nanoparticles interact with enzymes under normal protein–protein interactions through the evolution of a loosely attached protein 'corona' around the nanoparticles. The 'naked' nanoparticles tend to irreversibly bind and deform an initial layer of biomolecules that

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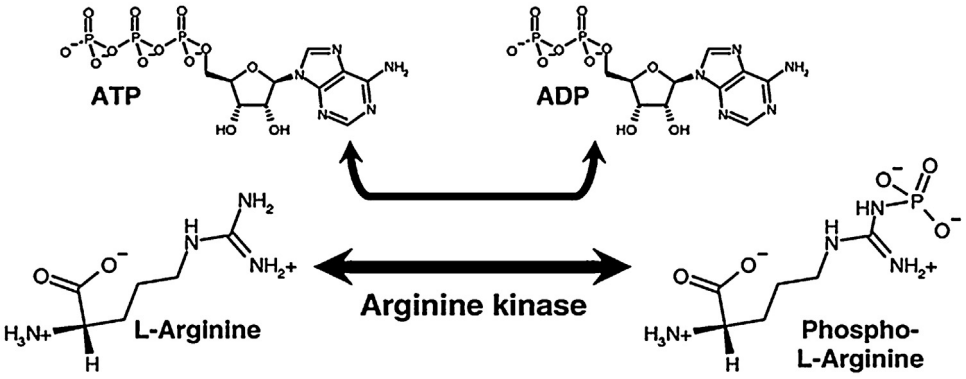

Fig. 1. Enzymatic reaction for arginine kinase.

form the first 'corona' layer, while functionalised nanoparticles, which already have an irreversible bound initial layer, act similarly by binding available biomolecules [14–16]. The binding between nanoparticles and the enzyme changes the structure of the latter and as the particle gets smaller not only does its interaction with the protein change but the composition of the protein itself changes. Consequently the 'nanoparticle-enzyme corona' has totally different biological properties in comparison to a native enzyme.

The nanoscale size of these metal nanoparticles allows their unique and remarkable properties to be exploited within the nanomedical fraternity [17–21] and to resolve several medical challenges in both infectious (malaria) and neurodegenerative diseases (Alzheimer). It is against this backdrop that the present study sought to investigate the interaction of silver and/or gold nanoparticles with a recombinant form of AK obtained from *T. brucei* (TbAK).

2. Materials and methods

2.1. Materials

Genomic DNA of *T. brucei* (strain: 927/4GUTat 10.1) was a gift from Professor Ullman, Department of Biochemistry & Biophysics, Oregon Health & Science University, Portland, Oregon, USA. Enzymes and PCR reagents were provided by Thermo Fischer Scientific, USA. A Bioflux kit was obtained from Separation Scientific (South Africa). Oligonucleotides and primers were from the Integrated DNA Technology (IDT) USA. The clone JET PCR kit, pSMART and pET-28b (+) as well as the BL21 DE3 and JM 109 strain of *E. coli* were obtained from Fermentas Life Sciences (USA). Absorbance spectroscopy was performed with a Synergy Mx (Monochromator Multi-Mode Microplate Reader, Biotek Instruments, Inc., USA). All other reagents were of analytical grade and obtained from Sigma, USA or from domestic suppliers unless otherwise stated.

2.2. Cloning of TbAK from genomic DNA [22]

The specific primers of TbAK including the forward (CAT ATG GGC TTC GGA TCA TCA AAA CCC) with *Nde*I restriction site underlined and reverse (CTC GAG CTG TTC CAC GTA CCT GC) with *Xho*I restriction site underlined were used to amplify the ORF of the TbAK gene. The PCR reaction was carried out with amplification conditions as follows: 98 °C at 3 min, 98 °C at 30 s, 62 °C for 30 s, 72 °C for 1 min, 72 °C, and held at 4 °C in a Biorad T100 thermal cycler. The 1000 bp product by PCR, excised and gel purified using a Bioflux kit was phosphorylated and blunt ligated into a pSMART vector using a T4 DNA ligase and then transformed into JM 109 cells. The plasmid containing TbAK was gel purified using the Bioflux kit and sequenced. This product showed 100% identity with the *Trypanosoma brucei* expressed product (Accession number – XP_826998.1) and a high identity with other related guanidino kinases. The plasmid was double digested and a fragment between *Nde*I and *Xho*I restriction sites containing the TbAK gene was excised from the plasmid and subcloned into pET-28b (+) expression vector previously treated with *Nde*I and *Xho*I. This plasmid transcribes under the control of T7 promoter and includes a polyhistidine tag.

2.3. Expression and purification of TbAK

A method previously described [23] was adopted with slight modification. The recombinant plasmid pET-28b (+) containing the TbAK gene was transformed into BL21 DE3 cells. The transformed cells were grown overnight, at 37 °C, in 50 ml LB culture medium containing kanamycin after which an aliquot (5.0 ml) was transferred into a 2 L flask containing auto-media culture (500 ml). This was grown at 20 °C, at 150 rpm for 36 h. Cells were then harvested (6000 × *g*, 10 min) washed twice with Tris buffered saline (Tris–HCl, 50 mM; NaCl, 150 mM; pH 7.5) and lysed (freeze-thawed [−80 °C/4 °C; 2 cycles]) in lysis buffer [NaH_2PO_4 buffer (50 mM, pH 7.6) containing NaCl (300 mM), glycerol (10%), Tween (0.25%), imidazole (10 mM), mercaptoethanol (10 mM), phenylmethylsulphonylfluoride (1 mM)]. The lysed cells were centrifuged (2700 × *g*, 30 min) and the supernatant centrifuged further (100,000 × *g*, 90 min) after which the supernatant (150 ml) was loaded onto a Ni-nitrilotriacetic acid affinity column. The column was washed with the same lysis buffer until a steady zero-baseline was obtained and then the fusion protein was eluted with increasing amounts of imidazole (0-500 mM) in Hepes buffer (10 mM, pH 7.5). The eluted protein was concentrated using vivaspin (GE Healthcare, Sweden) and the concentrated protein further purified by FPLC on a Superdex 200 HR 10/30 with Hepes buffer (25 mM, pH 7.6) containing glycerol (15%), EDTA (0.1 M), and KCL (1 M) at a flow rate (1 ml min^{-1}). Proteins were resolved by SDS-PAGE in order to confirm purity of fractions before pooling. All purification procedures were carried out at 4 °C.

2.4. SDS-PAGE

The effectiveness of the purification process was determined by SDS-PAGE [24] on samples exhibiting TbAK activity. Samples from each purification step (20 μl) and a standard molecular weight marker (10-170 kDa) were electrophoresed on 12% SDS-PAGE at 200 V. The gels were stained with Coomassie Brilliant Blue R-250, then destained in methanol:acetic acid:water (3:1:6 v/v/v). The molecular weight of the partially purified TbAK was determined using a standard curve of log molecular weight versus distance migrated.