Enzymatic properties and determination of amino acid residues essential for substrate catalysis of phosphagen kinase from *Schistosoma japonicum*

Shinji Tokuhiro

Department of Environmental Health Sciences, Kochi Medical School, Oko, Nankoku City, Kochi, 783-8505, Japan

1. Introduction

Phosphagen kinases (PKs) catalyze the reversible transfer of phosphate groups from MgATP to substrates containing a guanidine group to yield phosphagen and MgADP. To date, eight PKs have been identified, including creatine kinase (CK), arginine kinase (AK), taurocyamine kinase (TK), hypotaurocyamine kinase, lombricine kinase, glycocyamine kinase, thalassemine kinase, and opheline kinase. In trematodes, PK has been found in *Schistosoma mansoni* and *Paragonimus westermani*, which have unique two-domain TKs that show a different structure from monomeric and dimeric PKs, such as AK and CK. On the basis of the structural differences of

PKs between vertebrates and invertebrates, we hypothesized that invertebrate PKs from parasites and their intermediate hosts have potential as novel anthelmintic drug targets.

In the current study, we report the enzymatic properties and gene structure of PK in the trematode *Schistosoma japonicum*.

2. Materials and methods

Total RNA was extracted from adult worms of *S. japonicum* (Sorsogon strain). mRNA was then purified and cDNA was synthesized. The open reading frame (ORF) of a full-length contiguous dimeric construct [(domain1 (D1) domain2 (D2)] of the *S. japonicum* PK and its truncated domains, D1 and D2, were to be amplified via PCR. The ORF of each domain was subcloned into an expression vector through sequence determination by cycle sequencing.

Recombinant enzymes of D1, D2, and D1D2 were overexpressed in *Escherichia coli* by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside at 25°C, and the enzymes were purified for the enzyme assay. Enzyme activity was measured using the nicotinamide

adenine dinucleotide hydrate-linked spectrophotometric assay at 25 C determined for the forward reaction or phosphagen synthesis. The kinetic constants were calculated using the software written by Dr. R. Viola (Enzyme kinetic Program, ver. 2.0).

To compare the exon/intron organization between *S. japonicum* TK (SjTK) and other PKs, genomic DNA was isolated from the *S. japonicum* Sorsogon strain. The sequence of SjTK was determined.

SjTK mutants were constructed to determine the effect of mutation on the kinetics of substrate catalysts from SjTK-inserted plasmid DNA by site-directed mutagenesis. To identify amino acid residues that are critical for taurocyamine binding, we performed alanine scanning mutagenesis at positions 57–63 on the guanidino specificity (GS) region of the SjTK D1, which is believed to be involved in guanidino-substrate recognition. We also replaced the second glutamates of the NEED motif in SjTKD1 and the corresponding CEED motif in SjTKD2 with G residue. In addition, the Y84 residue of SjTK domain 1 was replaced with R, H, K, I, A, and G.

3. Results and discussion

3.1. Enzymatic properties of *S. japonicum* PK

S. japonicum has a two-domain TK consisting of 360 and 356 amino acid residues for D1 and D2, respectively. Each of the recombinant SjTK has specific activity for the substrate taurocyamine. A comparison of the enzymatic properties of the two domains of SjTK suggests that D1 has a higher turnover rate catalyzing the enzyme–substrate complex to obtain product and enzyme (k_{cat} = 52.91), and D2 has a higher affinity for taurocyamine (K_m^{Tauro} = 0.53 ± 0.06). The full-length protein exhibited higher affinity for taurocyamine (K_m^{Tauro} = 0.47 ± 0.03) than did the truncated domains (D1 = 1.30 ± 0.10, D2 = 0.53 ± 0.06). D1D2 also exhibited higher catalytic efficiency (k_{cat}/K_m^{Tauro} = 82.98) than D1 (40.70) and D2 (29.04).

3.2. *Evolutionary processes*

We determined the exon/intron organization (gene structure) of SjTK and compared its splicing sites with those of other PKs. SjTK has six introns in D1, four introns in D2, and a bridge intron between D1 and D2. All of these introns typically began with GT and ended with AG. Although a complete structure of SjTKD2 was not detected, these introns were partly conserved

between *S. mansoni* TK and mollusc AK.

3.3. *Role of both domains in contiguous dimer structure*

A mutation in SjTKD1 or SjTKD2 (D1E222G or D2E225G) caused a complete loss in activity for the substrate taurocyamine. Likewise, a double mutant (D1E222GD2E225G) in the D1D2 exhibited a complete loss in activity for the substrate taurocyamine. However, catalytic activity in the contiguous dimer remained in both D1 (D1D2D1E222G) and D2 inactive mutants (D1D2D2E225G), suggesting that efficient catalysis of SjTKD1D2 is dependent on the activity of D1 and D2. The catalytic efficiency of the mixture of both single domains (WTD1 + WTD2) showed similar enzymatic properties (K_m^{Tauro} = 0.68; V_{max}/K_m^{Tauro} = 137.04) to WTD1D2 (K_m^{Tauro} = 0.47; V_{max}/K_m^{Tauro} = 144.30). This result suggests that the contiguous dimeric structure is not essential for the catalytic efficiencies of both domains of SjTK.

3.4. *Identification of critical amino acid residues in the GS region of SjTKD1*

To identify amino acid residues critical for taurocyamine binding, we

performed alanine scanning mutagenesis at positions 57–63 on the guanidino specificity (GS) region of the SjTKD1, which is considered to be involved in guanidino-substrate recognition. Almost mutants of SjTK (P57A, K58A, L60A, L61A, and P62A) exhibited specific activity for the substrate taurocyamine. On the other hand, replacement of residue 63 mutants (R63A and R63Y) lost activity for taurocyamine, suggesting that these residues are associated with taurocyamine binding.

3.5. *Comparison of Y84 mutants of SjTKD1*

In the present study, we replaced Y84 with R, H, K, and I residues observed in native enzymes and with A and G residues. Enzymatic properties of the six mutants D1Y84R, D1Y84H, D1Y84K, D1Y84I, D1Y84A, and D1Y84G were determined. Although the activities of each mutant decreased (V_{max} = 2.36–67.50 μmol Pi/min/mg protein), they have specific affinity for taurocyamine (K_m^{Tauro} = 3.19–10.04 mM). However, these decreases in activity suggest that the Y84 residue has no effect on substrate specificity, despite its important role in substrate catalysis.