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Functional identity of the active sites of crustacean and viral thymidylate synthases

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ABSTRACT

Thymidylate synthase (TS) catalyzes the synthesis of deoxythymidine monophosphate (dTMP), which is an essential precursor for DNA synthesis. The rationale underlying drug design is to identify compounds that differentially inhibit a viral or parasite enzyme *vs.* the host homologue. We studied the TS of the white spot syndrome virus (WSSV TS) and the corresponding TS from the host, the marine invertebrate shrimp *Litopenaeus vannamei*. TS is the only *de novo* source of dTMP and is essential for host and viral DNA replication. To establish proof of principle, we cloned a full-length TS cDNA from the white shrimp *L. vannamei* (shrimp TS) that corresponds to a deduced sequence of 289 amino acids and over-expressed it to study inhibition of both shrimp and viral TSs. Steady-state kinetic parameters for both TSs are similar, and dissociation (K_d) or half maximal inhibitory concentration constants (IC_{50}) did not show differential inhibition between the folate analogues. Differences in their amino acid sequence are not reflected in theoretical molecular models of both TSs, since both appear to have identical active sites. These results suggest that the eukaryotic TS active site is very constrained into the functional residues involved in reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP).

1. Introduction

Thymidylate synthase (TS, E.C. 2.1.1.45) catalyzes the synthesis of 2′-deoxythymidine-5′-monophosphate (dTMP) from 2′-deoxyuridine-5′-monophosphate (dUMP) and 5,10-methylene-5,6,7,8-tetrahydrofolate (MTHF) as cofactor. TS is the only *de novo* source of dTMP in many organisms from all kingdoms, including some viruses, and its enzymatic inhibition becomes a hindrance for DNA replication (Carreras and Santi, 1995). TS is a strict dimer, since both active sites are formed by residues of both subunits, and has a non-allosteric ligand-induced conformational change (Stroud and Finer-Moore, 2003). Therefore, TS stands as an important model to understand the structure–function relationships and as a paradigm for structure-directed drug design against proliferative diseases.

TS has been thoroughly studied in many species by means of protein crystallography, site directed mutagenesis, and kinetics (Finer-Moore et al., 2003; Stroud and Finer-Moore, 2003), although it has not been examined much in invertebrates or non-model organisms. Since TS is present practically in all kingdoms, it was anticipated that it would be present in crustaceans, although it has not been described before in them. The presence of TS in the white spot syndrome virus genome, described as ORF54 (van Hulten et al., 2001) led to consideration of this gene as an antiviral target (Li et al., 2004). The white spot syndrome virus (WSSV) is a pathogen of cultivated shrimp and an economic menace to aquaculture in developing countries, so it is important to develop specific antivirals directed towards the elimination of this virus. WSSV has a circular double stranded DNA genome of approximately 300 kb, which encodes TS, among other gene products (Leu et al., 2009).

Differential targeting of enzymes as a drug target design approach is common in medicinal chemistry. Dihydrofolate reductase (DHFR) inhibitors that have antibacterial properties, such as trimethoprim, are still used, while investigations on TS inhibitors against parasites are currently underway (Atreya et al., 2003). Even if the simple inhibition of TS is not validated as a therapeutic approach, its inhibitors may potentiate the action of other compounds targeted to thymidine kinase or DNA polymerase, by reducing the deoxynucleotide pools that compete with these compounds, as shown in Scheme 1 (Panel A) (Prichard et al., 1993). Previous studies on WSSV TS show that it is expressed and translated during viral infection (Li et al., 2004). Much of

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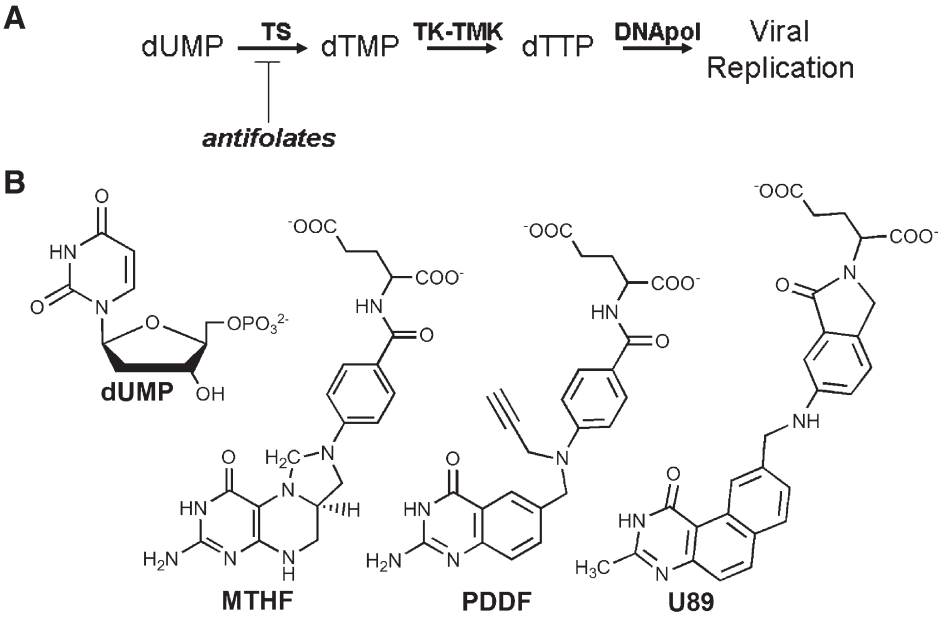

Scheme 1. Panel A. DNA replication pathway for the WSSV and drug targeting enzymes. Panel B. Chemical structure of substrates and inhibitors for thymidylate synthase.

the research in this area is focused on the innate immunity against the virus, including as an antiviral strategy the use of double stranded RNA to interfere with the expression of viral genes (RNAi) (Liu et al., 2009).

In order to assess the potential for WSSV TS inhibition as an antiviral strategy it is important to study nucleotide metabolism in crustaceans. Therefore, we cloned and over-expressed the Pacific white shrimp (*Litopenaeus vannamei*) TS to address differential inhibition studies against the white spot syndrome virus TS.

2. Materials and methods

2.1. Cloning and RACE

TS from white shrimp *L. vannamei* (shrimp TS) was amplified from a cDNA library constructed with the Zap Express cDNA Synthesis System (Stratagene) (Romo-Figueroa et al., 2004). Degenerate primers TSFw and TSRv (see Fig. 1) were designed over conserved regions of aligned TS amino acid sequences obtained from GenBank. The 5′ and 3′ ends of the shrimp TS coding sequence were completed by RACE, combining the specific primers TSFw1 and TSRv1 with universal primers located in the library's vector. Complete cDNA sequence was amplified using the primer set TSUTRFw and TSUTRRv from the cDNA library, thoroughly sequenced and deposited in GenBank under accession number **FJ972199.**

The cDNA of WSSV TS was amplified from a previously synthesized cDNA library of WSSV-infected shrimp (Clavero-Salas et al., 2007) constructed with the SMART System (Clontech). The primers for WSSV TS were designed from the nucleotide sequences reported in GenBank (accession numbers **AAK77723**, **AAL88992,** and **NP477589**).

2.2. Recombinant expression and purification

The amino acid sequence of both shrimp TS and WSSV TS were optimized by codon usage and obtained as synthetic genes (GeneArt AG). Both synthetic genes were cloned in pET11a (Novagen) into the *Nde*I and *Bam*HI sites. Once verified by DNA sequencing, the clones were used to transform *E. coli* BL21(DE3). The pET11a-shrimp TS clone was grown in Luria Broth medium at 37 °C in an orbital incubator until the A_{600nm} reached 0.6; then, IPTG was added to a final concentration of 0.5 mM and incubation was continued for16 h. For the viral enzyme, the pET11a-wssvTS clone was grown in Terrific Broth at 25 °C until the A_{600nm} reached 0.6; then, IPTG was added to a final concentration of 0.2 mM and incubation was continued for 16 h. In both cases, the cell pellet containing the recombinant TS was obtained by centrifugation at 4500×*g* for 20 min at 4 °C and washed with 0.9% NaCl. For the purification of the recombinant TSs, the cell pellet obtained from 1 L of culture was dissolved in 100 mL of 20 mM Tris–HCl, pH 7.5, plus 5 mM DTT and 0.5 mM PMSF, and then sonicated six times for 1 min in an ice-bath. The cell debris was removed by centrifugation at 35,000×*g* for 30 min at 4 °C. Nucleic acids were precipitated by adding 15 mL of 5% streptomycin sulfate to each 100 mL of the supernatant fraction and removed by centrifugation after stirring for 10 min in an ice-bath.

TSs were isolated in the supernatant fraction between 65% and 80% saturation of ammonium sulfate. The precipitated protein was extensively dialyzed against 25 mM potassium phosphate, pH 7.5, and loaded onto a Q-Sepharose column, on an AKTA chromatographer. The column was washed with 25 mM potassium phosphate, pH 7.5, plus 0.1 M NaCl and eluted with a 50 mL linear gradient of 0.1 to 0.2 M NaCl in 25 mM potassium phosphate, pH 7.5. The fractions containing pure TS were pooled and frozen at −80 ºC after precipitation with ammonium sulfate. The net yield was about 5 mg of pure TS from 1 L of culture. Since both TSs were purified without any affinity tag, we validated the identity of the over-expressed proteins by mass spectrometry at the Proteomics Facility of the Bio5 Institute, University of Arizona (Tucson, AZ, USA). All MS/MS samples were analyzed using Sequest (ThermoFinnigan, San Jose, CA, USA; version 27, rev. 12) and X!Tandem (www.thegpm.org; version 2007.01.01.1). Scaffold (version Scaffold_2_03_01, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications.

2.3. Steady-state kinetics and inhibition

TS activity was measured using the spectrophotometric assay for the reductive methylation of dUMP by MTHF (Wahba et al., 1962), with modifications (Maley et al., 1995). A unit of TS activity is defined as the amount of enzyme that converts one micromole of dUMP to dTMP per min at 30 °C under the conditions of the assay. Protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce). For the determination of kinetic parameters, initial velocities were obtained with 0.25 µM of shrimp or WSSV TS and combinations of various concentrations of MTHF (0–150 µM) and dUMP (0–20 µM). Analysis of steady-state kinetics was done with the GraFit software.