

**Kinetic Studies of Human Tyrosyl-DNA Phosphodiesterase, an Enzyme in the
Topoisomerase I DNA Repair Pathway**

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Running title: kinetic studies of tyrosyl-DNA phosphodiesterase

SYNOPSIS

Tyrosyl-DNA phosphodiesterase (TDP) cleaves the phosphodiester bond linking the active site tyrosine residue of topoisomerase I with the 3'-terminus of DNA in topoisomerase I-DNA complexes which accumulate during cancer treatment of camptothecin. In yeast, TDP mutation confers a 1000-fold hypersensitivity to camptothecin in the presence of an additional mutation of *RAD9* [Pouliot, J. J., Yao, K. C., Robertson, C. A. and Nash, H. A. (1999) *Science* **286**, 552-555]. Based on the recently solved crystal structure, human TDP belongs to a distinct class within the phospholipase D (PLD) superfamily in spite of very low sequence homology [Interthal, H., Pouliot, J. J. and Champoux, J. J. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12009-12014, and Davies, D. R., Interthal, H., Champoux, J. J. and Hol, W. G. J. (2002) *Structure* **10**, 237-248]. To understand enzymatic mechanism of this novel enzyme, and to facilitate inhibitor screening of human TDP, we have expressed and purified recombinant human TDP and a continuous colorimetric assay in a 96-well format was also developed using *para*-nitrophenyl thymidine-3'-phosphate as substrate. This assay system is able to detect enzymatic activity at enzyme concentrations as low as 15 nM. Purified recombinant human TDP cleaved *para*-nitrophenyl thymidine-3'-phosphate with K_m and k_{cat} values of $211.14 \pm 23.83 \mu\text{M}$ and 8.82 ± 0.57 per min in the presence of Mn^{2+} .

Key Words: Tyrosyl-DNA phosphodiesterase, Phospholipase D, High-throughput screening

INTRODUCTION

In eukaryotic cells, DNA topoisomerase I (Topo I) is an enzyme that relaxes DNA supercoiling and relieves torsional strain of DNA during replication, repair and transcription processes by making single stranded breaks on DNA, unwinding and religating the DNA ends in the cleaved strand [1]. After binding to DNA, Topo I cleaves double-stranded DNA on one DNA strand via a reversible trans-esterification reaction, in which the 5'-phosphorus of a phosphodiester bond is attacked by the hydroxy group of the active site tyrosine residue. Thus DNA becomes covalently linked to Topo I via the 3'-phosphate and forms a catalytic intermediate i.e. covalent Topo I-DNA complex. The phosphodiester bond formed between the tyrosine residue of Topo I and DNA is energy-rich and transient in nature. Under normal circumstances, Topo I is released from the catalytic intermediate after DNA becomes relaxed and religated. However, Topo I-linked DNA breaks would accumulate when Topo I acts on damaged DNA containing lesions such as thymine dimers, abasic site, and mismatched base pairs [2] or when Topo I-DNA complexes are bound by camptothecin or its derivatives rendering Topo I inactive in carrying out DNA religation [3]. Consequently, a normally transient break in DNA could become a long-lived double-stranded break upon collision of Topo I-DNA complex and DNA replication machinery. Accumulation of double-stranded DNA breaks above a threshold ultimately could cause cell death [2].

Camptothecin, a plant alkaloid originally isolated by Wani and Wall in 1966, inhibits Topo I at religation step selectively after cleaving the DNA [4]. Treatment of cancer cells with camptothecin-like analogs results in inhibition of DNA replication, chromosomal fragmentation, cell cycle arrest at G1 and G2 phase, and eventually programmed cell death [5]. However, non-mechanism related toxicity and adverse effects have limited the clinical utility of camptothecin [6]. Recent identification of tyrosyl-DNA phosphodiesterase (TDP) as the enzyme that resolved the Topo I-DNA covalent complexes might provide us another important enzyme target in the same pathway for therapeutic intervention.

TDP was first noted as an enzyme in yeast with activity that specifically cleaves the phosphodiester bond in Topo I-DNA complex [7]. Subsequently, the gene encoding TDP in *S. cerevisiae* was isolated and characterized [8]. In yeast, TDP mutation alone causes little change in phenotype. However, with an additional mutation of *RAD9* gene

providing repair-deficient background, mutant yeasts carrying null mutation of TDP was found to be hypersensitive to camptothecin treatment [8]. Similarly, a topoisomerase T722A mutation that increases the stability of Topo I-DNA covalent complex, thus mimicking the cytotoxic effect of camptothecin [9], has also rendered low viability of the yeast mutant carrying TDP mutation [10]. Results from genetic studies together with in vitro substrate specificity studies demonstrate that in vivo TDP cleaves Topo I from the Topo I-DNA complexes in Topo I-DNA repair pathway.

TDP homologs have been identified for several other species including *Drosophila melanogaster*, *C. elegans*, *S. pombe*, *Mus musculus* and *homo sapien*. Database searches showed that TDP does not share significant sequence homology with any other gene of known functions. On the basis of the presence of the signature HKD motifs, TDP was recently suggested to be a member in a distinct class of the phospholipase D (PLD) superfamily of enzymes that is comprised of a diverse set of proteins including PLDs from bacteria to mammals, a bacterial toxin, and some bacterial nucleases [11]. The PLDs hydrolyze the phosphodiester bond in the phospholipid such as phosphatidyl choline to produce phosphatidic acid and a free head group (often choline). The nucleases catalyze the hydrolysis of DNA phosphodiester bonds. Sequence alignments of PLDs revealed that, with the exception of two nucleases, most PLDs contain two copies of highly conserved HxK(x)₄D(x)₆GSxN sequence, termed HKD motif [12,13], which has been implicated in the catalytic mechanism. For human TDP, mutations of the most conserved histidines and lysines of tentatively assigned HKD motifs also rendered human TDP with reduced enzymatic activity [14]. Recently solved crystal structure of human TDP further confirms that TDP shares a similar protein fold with members of the PLD superfamily and its active site contains the pairs of conserved histidine and lysine residues of the HKD motifs [15].

In this study, we report the development of a sensitive colorimetric assay in 96-well format, the identification of a cofactor, and characterization of kinetic parameters of the human tyrosyl-DNA phosphodiesterase activity.

EXPERIMENTAL

Materials

All reagents were molecular biology grade unless otherwise indicated. The expression vectors pET-14b and pBAD/Thio-TOPO and pPICZB were purchased from Novagen (Madison, WI, U.S.A.) and Invitrogen (Carlsbad, CA, U.S.A.). The TrizolTM and reagents for PCR and RT-PCR were obtained from GIBCO Life Technologies, Inc (Rockville, MD, U.S.A.). Oligonucleotides used for PCR were from MWG Biotech (Charlotte, NC, U.S.A.). Protease inhibitor cocktail was from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). HiTrap chelating agarose was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). BCA reagent for protein concentration determination was from Pierce (Rockford, IL, U.S.A.).

Cloning of wild-type and mutant human TDP cDNA

Database searches identified a full-length human cDNA (National Center for Biotechnology Information Accession no. NM_018319) that shares substantial similarity to the yeast TDP sequence (gene YBR223c; Genbank Z36092.1). This full-length human TDP cDNA was amplified from cDNA pools prepared from total RNA of human cultured fibrosarcoma cells HT1080 (from ATCC CCL-121). Briefly, total RNA from HT1080 cells was isolated by cell lysis with TrizolTM Reagent followed with RNA precipitation by isopropyl alcohol. Next, the obtained RNA was reverse transcribed into cDNAs with ThermoScript RT-PCR system, and used as templates for PCR reactions to amplify the full-length human TDP cDNA. The resulting PCR product was cloned into the BamHI site of the vector pPICZB and the insert sequence was confirmed by nucleotide sequencing to encode human TDP. The human TDP coding sequence thus obtained differs in the following positions from the published sequence of the predicted human gene FLJ11090 (National Center for Biotechnology Information Accession no. NM_018319). Unlike nucleotide changes of 393 C to G and 1629 C to T that do not lead to amino acid changes, nucleotide changes of 378 A to T and 481 G to A lead to amino acid substitutions of R126S and G161R, respectively.

Two human TDP variants containing deletion of amino-terminal 39 (huTDPN Δ 39) and 174 (huTDPN Δ 174) amino acids were generated by the PCR mutagenesis method. For PCR amplification of human TDP variants, the full-length human TDP cDNA was provided as templates. To generate the huTDPN Δ 39 variant by PCR mutagenesis, oligonucleotides of 5'-GCAGCAAATGAGCCCAGGTACACCTGTTCC-3' and 5'-GGAGGGCACCCACATGTTCCCATGC-3' were used as the forward and reverse primer. Similarly, oligonucleotides of 5'-AAGTATAACTCTCGAGCCCTCCACATCAAGG-3' and 5'-GGAGGGCACCCACATGT TC CCATGC-3' were used as primers to generate the huTDPN Δ 174 variant.

Generation of expression constructs to produce wild-type and mutant human TDP

Full-length human TDP cDNA and PCR products of the human TDP deletion mutants were ligated into pBAD/Thio-TOPO vector separately according to the manufacturer's instruction. Restriction enzyme mapping and DNA sequencing confirmed the identity of the resultant plasmids pBAD/Thio-huTDP, pBAD/Thio-huTDPN Δ 39, and pBAD/Thio-huTDPN Δ 174.

Production and refolding of recombinant human TDP from *E. coli*

Wild-type and mutant human TDP enzymes were expressed in *E. coli* TOP10 cells bearing pBAD/Thio-huTDP, pBAD/Thio-huTDPN Δ 39 and pBAD/Thio-huTDPN Δ 174, respectively. After induction with 0.02% of arabinose for 2 h, *E. coli* cells were pelleted and broken in lysis buffer by sonication. Cell lysate was separated into the soluble and insoluble fractions by centrifugation. The expression levels and the solubility of recombinant human TDP proteins were analyzed by SDS-PAGE. The insoluble fraction containing human TDP was then solubilized in 8 M urea/20 mM sodium phosphate, pH 7.5/0.5 M NaCl/protease inhibitors. After centrifugation at 12,000g for 30 minutes to remove insoluble particulates, urea-denatured human TDP in solubilized lysate was purified with a Ni²⁺-charged metal chelating column equipped with AKTA prime purification system (Amersham Pharmacia Biotech). Briefly, the samples were loaded onto the NiCl₂-charged chelating column equilibrated with loading buffer (20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, and 8 M urea). The column was first washed with loading buffer followed by washing with wash buffer (20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 15 mM imidazole, 8 M urea) and the

final elution was carried out with the elution buffer (20 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 400 mM imidazole, 8 M urea). Individual fractions containing human TDP were pooled and analyzed by SDS-PAGE. Purified human TDP was refolded by stepwise dialysis against refolding buffer (100 mM NaCl, 100 mM Tris·HCl, pH 8.0, 2 mM DTT, 1% CHAPS) to lower the urea concentration by 2 M at each step. After refolding, purified human TDP protein was stored at -20°C.

Cloning, expression, and purification of recombinant yeast TDP as the control

The full-length coding sequence for yeast TDP (gene YBP223c; Genbank Z36092.1) was PCR-amplified directly from *S. cerevisiae* genomic DNA with the forward primer, 5'-GCTGGATCCCTCCCGAGAAACAAATTTCAA-TGG-3', and the reverse primer, 5'-TCGGGATCCATTACTAGTCGTTCTCATGACGAGCAAGG-3'. The amplified DNA fragments were digested with BamHI and then ligated into the BamHI sites of the vector pET14b (Novagen). The resultant expression construct pET14b-scTDP encodes a His tag and a thrombin cleavage site at the N-terminus of yeast TDP and was confirmed by restriction enzyme mapping and by nucleotide sequencing. The yeast TDP coding sequence obtained in our study differs from the one published in Genbank in the following two positions. Nucleotide changes of 148 G to A and 215 A to G confer the amino acid substitutions of V50I and E72G, respectively. Using this pET system, N-terminal His-tagged wild-type yeast TDP was produced in *E. coli* BL21(DE3)pLys cells grown in LB medium containing 50 µg/ml ampicillin at 37°C by the induction of 1 mM isopropyl-β-D-thiogalactoside. After being induced for 3 hours, *E. coli* were pelleted by centrifugation, then resuspended in cell lysis buffer (20 mM sodium phosphate, pH 8.0) containing protease inhibitor cocktail and lysed by sonication. After centrifugation, the supernatant was loaded onto a DEAE-sepharose column, and eluant containing his-tagged yeast TDP was then purified with HiTrap Chelating agarose equipped with AKTA Prime purification workstation (Amersham Pharmacia Biotech) as described above; except in the absence of 8 M urea. The purified protein was then dialyzed against the storage buffer (50 mM KCl, 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 2 mM DTT) and then stored in 20% glycerol at -20°C.

Protein identification by mass spectrum analysis

Purified proteins were verified by mass spectrometry (the Mass spectrometry core facility, Beckman Research Institute, City of Hope, Duarte, CA). Proteins were trypsinized, and the resultant peptides were loaded onto the LC/MS system (ThermoFinnigan, San Jose, CA, U.S.A). Fragmentation patterns detected in MS/MS spectra were used to assure protein identity by finding peptides with sequences matched. The analysis showed 7 matches (20% of amino acid sequence) for yeast TDP and 22 matches (36% of amino acid sequence) for both of the human TDP deletion variants.

Synthesis of the chromogenic substrate

To develop a chromogenic assay for TDP, we chose *para*-nitrophenyl thymidine-3'-phosphate (T3'P-*p*NP) as the substrate for detecting TDP activity. This compound contains a phosphodiester bond between the phosphate group at the 3' position of thymidine and the hydroxy group of the *para*-nitrophenol to mimic the phosphodiester bond in topoisomerase-DNA complex. The hydrolysis of phosphodiester bond in T3'P-*p*NP would release free *para*-nitrophenol that absorbs light at 415 nm. T3'P-*p*NP was synthesized from 5'-*O*-*para*-methoxytritylthymidine and *p*-nitrophenyl phosphodichloridate using the procedure reported by Turner and Khorana [16].

Development and optimization of Chromogenic Assay for TDP

The enzymatic reactions were performed in 96-well plates with a final volume of 200 μ l in each well, and in assay buffer containing 50 mM Tris-HCl, pH 7.5 and 100 mM NaCl at 37°C. The continuous changes in optical density at 415 nm were monitored with an Ultramark Microplate Imaging System (Bio-Rad, Hercules, CA, U.S.A.). The extinction coefficient (ϵ) of *para*-nitrophenol was determined to be 15,000 $M^{-1}\cdot cm^{-1}$ under assay conditions. The nmoles of the product i.e. *para*-nitrophenol were calculated from the absorbance at 415 nm with the equation $\Delta A = \epsilon \cdot \Delta C \cdot L$. The requirement of cofactor for the TDP enzymatic activity was examined by determining specific activity of TDP by following the cleavage of 1 mM of substrate by 0.125 μ M of enzyme in reaction buffer containing increasing concentrations of divalent ions. The optimum pH was examined with 100 mM NaCl, 5 mM $MnCl_2$, 1 mM of substrate, 0.125 μ M enzyme and 50 mM Tris-HCl at different pH values ranging from pH 7 to 9.

The dependence of enzymatic activity of TDP on salt and DTT was also examined separately in the presence of increasing concentrations from 25 to 500 mM NaCl, and 1-10 mM of DTT, respectively.

Determination of K_m , V_{max} , and k_{cat} of TDP activity

Enzymatic reactions were carried out in 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM MnCl₂, 1 mM DTT and 0.125 μM enzyme with different concentrations of substrate ranging from 25 μM to 2000 μM. Increases in optical density at 415 nm were monitored and the amount of released products was calculated as described above. The specific activity was determined as nmoles of product/min/μg of enzyme. K_m and k_{cat} values for various recombinant TDP enzymes were determined by the following procedure. Initial velocities (v) were determined after fitting the linear portion of the kinetic curve using MATLAB (The MathWorks Inc., Natick, MA, U.S.A.). The Lineweaver-Burk treatment of data gave a linear plot of $1/v$ versus $1/[\text{substrate}]$. According to rearranged Michaelis-Menten equation, $1/v = 1/V_{max} + K_m/V_{max} \cdot 1/[\text{substrate}]$, the K_m and V_{max} were determined from the Lineweaver-Burk plot and k_{cat} was determined by $k_{cat} = V_{max} / [\text{enzyme}]$.

RESULTS

Production of recombinant human TDP variants

Database searches of the human ortholog to the yeast TDP cDNA sequence revealed a full-length human cDNA, FLJ11090, that encodes a protein of 608 amino acids. By the MULTALIN program, the sequence of FLJ11090 shares a 14 % identity with the yeast TDP gene sequence, and a 97 % identity with the corresponding sequence of the partial human TDP cDNA sequence reported by Pouliot and coworkers (Figure 1, [8]). To obtain recombinant human TDP proteins in sufficient quantities for in vitro studies, we produced human TDP in an *E. coli* expression system using the pBAD/Thio-TOPO expression vector. The expression level of the full-length human TDP from our initial attempts was low. In contrast, we were able to produce yeast TDP abundantly from *E. coli* as soluble recombinant protein (data not shown).

Two human TDP variants were expressed in bacteria abundantly from cells bearing plasmid pBAD/THIO-huTDPN Δ 39 or pBAD/THIO-huTDPN Δ 174, however, as insoluble material forming inclusion bodies. Recombinant proteins were solubilized by urea, then purified as denatured protein with a metal chelating column to apparent homogeneity as shown by SDS-PAGE after Coomassie brilliant blue staining (Figure 2). Final yield of purified protein is approximately 5 mg/L of *E. coli* for both huTDPN Δ 39 and huTDPN Δ 174. Refolding was simply carried out by dialysis to remove the denaturant agent i.e. urea.

Development and optimization of chromogenic assay for TDP enzymatic activity

To overcome the inconvenience of gel-based assay previously used to measure the TDP enzyme activity in which substrate was a peptide fragment of Topo I containing active site tyrosine conjugated to the 3' phosphate group of oligonucleotide [14], we chose to develop a chromogenic enzymatic assay for TDPs with *para*-nitrophenyl thymidine 3'-phosphate (T3'P-*p*NP with molecular weight of around 443 Da) as substrate. The TDP enzymatic activity could be continuously monitored as an increase of optical density at the wavelength of 415 nm while the chromophore (i.e. *para*-nitrophenol) gets released upon hydrolysis of the phosphodiester bond (Figure 3). This

chromogenic assay was easily adapted to the 96-well plate format to facilitate high throughput screening of inhibitors in the future. When enzyme reactions were carried out with 0.125 μM of refolded human TDP (human TDPN Δ 39), and 1 mM of T3'P-*p*NP in Tris buffer at 8.5, the V_{max} was determined to be only 0.116 ± 0.021 $\mu\text{M}/\text{min}$ indicating a rather low enzymatic activity that is, however, comparable to that of soluble recombinant yeast TDP purified from *E. coli* (Table 1). We examined if small increase of OD₄₁₅ could be due to hydrolysis of substrate by water by heating the enzyme at 70°C for 15 minutes prior to the assay. The enzyme reactions carried out with heated TDPs produced no increase of OD₄₁₅, confirming that the hydrolysis of the tyrosine-DNA phosphodiester bond at this low basal level was indeed produced by the basal activity purified enzymes.

To optimize conditions for the TDP activity assay, we subsequently examined the dependence of human TDP activity on divalent ions, pH, salt, and reducing reagent. First, we examined the dependence of TDP activity on Mg²⁺ and Mn²⁺ concentration ranging from 0.1 to 10 mM of divalent ions. Magnesium showed minimal effect on the enzymatic activity of human TDP. In contrast, in the presence of 0.1 mM Mn²⁺ a 4-fold increase of human TDP activity was observed. As the Mn²⁺ concentration increased, human TDP activity increased. This Mn²⁺ concentration-dependent effect on TDP activity approached a plateau at 5 mM of Mn²⁺ where enzymatic activity was increased approximately by 10-fold. Similar effects of Mn²⁺ and Mg²⁺ to enzymatic activity were observed for the yeast TDP (Table 1). Altogether, data suggested that recombinant human TDP enzymes were refolded back to a conformation that possess comparable enzymatic activity of the recombinant yeast TDP which was produced and purified as soluble enzyme without undergoing denaturation and refolding.

Human TDP enzymatic activity was examined at various pH values within the buffering range of Tris buffer (pH 7–9) in the presence of Mn²⁺ (Figure 4). The optimum pH for huTDP enzymatic activity was determined to be 8.0–8.5.

Finally, the dependences on concentration of salt and reducing reagent were examined in Tris-HCl, pH 8.5 containing Mn²⁺ ions. It showed the enzymatic activity of human TDP did not change in the salt concentration from 25 mM to 500 mM and in the DTT concentration from 1 mM to 10 mM. The reaction condition for TDP activity

was optimized as 50 mM Tris·HCl, pH 8.5, 5 mM MnCl₂, 100 mM NaCl, 1 mM DTT. Under this condition, V_{max} was determined for huTDPNΔ39 and huTDPNΔ174 to be 1.079 and 0.182 μM/min, respectively (Table 1).

Initial velocities of enzymatic reactions carried out by human TDP i.e. human TDPNΔ39 at enzyme concentrations from 3 nM to 500 nM showed a linear relationship for enzyme concentrations from 15 nM to 500 nM (Figure 5) indicating that, unlike some obligatory homodimeric enzymes, the specific activity of human TDP stays constant and is independent of enzyme concentration. These data illustrates that this assay has a sensitivity concentration as low as 15 nM being comparable with the sensitivity of gel-based assay [14].

Kinetics parameters K_m , k_{cat} and V_{max} determined under optimal conditions

To determine the K_m and V_{max} of human TDP, initial rates of reaction were measured with increasing concentrations of substrate. The Michaelis-Menten plot of the data produced a typical hyperbolic curve. Based on the reciprocal Lineweaver-Burk plot (correlation coeff. $r^2=0.983$, Figure 6), human TDP displayed standard Michaelis-Menten kinetics with a K_m value of 211 μM and a V_{max} of 1.103 μM/min, and turnover number or rate constant of phosphodiester bond hydrolysis k_{cat} of 8.82 min⁻¹ in the presence of 5 mM of Mn²⁺.

DISCUSSION

TDP is a newly identified enzyme that cleaves the phosphodiester bond in Topo I-DNA covalent complexes. Clustal W analysis of all TDP protein sequences deduced from DNA sequences reveals a poorly conserved N-terminal region and a highly conserved C-terminal region containing two conserved sequence motifs of WxLxTSANLSxxAWG and YExGVL (residue 556-569 and 583-588, Figure 1). Blast and Psi-Blast searches showed that TDP does not share significant sequence identity/similarity with any other gene of known functions.

Initial attempts in producing full-length human TDP in *E. coli* were not successful. However, control yeast TDP could be produced abundantly as soluble recombinant protein in *E. coli*. The alignment of TDP protein sequences of human, yeast, and other organisms showed that sequences at amino-termini not only vary in length but also share little homology. In addition, protein structure prediction carried out using the bioinformatics tool 3D-PSSM suggests TDP could be composed of three-domains [17]. Hence, it is plausible that the poorly conserved N-terminal region is not needed for the phosphodiesterase activity of TDP enzymes, and forms a domain separate from the catalytic domain. Two human TDP variants, huTDPN Δ 39 and huTDPN Δ 174 were designed (Figure 1). HuTDPN Δ 39 is a deletion mutant of human TDP missing the first 39 amino acids that are absent in yeast TDP sequence. The other human TDP mutant, HuTDPN Δ 174, carries a deletion of the first 174 amino acids that are encoded by exon 1. Expression constructs carrying huTDPN Δ 39 and huTDPN Δ 174 led to higher expression levels of both human TDP enzyme variants as insoluble proteins. After protein purification using a metal-chelating column and protein refolding, the final yield of two human TDP variants was approximated to be 5mg/L of *E. coli* culture (Figure 2).

TDP is involved in the Topo I DNA repair pathway, and inhibitors of TDP may have therapeutic utility in treating cancers that are refractory to camptothecin treatment. In order to understand the structure-activity relationship of TDP and to facilitate inhibitor screening in a high throughput manner, we have developed an efficient assay system and studied kinetic properties of human TDP using chromogenic *para*-nitrophenyl thymidine-3'-phosphate as substrate in a 96-well format (Figure 3).

First, we demonstrated that yeast and two human TDP variants purified from *E. coli* showed low but comparable enzymatic activity in the absence of cofactors (Table 1). This result verified that the insoluble human TDP enzyme after refolding has recovered conformation and activity close to that of a native TDP with yeast origin.

The K_m value of human TDP toward T3'P-*p*NP was determined to be 211 μ M (Figure 6). We speculate this to be at least 1,000-fold higher than the K_m for the macromolecular natural substrate (Topo I-DNA complex) that offers more extensive surface for binding. A K_m value of 8.8 nM was reported for the yeast TDP toward the single-stranded oligonucleotide substrate of 18 bases in length [7].

Both human TDP variants with amino-terminal truncations of 39 or 174 amino acids had similar low base level enzymatic activity. Through efforts made to optimize the enzyme assay, we discovered that Mn^{2+} , but not Mg^{2+} , has a stimulatory effect on TDP. This stimulatory effect was, however, only observed for the human TDP Δ 39 variant. Addition of Mn^{2+} to enzyme reactions led to an increase of V_{max} and assay sensitivity level by 10-fold (Table 1). The lack of stimulation toward human TDP Δ 174 by Mn^{2+} suggests that human TDP with deletion of the first 174 amino acids has only retained the core of the catalytic domain, but has lost amino acid residues required for Mn^{2+} coordination. Therefore, the amino-terminal domain of TDP might serve a regulatory function. How Mn^{2+} regulates enzymatic activity of TDP and changes V_{max} or k_{cat} toward T3'P-*p*NP remains unknown in spite of the recent high-resolution structure of human TDP. Human TDP has a pH optimum between pH 8.0 and 8.5 (Figure 4). We speculate that cleavage of transition state TDP-oligonucleotide covalent complex detected by Interthal and co-workers [14] could occur more efficiently in an alkaline environment. Also, an optimum at pH 8.0-8.5 observed in this study (Figure 3) led us to speculate that the release of DNA and TDP from transition state complex might involve an activated water molecule in the active site. Requirement of manganese as cofactor and the pH profile of TDP enzymatic activity suggests that hydrolysis of the phosphodiester bond might involve water molecules bound in the active site similar to a catalytic mechanism proposed for arginase [18]. Indeed, recent crystal structure of the human TDP has two well-ordered water molecules bound in its active site [15]. Authors also suggest that one of the water molecules may become activated to carry out the hydrolysis of phosphodiester bond formed between TDP and DNA in the covalent intermediate. However, this structure of the human TDP apo enzyme does not contain any metal ion that is bound within the vicinity of the active site. It requires further investigation to see if protein

crystallization is carried out in manganese containing solution then a manganese cation could be detected in the active site to occupy one of the two water positions.

A linear relationship between initial velocity and enzyme concentration was demonstrated (Figure 5). That is the specific activity of human TDP remains constant and is independent of enzyme concentrations; as expected for monomeric enzymes. TDP being a monomeric enzyme is corroborated by the recent publication of human TDP crystal structure (PDB code: 1JY1) [15].

We also compared enzyme activity of the recombinant yeast TDP determined in this study with what was measured for TDP purified from yeast culture using the same chromogenic T3'P-*p*NP substrate. Surprisingly, recombinant yeast TDP enzyme purified from *E. coli* culture had a higher activity of 1.024 nmole of product per min per μ M enzyme (or 61 nmole of product per hour per μ M enzyme, Table 1) at 37°C as compared to only 2.1 nmole of product per μ M enzyme over a 16-hr reaction at 30°C [7]. Discrepancy in enzymatic activity from two different sources could be explained by several reasons 1) a temperature difference of 7°C, 2) different pH used, 3) the absence of Mn^{2+} as a cofactor, and 4) prolonged 16-hour enzyme reactions used in enzyme reactions performed with TDP purified from yeast culture. After normalizing data to account for all variations in assay conditions used by two groups, enzymatic activity determined for recombinant yeast TDP prepared from *E. coli* culture turned out to be more similar to that of the natural source.

Human TDP shares only 12.1% and 17.3% sequence identity with two sequences with known structures that are a PLD from *Streptomyces sp.*, and a bacterial nuclease from *Salmonella typhimurium* (Nuc) in the PLD superfamily. In spite of low sequence identity, the three-dimension structure of human TDP is remarkably similar to the known structures of the PLD superfamily with two similar domains that are related by a pseudo-2-fold axis of symmetry [15].

Alignments of PLD members showed a significant internal homology of a short sequence motif HXX(X)₄D(X)₆G^G/S, termed HKD motif [19]. The His263 of the human TDP serves as the nucleophile in the first step of the catalytic reaction and forms a transient covalent phosphoenzyme intermediate, while the other histidine

residue in the active site (i.e. His493) from the second HKD motif may function as a general base to activate a water molecule that cleaves the phosphodiester bond of the phosphoenzyme intermediate [12,15]. Because of impaired catalytic activity rendered by mutations of the conserved lysine residues of PLD superfamily member [12,13], it has been suggested that conserved Lys165 and Lys495 of the human TDP are involved in binding of the phosphate group of substrate and neutralizing its negative charges like the conserved lysine residues of other members of the PLD superfamily. For the human TDP, aspartic acid residues of the two HKD motifs are replaced by Gln294 and Glu538, respectively. The side chain of Glu538 in the active site of the human TDP is within the hydrogen bonding distance of His263 thus Glu538 is speculated to stabilize the developing positive charge on the imidazole ring of His263 to facilitate the formation of the phosphoenzyme intermediate [15]. Because of the lack of conserved aspartic acid residue in its active site, human TDP forms a new and distinct class of the PLD superfamily that is consistent with our observation that human TDP could not be inhibited by known inhibitors to PLD1 and PLD2 (data not shown).

When Topo I is inhibited by camptothecin and cancer chemotherapeutic agents, covalent Topo I-DNA complexes accumulate and cause cytotoxic effects if exceeding a threshold. It is yet unknown what is the exact nature of macromolecular substrate of Topo I-DNA covalent complex required by TDP for efficient catalysis to remove Topo I from DNA lesions caused by Topo I poisons. In this study, synthetic chromogenic substrate T3'-P-pNP was used to determine kinetic parameters of the human TDP which hydrolyzes the phosphodiester bond that links the Topo I enzyme and its DNA substrate during catalysis in the presence of Topo I inhibitors such as camptothecin. Inhibitors of the human TDP might potentiate or synergize the cytotoxic effect of Topo I inhibitors used as cancer therapeutic agents. The identification of manganese as a cofactor increased the sensitivity of this enzymatic assay. And, the 96-well format developed for this assay will facilitate drug screening in a high throughput manner. Recent solution of the human TDP apo enzyme reveals a structurally well-defined binding pocket for the tyrosine residue as well as DNA-binding cleft between the two domains. It would undoubtedly provide valuable structural insight for structure-based drug design for this new member of the PLD superfamily.

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ACKNOWLEDGEMENTS

* This work was supported by the start-up fund provided to Chen-Chen Kan by Keck Graduate Institute of Applied Life Sciences.

FOOTNOTES

Abbreviations used: TDP, tyrosyl-DNA phosphodiesterase; Topo I, topoisomerase I; PCR, polymerase chain reaction; RT, reverse transcriptase; scTDP, *S. cerevisiae* TDP; huTDP, human TDP; SDS-PAGE; sodium dodecyl-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DDT, dithiothreitol; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; T3'P-*p*NP; *para*-nitrophenyl-thymidine-3'-phosphate; PLD, phospholipase-D; DMSO, dimethyl sulfoxide;

LEGENDS

Figure 1 Sequence alignment of human and yeast TDP enzymes

MULTALIN program (Pole BioInformatique Lyonnais <http://npsa-pbil.ibcp.fr/>) was used to create the alignment. Identical residues were shaded in black and similar residues were shaded in gray. Exons of the human TDP were marked above the alignments. The recombinant human TDP variants, huTDPNΔ39 and huTDPNΔ174, used in these studies were indicated as bold, and thin line, respectively.

Figure 2 Expression and purification of two human TDP variants

The cells bearing plasmid pBAD/THIO-huTDPNΔ39 or pBAD/THIO-huTDPNΔ174 were grown to $A_{600\text{ nm}}$ mid log phase and induced with 0.02% arabinose for 2 hours. Cell pellets from induced cultures were sonicated, and soluble and insoluble fractions were collected separately after centrifugation. As described in Experimental Procedures, the insoluble fraction was solubilized with 8M urea and applied onto a Ni^{2+} chelating column. After collecting the flow-through (FT), the weakly bound proteins were washed off the column with buffer containing 15 mM imidazole, and the bound fractions were then eluted with 400 mM imidazole in the same buffer. Individual fractions were pooled for protein electrophoresis with 12.5 % SDS-polyacrylamide gels. Prior to electrophoresis, samples were boiled in reducing sample buffer. Shown were gels stained with Coomassie Brilliant Blue after electrophoresis. **(A)** huTDPNΔ39 variant, and **(B)** huTDPNΔ174 variant with Lane 1 and 2: uninduced (-) and induced (+) culture, Lane 3 and 4: soluble (S) and insoluble (I) fraction of the crude lysate, Lane 5, 6, 7, and 8: load, flow-through, wash and eluted fractions off the Ni^{2+} -chelating column, and *Lane M*: molecular mass markers. Arrowheads mark positions of huTDPNΔ39 and huTDPNΔ174. Molecular masses of markers are indicated in kDa.

Figure 3 Reaction mechanism of tyrosyl-DNA phosphodiesterase toward *para*-nitrophenyl-thymidine-3'-phosphate (T3'P-*p*NP)

As substrate for TDP in this study, T3'P-*p*NP was used to mimic Topo I-DNA complex. TDP attacks the phosphodiester bond in T3'P-*p*NP and form a transient reaction intermediate of TDP and thymidine emulating the TDP-DNA complex observed by Interthal and coworkers [14]. Meanwhile, a chromogenic *para*-nitrophenol group is released. To complete a reaction cycle, the water molecule in the active site of TDP hydrolyzes the covalent bond between TDP and DNA to release TDP as free enzyme for subsequent rounds of catalysis.

Figure 4 pH dependence of human TDP enzyme activity

The reactions were carried out in a total volume of 200 μl of 50 mM Tris-HCl, 100 mM NaCl, 5 mM Mn^{2+} , 0.125 μM of human TDPN Δ 39 enzyme, and 1 mM of substrate in Tris-HCl buffer at varying pH. Increases in optical density at 415 nm were monitored and the amount of released products was calculated based on the extinction coefficient (ϵ) of 15,000 $\text{M}^{-1}\cdot\text{cm}^{-1}$. The rate was then determined as the amount of released *para*-nitrophenol per min. The pH profile represents the results from three separate assays with duplicated samples in each experiment.

Figure 5 Dependence of human TDP activity on enzyme concentration

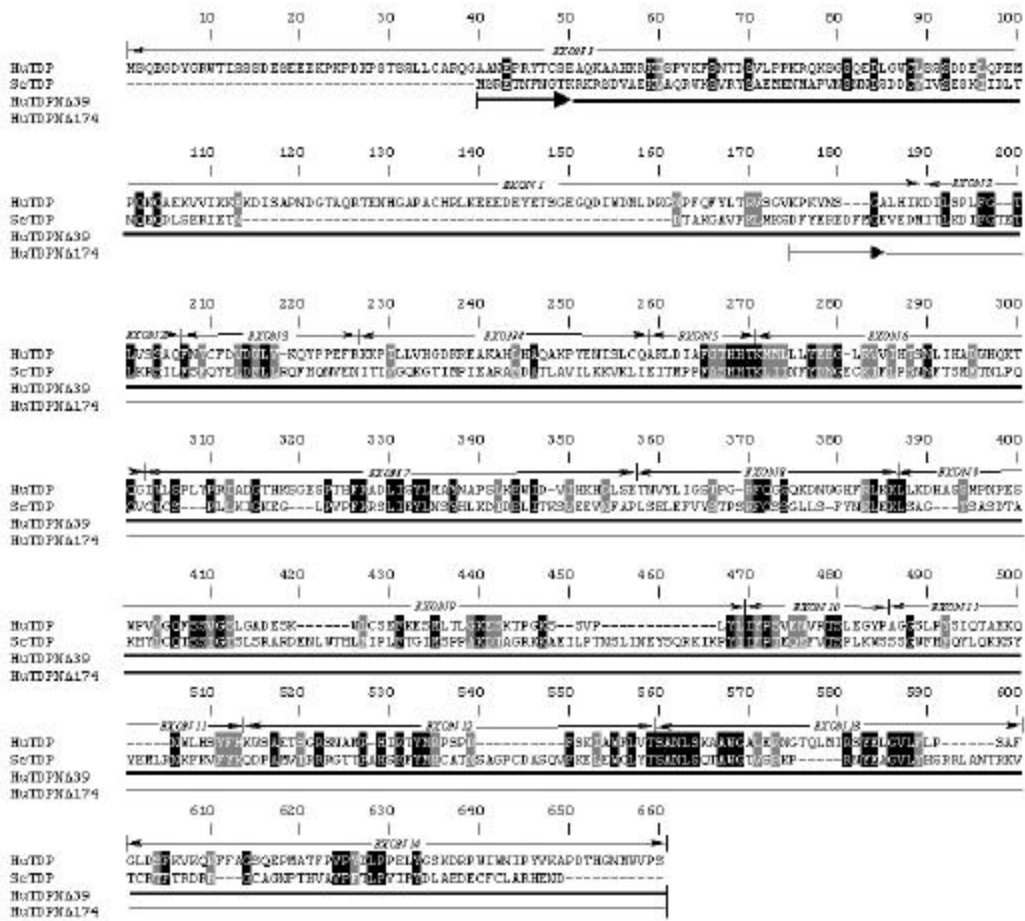
Enzymatic reactions were carried out with different amounts of human TDPN Δ 39 enzyme in the presence of 1 mM substrate in 50 mM Tris-HCl, at an optimal pH of 8.5, 100 mM NaCl, 1 mM DTT, and 5 mM MnCl_2 as cofactor. Increases in optical density at 415 nm were used to calculate the amount of released products based on the extinction coefficient (ϵ) of 15,000 $\text{M}^{-1}\cdot\text{cm}^{-1}$. Initial velocity for each reaction was determined with data collected from three separate assays with duplicate set of samples in each experiment.

Figure 6 Determination of the kinetic parameters K_m , V_{max} , and k_{cat} for human TDP

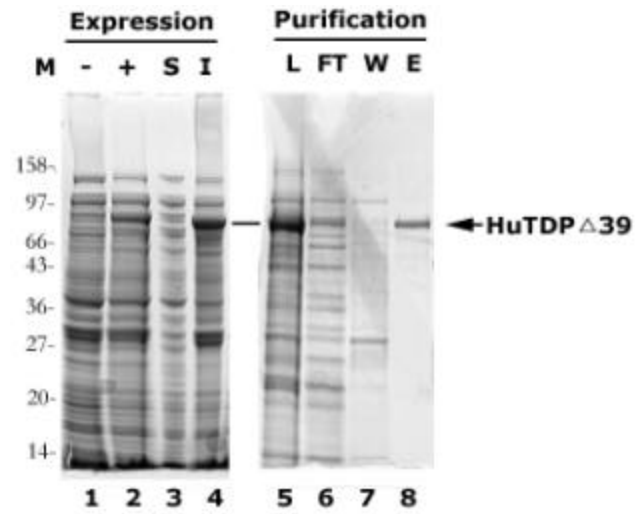
The reactions were carried out with different concentrations of substrate ranging from 25 to 2000 μM in reaction mixtures containing 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM MnCl_2 , 1 mM DTT, and 0.125 μM of human TDPN Δ 39 enzymes. The dependence of initial rates on substrate concentration are shown in (A) Michaelis-Menton plot, as well as (B) Lineweaver-Burk plot that is used to determine the values of the kinetic parameters K_m , V_{max} , and

k_{cat} for human TDP enzyme. Data were collected from four separate assays (depicted by Δ , \circ , \diamond , \square) performed with quadruplicate sets of samples.

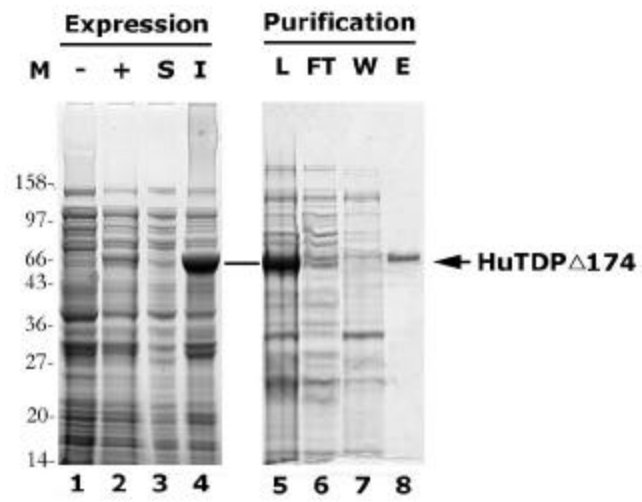
Cheng et al., Figure 1



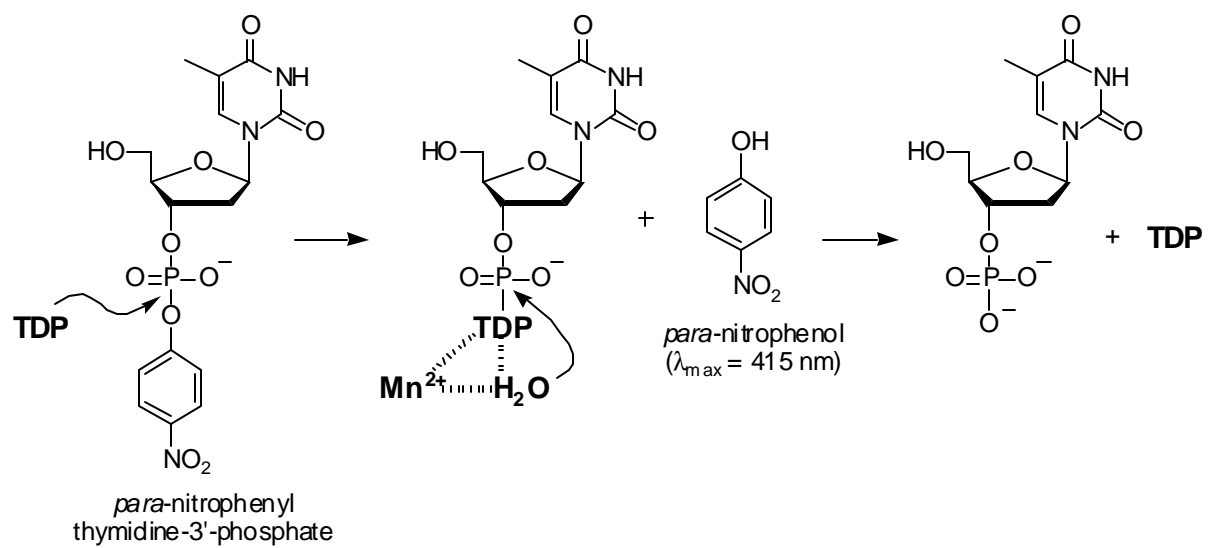
A Human TDPN Δ 39



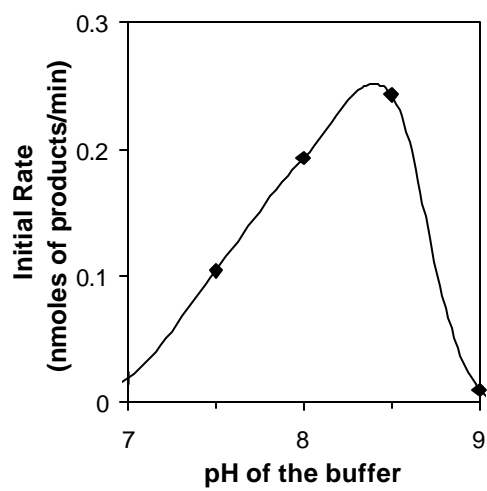
B Human TDPN Δ 174



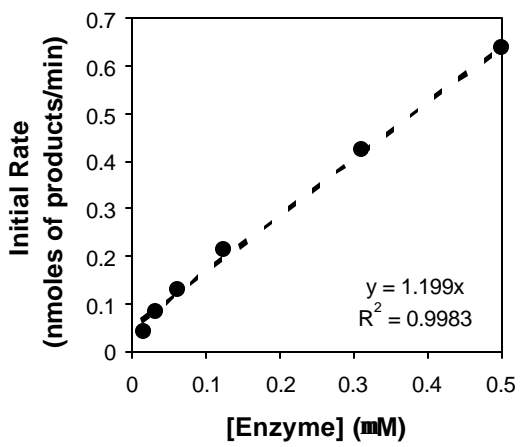
Cheng et al., Figure 3



Cheng et al., Figure 4

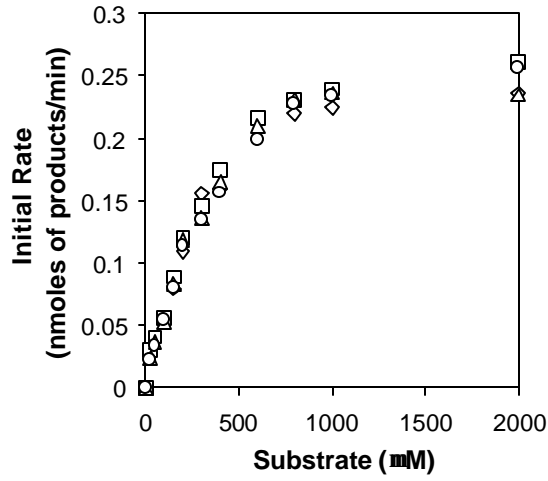


Cheng et al., Figure 5



A

Michaelis-Menten plot



B

Lineweaver-Burk plot

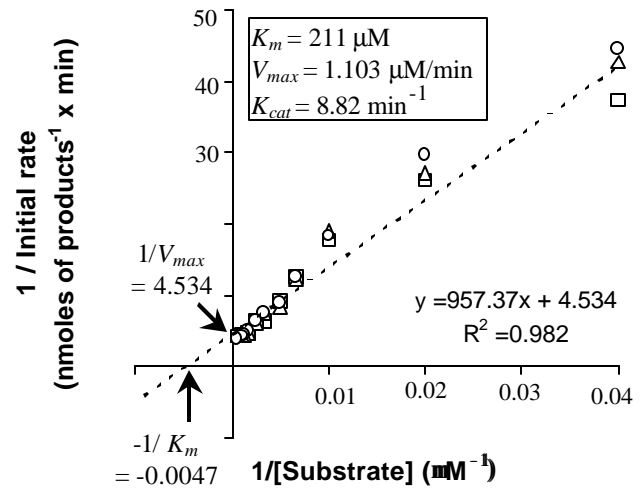


Table 1 The effects of divalent metal cations on V_{max} of TDP enzymes.

Enzyme ^a	V_{max} ($\mu\text{M}/\text{min}$) ^b		
	control	+ 5 mM Mn^{2+}	+ 5 mM Mg^{2+}
Yeast TDP	0.130 ± 0.008	1.024 ± 0.128	0.334 ± 0.018
Human TDPN Δ 39	0.116 ± 0.021	1.079 ± 0.072	0.200 ± 0.015
Human TDPN Δ 174	0.114 ± 0.011	0.182 ± 0.003	0.146 ± 0.013

^a All enzymes used in this experiment were at 0.125 μM concentration in 50 mM Tris·HCl, pH 8.5, 100 mM NaCl, 5 mM MnCl_2 , and 1 mM DTT.

^b Data were obtained from three assays performed with duplicate sets of samples.