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]. During the process, DNA becomes covalently linked to Topo I via the 3¢ terminus of DNA in topoisomerase I–DNA complexes [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, Topo I-linked DNA breaks would accumulate when Topo I acts on damaged DNA containing lesions such as thymine dimers, abasic sites, 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 & 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 with another important enzyme target in the topoisomerase I 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 were 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].

TDP homologs have been identified for several other species including Drosophila melanogaster, Caenorhabditis elegans, Saccharomyces pombe, Mus musculus and Homo sapiens. Database searches showed that TDP does not share significant sequence homology with any other genes 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]. The 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 a 96-well format, the identification of a cofactor, and characterization of kinetic parameters of the human tyrosyl-DNA phosphodiesterase activity.

**EXPERIMENTAL PROCEDURES**

**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, USA) and Invitrogen (Carlsbad, CA, USA). The Trizol™ and reagents for PCR and RT-PCR were obtained from Gibco Life Technologies, Inc (Rockville, MD, USA). Oligonucleotides used for PCR were from MWG Biotech (Charlotte, NC, USA). Protease inhibitor cocktail was from Roche Molecular Biochemicals (Indianapolis, IN, USA). HiTrap chelating agarose was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). BCA reagent for protein concentration determination was from Pierce (Rockford, IL, USA).

**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 cultured human fibrosarcoma cells HT1080 (from ATCC CCL-121). Briefly, total RNA from HT1080 cells was isolated by cell lysis with Trizol™ Reagent followed by RNA precipitation with 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. 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 C393 to G and C1629 to T that do not lead to amino acid changes, nucleotide changes of A378 to T and G481 to A lead to amino acid substitutions of R126S and G161R, respectively.

Two human TDP variants containing deletion of N-terminal 39 (huTDPNA39) and 174 (huTDPNA174) 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 huTDPNA39 variant by PCR mutagenesis, oligonucleotides of 5'-GCAGCAAATGAGCCAGGTACACCTGTTCC-3' and 5'-GGAGGGGCACCACATGTTCCATGC-3' were used as the forward and reverse primers. Similarly, oligonucleotides of 5'-AAGTATACCCTCGAGCCCTCAATCAAGG-3' and 5'-GGAGGGCACCCACATGTTCCATGC-3' were used as primers to generate the huTDPNA174 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-huTDPNA39, and pBAD/Thio-huTDPNA174.

**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-huTDPNA39 and pBAD/Thio-huTDPNA174, respectively. After induction with 0.02% 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 12 000 g for 30 min to remove insoluble particulates, urea-denatured human TDP in solubilized lysate was purified with a NiCl2-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 analyzed and pooled 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 dithiothreitol, 1% Chaps) to lower the urea concentration.
by 2 M at each step. After refolding, purified human TDP protein was stored in 20% glycerol 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′-GCTGGATCCCTCCCGAGAAACAATTTCATGG-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)pLysS cells grown in Luria–Bertani medium containing 50 μg/ml ampicillin at 37 °C by the induction of 1 mM isopropyl-β-D-thiogalactoside. After being induced for 3 h, 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-protein A column, and the resultant proteins were loaded onto the HiTrap Chelating agarose column. Hydrolysis of the phosphodiester bond in the substrate by the enzyme releases free p-nitrophenol that absorbs light at 415 nm. T3′P-pNP was synthesized from 5′-O-p-methoxytryethylthymidine 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, in assay buffer containing 50 mM Tris/HCl, pH 7.5 and 100 mM NaCl at 37 °C at a final volume of 200 μL in each well. The continuous changes in absorbance at 415 nm were monitored using an Ultramark Microplate Imaging System (Bio-Rad, Hercules, CA, USA). The extinction coefficient (c) of p-nitrophenol was determined to be 15 000 M⁻¹ cm⁻¹ under assay conditions. The nmol of the product, i.e. p-nitrophenol were calculated from the absorbance at 415 nm using the equation \( A = \frac{\text{absorbance}}{\text{molar extinction coefficient}} \times \text{concentration} \times \text{path length} \). The requirement of cofactor for TDP enzymatic activity was examined by determining the specific activity of TDP by following the cleavage of 1 μM of substrate by 0.125 μM of enzyme in reaction buffer containing increasing concentrations of divalent ions. The optimum pH was examined with 100 mM NaCl, 5 mM MnCl₂, 1 μM of substrate, 0.125 μM enzyme and 50 mM Tris/HCl at different pH values ranging from pH 7–9. The dependence of the enzymatic activity of TDP on salt and dithiothreitol was also examined separately in the presence of increasing concentrations from 25 to 500 mM NaCl, and 1–10 mM of dithiothreitol, 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 dithiothreitol and 0.125 μM enzyme with different concentrations of substrate ranging from 25 μM to 2000 μM. Increases in absorbance at 415 nm were monitored and the amount of released products was calculated as described above. The specific activity was determined as nmol of product min⁻¹ μg⁻¹ 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, USA). The Lineweaver–Burk treatment of data gave a linear plot of 1/V vs. 1/[substrate]. According to the rearranged Michaelis–Menten equation, 1/V = 1/V_{max} + \frac{K_m}{V_{max}} 1/[substrate], the \( K_m \) and \( V_{max} \) were determined from the Lineweaver-Burk plot and \( k_{cat} \) was determined by \( k_{cat} = \frac{V_{max}}{[enzyme]} \).

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 [17], the sequence of FLJ11090 shares a 14% identity with the yeast TDP gene sequence, and a 97% identity with the corresponding sequence of the

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, USA). Fragmentation patterns detected in MS/MS spectra were used to assure protein identity by finding peptides with sequences matched. The analysis showed seven matches (20% amino acid sequence) for yeast TDP and 22 matches (36% amino acid sequence) for both human TDP deletion variants.

Synthesis of the chromogenic substrate

To develop a chromogenic assay for TDP, we chose p-nitrophenyl-thymidine-3′-phosphate (T3′P-pNP) as the substrate. This compound contains a phosphodiester bond between the phosphate group at the 3′ position of thymidine and the hydroxy group of the p-nitrophenol to mimic the phosphodiester bond in the topoisomerase–DNA complex. Hydrolysis of the phosphodiester bond in T3′P-pNP releases free p-nitrophenol that absorbs light at 415 nm. T3′P-pNP was synthesized from 5′-O-p-methoxytritylthymidine and p-nitrophenyl phosphodichloridate using the procedure reported by Turner and Khorana [16].
partial human TDP cDNA sequence reported by Pouliot and coworkers [8] (Fig. 1). 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 abundantly expressed in bacterial cells bearing plasmid pBAD/THIO-huTDP39 or pBAD/THIO-huTDP174. The recombinant proteins obtained were insoluble and formed 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 (Fig. 2). The final yield of purified protein was approximately 5 mg L⁻¹ of E. coli for both huTDP39 and huTDP174. Refolding was simply carried out by dialysis to remove the denaturant, i.e. urea.

Development and optimization of chromogenic assay for TDP enzymatic activity

To overcome the inconvenience of the gel-based assay previously used to measure TDP enzyme activity, in which the substrate was a peptide fragment of Topo I containing active site tyrosine conjugated to the 3’ phosphate group of the oligonucleotide [14], we chose to develop a chromogenic assay for TDPs using p-nitrophenyl-thymidine-3’-phosphate (T3’P-pNP, molecular weight approximately 443 Da) as a substrate. The TDP enzymatic activity could be continuously monitored as an increase in absorbance at the wavelength 415 nm during chromophore (i.e. p-nitrophenol) release upon hydrolysis of the phosphodiester bond (Fig. 3). This chromogenic assay was easily adapted to the 96-well plate format to facilitate high throughput screening of inhibitors. When enzyme reactions were carried out with 0.125 μM refolded human TDP (human TDP39), and 1 mM T3’P-pNP in Tris buffer at 8.5, the changes in absorbance at 415 nm showed a linear relationship in a time-dependent manner (Fig. 4). We further examined if increase in A₄₁₅ could be due to the hydrolysis of substrate by water, by heating the enzyme at 70 °C for 15 min prior to the assay. The enzyme reactions carried out with heated
TDPs produced no increase in $A_{415}$, confirming that the hydrolysis of the tyrosine-DNA phosphodiester bond detected before was indeed produced by the activity of purified recombinant huTDPN$_{39}$ enzymes.

The $V_{\text{max}}$ of huTDPN$_{39}$ determined as described above was only 0.116 ± 0.021 μM min$^{-1}$ indicating a rather low enzymatic activity, however, this was comparable to that of soluble recombinant yeast TDP purified from *E. coli* (Table 1). 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 Mg$^{2+}$ and Mn$^{2+}$ 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 Mn$^{2+}$ a fourfold increase in human TDP activity was observed. This Mn$^{2+}$ concentration-dependent effect on TDP activity approached a plateau at 5 mM Mn$^{2+}$ where enzymatic activity was increased approximately 10-fold. Similar effects of Mn$^{2+}$ and Mg$^{2+}$ to enzymatic activity were observed for the yeast TDP (Table 1). Altogether, the data suggests that recombinant human TDP enzymes were refolded back to a conformation that possess comparable enzymatic activity to 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 Mn$^{2+}$ (Fig. 5). The optimum pH for human TDP enzymatic activity was determined to be 8.0–8.5.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control + 5 mM Mn$^{2+}$</th>
<th>+ 5 mM Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast TDP</td>
<td>0.130 ± 0.008</td>
<td>1.024 ± 0.128</td>
</tr>
<tr>
<td>Human TDPN$_{39}$</td>
<td>0.116 ± 0.021</td>
<td>1.079 ± 0.072</td>
</tr>
<tr>
<td>Human TDPN$_{174}$</td>
<td>0.114 ± 0.011</td>
<td>0.182 ± 0.003</td>
</tr>
</tbody>
</table>

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

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

Fig. 3. Suggested reaction mechanism of tyrosyl-DNA phosphodiesterase toward $\beta$-nitrophenyl-thymidine 3'-phosphate (T3'pNP). As substrate for TDP in this study, T3'pNP was used to mimic Topo I-DNA complex. TDP attacks the phosphodiester bond in T3'pNP and forms a transient reaction intermediate of TDP and thymidine emulating the TDP-DNA complex observed by Interthal and coworkers [14]. Meanwhile, a chromogenic $\beta$-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 [15].

Fig. 4. Time-dependence of human TDP activity. Enzymatic reactions were performed by incubating 0.125 μM of human TDPN$_{39}$ enzyme and 1 mM substrate in the presence of 50 mM Tris/HCl, pH 8.5 and 100 mM NaCl. Increases in absorbance at 415 nm were detected and used to calculate the amount of products based on the extinction coefficient of $\beta$-nitrophenol being 15 000 M$^{-1}$ cm$^{-1}$. The data shown represents three separate experiments with a duplicate set of samples used in each experiment.
Finally, the dependencies on concentration of salt and reducing reagent were examined in Tris/HCl, pH 8.5 containing Mn$^{2+}$ ions. It showed the enzymatic activity of human TDP did not change in salt concentrations from 25 mM to 500 mM and in dithiothreitol concentrations from 1 mM to 10 mM. The reaction conditions for TDP activity were optimized as 50 mM Tris/HCl, pH 8.5, 5 mM MnCl$_2$, 100 mM NaCl, 1 mM dithiothreitol. Under these conditions, $V_{\text{max}}$ was determined for huTDPN$_{39}$ and huTDPN$_{174}$ to be 1.079 and 0.182 $\mu$mol min$^{-1}$, respectively (Table 1).

Initial velocities of enzymatic reactions carried out with human TDP, i.e. human TPDN$_{39}$ at enzyme concentrations from 3 nM to 500 nM showed a linear relationship for enzyme concentrations from 15 nM to 500 nM indicating that, unlike some obligatory homodimeric enzymes, the specific activity of human TDP stays constant and is independent of enzyme concentration, as expected for a monomeric enzyme. TDP being a monomeric enzyme is corroborated by the recent publication of the crystal structure of human TDP (PDB accession no. 1JY1) [15].

These data illustrate that this assay has a sensitivity concentration as low as 15 nM, which is comparable with the sensitivity of the gel-based assay [14].

**Kinetic parameters $K_m$, $k_{\text{cat}}$ and $V_{\text{max}}$ determined under optimal conditions**

To determine the $K_m$ and $V_{\text{max}}$ 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 coefficient $r^2 = 0.983$, Fig. 6), human TDP displayed standard Michaelis–Menten kinetics with a $K_m$ value of 211 $\mu$M and a $V_{\text{max}}$ of 1.103 $\mu$mol min$^{-1}$, and turnover number or rate constant of phosphodiester bond hydrolysis $k_{\text{cat}}$ of 8.82 $\text{min}^{-1}$ in the presence of 5 mM Mn$^{2+}$.

**DISCUSSION**

TDP is a newly identified enzyme that cleaves the phosphodiester bond in Topo I–DNA covalent complexes.

![Fig. 6. Determination of the kinetic parameters $K_m$, $V_{\text{max}}$, and $k_{\text{cat}}$ for human TDP.](image)

Fig. 6. Determination of the kinetic parameters $K_m$, $V_{\text{max}}$, and $k_{\text{cat}}$ for human TDP. The reactions were carried out with different concentrations of substrate ranging from 25 to 2000 $\mu$M in reaction mixtures containing 50 mM Tris/HCl, pH 8.5, 100 mM NaCl, 5 mM MnCl$_2$, 1 mM dithiothreitol, and 0.125 $\mu$M of human TDPNA39 enzymes. The dependence of initial rates on substrate concentration are shown in (A) Michaelis-Menten plot, as well as (B) Lineweaver-Burk plot used to determine the values of the kinetic parameters $K_m$, $V_{\text{max}}$, and $k_{\text{cat}}$ for human TDP enzyme. Data were collected from four separate assays (depicted by △, ○, ◇, □) performed with quadruplicate sets of samples.
natural substrate (Topo I–DNA complex) that offers a 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 but low basal level enzymatic activity. Through efforts made to optimize the enzyme assay, we discovered that Mn$^{2+}$, but not Mg$^{2+}$, had a stimulatory effect on TDP. This stimulatory effect was, however, only observed for the human TDPNA39 variant. Addition of Mn$^{2+}$ to enzyme reactions led to an increase in $V_{max}$ and assay sensitivity level by 10-fold (Table 1). The lack of stimulation toward human TDPNA174 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 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 $T3P$-$pNP$ 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 (Fig. 4). We speculate that cleavage of the transition state TDP-oligonucleotide covalent complex detected by Internal and coworkers [14] could occur more efficiently in an alkaline environment. Also, an optimum at pH 8.0–8.5 observed in this study (Fig. 3) led us to speculate that the release of DNA and TDP from the 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, the recently determined crystal structure of human TDP has two well-ordered water molecules bound in its active site [15]. The authors also suggested 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 [15]. However, this structure of the human TDP apoenzyme does not contain any metal ion that is bound within the vicinity of the active site. Further investigation is required to determine if protein crystallization of human TDP were carried out in the presence of manganese, manganese ion would be detected at one of the two water molecule positions in the active site.

We also compared enzyme activity of the recombinant yeast TDP determined in this study with the value determined for TDP purified from yeast culture using the same chromogenic $T3P$-$pNP$ substrate. Surprisingly, recombinant yeast TDP enzyme purified from E. coli culture had a higher activity of 1.024 nmol of product per min per $\mu$m enzyme (or 61 nmol of product per hour per $\mu$m enzyme, Table 1) at 37 °C as compared to only 2.1 nmol of product per $\mu$m native yeast enzyme over a 16-h reaction at 30 °C [7]. Discrepancy in enzymatic activity from the two different sources could be explained by several reasons: (a) a temperature difference of 7 °C; (b) different pH used; (c) the absence of Mn$^{2+}$ as a cofactor; and (d) prolonged 16-h 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 the 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 S. typhimurium (Nuc) in the PLD superfamily. In spite of low sequence identity, the three-dimensional 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-twofold axis of symmetry [15]. Alignments of PLD members showed a significant internal homology of a short sequence motif HXK(X)4D(X)6GG/S, termed HKD motif [19]. Notably, the TDP homologs lack the otherwise invariably conserved aspartate [14]. Because of the lack of a 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. The exact nature of the macromolecular substrate, i.e. Topo I–DNA covalent complex required by TDP for efficient catalysis is yet unknown. In this study, the synthetic chromogenic substrate $T3P$-$pNP$ was used to determine the kinetic parameters of 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 apoenzyme reveals a structurally well-defined binding pocket for the tyrosine residue as well as DNA-binding cleft between the two domains. It undoubtedly provides valuable insight for structure-based drug design for this new member of the PLD superfamily.

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**REFERENCES**

cycle of lymphocytic (L1210, MOLT-4) and myelogenous (HL-60, KG1) leukemic cells. *Cancer Res.* 50, 5746–5750.


