A *Tetrahymena thermophila* G4-DNA Binding Protein with Dihydrolipoamide Dehydrogenase Activity†

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ABSTRACT: G4-DNA is a four-stranded structure that is formed by guanine-rich sequences. We report here the purification and characterization of a novel G4-DNA binding protein from *Tetrahymena thermophila*, designated TGP2. TGP2 was found to preferentially bind to G4-DNA oligonucleotides with adjacent single-stranded domains containing phosphorylated 5′ ends and the sequence element, 5′-ACTG-3′. The amino acid sequence of TGP2 has high similarity to dihydrolipoamide dehydrogenase (DLDH) from a variety of species, and TGP2 was shown to have DLDH activity. Purified DLDH from porcine heart and bovine intestinal mucosa were shown to bind specifically to G4-DNA oligonucleotides. On the basis of these results we conclude that TGP2 is DLDH in *T. thermophila* and suggest that the G4-DNA binding capability of TGP2/DLDH may be biologically relevant.

DNA can adopt a variety of structures in addition to the classical double helix. G4-DNA is one of these structures. It is a four-stranded DNA structure containing a G-quartet structural motif and is characterized by high stability and selective cation binding (*1*). Many G-rich sequences are capable of forming G4-DNA structures in vitro. These include telomeric sequences (*2, 3*), the immunoglobulin switch region (*4*), a retrovirus dimerization domain (*5*), recombination hot spots (*6*), and gene regulatory regions (*7, 8*). Nevertheless, direct evidence for a biological role for G4 structures has yet to be reported.

One approach to assessing the biological relevance of G4-DNA is to search for proteins that bind specifically to this structure. Several such G4-DNA binding proteins have been reported, including MyoD, a transcription factor that induces myogenesis (*9*), QUAD, a protein from hepatocyte chromatin of rabbits (*10*), topoisomerase II (*11*), and TGP1 (*12*), which was found to bind to G4-DNA with single-stranded domains. Other proteins have been shown to bind and interact dynamically with G4-DNA substrates. The β-subunit of the telosome in the ciliate Oxytricha and RAP1 in yeast were both found to promote formation of the G4 structure in telomeric DNA (*13, 14*). The KEM1 gene product in yeast was identified as a G4-DNA-dependent nuclease, and a homozygous deletion of the KEM1 gene blocks meiotic cells at the 4N stage (*15, 16*). Taken together, these findings suggest that G4-DNA has important biological functions in vivo.

Dihydrolipoamide dehydrogenase (DLDH)[1] is a ubiquitous and highly conserved enzyme that participates in the oxidation of dihydrolipoamide and the reduction of NAD^+ (for review, see ref 17).

$$\text{protein}-\text{dihydrolipoamide} + NAD^+ \Leftrightarrow \text{protein}-\text{lipoamide} + NADH + H^+$$

This enzyme is an integral part of the pyruvate dehydrogenase multienzyme complex catalyzing the formation of acetyl-CoA from pyruvate. In addition to this role, this disulfide oxidoreductase also catalyzes the conversion of glycine to N^5,N^{10}-methylenetetrahydrofolate, CO_2, and NH_4^+ (*18*). Several new findings suggest additional functions of this enzyme. In archaebacteria, where 2-oxoacids are metabolized by different enzymes, DLDH is still present, but as a noncomplexed enzyme (*19*). Moreover, in the parasitic protozoan, *Trypanosoma brucei*, DLDH has been found in the mammalian bloodstream form of the parasite, despite the absence of the multienzyme complexes with which this enzyme is usually associated (*20*). The *Trypanosoma* enzyme is found to be loosely associated with the inner surface of the plasma membrane, which is an unusual location compared with the more common location in mitochondria (*21*).

We report here the purification and characterization of a second *Tetrahymena thermophila* G4-DNA binding protein, designated TGP2. The amino acid sequence from the amino terminal of TGP2 has high similarity with DLDH from a variety of species, including humans. We show that TGP2 has DLDH activity and that two purified commercial preparations of DLDH from other sources have NAD^+-dependent G4-DNA binding activity. These data strongly suggest that TGP2 is DLDH in *Tetrahymena* and that this enzyme has G4-DNA binding activity. Possible roles for

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[1] Abbreviations: TGP2, *Tetrahymena thermophila* G4-DNA binding protein 2; DLDH, dihydrolipoamide dehydrogenase.

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TGP2 in the context of these observations are discussed.

EXPERIMENTAL PROCEDURES

DNA Oligonucleotides. All oligonucleotides (Tet4, Oxy4, T, M, Y, Y-G, R, G, P, Z, XYa, XYb, and XYc) were synthesized by phosphoramidite chemistry (Nucleic Acid Research Facility at Iowa Sate University and Midland Certified Reagent Co.) and purified as previously described (*2*).

Chromatography Media. Chromatofocusing was carried out using matrix PBE94 and Polybuffer96 (Pharmacia Biotech). Protein samples were concentrated using Centricon 10 spin concentrator devices (Amicon).

DLDH Enzymes and Substrates. Dihydrolipoamide dehydrogenase (DLDH) assays were conducted using NAD^+, DL-lipoamide (DL-thioctic acid amide), and DLDH from porcine heart, torula yeast, and bovine intestinal mucosa (Sigma). *Escherichia coli* DLDH was the generous gift of Dr. C. H. Williams.

Tetrahymena Cell Culture and Extract Preparation. Cells were cultured and extracted as previously described (*12*). Briefly, *T. thermophila* strain C3V was grown to midlog phase and harvested without mating. The whole cell lysate was obtained as previously described (*12*), and the 100000*g* supernatant (S100) was aliquoted and immediately frozen in liquid nitrogen. The protease inhibitors leupeptin (0.01 mM), pepstatin (0.01 mM), and Pefabloc (0.1 mM) (Boehringer Mannheim) were added to all S100 aliquots before they were frozen.

DNA Oligonucleotide Purification and 5′ End Radiolabeling. DNA oligonucleotides were gel purified and desalted as previously described (*12*). Briefly, DNA nucleotides were gel purified by 20% polyacrylamide gels containing 7 M urea and 1× TBE buffer. The desired oligonucleotides were visualized by UV shadowing, excised, and desalted by C18 chromatography (*2*). Oligonucleotides were radiolabeled at the 5′ end with T4 polynucleotide kinase (New England Biolabs) according to standard procedures (*22*).

Electrophoretic Mobility Retardation and Competition Assay. An amount of 312.5 pmol of 5′ ^{32}P-labeled probe was boiled for 5 min in the presence of 67 mM KCl, 17 mM $MgCl_2$, and 17 mM Tris-HCl (pH 7.5) and allowed to cool to room temperature for at least 30 min to permit formation of G4 structures. Fifty micrograms of poly(dI-dC)/poly(dI-dC) (nonspecific competitor) and 20% (v/v) glycerol were added after cooling. The mixture was then diluted 10-fold in doubly distilled water, making the ^{32}P-probe final concentration 0.125 μM. Based on the result of mobility retardation assays, over 97% of ^{32}P-Y formed a G4 structure (Y4) under these conditions (Figure 1). Five microliters of 0.125 μM ^{32}P-probe was mixed with the protein solution to produce a final volume of 20 μL. Final concentrations in the reaction mixture were 31.25 nM ^{32}P-Y, 0.8 mM KCl, 0.2 mM $MgCl_2$, and 0.2 mM Tris-HCl. Unless otherwise noted, all oligonucleotides used in competition assays were 5′ phosphorylated using ATP and T4 polynucleotide kinase (New England Biolabs). Labeled or unlabeled oligonucleotides were individually heated and cooled under conditions identical to those of the probe to allow individual G4 formation. Once the probe and competitor were combined, protein solution was added to produce a final volume of 20 μL. The reaction mixtures were incubated on ice for 20 min and loaded onto a 6% nondenaturing polyacrylamide gel in 0.6× TBE. Electrophoresis at 10 V/cm was carried out for 90 min at room temperature. The gels were then dried and exposed to Kodak XAR-5 film or phosphor screens. Radioactivity in individual bands was quantitated using a Molecular Dynamics PhosphorImager. The ability of a variety of oligonucleotides to compete with ^{32}P-Y4 for TGP2 binding (Figure 5D) was evaluated by comparison of the average slopes of a line connecting each molar excess point of pY. For example, pZ decreased the ^{32}P-Y4 binding activity to 100%, 89%, 78%, 65%, and 62%, when pZ was added at the molar excess

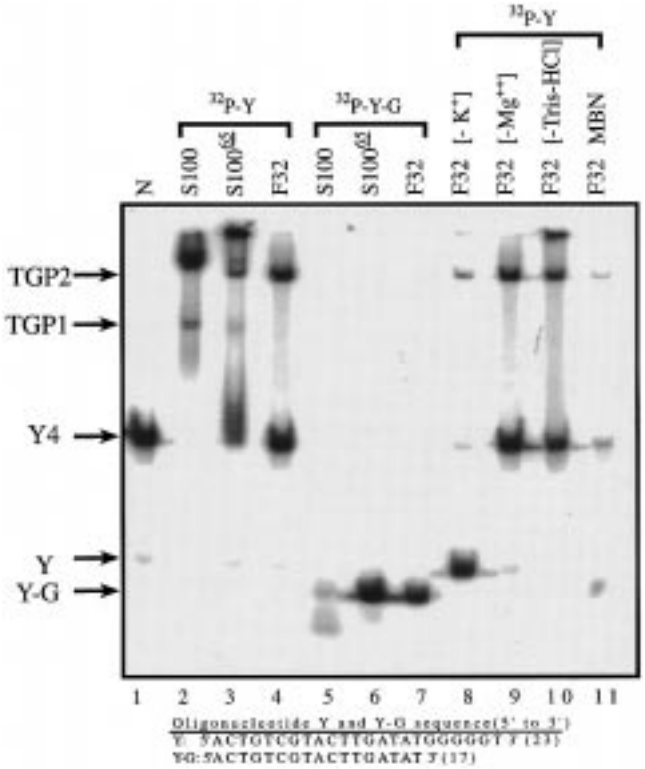


FIGURE 1: TGP2 specifically binds to the G4-DNA molecule Y4. Crude cell extract from *T. thermophila* (S100) and purified TGP2 ($S100^{65}$ and F32) were complexed to ^{32}P-labeled Y or Y4 and analyzed by a gel mobility retardation assay. Lane 1: Negative control (N) lacking any protein but containing the reaction mixture and ^{32}P-Y. The monomeric (Y) and tetrameric forms (Y4) of the substrate have significantly different mobilities in the gel (arrows). Lane 2: S100 shifted ^{32}P-Y4 to several lower mobility positions corresponding to G4-DNA binding proteins TGP1 and TGP2. Lane 3: Heat-purified TGP2 ($S100^{65}$) illustrating that TGP2 is heat stable. Lane 4: TGP2 from chromatofocusing column Fraction 32 (F32) plus the ^{32}P-Y reaction mixture showed a single-shifted band. Lanes 5, 6 and 7: S100, $S100^{65}$, and F32, respectively, were incubated with the ^{32}P-Y−G reaction mixture. Oligo Y−G lacks the guanine-rich region necessary for Y4 formation, so no Y4 is formed. No shifted bands were observed. Substrate degradation seen in Lanes 5 resulted from nucleases in this relatively impure protein preparation. Lane 8, 9, and 10: The effects of altering reaction conditions on TGP2−Y4 complex formation. As would be expected for a G4-DNA molecule, potassium ions profoundly enhance Y4 formation. In the absence of potassium, very little Y4 forms, and consequently only a small amount of TGP2−Y4 complex is observed. Lane 11: 85 units of mung bean nuclease was incubated with the reaction mixture after formation of the TGP2−Y4 complex. Over 90% of ^{32}P-Y was digested, presumably in the single-stranded domain, suggesting that the single-stranded portion is not well protected by TGP2 in the complex.

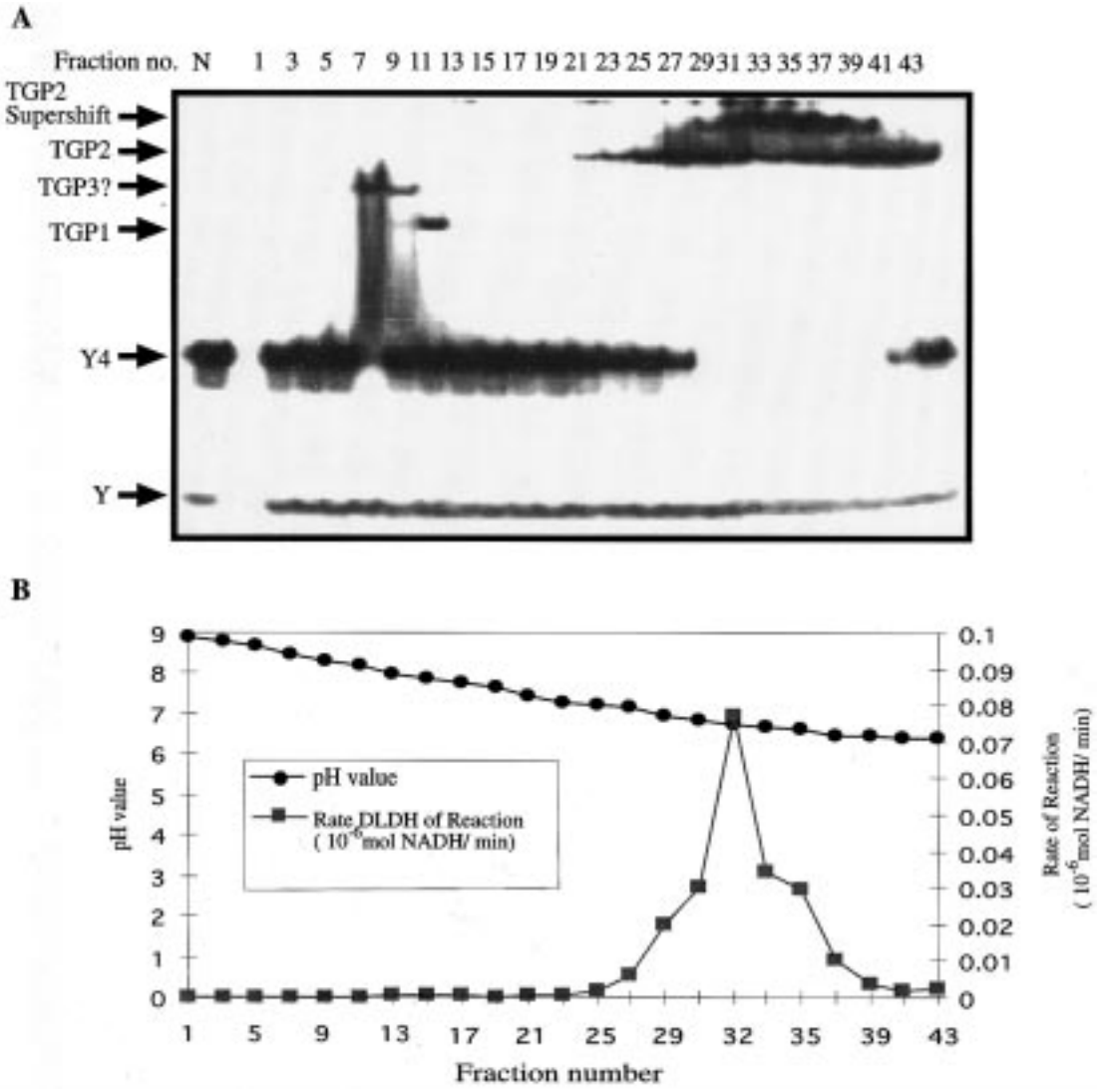
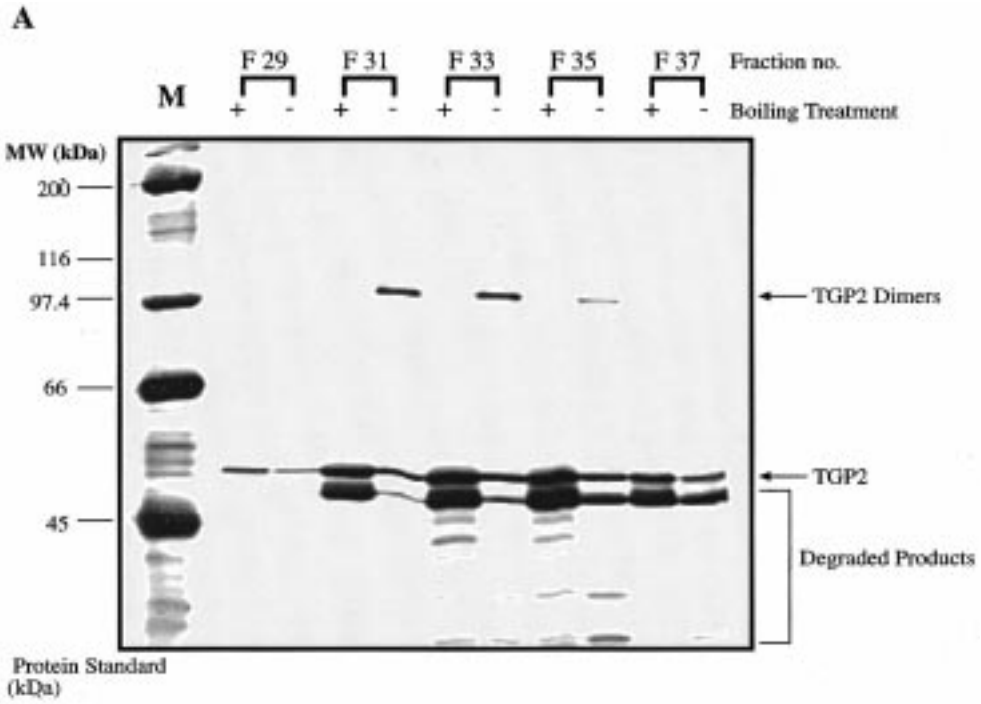


FIGURE 2: (A) TGP2 can be separated from other TGPs by chromatofocusing. Fractions with descending pH [shown in (B)] were tested for Y4 binding activity. Several Y4 binding proteins, including TGP1 (*12*) and an as yet uncharacterized protein, TGP3, appeared in earlier fractions (F7 to F11). TGP2 was concentrated in fractions 21−43. Fractions containing higher concentrations of TGP2 showed an additional band which was determined to be a supershift caused by TGP2. Fraction 32 (F32) was found in further analysis to contain the highest Y4 binding activity. (B) TGP2 has dihydrolipoamide dehydrogenase (DLDH) activity. After determination of a portion of the amino acid sequence for TGP2 and alignment with known sequences, very high similarity was observed between TGP2 and DLDH from a variety of species. DLDH assays confirmed that the chromatofocusing fraction containing TGP2 with the highest Y4 binding activity also had the highest DLDH activity. Thus, TGP2 is likely to be identical to DLDH in *Tetrahymena*.

of 0-fold, 2-fold, 4-fold, 12.5-fold, and 50-fold, respectively (Figure 5B). Since a more negative slope correlates with more efficient competition, the percent of decrease of the binding activity was converted to the slope between two molar excess points. Hence, pZ had slopes of −5.50, −5.48, −2.77, and −0.75. The average slope of the first three slopes for −4.58, pZ, was then compared to the average slope of pY, 23.87, and represented as percent competition (Figure 5D; all the fourth slopes were not included for the averages because some of the fourth slopes showed saturation). The percent competition of pZ was found to be about 19% of that of pY.

Purification of TGP2. S100 (20 mL) was thawed and immediately incubated in a 65 °C water bath for 20 min. Heat-treated S100, which contained a large amount of denatured protein, was centrifuged at 14 000 rpm for 15 min. Supernatant ($S100^{65}$) was collected, and white pellets were discarded. A chromatofocusing column packed with PBE94 matrix was equilibrated with 25 mM ethanolamine-HCl, pH 9.05, at the rate of 1 mL/min until the pH of effluent reached 9.00. Polybuffer96 was diluted 10-fold with ddH_2O and adjusted to pH 6.5 as the eluting buffer. Five milliliters of eluting buffer was loaded onto the column at 0.4 mL/min followed by ∼18 mL of $S100^{65}$. TGP2 was then eluted at a flow rate of 0.4 mL/min and collected in 7 mL fractions. The average difference of pH between each fraction was approximately 0.07 unit.

SDS−PAGE Analysis. Chromatofocusing fractions were tested for TGP2 activity in mobility retardation assays. Fractions with TGP2 activity were concentrated ∼29-fold using Centricon 10 spin concentrators. Samples were electrophoresed through a 10% polyacrylamide gel using the standard Laemmli method (*23*). The sample buffer contained 25% 4× Tris-HCl/SDS, pH 6.8 (v/v, 0.5 M Tris-HCl containing 0.4% SDS), 4% SDS (w/v), 2% 2-mercaptoethanol (v/v) or 3% DTT (w/v), and 0.001% bromophenol blue



B

Protein-Species		Amino Acid Sequence (N' to C')		Score	Predicted No.
TGP2	1	STSTQYDVV_IGG_PGGYVA_IKAGQLGL_TAT	33	-	-
DLDH-TRYBB	12	YDVVVVGGGPGGYVAAIKAAQLGLKTA	38	104	7.72e-15
DLDH-AZOVI	3	SQKFDVIVIGAGPGGYVAAIKSAQLGLKTA	31	95	2.82e-12
DLDH-PEA	33	SGSDENDVVIIGGGPGGYVAAIKAAQLGFKT	63	95	2.82e-12
DLDH-YEAST	27	HDVVVJGGGPAGYVAAIKAAQLGFNTA	53	90	7.04e-11
DLDH-PIG	43	DVTVIGSGPGGYVAAIKAAQLGFKT	67	80	3.74e-08
DLDH-HUMAN	43	DVTVIGSGPGGYVAAIKAAQLGFKT	67	80	3.74e-08

FIGURE 3: (A): TGP2 has a molecular mass of approximately 53 kDa and dimerizes in SDS in the absence of boiling. SDS−PAGE (10% polyacrylamide gel) analysis of concentrated chromatofocusing column fractions showed a major species with an apparent molecular mass of 53 kDa which was assigned to TGP2. The intensity of TGP2 increased from F29 to F33 and decreased thereafter. An equal amount of fractions was loaded in both plus and minus boiling treatment lanes. Degradation products were pronounced in fractions that were boiled in SDS prior to loading on the gel. In the absence of boiling, a band appeared at 110 kDa, and the TGP2 and degradation product bands decreased in intensity significantly. The 110 kDa band was tentatively identified as the previously described dimer form of TGP2. (B) The sequence of the first 33 amino acids of TGP2 was determined by Edman degradation sequencing. The underscore (_) indicates ambiguous amino acids. Alignment with protein sequences in the SWISSPRO database revealed high similarity with DLDH from a variety of species. The "Score" column indicates the degree of similarity, with the perfect score being 182. The "Predicted No." column is the number of results expected by chance to have a score greater than or equal to the score of the result obtained.

(w/v). All samples were mixed with sample buffer in a 1 to 1 ratio, followed by either heating to 95 °C or room-temperature incubation for 5 min, before loading onto the gel. The gel was run at 15 mA through a stacking gel (4% polyacrylamide), and at 20 mA in the separating gel (10% polyacrylamide). Electrophoresis was stopped when the bromophenol blue reached the bottom edge of the gel. The gel was then silver stained according to standard procedures.

Protein Sequencing. The concentrated chromatofocusing fraction showing the TGP2 band with the fewest degradation products (F29, Figure 3) was used for amino terminal sequencing. The concentrated fraction was separated by 10% SDS−PAGE, followed by 1 h electrotransferring to Transblot PVDF membrane (Bio-Rad). The membrane was then stained with 0.1% Coomassie Brilliant Blue (w/v) in 10% methanol (15 min), and destained in 45% methanol, 7% acetic acid (15 min) followed by a second destaining in 90% methanol, 7% acetic acid (2 min). The 53 kDa band was sliced out after air-drying overnight and subjected to Edman degradation sequence determination at the Protein Facility at Iowa State University. Protein concentration was measured by the method of Bradford using bovine serum albumin (BSA) as a standard (*23*).

Dihydrolipoamide Synthesis and DLDH Assay. Dihydrolipoamide was synthesized, and the DLDH activity assay was performed as described by Patel et al., with minor modification (*24*). DL-dihydrolipoamide was synthesized as described, except that the dried dihydrolipoamide was not further purified by HPLC. The final concentrations of components in the DLDH assay were 100 mM potassium phosphate, pH 8.0, 1.5 mM EDTA, 3.0 mM DL-dihydrolipoamide in 95% ethanol, and 3.0 mM NAD^+. After the addition of 50 μL of enzyme solution in a total reaction volume of 1 mL, the increase in NADH concentration was monitored at 340 nm using a DU-7400 spectrophotometer (Beckman). Only the absorbances obtained during the first three minutes were used for measurement of the DLDH reaction rate, since the increase of absorbances in this period was found to be linear (NADH is an inhibitor of DLDH).

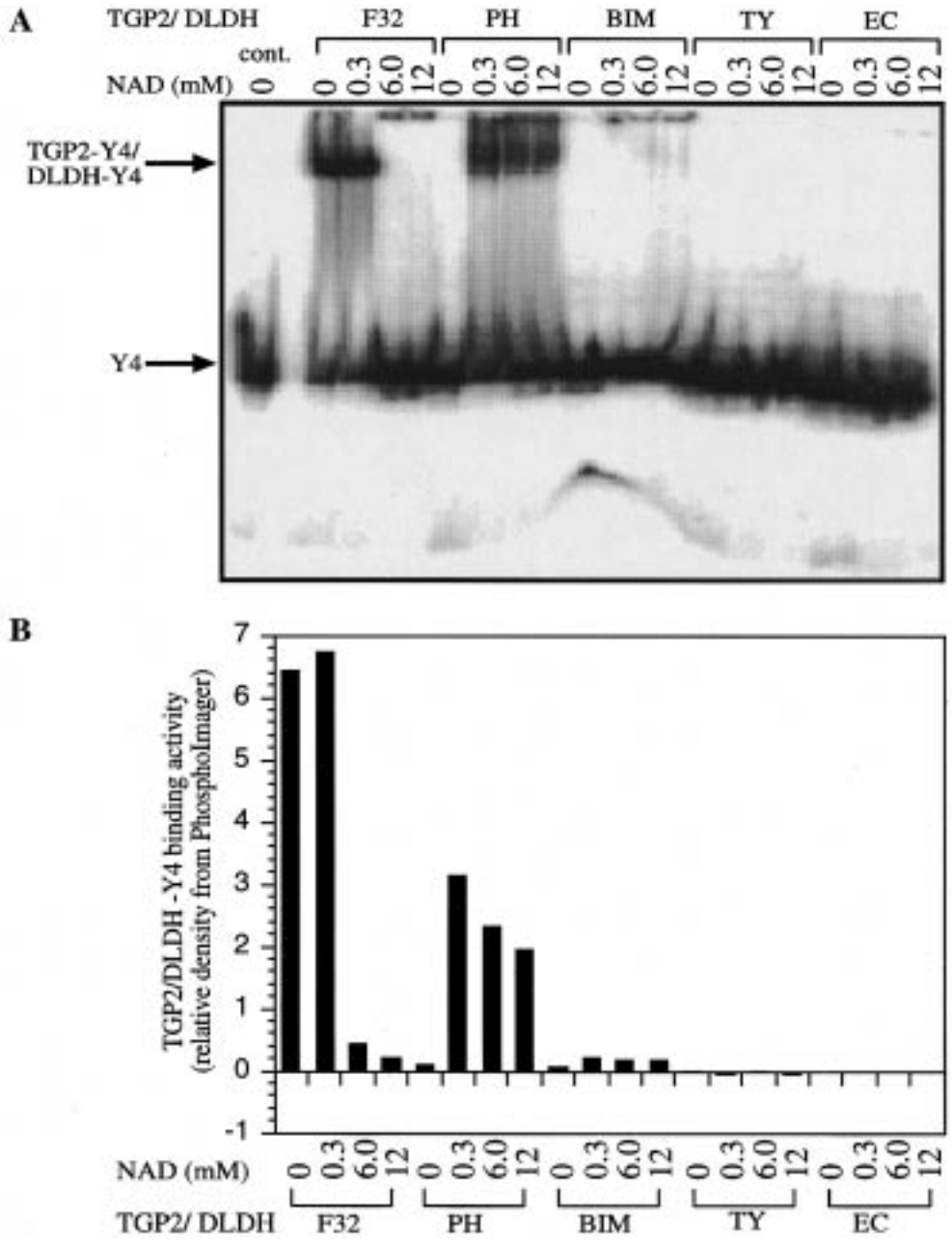


FIGURE 4: Commercial preparations of DLDH from two other species also showed Y4 binding activity in the presence of NAD^+. (A) Mobility retardation assay using several DLDH preparations from different species. TGP2 (F32, 0.013 mg/mL), type III porcine heart DLDH (PH, 0.034 mg/mL), bovine intestinal mucosa DLDH (BIM, 0.022 mg/mL), torula yeast DLDH (TY, 0.025 mg/mL), and *E. coli* DLDH (EC, 0.028 mg/mL) were incubated with ^{32}P-Y and analyzed on a 6% nondenaturing polyacrylamide gel. NAD^+ was added to the indicated final concentrations. NAD^+ concentration affected the degree of binding in all cases, with TGP2 being most sensitive to NAD^+ inhibition of Y4 binding. (B) Quantitation of the autoradiogram shown in panel A. All readings were subtracted from a background reading taken from the region corresponding to the TGP−4 complex mobility in the lane labeled "cont.", which did not contain any protein.

Calculation of Apparent K_d. Bound and unbound oligonucleotides were quantified using a Molecular Dynamics PhosphorImager. A constant amount of ^{32}P-Y mixture (4.7 nmol ^{32}P-Y4) was added to increasing amounts of TGP2 (chromatofocusing fraction 32). An apparent K_d was estimated from the resultant hyperbolic binding isotherm as described previously (Figure 6B) (*23*).

RESULTS

TGP2 Specifically Binds to a Parallel G4-DNA Structure. An oligonucleotide designated Y (*1*) was used for this study. This oligonucleotide has been well studied and shown to form a stable parallel-stranded G4 structure, Y4 (*1*, *12*). At low concentrations of ^{32}P-Y (31.25 nM) and low concentration of potassium ion (0.8 mM), over 97% of ^{32}P-Y forms the G4 structure ^{32}P-Y4 (Figure 1, lane 1). Crude cell extracts from *T. thermophila* (S100) were found to contain more than one ^{32}P-Y4 binding protein, all designated with the prefix TGP (*Tetrahymena thermophila* G4-binding Protein). These proteins included TGP1, which has already been reported (*12*), TGP2, described herein, and an as yet uncharacterized protein, TGP3 (Figure 2A). All TGPs bound to ^{32}P-Y4, but not the monomer, ^{32}P-Y. This is clearly shown by the mobility shift assay depicted in Figure 2A, F29 to F39, in which all ^{32}P-Y4 (tetramer) was shifted to the top of the gel but the monomeric ^{32}P-Y species remained at the

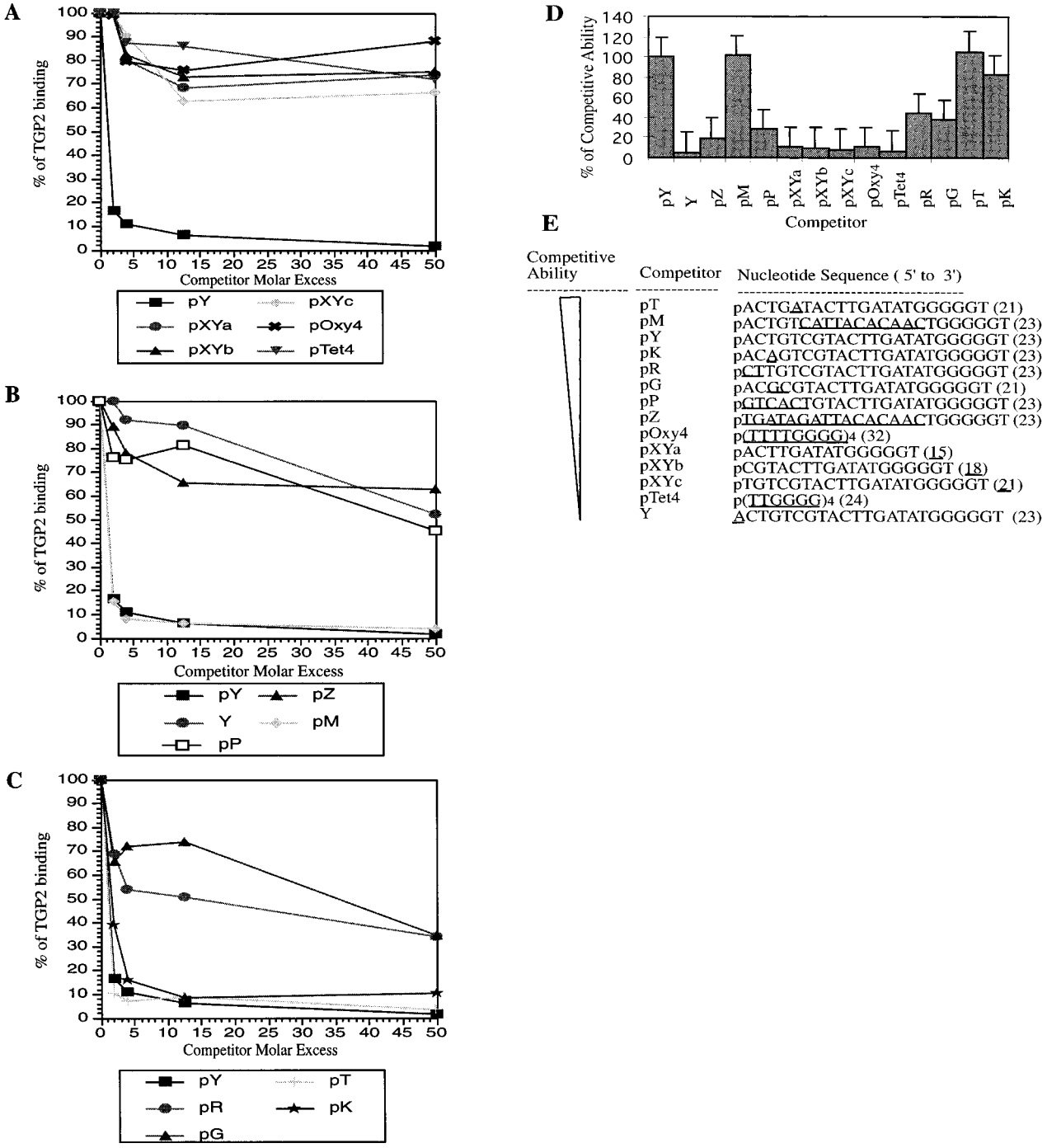


FIGURE 5: TGP2 specifically binds to parallel-stranded G4-DNA and shows sequence specificity at the 5′ end. Panels A, B, and C show the results of competition experiments analyzed by mobility shift assays. All oligonucleotides used are shown in panel E. (A) Two telomeric oligonucleotides (pTet4, pOxy4) with G-rich sequences and three oligonucleotides (pXYa, pXYb, pXYc) with different lengths were used to test telomeric binding ability of TGP2 and the substrate length requirement. (B) Competition with Y oligonucleotide with and without a 5′ phosphate showed that TGP2 bound specifically to 5′ phosphorylated species. Evaluation of the competitive ability of pZ, pM, and pP showed that TGP2 binding was sensitive to changes in the 5′ proximal 6 nucleotides. (C) competition with pT showed that 21 nucleotides were sufficient for TGP2 binding. Competition with pG, pR, and pK showed that changing 5′ nucleotides 1−4 reduces TGP2 binding to different levels. Combining these results revealed a preferred 5′ binding sequence, 5′-ACTG-3′. (D) Summary of the competitive abilities of the oligonucleotides used in this set of experiments. (E) The sequences of all the oligonucleotides used and their changes compared to pY. Nucleotide changes or deletions are underlined. pXYa, pXYb, and pXYc are truncated forms of pY.

bottom of the gel. The binding specificity was further confirmed by the fact that the molecule ^{32}P-Y-G, which lacks a G-rich domain and therefore cannot form G4-DNA, was not shifted by TGP1 or TGP2 (Figure 1, lanes 5, 6, and 7). As with most G4-DNA structures, potassium ions were found to be important for stabilizing the tetramer structure while magnesium ions were not. In the absence of potassium, over 93% of ^{32}P-Y remained monomeric and very little TGP2 binding activity was observed (compare TGP2, ^{32}P-Y4, and ^{32}P-Y radioactivity on lanes 4 and 8 in Figure 1). This further strengthened the proposal that without the formation of tetramer ^{32}P-Y4, TGP2 could not bind to the ^{32}P-Y monomer. Digestion of ^{32}P-Y4 after formation of the TGP2−^{32}P-Y4 complex with mung bean nuclease (Figure

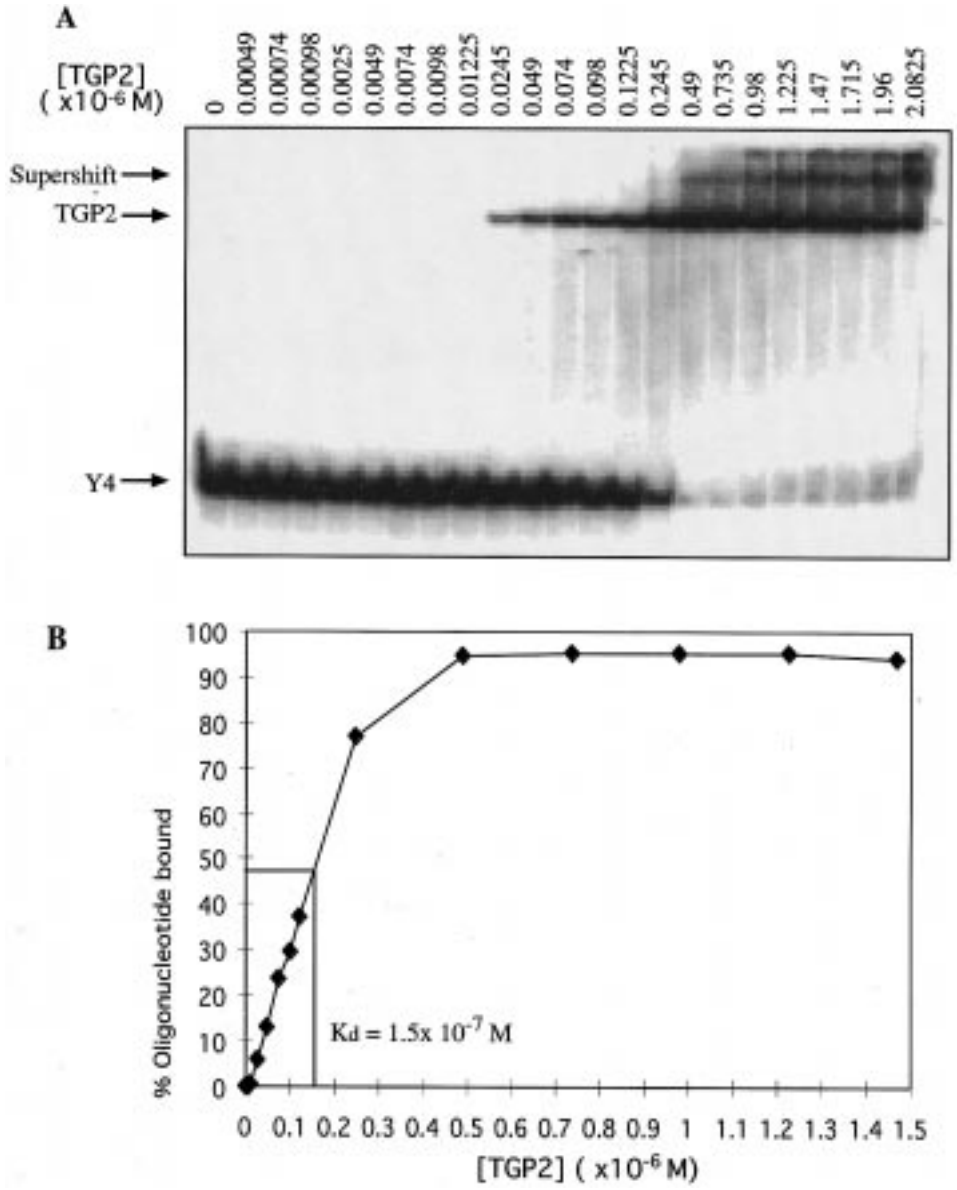


FIGURE 6: Determination of the apparent equilibrium dissociation constant for the complex of TGP2 and ^{32}P-Y. (A) Mobility shift assay in which ^{32}P-Y (4.7 nM) was incubated with the indicated concentrations of TGP2 (~0.49 to ~2080 nM). (B) An apparent K_d was estimated from the resultant hyperbolic binding isotherm (50% of the maximal binding).

1, lane 11) and with proteinase K (data not shown) both resulted in the disappearance of the shifted bands, confirming that the shifted band was a complex of protein and DNA.

Purification and Amino-Terminal Sequencing of TGP2. After S100 was heat treated as described in Experimental Procedures, ^{32}P-Y4 binding activity remained in the supernatant, a characteristic of the heat stable DLDH enzyme (Figure 1, lane 3). In the heat purification, at least 90% of the total cellular protein was precipitated at 65 °C, as calculated by comparing protein concentrations in S100 and S100^{65} (supernatant of heat-denatured S100).

TGP2 was further purified by chromatofocusing, a method that fractionates protein components according to their p*I*, with resolution of 0.07 pH unit. TGP2 was found to elute between pH 7.30 and 6.50 (Figure 2A,B). The fraction that showed highest TGP2 activity was fraction 32 (F32, data not shown) with a pH value of 6.75. SDS−PAGE analysis of F29 to F37 showed that the intensity of a predominant band at 53 kDa increased from F29 to F33 and decreased by F37 (Figure 3A). The intensities of one predominant slightly lower band (~47 kDa) and several minor lower bands increased and decreased in the same pattern as the 53 kDa band. Concentrated fractions that were not boiled prior to analysis showed a clear band at 110 kDa with the same intensity changes as the 53 kDa band. Furthermore, samples without boiling treatment all showed a decrease in intensities of bands equal to or lower than 53 kDa. Taken together, these data suggest that the 53 kDa band was TGP2 and that the lower bands (<53 kDa) were degradation products resulting from the heat treatment or preexisting in a multimeric form prior to heat denaturation in SDS. The appearance of the 110 kDa band was consistent with the dimerization of TGP2, a previously reported characteristic of DLDH (*25*). UV cross-linking experiments carried out using standard protein−Y4 reaction mixtures showed that both the 53 and 47 kDa bands bound to ^{32}P-Y4 (data not shown), supporting the notion that they were different forms of the same protein.

Table 1: Specific Activities of Dihydrolipoamide Dehydrogenases from Different Species

species/source	preparation	specific activity (units/mg)
T. thermophila	crude extract (S100)	0.44
T. thermophila	heat purified ($S100^{65}$)	1.68
T. thermophila	chromatofocusing (F32)	13.78
Haloferax volcanii	crude extract	0.1[37]
Haloferax volcanii	purified enzyme	16.1[37]
porcine heart	purified enzyme	100−200[38]
torula yeast	purified enzyme	40−100[38]
bovine intestinal mucosa	purified enzyme	100−200[38]
E. coli	purified enzyme	120[39]

Edman degradation sequencing of the 53 kDa protein revealed the sequence of 33 amino acids from the amino terminus (Figure 3B). After searching in the SWISSPRO database using the BLITZ server and MPsrch program (*26*), it was found that dihyrolipoamide dehydrogenases (DLDH) from a variety of species had high similarity to TGP2 (Figure 3B). The heat stability of DLDH (*27*) and its ability to form homodimer functional units (*17*) correlated well with the observed characteristics of TGP2, strongly suggesting that TGP2 was DLDH in *T. thermophila*.

DLDH Activity of TGP2. To test further the idea that DLDH and TGP2 were the same protein, a DLDH activity assay was performed on the chromatofocusing fractions. This assay showed that a DLDH activity gradient existed which increased from F21 to F32 and decreased from F32 to F43 (Figure 2B). The highest DLDH activity appeared in F32. Negative controls lacking either dihydrolipoamide or NAD^+ showed no increased enzyme activity after the addition of F32, confirming the substrate specificity of F32. The fractions that expressed DLDH activity were strongly correlated with the fractions showing ^{32}P-Y4 binding activity. The fraction which showed the highest ^{32}P-Y4 binding activity among the fractions, was also the fraction expressing highest DLDH activity. This further supported the proposal that TGP2 is DLDH in *T. thermophila*. On the basis of measured specific activities of DLDH/TGP2 in purification stages S100, $S100^{65}$, and F32, we attained a minimum purification of 30-fold for DLDH by the protocol described here (Table 1). The fractions collected from the cation exchange column also showed the same pattern of activities and correlation between Y4 binding and DLDH activity. The 53kD band also appeared at the highest concentration in the fraction with the highest amount of DLDH activity and Y4 binding activity (data not shown).

DLDH of Other Species Show NAD^+-Dependent Binding to ^{32}P-Y4. To determine whether DLDHs of other species have ^{32}P-Y4 binding activity, several purified DLDHs from different species were tested in gel mobility retardation assays. DLDHs from porcine heart, bovine intestinal mucosa, torula yeast, and *E. coli* showed no ^{32}P-Y4 binding activity without addition of NAD^+ to the reaction mixtures. However, when 0.3 nM NAD^+ was added to the reaction mixtures, porcine heart and bovine intestinal mucosa DLDH showed significant ^{32}P-Y4 binding activity, but torula yeast and *E. coli* DLDHs still showed no significant activity (Figure 4A,B). When the NAD^+ concentration was increased from 6 to 12 mM, ^{32}P-Y4 binding activities of TGP2, porcine heart, and Bovine Intestinal Mucosa DLDH all decreased. The extent of decrease in the activities varied from species to species, with the greatest extent in TGP2, followed by porcine heart DLDH, and with the least decrease seen with bovine intestinal mucosa DLDH. These results showed that DLDHs of certain species could bind to ^{32}P-Y4 and that this binding was dependent upon NAD^+ concentration.

Sequence and Structural Requirements for TGP2 Binding. TGP2 requires a substrate with both a G4-DNA and single-stranded domain for optimal binding. We designed a series of oligonucleotides to further explore this characteristic. pXYa, pXYb, and pXYc are truncated forms of pY (the lowercase p indicates the presence of a nonradioactive 5′ phosphate) with nucleotides removed from the 5′ end, and pT contains an internal sequence change and deletion (Figure 5E). These oligonucleotides were used as competitors to investigate the length specificity of TGP2 binding with regard to the single-strand domain. All three truncated oligonucleotides were found to have low competitive abilities (Figure 5A,D). However, pT, which was 21 nucleotides long but retained the 5′ end sequence of pY, was as efficient a competitor as was pY. Therefore, we concluded that length was a less critical factor than was the sequence at the 5′ end of the single-stranded domain.

In another set of experiments it was observed that the nonphosphorylated form of Y was a poor competitor for TGP2 binding relative to ^{32}P-Y. However, when the nonradiolabeled Y competitor was 5′ phosphorylated (pY), its ability to compete with ^{32}P-Y4 for binding to TGP2 increased dramatically (Figure 5B). Thus, TGP2 requires the 5′ phosphate for optimal binding activity and may recognize this phosphate group directly.

On the basis of the experiments described above, TGP2 requires a substrate with a G4 domain adjacent to a single-stranded domain for binding. Furthermore, the single stranded domain has strong sequence constraints at the 5′ end and must be 5′ phosphorylated. The sequence specificity of TGP2 was further tested using a series of oligonucleotides related to the pY4 substrate. pZ contained a 16 nucleotide random sequence at the 5′ end of the single-stranded domain, but it retained the G-rich domain and the ability to form G4-DNA (Figure 5E). pZ showed very low competitive ability with pY (Figure 5B,D). Further investigation of sequence specificity by using pM (altered middle portion of the single-stranded sequence), pP (altered the last 5 nucleotides from the 5′ end) as competitors, clearly showed that changing the last five nucleotides of the original pY sequence decreased the ability of the altered molecules to compete with ^{32}P-Y4 for TGP2 binding. These data support the proposal that TGP2 specifically binds to oligonucleotides that have a defined sequence in the 5′ single-stranded domain, but indicate that the sequence requirements starting from nucleotide 6 to 16 were less specific.

To narrow further the sequence requirements for TGP2 binding, another series of oligonucleotides, pT, pR, pG, and pK, was used as competitors (Figure 5E). The ability of pT to compete effectively indicated that nucleotide number 5 from the 5′ was not highly sequence constrained. In contrast, the inability of oligonucleotides pR and pG to compete effectively showed that nucleotides 1−4 were critical (Figure 5C). Oligonucleotide pK, which has a single nucleotide change at position 3 from the 5′ end, showed a minor but significant decrease in competitive ability, indicating that nucleotide 3 was also sequence specific. Oligonucleotide

pG which had two nucleotide changes at positions 3 and 4 showed much less competitive ability compared to pK, which had a change at position 3, indicating that nucleotide 4 was also important for binding specificity.

Considering the results of all the competition assays and the results using ^{32}P-Y-G as probe (Figure 1), we conclude that TGP2 binds to a substrate with a G4-DNA domain at the 3′ end, a single-stranded domain at the 5′ end, requires a 5′ phosphate, and requires the 5′ sequence 5′-ACTG-3′ for optimal binding.

Dimerization and Apparent K_d of TGP2 Using ^{32}P-Y4. When relatively high concentrations of TGP2 were added to a typical ^{32}P-Y4 reaction mixture, a band of lower mobility appeared above the characteristic TGP2−Y4 complex band (Figure 6, 0.49−2.08 μM). This band was designated the TGP2 supershift band. Since the intensities of the characteristic TGP2 band decreased when the supershift appeared (Figure 2A, F25−F35; Figure 6A, 0.49−2.08 μM), it was proposed that TGP2 was shifted to the higher position. Referring to the resultant hyperbolic binding isotherm (Figure 6B), the supershift band appeared after the binding activity had reached its maximum (95%). Therefore, it was reasonable to postulate that since the supershift appeared when all available ^{32}P-Y4 had been bound, it represents the binding of additional TGP2 to Y4−TGP2 complexes. Since DLDH forms dimers, it is likely that this supershift represents the dimerization event. The K_d of TGP2 with ^{32}P-Y4 was estimated from the isotherm curve (Figure 6B) to be approximately 1.5×10^{-7} M.

DISCUSSION

Our studies showed that a G4-DNA binding protein, TGP2, copurified with DLDH enzyme activity (Figure 2A,B). Moreover, purified DLDH from porcine heart and bovine intestinal mucosa bound to the G4-DNA substrate ^{32}P-Y4 and shifted it to the same position as did TGP2 in mobility retardation assays (Figure 4A), indicating a shared G4-DNA binding activity for DLDH from different species. The high similarity between amino acid sequences of TGP2 and a variety of DLDHs (Figure 3B) provided additional evidence that TGP2 was DLDH in *T. thermophila*. Hence, we propose that TGP2 has dual functions as a G4-DNA binding protein and the metabolic enzyme DLDH.

Purification and characterization of DLDH from *T. thermophila* has not been previously reported. DLDH from several species, including humans, has been purified and is apparently encoded by a single gene (*28*, *29*). If TGP2 is DLDH in *T. thermophila* and if the DLDH is coded by a single gene, TGP2 and DLDH will have the same gene sequence. Cloning the TGP2 gene and testing the expressed gene product for DLDH activity and G4-DNA binding will definitively demonstrate that TGP2 is DLDH in *T. thermophila*.

The effect of NAD^+ on the binding of TGP2 and other DLDHs to ^{32}P-Y4 is significant and interesting. NAD^+ is the oxidizing substrate in the forward reaction of DLDH (*17*). In our studies, 0.3 mM NAD^+ increased the binding of TGP2, porcine heart DLDH, and bovine intestinal mucosa DLDH to ^{32}P-Y4. However, 6 and 12 mM NAD^+ significantly decreased the binding of TGP2 and PH-DLDH to ^{32}P-Y4 (Figure 4A,B). Since pY4 was not an inhibitor of the DLDH forward reaction (data not shown), it is likely that the G4-DNA binding site is different from the enzymatic active site. These data suggest that NAD^+ affects the binding of ^{32}P-Y4 in a complex way that is neither a straightforward inhibition nor allosteric facilitation. Further investigation is needed to understand the role of NAD^+ in TGP2 binding activity.

Dimerization and Supershift of TGP2. Another interesting characteristic of TGP2 was the apparent dimerization in SDS−PAGE analysis (Figure 3A) and the supershift that appeared when TGP2 reached a certain concentration (Figures 2A and 6A). The crystal structure of lipoamide dehydrogenase from *Azotobacter vinelandii* revealed that there are hydrogen bond interactions between two subunits/monomers (*25*). Moreover, DLDH is inactive as a monomer because the active site is composed of residues from both subunits (*17*). A sufficiently strong native interaction between the monomers might stabilize DLDH, even in the presence of SDS at concentrations typically used in SDS−PAGE. The supershift of TGP2 in mobility shift experiments can then be explained by a predisposition of the protein to form dimers and is consistent with the apparent doubling in size of the observed complex. Thus, at low concentration, TGP2 might bind to ^{32}P-Y4 as a monomer, but raising the concentration drives dimer formation, resulting in the observed supershift. An interesting consequence of this model is that the dimerization domain is not likely to be close to the G4-DNA binding domain since this would disrupt dimerization. Moreover, this positions the G4-DNA binding domain away from the enzyme active site, which is at the dimerization interface. This is consistent with our enzyme activity results indicating that the G4-DNA substrate did not inhibit DLDH activity (data not shown). Finally, this model would provide a mechanism for the enzyme to bind to defined DNA domains and retain enzyme activity to control the local redox environment as speculated below. However, an alternative explanation for the supershift is that a second protein binds to the DNA component of the TGP2−Y4 complex. This seems less likely since the G4 domain is quite small and the mechanism for two proteins binding to this target is not obvious. However, if single-strand binding proteins are present in low concentration, they could bind to the single-stranded domains (four per Y4 molecule) and the result would be a mobility decrease. In this case, we would expect a variety of lower mobility complexes to result, but this is not observed.

TGP2 Binding Specificity. Some of the previously reported G4-DNA binding proteins appear to require only the G4 structure. These include MyoD (a transcription factor that regulates myogenesis) (*9*), QUAD (a hepatocyte chromatin protein) (*30*), and the β-subunit of the Oxytricha telomeric end binding protein (*13*). Others require a single-stranded domain along with the G4-structure. These include topoisomerase II from chicken blood (*11*), the KEM1 gene product in yeast (*15*), and the *Tetrahymena* G4 DNA binding protein TGP1 (*12*). It was reported that topoisomerase II can only cleave parallel-stranded G4-oligonucleotides whose sequence corresponds to the human immunoglobulin switch region, but the KEM1 gene product has no sequence requirement for its G4-dependent nuclease activity. Though TGP1 was found to require a single-stranded region in addition to the G4 structure (*12*), no other sequence specific-

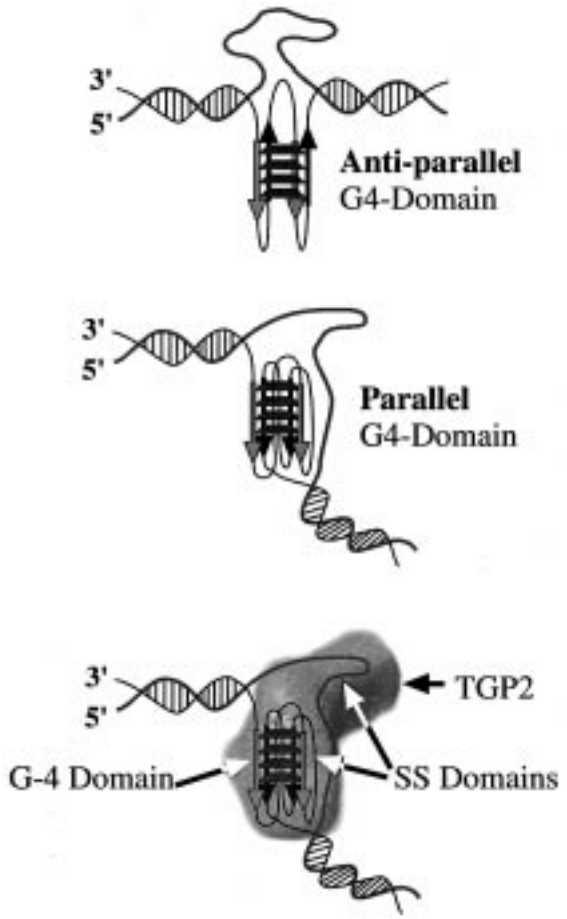


FIGURE 7: Speculative diagram illustrating the concept of extrusion of intramolecular parallel or antiparallel G4-DNA domains in a Watson−Crick duplex DNA environment. Local denaturation of the duplex allows formation of G4-DNA between runs of Gs in one strand of the duplex. G4-DNA binding proteins such as TGP2 could then bind to both the G4 domain and the single-strand domains, thereby further stabilizing the non-Watson−Crick structure.

ity was apparent for this protein. TGP2 is the first G4-DNA binding protein reported to require a specific sequence element at the 5′ end of a single-strand domain. Moreover, phosphorylation of the 5′ end was also required for optimal binding. Thus, TGP2 binds to a substrate with the following characteristics: 5′-PO_4-ACTG$(X)_n$-G4-3′ (Figure 5E). The 17 nucleotides forming the 5′ end of Y were originally randomly selected in the design of oligonucleotides capable of forming parallel-stranded G4 structures (D. Sen, personal communication). The reason for the nucleotide sequence and structural specificity of TGP2 is not clear. A search of the Genbank database using the program BLASTN revealed no strong match of Y sequence (*31*). However, there are many nucleotide sequences which have the motif 5′-PO_4-ACTG$(X)_n$-G4-3′, and we are currently investigating the possibility that this sequence motif has functional relevance with regard to TGP2 binding.

Why Does TGP2/DLDH Bind DNA? Thus far, no DLDH from any species has been reported to have a direct interaction with DNA. Interestingly, it has been reported (*32*) that the substrate of DLDH, dihydrolipoamide, enhanced binding of the transcription factor NF-kB to the enhancer sequence of HIV-1. Moreover, several other thiol reductants have been found to enhance the binding of NF-kB to the enhancer element (*32*), consistent with NF-kB being a reduction/oxidation-sensitive transcription factor. Several findings have suggested that binding of transcription factors, including NF-kB and Sp1, mediates structural changes in chromatin at the enhancer region bound by the transcription factors (*33*, *34*). These rearrangements create nuclease hypersensitive sites, consistent with the idea of alterations in the DNA structure, which could include formation of G4-DNA domains. Thus, we suggest the provocative (albeit speculative) hypothesis that enhancers containing blocks of guanines, such as the HIV-1 enhancer, might form G4 structures to which TGP2−DLDH could bind and modulate binding by NF-kB or other redox sensitive transcription factors (*35*, *36*) by regulating the local redox environment, or some other mechanisms. We have obtained preliminary evidence that the HIV-1 enhancer can form G-4 DNA to which TGP2 binds, and these data will be presented in a subsequent report.

The formation of single-stranded domains occurs in genomic DNA during replication, transcription, recombination, gene rearrangement, and possibly other processes, including regulation of gene expression. There is substantial precedent for the existence of single-stranded DNA binding proteins that bind to transient single-stranded domains formed during these processes. It is possible that some of these single-stranded domains may be "protected" by other mechanisms, including formation of G4-DNA and binding by G4-DNA-specific proteins. The scenario of interstrand G4-DNA formation has been previously proposed (*1*, *15*). In the case of a transiently denatured single duplex, a G-rich strand could form an intrastrand G4 domain, while the C-rich complementary strand would be single stranded (Figure 7). A protein with the characteristics of TGP2, capable of binding parallel or antiparallel G-DNA and single-stranded DNA, could bind to this domain. This process could function as a molecular switch, as previously proposed by Sen and Gilbert (*1*). While these types of models are interesting and provide the basis for continued experimentation, definitive demonstration of the existence and role of G4-DNA in vivo remains an elusive goal.

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