Substrate and DNA Binding to a 50-residue Peptide Fragment of DNA Polymerase I

COMPARISON WITH THE ENZYME*

Gregory P. Mullen, P. Shenbagamurthi, and Albert S. Mildvan†

From the Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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The fluorescent nucleotide 2′,3′-trinitropheryl-ATP (TNP-ATP) binds to the triphosphate substrate binding site of the large (Klenow) fragment of DNA polymerase I (Pol I) as detected by direct binding studies measuring the increase in fluorescence of this ligand (n = 1.0, KD = 0.07 μM). The enzyme-TNP-ATP complex binds Mg2+ and Mn2+ tightly (KD = 0.05 μM) as measured by an increase in fluorescence on titrating with these metals. The substrate dGTP competitively displaces TNP-ATP from the enzyme (KD = 5.7 μM) de-enhancing the fluorescence. The polymerase reaction is half-maximally inhibited by 0.8 μM TNP-ATP in the presence of dATP (10 μM) as substrate.

A region of the amino acid sequence of Pol I (peptide I) consisting of residues 728–777 has been synthesized and found to contain significant secondary structure by CD both in water and 50% methanol/water. In water at 3 °C, peptide I binds the substrate analog TNP-ATP (KD = 0.03 μM) with a stoichiometry of 2. In 50% methanol at 3 °C, peptide I binds TNP-ATP with a higher stoichiometry than in water, consistent with a 1:1 complex, but biphasically (16% of the peptide, KD = 0.09 μM; 84% of the peptide, KD = 5.0 μM), and competitively binds the Pol I substrates dATP, TTP, and dGTP (KD = 230–570 μM). Evidence from size exclusion high performance liquid chromatography suggests that these two forms of the peptide are monomer and dimer, respectively. Significantly, the peptide I-TNP-ATP complex binds duplex DNA, tightly (KD = 0.1–0.5 μM) and stoichiometrically, and single stranded DNA more weakly. The peptide I-duplex DNA complex binds both TNP-ATP (KD = 0.5–1.5 μM) and Pol I substrates (KD = 350–2100 μM) stoichiometrically. In a control experiment, a second peptide, peptide II, based on residues 840–888 of the Pol I sequence, retains secondary structure, as detected by CD, but displays no binding of TNP-ATP. The ability of peptide I, which represents only 8% of the large fragment of Pol I, to bind both substrates and duplex DNA indicates that residues 728–777 constitute a major portion of the substrate binding site of this enzyme.

The residues participating in substrate binding on DNA polymerase I from Escherichia coli and the mechanism of subsequent chain elongation of a DNA primer terminus, catalyzed by this enzyme, remain to be experimentally elucidated. Clues to the location of the substrate binding site in the x-ray structure of the large fragment of Pol I (Ollis et al., 1985) have been provided by intermolecular nuclear Overhauser effect studies which suggest the presence of Tyr, Ile, and other hydrophobic residues in close proximity to the bound substrate (Ferrin and Mildvan, 1985, 1986), and by photoaffinity labeling with 8-azido-dATP of Tyr-766 (Joyce et al., 1985) which is in the hydrophobic sequence Leu-Ile-Tyr (Joyce et al., 1982). Additionally, Lys-758 has been modified by pyridoxal phosphate functioning as an affinity label (Basu and Modak, 1987), and His-851 has been photoaffinity labeled with TTP (Pandey et al., 1987). Three of these residues, Lys-758, Ile-765, and Tyr-766 are part of the Ω-helix in the enzyme crystal structure, while His-851 is in the turn between β-strands 12 and 13 (Fig. 1). Based on the labeling experiments and on the size of the DNA-binding site estimated by footprinting experiments, Joyce et al. (1985) and Joyce and Steitz (1987) have suggested that the dNTP binding site on the large fragment of Pol I, lies somewhere between the C-terminal end of the Ω-helix, the N-terminal end of the Ω-helix and the bed formed by strands 7, 8, 12, and 13 of the antiparallel β-sheet shown in the schematic three-dimensional structure of the enzyme (Fig. 1).

It appeared feasible that a peptide segment from one of these regions of Pol I could provide a significant part of the substrate binding site. Such peptide fragments of enzymes have been shown to functionally and structurally mimic the corresponding sequence of residues in their enzyme counterparts. One example of this is the 45-residue adenylate kinase peptide (Hamada et al., 1979; Fry et al., 1985, 1986), which binds Mg2+ATP with an affinity and in a conformation similar to those found on adenylate kinase. Another example is the homologous peptide from F1 ATPase (Garboczi et al., 1988). While adenylate kinase shows regions of amino acid sequence homology with a number of other nucleotide-binding enzymes (Walker et al., 1982; Fry et al., 1985, 1986), DNA polymerases are not among them (Joyce et al., 1982). The approach we are using for elucidating the substrate binding site on Pol I is one similar in principle to that used for adenylate kinase. Accordingly, two peptides from Pol I were synthesized, one comprising

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†To whom correspondence should be addressed: Dept. of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

1 The abbreviations used are: Pol I, DNA polymerase I; TNP-ATP, 2′,3′-trinitropheryl adenosine triphosphate; HPLC, high pressure liquid chromatography; CD, circular dichroism; Boc, t-butoxycarbonyl; dNTP, deoxynucleoside triphosphates; Pipes, 1,4-piperazine-diethylasulfonic acid; AMP-CPP, adenosine 5′-(α,β-methylene) triphosphate.

19637
Binding Properties of a Peptide Fragment of Pol I

**Methods**

**Kinetic Studies of the Effects of TNP-ATP on the DNA Polymerase Reaction**—Kinetic experiments were performed at 29 °C in 10 mM Tris-HCl, pH 7.5, 35 mM KCl with a total Mg²⁺ concentration of 1.7 mM. The concentrations of poly(dT), oligo(dA)₃₄₅, and enzyme were 0.13 mg/ml, 0.033 mg/ml, and 5.8 nM, respectively, in a reaction volume of 150 μl. Bovine serum albumin (67 μg/ml) was used to stabilize the enzyme. For kinetic experiments, the D355A, E357A exonuclease-deficient mutant of the large fragment of Pol I from the E. coli strain CJ-375 was used. This engineered E. coli strain CJ-375 was generously provided by C. Joyce.

Assays were performed with GFC glass fiber filters as described by Setlow (1974) using 20-min reaction times. At a constant concentration of 10 μM dATP, 10 concentrations of TNP-ATP ranging from 0.22 to 54.6 μM reduced the velocity of the reaction asymptotically toward zero.

**Synthesis and Purification of the 50-Amino Acid Peptide (Residues 728-777) from Pol I (Peptide I)**—The peptide was synthesized using an Applied Biosystems Inc. model 430A peptide synthesizer which automates the solid phase method of Merrifield (1963). The procedure for the synthesis and purification was essentially the same as described previously (Garboczi et al., 1988) except for the following. Synthesis was initiated with the Boc-Leu-OCH₂-phenylacetamido methyl resin, and 40 mg of the 50-residue peptide was obtained which, after purification by HPLC, was >85% pure. A second purification by HPLC yielded 17 mg of peptide I with >95% purity on pooling and lyophilizing the appropriate fractions. In both HPLC purifications, a semipreparative C₁₈ column (10 μm, 30 × 0.78 cm) was used. Buffer A contained water and 0.03% trifluoroacetic acid, and buffer B contained acetonitrile and 0.03% trifluoroacetic acid. The peptide was purified by loading 4-mg quantities of the peptide and using a gradient of 30-50% buffer B in buffer A over 30 min.

**Synthesis and Partial Purification of the 49-Amino Acid Peptide (Residues 840-888) from Pol I (Peptide II)**—The conditions for the synthesis of this peptide were the same as those described for peptide I. HPLC purification of the crude peptide using the gradient conditions described above followed by lyophilization of the appropriate fractions gave 40 mg of peptide II with 50% purity as estimated by HPLC. A second attempted purification yielded 11 mg of the peptide without significantly increasing the peptide purity. UV and fluorescence measurements indicated that the single tryptophan in the 49-amino acid peptide had undergone partial degradation in the purification procedure. In these spectroscopic quantitations, N-acetyltryp- tophan amide was used as a standard. The amount of intact tryptophan in the sample was 46% by tryptophan fluorescence, and the UV spectrum showed additional absorbing species below 270 nm.

**Amino Acid Analyses**—For amino acid analyses, the peptides (~50 μg) were hydrolyzed in 6 N HCl containing 1% phenol (v/v) for 24 h, and the resulting amino acids were derivatized with phenyl isothio- cyanate. The solutions of the phenylthiocarbamyl derivatives were separated by reverse phase HPLC and their absolute quantity calibrated with respect to phenylthiocarbamyl derivatives of amino acid standards (250 pmol) using the Millipore-Waters PICO- TAG amino acid analysis system. The percentage of picomoles of each amino acid in the peptides was multiplied by the total number of residues in the peptides to yield the experimental number of each amino acid in the two peptides.

**Preparation of Samples for Use**—Before use, all solutions except the peptide, enzyme, and Mg²⁺ solutions were passed through Chelex 100. The purity of nucleoside triphosphates was checked using polyethyleneimine-cellulose TLC in 1 M LiCl or 0.5 M LiCl, 2 M HCOOH. In preparation of the stock solution of peptide I, the solid peptide

**Experimental Procedures**

**Materials**

The Boc-protected amino acids, the Boc-Leu-OCH₂-phenylacetamido methyl resin, and the reagents for peptide synthesis were purchased from Applied Biosystems, Inc. Other reagents for peptide synthesis and purification and reagents for amino acid analysis have been previously described (Garboczi et al., 1988). Tris-HCl, Pipes, Sephadex G-15, and Sephadex G-25 were purchased from Sigma. TNP-ATP was a product of Molecular Probes and was purified by scaling up the procedure described by Grubmeyer and Paneksky (1981). All deoxyribonucleotide triphosphates and oligodeoxynucleotides were purchased from Pharmacia LKB Biotechnology Inc. Utrapure urea was a product of Mann Research Laboratories. Polyethyleneimine-cellulose plates were purchased from Brinkman Instruments. Chelex 100 was purchased from Bio-Rad and was converted to the form before use. Spectroscopically pure (99.999%) magnesium chloride hexahydrate was purchased from Aldrich. E. coli strain (CJ155), which overproduces the large fragment of DNA polymerase I, was generously provided by C. Joyce and N. Grindley of Yale University School of Medicine. The large fragment of DNA polymerase I from CJ155 was previously purified in this laboratory (Ferrin and Midvand, 1985, 1986) using the method of Joyce and Grindley (1983).
was dissolved, adjusted to pH 6.9, and denatured at 0 °C in 6.4 M urea for 1 h at pH 6.8. The peptide was separated from the urea, trifluoroacetic acid, and metal impurities by eluting it from a Sephadex G-15 column (1 × 18 cm), at 4 °C, which had been washed with 20 mM EDTA and equilibrated with 10 mM Pipes, pH 6.9, 35 mM KC1. As described, the basis of the weight of the dry peptide and a M, of 6300 calculated from the amino acid composition and the expected number of trifluoroacetic acid counter-ions. This 1/20 was used in the calculation of all peptide concentrations. All studies on peptide I were performed at pH 6.9 due to a cloudiness of the peptide solution at pH 7.45 which is indicative of a result of peptide precipitation near the isoelectric point. The isoelectric point is approximately pH 8.0 on the basis of its composition.

The state of oligomerization of peptide I was studied by HPLC using a Bio-Sil TSK-125 size exclusion column obtained from Bio-Rad both in water containing 10 mM Pipes, pH 6.9, 35 mM KC1 at 25 °C and in the same buffer containing 50% methanol at 0 °C.

The enzyme, which was stored in 50 mM K+ phosphate buffer, pH 7.0, 0.5 mM diithiothreitol buffer in 50% (v/v) glycerol, was eluted from a Sephadex G-25 column (1.4 × 19 cm) at 4 °C, which had been washed with 20 mM EDTA and equilibrated with 10 mM Tris, pH 7.5, 32 mM KC1 at 0.9-6.3 pM concentrations of the large fragment of Pol I was 11,000 units/mg. The concentration of the large fragment was determined using A280 = 9.3 (Setlow et al., 1972) and a M, of 68,000.

The concentrations of TNP-ATP, dGTP, dATP, and TTP were determined using ε266 = 56,400 (Hiratsuka, 1982), ε9390 = 15,700, ε8390 = 15,200, and ε7590 = 9600 M-1 cm-1, respectively (Dawson et al., 1986). The concentrations of p(dA)6, p(dA)18, p(dT)18, and p(dTh1, were determined using ε7590 = 15,700, ε8390 = 9600 (Cassoni and Bollum, 1969), and ε9390 = 9600 M-1 cm-1 (Iman and Baldwin, 1962), respectively, which are per residue extinction coefficients.

**CD Spectroscopy**—CD spectra were obtained on an AVIV 60DS spectropolarimeter with the cell chamber flushed with nitrogen using 0.5-6 mM sample at 2°C contained with 164 μM peptide I in 10 mM Pipes, pH 6.9, 35 mM KC1 in a 0.1-mm cell. The two samples at 2°C contained 2 μM peptide I and 2 μM peptide I together with 3.6 μM TNP-ATP, respectively, in 5 mM Pipes, pH 6.9, 17 mM KC1, 50% methanol in a 1-cm cell. With peptide II, the 30-μl sample at 24°C contained 20 μM peptide II in 10 mM Tris-HCl, pH 8.0, 32 mM KC1 in a 0.1-mm cell. At 3°C the sample contained 19 μM peptide II, 5 mM Tris-HCl, pH 8.0, 16 mM KC1, 50% methanol in a 1-mm cell. The spectra obtained from the samples were an average of 5 scans and were smoothed and base-line-corrected after the appropriate control spectrum without the peptide present was subtracted. The spectra obtained were smoothed and base-line-corrected after the appropriate multiplication factor, obtained by measuring a fluorescence emission at both settings.

**Fluorescence Measurements**—The fluorescence emission spectra of TNP-ATP and TNP-ATP in its complexes with peptide I or the enzyme were obtained on an Amico Bowman spectrophotofluorimeter by exciting at 412 nm and recording the trace of the emission spectrum from 250 to 650 nm. The height of the fluorescence emission maximum at 540 nm was measured from the base line. Tryptophan fluorescence of peptide II was measured by excitation at 295 nm and monitoring the emission at 355 nm. When necessary, correction for dilution were made. In all titrations, the total increase in volume never exceeded 20% and was typically less than 15%. In the analysis of the titrations, the height of the baseline, measured from the fluorescence emission maximum. Measurements requiring the photomultiplier to be reduced due to a large enhancement in fluorescence, were normalized by the appropriate multiplication factor, obtained by measuring a fluorescence emission at both settings.

**Determination of the Dissociation Constants and Stoichiometries**—The monophasic binding curves were fit using the equation

\[
\Delta F = \Delta F_{\text{max}} S / (S + K_D)
\]

where

\[
\]

In these equations, ΔF is the observed increase in the fluorescence of TNP-ATP in the presence of the peptide or enzyme over that of TNP-ATP in the buffer, or the change in fluorescence on titrating a fixed concentration of TNP-ATP enzyme or TNP-ATP-enzyme complex with an additional ligand, ΔF_{\text{max}} is the maximal change in fluorescence for a one-site fit, S is the free substrate or peptide concentration, and K_D is the dissociation constant for a one-site fit. Biphase binding curves were fit using Equation 3:

\[
\Delta F = \Delta F_{\text{max}} S / (S + K_D) + \Delta F_{\text{p}} S / (S + K_p)
\]

in which the value for S is determined using the cubic equation

\[
S_T = (K_D + C_t + C_e + C_t - S_T)S_T + (K_tC_e + C_t)C_t - S_T - K_tC_t + K_e - S_T E_T = 0
\]

In these equations, ΔF_1 and ΔF_2 are the maximal changes in fluorescence for a two-site fit, and K_D and K_p are the two dissociation constants for a biphase binding curve. The parameters C_i and C_e are the concentrations of the tight and weak sites, respectively, and are equal to n[peptide] where n is the stoichiometry. Roots to Equation 4 were obtained using the program MACSYMA. In fitting the titration curves, ΔF_{\text{max}} was estimated from extrapolating to infinite substrate concentration (S), (n) were estimated from the break point in the titration curve, which were obtained by drawing asymptotes through the first and last points of the titration curves. Minimal iterations of these values were required to yield the best fit to the data using Equation 1 or 3.

In the dNTP titrations, the data were fit exactly by assuming simple competition between dNTP and TNP-ATP for enzyme (or peptide) binding, yielding Equation 5, a cubic in bound TNP-ATP (S_b).

\[
S_T = (K_D - K_{d0}) + S_T (K_D E_T + 2K_{d0} S_T + K_{d0} N_T)
\]

In Equation 5, N_T is the total concentration of added dNTP. The value of S_T during the titration was solved for the program MACSYMA using the measured dissociation constant of TNP-ATP under the conditions of the displacement (K_{d0}), optimizing the dissociation constant of dNTP (K_{d0}). The ratio of the calculated S_T during the titration to the initial S_T yielded the fractional residual fluorescence.

Additionally, the data were well approximated, within the uncertainty of K_{d0}, by a simple hyperbola (Equation 1). In this approximation, K_{d0} was calculated using Equation 6:

\[
K_{d0} = K_{d0}^b / (1 + [\text{TNP-ATP}])
\]

where K_{d0}^b is the apparent K_{d0} determined from the displacement titration. In correcting the apparent K_{d0}, the concentration of free
RESULTS AND DISCUSSION

Interaction of TNP-ATP with the Large (Klenow) Fragment of Pol I—Titration of the large fragment of Pol I with TNP-ATP measuring the increase in fluorescence of this ligand were performed at 29 °C with and without saturating concentrations of Mg²⁺ at pH 6.9 (Fig. 2) and at pH 7.5. These titrations were followed by the complete displacement of TNP-ATP from the enzyme by dGTP, an authentic substrate for the large fragment, at pH 6.9 (Fig. 2) and at pH 7.5. TNP-ATP binds stoichiometrically with very high affinity, and equally well to the enzyme, with and without Mg²⁺ at both pH 6.9 and 7.5 (Table I). Moreover, dGTP binds to the enzyme in simple competition with TNP-ATP with high affinity with and without Mg²⁺ at pH 6.9 and 7.5 (Table I). Hence, TNP-ATP serves as an excellent probe for the substrate binding site of the large fragment of Pol I, despite the bulky trinitrophenyl ring on this substrate analog.

A kinetic study at pH 7.5 and 29 °C showed the fluorescent nucleotide TNP-ATP to be a potent inhibitor of the polynucleotidase reaction. With poly(dT)-oligo(dA)₉₋₁₅ as template-primer and dATP (10 μM) substrate, half-maximal inhibition was observed at 0.8 μM TNP-ATP. The detailed mechanism of inhibition and possible chain termination are under investigation.

Mg²⁺ and Mn²⁺ Binding to the Enzyme-TNP-ATP Complex—The fluorescence of TNP-ATP bound to the large fragment of Pol I, is increased either by lowering the pH from 7.5 to 6.9, or by adding Mg²⁺, and the effects of both cations, H₂O⁺ and Mg²⁺ on the fluorescence enhancement are additive (Table I). Control experiments in the absence of enzyme showed no change in the fluorescent of TNP-ATP between pH 6.9 and 7.5, but a similar 20% enhancement on adding Mg²⁺ to the free nucleotide at pH 6.9. The mechanism of metal enhanced fluorescence appears to be charge neutralization. A fluorescence titration with Mg²⁺ yielded a dissociation constant of 294 μM for the binary Mg₂⁻TNP-ATP complex at pH 6.9. Fluorescence titrations of the enzyme-TNP-ATP complex with Mg²⁺ or Mn²⁺ yielded the 5900-fold tighter dissociation constants, and stoichiometries given in Table I. Both Mg²⁺ and Mn²⁺ have high affinity for the enzyme-TNP-ATP complex under all conditions studied. The slightly reduced stoichiometry of metal binding to the enzyme-TNP-ATP complex may be due to slow decomposition of TNP-ATP in the presence of enzyme as suggested by a slow time-dependent loss in the enzyme-TNP-ATP fluorescence. When an excess of TNP-ATP was present with the enzyme, higher stoichiometries for metal binding were obtained, approaching unity (Table I). The presence of Mg²⁺ does not significantly alter the affinity of enzyme for TNP-ATP. However, the enzyme raises the affinity of TNP-ATP for Mg²⁺. This difference can be explained by assuming that the binding of Mg²⁺ to the enzyme-TNP-ATP complex changes the conformation of the system to one which has a much greater affinity for Mg²⁺ but not for TNP-ATP.

Purity and Amino Acid Composition of Peptide I—Peptide I elutes as a single symmetrical peak on a reverse phase C₁₈ column and shows no chromatographic evidence of impurities. In Table II the results of the amino acid analysis of peptide I are given. With the exception of isoleucine, all of the experimentally determined numbers of amino acids are within 20% of the predicted number. The lower than predicted number of isoleucines is attributed to the overlap of the Ile and Leu peaks in the chromatogram, resulting in an equivalently greater number of leucines.

CD Spectroscopy of Peptide I—The CD spectra of peptide I in water at 24 °C and in 50% methanol at 2 °C (Fig. 3) are indicative of either a highly structured peptide or an ensemble of peptide structures. In water, the computed percentages of α-helix, β-structure, β-turn, and coil or aperiodic structure consistent with the spectrum of Fig. 3a (Table III) show a decrease in α-helical content, an increase in β-structure and in coil, and an absence of turns in comparison with residues 728–777 of the crystalline enzyme (Ollis et al., 1985). In 50% methanol, turns are detected and the coil content decreases, better approximating those components of the crystalline enzyme, although the helix content may have further decreased and that of β-structure may have increased. The addition of TNP-ATP to peptide I in 50% methanol at 2 °C produced little change in the CD spectrum (Fig. 3, B and C) or in the estimated secondary structure (Table III). The mean secondary structural content of peptide I is quite high, considering that it lacks the structural support of the remainder of the protein. In the ATP binding peptide 1–45 from adenylate kinase, only 42% of the residues showed sequential nuclear Overhauser effects indicative of ϕ- and ψ-angles corresponding to those found in the complete protein, indicating that less than half of the secondary structure of the enzyme was retained in the peptide (Fry et al., 1988).

TNP-ATP Binding to Peptide I in H₂O—Titration of peptide I with the fluorescent substrate analog TNP-ATP in aqueous buffer at 3 °C revealed by 38-fold fluorescence enhancement, that TNP-ATP bound tightly to peptide I (K₀ =
**TABLE I**

Fluorescence titrations of the large (Klenow) fragment of Pol I at 29 °C

<table>
<thead>
<tr>
<th>Titrant</th>
<th>System*</th>
<th>[Enzyme]</th>
<th>[TNP-ATP]</th>
<th>n*</th>
<th>Kd/Ko</th>
<th>Enhancement factor</th>
<th>Change in ΔF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNP-ATP</td>
<td>Enzyme, pH 6.9</td>
<td>0.9</td>
<td>1.0</td>
<td>0.05</td>
<td>29.4</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Enzyme, pH 7.5</td>
<td>0.9</td>
<td>1.0</td>
<td>0.05</td>
<td>22.8</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Enzyme, Mg**, pH 6.9</td>
<td>0.9</td>
<td>1.0</td>
<td>0.05</td>
<td>37.1</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Enzyme, Mg**, pH 7.5</td>
<td>0.9</td>
<td>1.0</td>
<td>0.07</td>
<td>30.0</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>dGTP</td>
<td>Enzyme-TNP-ATP, pH 6.9</td>
<td>0.9</td>
<td>4.8</td>
<td>1.0</td>
<td>2.3</td>
<td>1.0</td>
<td>-100</td>
</tr>
<tr>
<td>dGTP</td>
<td>Enzyme-TNP-ATP, pH 7.5</td>
<td>0.9</td>
<td>4.8</td>
<td>1.0</td>
<td>2.3</td>
<td>1.0</td>
<td>-100</td>
</tr>
<tr>
<td>dGTP</td>
<td>Enzyme-TNP-ATP, Mg**, pH 6.9</td>
<td>0.9</td>
<td>4.6</td>
<td>1.0</td>
<td>10</td>
<td>1.0</td>
<td>-100</td>
</tr>
<tr>
<td>dGTP</td>
<td>Enzyme-TNP-ATP, Mg**, pH 7.5</td>
<td>0.9</td>
<td>4.6</td>
<td>1.0</td>
<td>10</td>
<td>1.0</td>
<td>-100</td>
</tr>
<tr>
<td>Mg**</td>
<td>Enzyme-TNP-ATP, pH 6.9</td>
<td>6.3</td>
<td>6.8</td>
<td>0.40</td>
<td>0.05</td>
<td>+41</td>
<td></td>
</tr>
<tr>
<td>Mg**</td>
<td>Enzyme-TNP-ATP, pH 6.9</td>
<td>6.3</td>
<td>6.8</td>
<td>0.40</td>
<td>0.05</td>
<td>+102</td>
<td></td>
</tr>
<tr>
<td>Mn**</td>
<td>Enzyme-TNP-ATP, pH 6.9</td>
<td>6.3</td>
<td>6.8</td>
<td>0.40</td>
<td>0.05</td>
<td>+30</td>
<td></td>
</tr>
<tr>
<td>Mn**</td>
<td>Enzyme-TNP-ATP, pH 7.5</td>
<td>3.4</td>
<td>10.5</td>
<td>0.79</td>
<td>0.05</td>
<td>+46</td>
<td></td>
</tr>
</tbody>
</table>

* When present, the concentration of Mg** was 3.8 mM.
* Errors in the stoichiometries for TNP-ATP binding are less than ±5%. The dGTP displacement curves were fit, most simply, by assuming a stoichiometry of 1 dGTP/enzyme-TNP-ATP complex in agreement with the 100 ± 5% increase in fluorescence. The stoichiometries of metal binding are with respect to [enzyme-TNP-ATP]. The errors in the stoichiometries of metal binding are less than ±10%.
* The errors of Kd for TNP-ATP binding are less than ±20%. The errors in Kd for dGTP binding are less than ±30%.
* The enhancement factor of the fluorescence (F) is defined as Fenzyme-TNP-ATP/FTNP-ATP where the concentrations of enzyme-TNP-ATP and TNP-ATP are equal and Fenzyme-TNP-ATP has no contribution from free TNP-ATP. Errors in the enhancement factors are less than ±5%.
* ΔF is the increase in fluorescence above that of TNP-ATP in buffer. Errors in the change in ΔF are less than ±5%.

**TABLE II**

Amino acid analysis of peptide I and II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide I*</th>
<th>Peptide II**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Predicted value</td>
<td>Number</td>
</tr>
<tr>
<td>Asx</td>
<td>1.59</td>
<td>2</td>
</tr>
<tr>
<td>Glx</td>
<td>5.96</td>
<td>6</td>
</tr>
<tr>
<td>Ser</td>
<td>3.10</td>
<td>3</td>
</tr>
<tr>
<td>Gly</td>
<td>5.29</td>
<td>5</td>
</tr>
<tr>
<td>His</td>
<td>0.83</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>4.40</td>
<td>4</td>
</tr>
<tr>
<td>Thr</td>
<td>2.98</td>
<td>3</td>
</tr>
<tr>
<td>Ala</td>
<td>8.22</td>
<td>8</td>
</tr>
<tr>
<td>Pro</td>
<td>1.10</td>
<td>1</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>1.92</td>
<td>2</td>
</tr>
<tr>
<td>Met</td>
<td>1.15</td>
<td>1</td>
</tr>
<tr>
<td>lle</td>
<td>2.20</td>
<td>3</td>
</tr>
<tr>
<td>Leu</td>
<td>5.53</td>
<td>5</td>
</tr>
<tr>
<td>Phe</td>
<td>2.87</td>
<td>3</td>
</tr>
<tr>
<td>Lys</td>
<td>1.77</td>
<td>2</td>
</tr>
</tbody>
</table>

* Tyr undergoes total damage during hydrolysis and is not detected.

0.03 μM with low stoichiometry (n = 0.2). This stoichiometry was increased by the addition of methanol to 50% by volume as described in the next section.

* Preliminary 250 MHz proton NMR studies of 2.5 mM peptide I in 50 mM KCl and 2H2O at 24 °C give line widths indicative of peptide aggregation throughout the pH range of 3.1–6.1. However, this concentration was much higher than those used in fluorescence titrations with TNP-ATP. In 50% deuteromethanol in 2H2O at 2 or 24 °C, at pH 6.3, the narrower resonances observed for 0.1 mM peptide I are consistent with a dissaggregated state of the peptide.
Theoretical spectrum did not significantly alter the contributions. Peptide I (HzO, solid uncertainties in the contributions are of the α-carbon atoms in the x-ray structure (Ollis et al., 1985). Enzyme (residues 728-777) consists in 2-nm increments. Using data from methanol. The secondary structure of peptide I and corresponds to monomeric peptide. All other standards were found to elute as symmetrical peaks. Under conditions comparable to those of the peptide titrations with TNP-ATP, namely 50% methanol at 3 °C in 10 mM Pipes, pH 6.9, 35 mM KCl, the same elution profile as observed in water was obtained. A major peak followed by a minor eluting shoulder (3800 ± 1000 daltons) corresponds to monomeric peptide. All other standards were found to elute as symmetrical peaks. Under conditions comparable to those of the peptide titrations with TNP-ATP, namely 50% methanol at 3 °C in 10 mM Pipes, pH 6.9, 35 mM KCl, the same elution profile as observed in water was obtained. A major peak followed by a minor shoulder was observed, consistent with a dimer and monomer (Fig. 5B). In 50% methanol at 3 °C, addition of TNP-ATP to peptide I, both in the injection volume and in the buffer, produced a more symmetrical and slower moving elution band, which we attribute to
and the peptide-TNP-ATP complex (Fig. 6). The biphasic binding curves are not significantly different from simple gel filtration. Similarly, the faster elution of TNP-ATP than peptide-TNP-ATP reflects these effects.

**TABLE IV**

Fluorescence TNP-ATP titrations in 50% methanol/water of peptide I (residues 728-777 of Pol I) at 3 °C

<table>
<thead>
<tr>
<th>Titrant</th>
<th>System</th>
<th>[Peptide]</th>
<th>n*</th>
<th>$K_D$</th>
<th>Enhancement factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNP-ATP</td>
<td>Peptide</td>
<td>0.62</td>
<td>0.09</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Peptide</td>
<td>2.8</td>
<td>0.05</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Peptide-p(dT)$<em>{16}$-p(dA)$</em>{12}$</td>
<td>2.8</td>
<td>0.30</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Peptide-p(dA)$<em>{16}$-p(dT)$</em>{12}$</td>
<td>2.8</td>
<td>0.32</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>Peptide-p(dT)$<em>{16}$-p(dA)$</em>{12}$</td>
<td>2.7</td>
<td>0.50</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Errors in the stoichiometries of the fractional sites with $K_D$ values of 0.09 and 0.05 are less than ±5%. Errors in the stoichiometries of the peptide-DNA complexes are less than ±30%. The biphasic binding curves are not highly sensitive to changes in the stoichiometries of the weaker sites, and these curves were fit, most simply, by assuming the weak site stoichiometries to be the difference between $n = 1$ and the tight site stoichiometry.

$^b$ Errors in $K_D$ are typically less than ±30%.

$^c$ The enhancement factor of the fluorescence ($F$) is defined as $F_{peptide}$-TNP-ATP/$F_{TNP-ATP}$, where the concentrations of peptide-TNP-ATP and TNP-ATP are equal and $F_{peptide}$-TNP-ATP has no fluorescence contribution from free TNP-ATP. Errors in the enhancement factors are less than ±20%.

$^d$ $[p(dT)$_{16}$-p(dA)$_{12}$] = 2.8 μM.

$^e$ $[p(dA)$_{16}$-p(dT)$_{12}$] = 2.8 μM.

$^f$ $[p(dT)$_{16}$-p(dA)$_{12}$] = 5.6 μM.

**SCHEME 1**

TNP-ATP-bound monomeric peptide I (Fig. 5B). Smaller effects on dimeric peptide were observed when TNP-ATP was included only in the injection volume (data not shown). Ubiquitin, a structured peptide in 50% methanol (Wilkinson and Mayer, 1986) with a molecular mass of 8500 daltons, was found to elute between the peak ascribed to dimeric peptide and the peptide-TNP-ATP complex (Fig. 5B). In 50% methanol, a precise determination of the relative molecular weights of the peptide forms was not possible since other protein standards did not elute normally, possibly reflecting chromatographic effects in addition to simple gel filtration. Similarly, the faster elution of TNP-ATP than peptide-TNP-ATP reflects these effects.

**TABLE V**

Displacement titrations of TNP-ATP from peptide I (residues 728-777 of Pol I) by deoxynucleoside triphosphate substrates, in 50% methanol/water at 3 °C

<table>
<thead>
<tr>
<th>Titrant</th>
<th>System</th>
<th>[Peptide]</th>
<th>[TNP-ATP]</th>
<th>$n^*$</th>
<th>$K_D$</th>
<th>Decrease in $\Delta F$%</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>Peptide-TNP-ATP</td>
<td>0.64</td>
<td>0.86</td>
<td>1.0</td>
<td>230</td>
<td>110</td>
</tr>
<tr>
<td>TTP</td>
<td>Peptide-TNP-ATP</td>
<td>0.64</td>
<td>0.86</td>
<td>1.0</td>
<td>390</td>
<td>65</td>
</tr>
<tr>
<td>dGTP</td>
<td>Peptide-TNP-ATP</td>
<td>0.62</td>
<td>0.72</td>
<td>1.0</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>dGTP</td>
<td>Peptide-TNP-ATP</td>
<td>2.7</td>
<td>8.1</td>
<td>1.0</td>
<td>570</td>
<td>107</td>
</tr>
<tr>
<td>dATP</td>
<td>Peptide-TNP-ATP</td>
<td>2.7</td>
<td>4.5</td>
<td>1.0</td>
<td>470</td>
<td>120</td>
</tr>
<tr>
<td>TTP</td>
<td>Peptide-TNP-ATP</td>
<td>2.7</td>
<td>4.5</td>
<td>1.0</td>
<td>730</td>
<td>110</td>
</tr>
<tr>
<td>dGTP</td>
<td>Peptide-TNP-ATP</td>
<td>2.6</td>
<td>7.1</td>
<td>1.0</td>
<td>350</td>
<td>110</td>
</tr>
<tr>
<td>dGTP</td>
<td>Peptide-TNP-ATP</td>
<td>2.7</td>
<td>6.8</td>
<td>1.0</td>
<td>620</td>
<td>105</td>
</tr>
<tr>
<td>dGTP</td>
<td>Peptide-TNP-ATP</td>
<td>2.6</td>
<td>2.6</td>
<td>1.0</td>
<td>610</td>
<td>125</td>
</tr>
<tr>
<td>dGTP</td>
<td>Peptide-TNP-ATP</td>
<td>2.6</td>
<td>2.7</td>
<td>1.0</td>
<td>2100</td>
<td>140</td>
</tr>
</tbody>
</table>

$^a$ The displacement curves were fit most simply by assuming a stoichiometry of 1 dNTP per peptide-TNP-ATP complex in agreement with the 100 ± 40% decrease in $\Delta F$ observed in almost all titrations, reflecting the complete displacement of TNP-ATP.

$^b$ Errors in $K_D$ are typically less than ±30%.

$^c$ $\Delta F$ is defined in Table I. Errors in the decrease in $\Delta F$ are less than ±20%, except in the titration with TTP where the error was ±35%.

$^d$ $[p(dT)$_{16}$-p(dA)$_{12}$] = 2.8 μM.

$^e$ $[p(dA)$_{16}$-p(dT)$_{12}$] = 2.8 μM.

$^f$ $[p(dT)$_{16}$-p(dA)$_{12}$] = 5.6 μM.

$^g$ $[p(dA)$_{16}$-p(dT)$_{12}$] = 6.1 μM.
Binding Properties of a Peptide Fragment of Pol I

**Fig. 5.** HPLC analysis of the molecular weight of peptide I. A, plot of molecular weight versus $K_v$ for peptide I in 10 mM Pipes, pH 6.9, 35 mM KCl at 25 °C. $K_v$ is defined as $(u_v - u_w)/(u_v - u_p)$ where $u_v$, $u_p$, and $u_w$ are elution volume, void volume, and total volume, respectively. The $K_v$ for the major band of peptide I and the minor shoulder are marked with arrows. The molecular weight markers in decreasing molecular size are thyroglobulin, $\gamma$-globulin, bovine serum albumin, ovalbumin, myoglobin, cytochrome c, ubiquitin, bovine pancreatic trypsin inhibitor, the peptide LRR(NO$_2$)$_2$YSLG, vitamin B$_{12}$, and N-acetyltryptophan amide. B, elution profile of peptide I at 25 °C in 10 mM Pipes, pH 6.9, 35 mM KCl. Major band (peptide dimer) $u_v = 12.36$ ml, minor shoulder (peptide monomer) $u_v = 13.69$ ml (a); elution profile of peptide I at 0 °C in the same buffer as in a with methanol added to 50% by volume, major band (peptide dimer) $u_v = 13.45$ ml, minor shoulder (peptide monomer) $u_v = 14.85$ ml (b); elution profile of peptide I and TNP-ATP at 0 °C in the same buffer as in a with methanol added to 50% by volume and 14 $\mu$M TNP-ATP throughout the buffer, TNP-ATP $u_v = 14.75$ ml, peptide-TNP-ATP $u_v = 15.55$ ml (c). In the 98-ml injection volume the peptide I concentration was 130 $\mu$M and was diluted during chromatography ~20-fold. In b, the concentration of TNP-ATP in the injection volume was 186 $\mu$M. The designations 8.4K and 6.5K are at the elution volumes of ubiquitin and bovine pancreatic trypsin inhibitor, respectively.

**Fig. 6.** Fluorescence titrations of peptide I-DNA complexes with TNP-ATP followed by displacement of TNP-ATP with dGTP. A, titration of a mixture of 2.8 $\mu$M peptide I and 2.8 $\mu$M p(dT)$_{16}$-p(dA)$_{12}$ with TNP-ATP (C) ($K_o = 0.85$ $\mu$M, n = 0.30) and displacement of TNP-ATP by dGTP ($\Delta$) ($K_o = 350$ $\mu$M). B, titration of 2.8 $\mu$M peptide I and 2.8 $\mu$M p(dA)$_{12}$-p(dT)$_{16}$ with TNP-ATP (C) ($K_o = 1.5$ $\mu$M, n = 0.32) and displacement of TNP-ATP by dGTP ($\Delta$) ($K_o = 620$ $\mu$M). Conditions are otherwise as described in Fig. 4. Corrections in $\Delta F$ for free TNP-ATP were made and the final three points in the TNP-ATP titration curves were corrected for the inner filter effect.

The dimeric peptide, but a lower affinity for TNP-ATP than the monomeric peptide. The results suggest that 30% of the peptide forms a peptide-DNA complex and that the DNA dissociates peptide dimers. To further test this point, a solution containing 2.7 $\mu$M peptide and excess (5.6 $\mu$M) p(dT)$_{16}$-p(dA)$_{12}$ was titrated with TNP-ATP. Peptide I was found to bind TNP-ATP with a stoichiometry of 1.0 ± 0.3 with little change in the dissociation constant, indicating nearly complete occupancy of peptide with DNA. From the 0.3 ± 0.1 stoichiometry and the lower limit stoichiometry of 0.7 for DNA binding obtained in these experiments, overlapping dissociation constants ($K_o$ in Scheme 1) of 2-10 $\mu$M and ≤2 $\mu$M were calculated for the peptide-DNA complex. The titrations of peptide-p(dT)$_{16}$-p(dA)$_{12}$ and peptide-p(dA)$_{12}$-p(dT)$_{16}$ with TNP-ATP (Fig. 6) were fit with dissociation constants for TNP-ATP which favored nucleotide binding to the complex with the complementary (dT)$_n$ overhang by a factor of ~2.

Both dATP and dGTP displaced TNP-ATP from the peptide-p(dT)$_{16}$-p(dA)$_{12}$ complex with high and comparable affinities (Table V). Thus, the correct nucleotide did not display a higher affinity for the peptide-p(dT)$_{16}$-p(dA)$_{12}$ complex. Similarly, both TTP and dGTP displaced TNP-ATP from the
peptide-p(dA)_{16}-p(dT)_{12} complex, and TTP did not display a higher affinity for the peptide-p(dA)_{16}-p(dT)_{12} complex. Hence duplex DNA does not greatly affect the binding of dGTP, dATP, or TTP to the peptide, regardless of the complementarity or non-complementarity of the template. Similar behavior has been found with the large fragment of Pol I (Ferrin and Mildvan, 1986). The dissociation constants for dATP, TTP, and dGTP from the peptide-DNA complexes were ~2-fold weaker than those obtained in the absence of DNA.

DNA Titrations of the Peptide I-TNP-ATP Complex and Displacement of TNP-ATP from These Complexes with dGTP—Titrations of the peptide-TNP-ATP complex with p(dT)_{16}-p(dA)_{16}-p(dA)_{16}-p(dT)_{12}, and with the single stranded p(dT)_{12} or p(dA)_{12} are shown in Fig. 7. Double stranded DNA produced partial quenching of the TNP-ATP-fluorescence with typical saturation behavior, indicating the formation of a ternary peptide I-DNA-TNP-ATP complex, while single stranded DNA produced little fluorescence quenching (Fig. 7). Titrations with double stranded DNA, with or without an overhang gave stoichiometries of ~1.0 with respect to the peptide-TNP-ATP complex and high affinities of the duplex DNA for the complex (Table VI). In control experiments, the addition of p(dA)_{16}-p(dT)_{12} to 2.8 μM TNP-ATP in the absence of peptide produced no change in the fluorescence intensity of TNP-ATP to within ±5%. The partial quenching of the TNP-ATP fluorescence by duplex DNA may be due either to an increase in local polarity resulting from the proximity of the additional negative charges of DNA, or an increase in mobility of bound TNP-ATP. Alternative explanations for the fluorescence quenching, such as a decrease in TNP-ATP mobility in the complex, or a decrease in local polarity are unlikely, since these are observed to produce the opposite effect. Thus, lower temperatures or changes in solvent from water to methanol/water enhance the fluorescence of TNP-ATP. The dissociation constant of p(dT)_{16}-p(dA)_{12} from the peptide-TNP-ATP complex was 3.5 times tighter than the dissociation constant of p(dA)_{16}-p(dT)_{12} from peptide-TNP-ATP, indicating a slight preference for the complementary template.

In Fig. 7 is shown the dGTP displacement of TNP-ATP on saturating the TNP-ATP-peptide complex with p(dA)_{16}-p(dT)_{12} or p(dT)_{16}-p(dA)_{12} and in Table V are given the corresponding dissociation constants. Again, the resulting dGTP dissociation constants are 2-6-fold greater in the presence of a stoichiometric amount of DNA, than in its absence, probably due to charge repulsion (Table V). The simultaneous and strong interactions of negatively charged (~4) substrates and polyanionic (~26) DNA duplexes with peptide I very likely represent specific binding rather than nonspecific charge neutralization, since the net charge of peptide I with 5 anionic and 7 cationic residues is only +2.

On titrating the peptide-TNP-ATP complex with up to 7.5 μM p(dAT)_{14}, which is single stranded in methanol at 3°C, 4° no change in the TNP-ATP fluorescence was observed. However, a competition experiment, in which 7.5 μM p(dAT)_{14}, 2.7 μM peptide, and 2.7 μM TNP-ATP was titrated with p(dT)_{16}-p(dA)_{12}, revealed p(dAT)_{14} to be a potent inhibitor of p(dT)_{16}-p(dA)_{12} binding (K_i = 0.1 μM), suggesting either that p(dAT)_{14} might form a duplex on the peptide which does not quench the fluorescence of TNP-ATP, or that the single p(dAT) strand has an unusually high affinity for the peptide.

Further evidence for the binding of single stranded DNA to the peptide I-TNP-ATP complex was sought by competition with duplex DNA binding in the following way. The peptide-TNP-ATP complex (2.7 μM), in the presence of an excess (9–13 μM) of single stranded DNA, was titrated with the complementary DNA strand (Table VI). Thus, the prior presence of excess p(dT)_{12} did not inhibit p(dA)_{12} binding as the p(dA)_{12}-p(dT)_{12} duplex, as shown by a K_i of 0.2 μM for p(dA)_{12} compared to that obtained by titration with the duplex (K_i = 0.3 μM) (Table VI). Similarly, excess p(dA)_{14} had little effect on the subsequent binding of the p(dA)_{16}-p(dT)_{12} duplex, while excess p(dA)_{12} slightly inhibited the binding of p(dA)_{12}-p(dT)_{12} to the peptide-TNP-ATP complex, indicating weak binding of the single stranded DNA to the peptide-TNP-ATP complex. However, excess p(dT)_{12} promoted the binding of p(dA)_{12} as shown by a 7-fold tightening of p(dA)_{12} when the strands are added separately, over that found with the duplex, raising the possibility of triple helix formation (Felsenfeld and Miles, 1967) followed by tighter binding of the triple helix to the peptide.

**CD Spectroscopy and TNP-ATP Titrations of Peptide II**—Peptide II showed low solubility at pH values ≤7.0. Hence all studies of this peptide were carried out at pH 8.0 as described under “Methods.” With the exception of leucine, due to over-
Binding Properties of a Peptide Fragment of Pol I

**TABLE VI**
DNA titrations of the complex formed by peptide I (residues 728-777 of Pol I) and TNP-ATP in 50% methanol/water at 3°C

<table>
<thead>
<tr>
<th>Titrant System</th>
<th>[Peptide]</th>
<th>[TNP-ATP]</th>
<th>n°</th>
<th>K(_D)</th>
<th>Decrease in (\Delta F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p(dT)(<em>{14}), p(dA)(</em>{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>2.8</td>
<td>1.0</td>
<td>0.10</td>
<td>16</td>
</tr>
<tr>
<td>p(dT)(<em>{14}), p(dA)(</em>{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>7.6</td>
<td>1.0</td>
<td>0.50</td>
<td>37</td>
</tr>
<tr>
<td>p(dA)(<em>{12}), p(dT)(</em>{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>2.8</td>
<td>1.0</td>
<td>0.35</td>
<td>40</td>
</tr>
<tr>
<td>p(dA)(<em>{12}), p(dT)(</em>{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>2.8</td>
<td>1.0</td>
<td>0.30</td>
<td>21</td>
</tr>
<tr>
<td>p(dA)(<em>{12}), p(dT)(</em>{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>2.8</td>
<td>1.0</td>
<td>0.30</td>
<td>21</td>
</tr>
<tr>
<td>p(dT)(_{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>2.8</td>
<td>1.0</td>
<td>0.30</td>
<td>21</td>
</tr>
<tr>
<td>p(dT)(_{12}) Peptide-TNP-ATP</td>
<td>2.8</td>
<td>2.8</td>
<td>1.0</td>
<td>0.30</td>
<td>21</td>
</tr>
<tr>
<td>p(dT)(_{12}) Peptide-TNP-ATP</td>
<td>2.6</td>
<td>2.6</td>
<td>1.0</td>
<td>0.70</td>
<td>23</td>
</tr>
<tr>
<td>p(dT)(<em>{12}) Peptide-TNP-ATP, p(dA)(</em>{12}) Peptide-TNP-ATP</td>
<td>2.7</td>
<td>2.7</td>
<td>1.0</td>
<td>1.5</td>
<td>19</td>
</tr>
<tr>
<td>p(dA)(<em>{12}), p(dT)(</em>{12}) Peptide-TNP-ATP, p(dA)(_{12}) Peptide-TNP-ATP</td>
<td>2.7</td>
<td>2.7</td>
<td>1.0</td>
<td>0.20</td>
<td>19</td>
</tr>
<tr>
<td>p(dA)(<em>{12}), p(dT)(</em>{12}) Peptide-TNP-ATP, p(dT)(_{12}) Peptide-TNP-ATP</td>
<td>2.7</td>
<td>2.7</td>
<td>1.0</td>
<td>0.50</td>
<td>19</td>
</tr>
</tbody>
</table>

* Stoichiometries are for DNA binding with respect to [peptide-TNP-ATP]. Errors in stoichiometries are less than ±30%.
* Dissociation constant of DNA from ternary peptide-TNP-ATP-DNA complex. Errors in \(K_D\) are typically less than ±30%.
* \(\Delta F\) is defined in Table I. Errors in the decrease in \(\Delta F\) are less than ±20%.
* Could not be determined due to negligible change in \(\Delta F\).

Despite the preservation of a significant amount of secondary structure, peptide I revealed no evidence of binding the substrate analog TNP-ATP in either solvent system. Thus, a TNP-ATP titration of 8.8 \(\mu M\) peptide I in buffer at 24°C produced no change in the fluorescence at 540 nm compared to the fluorescence of TNP-ATP in buffer alone for TNP-ATP concentrations as high as 13.5 \(\mu M\), indicating no TNP-ATP binding by peptide I. A second titration produced virtually identical results. Similarly, no significant effect of TNP-ATP, up to 13 \(\mu M\), on the cryptophan fluorescence of peptide II (8 \(\mu M\)) was observed after correction for the large inner filter effect. A peptide II titration of 8.0 \(\mu M\) TNP-ATP in 5 mM Tris-HCl, pH 8.0, 16 mM KCl, and 50% methanol at 3°C, also produced no change in TNP-ATP fluorescence.

**CONCLUSIONS**

The fluorescent ligand TNP-ATP serves as a probe of the dNTP-binding site of DNA polymerase I, as judged by kinetic and direct binding studies. It also interacts with peptide I, based on residues 728-777 of the Pol I sequence, but in a complicated manner unless DNA has previously bound, as shown in Scheme I. In this scheme, peptide I is depicted as a monomer and dimer which are not in rapid equilibrium. Either TNP-ATP or duplex DNA bind to both monomer and dimer, and the binding of either TNP-ATP or DNA to the peptide dimer induces dissociation. Subsequently, either TNP-ATP or duplex DNA can bind classically to form a ternary peptide-DNA-TNP-ATP complex. Binding cooperativity is the same for both TNP-ATP and DNA binding to the major dimeric form of the peptide. Thus, TNP-ATP binding to dimeric peptide results in dissociation of the peptide dimer yielding a monomeric peptide-TNP-ATP complex which displays higher affinity for DNA than dimeric peptide. Similarly, DNA binding to dimeric peptide results in dissociation of the peptide dimer and a monomeric peptide-DNA complex which displays higher affinity for TNP-ATP than dimeric peptide. Authentic dNTP substrates compete for binding with TNP-ATP both in binary peptide I-TNP-ATP and ternary peptide I-DNA-

![Circular dichroism spectra of peptide II. A, peptide II (221 \(\mu M\)) in 10 mM Tris-HCl, pH 8.0, 32 mM KCl at 24°C. B, peptide II (19 \(\mu M\)) in 50% methanol containing 5 mM Tris-HCl, pH 8.0, 16 mM KCl at 3°C. Optical path lengths were 0.1 mm in A and 1.0 mm in B. The data are shown as points and the theoretical fits as solid curves.](image-url)
TNP-ATP complexes, as found with the complete enzyme. Peptide II, consisting of residues 840–888 of the Pol I sequence, retains structure but does not detectably bind TNP-ATP, thus providing a negative control for the location of the dNTP substrate binding site on the enzyme.

From the ability of peptide I to bind TNP-ATP, dATP, dTTP, and dGTP with high affinity in a manner analogous to the large fragment of Pol I, we conclude that residues 728–777 of Pol I comprise many of the structural and contact residues necessary for substrate binding to the enzyme. Since the large fragment of Pol I binds only one TNP-ATP per enzyme, it is unlikely that the TNP-ATP binding to peptide I is due to a secondary nucleotide-binding site. From the binding of duplex DNA to peptide I, we conclude that residues 728–777 of the enzyme also contribute significantly to the binding of double-stranded DNA to the enzyme. The partial fluorescence quenching of the peptide-TNP-ATP complex by duplex DNA, but not by single-stranded DNA, suggests a close interaction of the DNA duplex with the bound TNP-ATP, possibly resulting from the alignment of TNP-ATP with the incoming DNA duplex. Although peptide I represents only 8% of the large fragment of Pol I, it appears to retain the ability to bind and arrange the substrate and template-primer, thus mimicking the properties of the complete enzyme.

The first use of a peptide fragment to locate the substrate binding site on an enzyme was in the case of adenylate kinase, where such studies corrected the location of the ATP binding site deduced from x-ray studies, and established that sequence homologies among nucleoside triphosphate-binding enzymes occurred at the nucleoside triphosphate binding site (Fry et al., 1985). Our present findings that peptide I from Pol I, with the homologous sequence Leu(or Val)-Ile-Tyr-Gly, preserves only 8% of the large fragment of Pol I, it appears to retain the ability to bind and arrange the substrate and template-primer, thus mimicking the properties of the complete enzyme.

Acknowledgments—We are grateful to Dr. Lance Ferrin for purifying the large fragment of Pol I to Dr. Tian Tsong for the use of the fluorimeter, to Dr. David Shortle for assistance in the use of the spectropolarimeter, to Dr. Eleanor M. Brown for generous help in the analysis of the CD spectra, to David Garboczi and Dr. Peter Pedersen for valuable discussions, and to Peggy Ford for typing this manuscript.

REFERENCES