Nucleotide Binding by the Poliovirus RNA Polymerase*

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Cross-linking of ribonucleoside triphosphates (NTPs) to specific binding sites on the poliovirus RNA-dependent RNA polymerase has been performed by ultraviolet irradiation and by reduction of oxidized nucleotide-protein complexes. The latter method approached a cross-linking efficiency of 1 NTP/molecule of enzyme. Nucleotide competition experiments suggested that the same binding site is occupied by all NTPs. Analysis of peptides produced by proteinase K and trypsin digestion and labeled with [32P]GTP indicated that a lysine residue between Met-189 and Lys-228 in the polymerase was cross-linked to NTP. Nucleotide binding was exploited for rapid purification of the enzyme by GTP-agarose affinity chromatography. In addition, a set of cloned, modified polymerase molecules with reduced or absent polymerization activity was analyzed for binding efficiency to a GTP-agarose column. Some mutations eliminated GTP binding, whereas others generated proteins with varying affinities for GTP. Incubation of the poliovirus polymerase with high concentrations of NTP, particularly GTP, resulted in a dramatic protection against heat denaturation and activity loss. These data suggest that nucleotide binding results in an alteration of the enzyme conformation or the stabilization of an ordered conformation.

The genomes of single-stranded RNA viruses all encode an RNA-dependent RNA polymerase which catalyzes the synthesis of both minus and plus strand RNAs, required for replication of the viral genome. Little is known about the biochemical mechanisms of these activities. The most intensively studied of these enzymes is the RNA polymerase encoded by poliovirus RNA, called 3Dpol. It is a 52-kDa polypeptide that has been shown to contain an RNA chain elongation activity that is dependent upon both a template and a primer (for review, see Refs. 1–3). Other factors and/or activities appear to be required for RNA chain initiation, but these have not been identified, and their catalytic roles have not been elucidated.

Biochemical studies of 3Dpol have been hampered by the small yields of enzyme that can be obtained from virus-infected cells. To overcome this, we and others have cloned 3Dpol in *Escherichia coli* (4–6) and in recombinant baculovirus-infected insect cells (7), so that large amounts of enzyme are now available for biochemical and structural analyses. Amino acid sequence comparisons of the poliovirus 3Dpol protein with RNA-dependent RNA polymerases of other animal and plant viruses revealed a short, highly conserved sequence of amino acids (YGDD) flanked by a region of hydrophobic residues that is present in many RNA polymerases (8, 9). A recent study in which the G residue within the conserved YGDD sequence was substituted by other amino acids suggested that this region of the poliovirus polymerase is essential for enzyme activity (10). This region has been proposed to comprise an essential component of the catalytic site or of a metal or nucleotide or RNA binding site (8). Studies of a small set of engineered mutations scattered throughout the 3D gene sequence failed to reveal specific domains of the protein associated with individual activities (11). RNA binding to purified 3Dpol protein was demonstrated by a filter binding assay (12); some specificity of binding to poliovirion RNA over non-viral RNAs was observed, and poly(G) binding was highly preferred over other homopolymers, but binding sites on the protein were not identified.

As part of a long term study to characterize the structure-function relationships of the poliovirus 3Dpol protein, we have initiated experiments to identify the enzyme’s ribonucleoside triphosphate (NTP) binding site(s). In this report, we demonstrate the specific binding of NTP to purified 3Dpol, isolated from *E. coli*, by several methods. Binding of a given nucleotide was readily competed by other nucleotides, suggesting that the same site or overlapping site(s) is utilized by all NTPs. Preliminary results indicate that this site utilizes one of three lysine residues in the middle portion of 3Dpol. This binding has been exploited for rapid purification of 3Dpol by NTP affinity chromatography, and for the analysis of 3Dpol mutants with reduced or absent polymerase activity. In addition, incubation of the enzyme with high concentrations of NTP resulted in a dramatic protection against heat denaturation and activity loss, suggesting that nucleotide binding causes a conformational alteration of the enzyme’s structure.

**EXPERIMENTAL PROCEDURES**

*Expression of Poliovirus Polymerase and Enzyme Purification—*

Single colony isolates of *E. coli* harboring the plasmid, pEXC-3D, were expressed at 30°C and crude sonicates were prepared as described by Rothstein et al. (13). The lysates were centrifuged at 29,000 rpm in a Beckman 50.2 fixed-angle rotor at 5°C for 2 h, and the supernatant (S-100) was collected. Poliovirus RNA polymerase was purified from the S-100, basically as described elsewhere (14), and included 0–40% saturation ammonium sulfate precipitation, phosphocellulose (Whatman), Mono Q (Pharmacia LKB Biotechnology Inc.), phenyl-Sepharose (Pharmacia), poly(U)-Sepharose 4B (Pharmacia), and a second Mono Q chromatography to yield a polymerase preparation which was greater than 95% pure. The last column was used to concentrate the enzyme, remove Nonidet P-40, and change
the buffer to 50 mM HEPES, pH 8.0. The common buffer used in most column purifications was Buffer A (0.05 M Tris-HCl, pH 8.0, 0.1% Nonidet P-40, 10% glycerol, 5 mM β-mercaptoethanol) with modifications of salt concentration as indicated under "Results."

**Polymerase Activity Assay**—The standard poliovirus polymerase assay used in these studies was the poly(U) polymerase activity on a poly(U) template described by Hey et al. (15); incubations were performed at 30 °C for 30 min, and 20-μl aliquots from a 50-μl reaction mix were assayed for acid-precipitable material.

**Protein Analyses**—Samples were fractionated in 10% polyacrylamide-SDS gels (16), and gels were examined by immunoblot analysis (17, 18) using transfer to nitrocellulose sheets and detection of polymerase antibodies with rabbit anti-polymerase serum (4) and anti-rabbit alkaline phosphatase conjugate (Promega) as the secondary antibody. Color development utilized nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Radiolabeled protein was detected by autoradiography with Kodak SB-5 film.

Total protein concentrations were determined by the Bradford assay (19), and poliovirus polymerase concentrations were determined by immunoblots using a series of enzyme dilutions and direct comparison with known concentrations of a standard polio polymerase preparation.

**Cross-linking NTPs to Polymerase by Ultraviolet Irradiation**—Poliovirus polymerase (3Dpol) was incubated with NTPs in a 50-μl reaction mix (Buffer A, 3 mM magnesium acetate, between 10 and 200 μM [α-32P]NTP, 60 μCi, and usually 8 μM 3Dpol) at 30 °C for 2 min. The mix was cooled on ice, adjusted to 5% acetone (20), and transferred to a parafilm sheet placed on an aluminum block submerged in ice water. The mix was irradiated for 2 min at a 3-cm distance from an ultraviolet light with 313 nm emission (Chromato-Vue Transilluminator, model TL-33; Ultraviolet Products Inc.). Subsequently, non-cross-linked NTPs were removed in a Sephadex G-50 spin column (21) in Buffer A, 0.3 M NaCl; the excluded material was collected as NTP-3Dpol complexes.

**Cross-linking Oxidized GTP with Poliovirus 3Dpol**—As described by Cleland and Cuzin (22). [32P]GTP-3Dpol complexes were separated from non-cross-linked oxidized GTP in a Sephadex G-50 column (1 × 45 cm) or in a Sephadex G-50 spin column in Buffer A, 0.3 M NaCl. In other experiments the reaction mixtures containing [32P]GTP-3Dpol complexes were loaded directly onto gradient SDS-PAGE gels to separate free nucleotides from complexes. The 32P recovered in the excluded material from G-50 columns or the 32P found in selected bands from SDS-PAGE gels plus the specific activity of the [32P]GTP (radiolabeled plus cold carrier GTP) used at the oxidation step were used to calculate the efficiency of cross-linking of GTP to 3Dpol.

**Proteolytic Digestion of 3Dpol and Peptide Resolution**—Complexes of 35P[GTP-3Dpol were alkylated with iodoacetamide as described previously (23). Digestions with endoproteinase Glu-C (Boehringer Mannheim) were done in 30 mM NH4HCO3, pH 7.8, 0.2% SDS at room temperature for 5 h at a 3DP-:Glu-C ratio of 12:1. Digestions with TPCK-treated trypsin (Sigma, type XIII) were at 37 °C for 1.5 h at a 3DP-:trypsin ratio of 50:1. Peptides were resolved by addition of gel sample buffer, boiling, and direct application of samples to 8-20% SDS-PAGE gels. Unincorporated, labeled nucleotides were just run off the gel, and the peptides in the gel were transblotted to ProBlott (Applied Biosystems) as recommended by the vendor at 50 V, 4 °C, for 1.5 h. The sheets of ProBlott were analyzed by autoradiography using Kodak SB-5 film. Radioactive bands (peptides) were cut out and submitted to direct amino acid sequence analysis for a minimum of five cycles in an ABI 477A pulsed liquid sequenator to determine the amino terminus of the labeled peptide.

**GTP-Agarose Affinity Chromatography of Poliovirus 3Dpol**—A 1-ml column of GTP-agarose (Sigma; linked to GTP on ribose via a 22-atom spacer) was equilibrated in Buffer A, 0.05 M KCl. Polio 3Dpol was applied to the column in the same buffer and the column was washed with four 0.5-ml aliquots of the same buffer. Elution was mediated with four 0.5-ml washes with Buffer A, 0.05 M KCl, and usually 5 mM ATP or GTP. All buffers were supplemented with phosphatase inhibitors (10 μM NaF, 100 μM orthovanadate, and 10 μM molybdic acid). The fractionation of polymerase was monitored by SDS-PAGE and immunoblotting.

**Heat Stability of Polymerase**—Purified 3Dpol was incubated in Buffer A, 0.1 M KCl with or without 5 mM UTP, CTP, ATP, or GTP for various times at 42 °C. Aliquots were removed from the incubation mix at designated times and stored on ice prior to assay for polymerase activity.

**RESULTS**

**Cross-linking of NTPs to Poliovirus RNA Polymerase by Ultraviolet Irradiation**—Substrate binding to poliovirus RNA polymerase (3Dpol) was examined by incubation with NTP and subsequent stabilization of the bound NTP by ultraviolet irradiation. The enzyme used in these studies was >95% pure, as shown by silver staining of the protein preparation fractionated in a SDS-PAGE gel (Fig. 1A). UTP or GTP was bound to the polymerase using a constant amount of [α-32P] NTP and increasing concentrations of carrier NTP (10–200 μM). The molar NTP to polymerase ratio varied between 2:1 and 40:1. Nucleotide binding to polymerase was detected as labeled protein co-migrating with poliovirus polymerase, as determined by immunoblotting following SDS-PAGE. Fig. 1C (lane 11) shows the labeling of a 52-kDa protein in the polymerase preparation by incubation with 10 μM [α-32P]UTP and irradiation with ultraviolet light. The labeled band co-migrates with the anti-polymerase immunoreactive protein in the same preparation (Fig. 1B, lane 6). GTP was found to compete with [α-32P]UTP for binding to polymerase (Fig. 1C), one interpretation being that GTP binds at the same or a sterically close site(s) to UTP. Increasing the GTP concentration resulted in decreased labeled UTP associated with the enzyme (Fig. 1C), although the amount of recovered polymerase was constant (Fig. 1B).

The stoichiometry of NTP binding to polymerase is low. When the 32P associated with the polymerase band was quantified (accounting for losses during preparation for gel analy-
polymerase activity decreased with increased irradiation time in the presence or absence of NTP, suggesting protein degradation or protein-protein cross-linking (data not shown). With increasing irradiation time, polymerase protein was detectably degraded, as shown by the appearance of faster migrating immunoreactive species and a smearing of the polymerase band upon polyacrylamide gel electrophoresis and immunoblotting. It was decided, therefore, to try another method of cross-linking NTP to polymerase.

Cross-linking of Oxidized NTPs to Poliovirus Polymerase and Stabilization by Reduction—The vicinal hydroxyls in the ribose ring of NTPs, e.g. GTP, can be oxidized to reactive aldehyde residues with sodium periodate (22). Subsequent incubation with protein which has NTP binding sites, but not with random proteins, results in reaction with the activated NTP at lysine residues at or near the binding site to form an unstable Schiff base. Reduction of this bond forms a stable, covalent NTP-protein linkage. GTP binding to poliovirus polymerase was investigated using this procedure. Unreacted GTP was removed by filtration on Sephadex G-50. Residual polymerase activity coincided with excluded label from the column, and the cross-linking efficiency of [α-32P]GTP to polymerase was calculated from this material.

Fig. 2 illustrates the cross-linking efficiency of GTP to polymerase as a function of GTP concentration. Cross-linking was strongly dependent upon GTP concentration, and at 5 mM GTP the efficiency approached 1 GTP cross-linked per polymerase molecule. The absolute efficiency was difficult to determine due to potential losses of protein by column filtration. Coincident with increased binding of GTP to polymerase was a decrease in polymerase activity, implying that covalent binding of GTP inactivates RNA elongation activity. The fact that GTP binding does not exceed 1 molecule per polymerase molecule suggests a single GTP site per polymerase molecule, but a unique site has not been demonstrated.

Localization of the Cross-linked Polymerase Peptide—To localize the region in the 3Dpol molecule which bound NTP, a method of overlapping peptide analysis was used to identify NTP-cross-linked polymerase peptides. For example, cross-linked polymerase (Fig. 3, lane 2) was partially digested with endoproteinase Glu-C, and the resulting peptides were resolved on a preparative SDS-polyacrylamide gel (Fig. 3, lane 1). A labeled peptide (indicated by arrow) of 35-40 kDa was excised and subjected to N-terminal amino acid sequencing analysis. The results showed that the peptide started with Ala109, and the size suggested that it contained the complete carboxyl terminus of 3Dpol. Digestion with TPCK-treated trypsin generated a 32P-labeled peptide of about 20 kDa (Fig. 3B, lane 4, arrow) which had an NH2 terminus of Leu50-Lys218-Thr-Asp-Phe-Glu-Glu-Ala and extended to approximately Lys228. Assuming that there is only one reactive lysine, the overlap between these two peptides localizes the reactive residue between Ala109 and Lys228 (there are 13 lysine residues in this region). Fig. 3B (lane 4) also shows an intensely labeled doublet of about 30 kDa. The faster migrating band of the doublet has an amino terminus of Met158-Ala-Phe-Gly-Asn-Leu and likely includes the carboxyl terminus of the protein. The overlap between this peptide and the smaller trypsin-generated peptide indicates a reactive lysine residue between Met158 and Lys228 (Fig. 4). There are only 3 lysine residues in this region, any one of which could participate in the GTP binding site.
Affinity of Poliovirus Polymerase for GTP-Agarose—The ability to cross-link stoichiometric amounts of GTP to the poliovirus polymerase suggested that binding of the enzyme to a GTP-affinity column might be a useful method for rapid purification of polymerase from cruder preparations and for rapid screening of modified polymerase molecules for affinity binding. Thus, binding of the enzyme to GTP-agarose was examined. The polio polymerase bound GTP-agarose under conditions (Buffer A, 0.05 M KCl) used for polymerase purification. Subsequently the polymerase could be eluted in the same buffer supplemented with 5 mM of any of the NTPs (GTP, CTP, UTP, or ATP), dATP, or even 25 mM PPI. Significant retention of the polymerase on the GTP-agarose column required some preliminary fractionation presumably due to saturation of the column with a large number of GTP-binding proteins present in crude bacterial sonicates. We routinely used the 40% fractional ammonium sulfate precipitate of the S-100 fraction for GTP affinity chromatography; however, highly purified 3DP0' behaved similarly. Fig. 5A demonstrates the pattern displayed by a silver-stained SDS-polyacrylamide gel of a polymerase preparation fractionated on a GTP-agarose column. Most of the proteins in this preparation did not stick to the affinity column (Fig. 5, lanes 1–4). In contrast, 3DP0' and very few other proteins were retained on the column and could be eluted with 5 mM ATP (Fig. 5, lanes 5–8). Thus, a high degree of purification of 3DP0' was achieved in a single step of affinity chromatography on GTP-agarose. Fragmentation of 3DP0' by protease V8 (Boehringer Mannheim) prevented any binding of the resulting 3D peptides to GTP-agarose. Fig. 5B illustrates an immunoblot of fractions identical to those used in Fig. 5A and demonstrates that 3DP0' is the predominant immunoreactive species (anti-3D serum) which has an affinity for GTP-agarose (lanes 6–8). Other immunoreactive species do not bind to the column (lanes 2–4), including some smaller degradation products of 3D, as well as the larger protein, 3CD (72 kDa). 3CD consists of a fusion of polioviral protease (3C) sequences with the polymerase (3D). It accumulates as a relatively abundant protein in infected cells and manifests essential protease activity. The inability of 3CD to bind to the column is consistent with the absence of polymerase activity associated with 3CD (24) and suggests that the NTP binding site in 3D is altered or unavailable.

Analysis of the GTP-agarose column fractions for polymerase activity showed that the majority of active enzyme was retained on the column (Fig. 6). A small portion of activity was detected in the wash fractions (Fig. 6, fractions 2–4), while most was eluted with 5 mM ATP (Fig. 6, fractions 6–8). In different preparations, variable amounts of 3D protein failed to bind GTP-agarose (generally does not exceed 20% of the total 3D protein recovered) and were detected as immunoreactive, 52 kDa protein in the wash fractions (Fig. 5B, lanes 2–4). If the portion which did not bind to the column was rerun over the column, all polymerase activity was bound, but there was always some immunoreactive material which washed through the column (even after a second rerun), and which had no detectable activity. It is possible that this latter material is denatured enzyme, which is present in every preparation and which is unable to bind GTP.

An experiment was performed to determine whether cross-linking of oxidized [32P]ATP to 3DP0' interfered with the enzyme’s ability to bind GTP-agarose. All cross-linked, labeled enzyme washed through the column (data not shown). These results suggested that both ATP and GTP bound the polymerase at the same site, consistent with the nucleotide competition data shown in Fig. 1.

The capacity of GTP-agarose for poliovirus polymerase was determined on a 1.5 × 8-cm column. This column could retain the polymerase present in the equivalent of 100–200 ml of E. coli culture expressing polio polymerase from the expression plasmid pEXC-3D and collected at early stationary phase. Thus, larger columns would be required for rapid purification of polymerase on a larger scale.

**Fig. 5. Analysis of GTP-agarose column fractions.** A. A 0.40% fractional ammonium sulfate precipitate of a S-100 from a sonicate of an E. coli culture containing pEXC-3D (see "Experimental Procedures") was fractionated on a GTP-agarose column. Eluates from the column were fractionated by 10% SDS-PAGE, and proteins were detected by silver staining (A) or by immunoblotting with anti-3D serum (B). An aliquot of the sample applied to the column is depicted on the left of each panel. Lanes 1–4 show the protein profile of equal volumes of successive 0.5-ml washes of the column with Buffer A, 0.05 M KCl. Lanes 5–8 show corresponding profiles of the same levels of successive 0.5-ml elutions from the column with Buffer A, 0.05 M KCl, 5 mM ATP. The arrows to the right denote the position of 3DP0' in each gel.

**Fig. 6. Bar graph of the total polymerase activity associated with fractions from the GTP-agarose column depicted in Fig. 5.** Each of the wash (fractions 1–4) and elution (fractions 5–8) fractions from the GTP-agarose column depicted in Fig. 5 was assayed for poly(U) polymerase activity (see "Experimental Procedures"), and the total [3H]UIMP incorporated into acid-precipitable material is indicated on the ordinate.
Studies of nucleotide binding by RNA (or DNA) polymerases have been conducted for a number of different enzymes and have generated valuable information about enzyme mechanism and substrate interactions. Specific binding sites can be localized on the polypeptide, and specific reactive groups involved in nucleotide binding can be defined (25–34). For example, covalent attachment of radiolabeled nucleotides to amino acid residues that comprise specific binding sites has been achieved by several procedures: ultraviolet irradiation with or without photoreactive analogs (32, 35–38), reduction of oxidized nucleotide-protein complexes (22, 38, 39), reduction of pyridoxal phosphate-protein complexes concomitant with competition of NTP for these binding sites (25, 27, 30), and linkage of benzaldehyde or alkyl halide derivatized nucleotides to protein followed by incorporation of an additional radiolabeled nucleotide (33, 34, 40, 41). It is toward these aims that the current work was initiated.

Ultraviolet irradiation was relatively inefficient at inducing protein-nucleotide cross-links and caused extensive protein degradation, even in the absence of nucleotide (Ref. 42 and this study), but NTP binding to 3Dpol could be demonstrated. Altering the conditions of the cross-linking reaction may minimize or alleviate some of these difficulties (29, 37, 43). The utilization of oxidized nucleotides to form reactive Schiff bases with protein amino groups was quite efficient and appears to be specific for functional binding sites, since cross-linking approaches but never exceeds 1:1. An unexpected difficulty with this procedure is that we experienced very high losses of polymerase during subsequent handling of the cross-linked complexes. In the preliminary studies described here, however, overlapping peptide analyses, using proteinases of different specificity, have localized the NTP binding site for poliovirus RNA polymerase to the region between Met100 and Lys238 (the carboxyl limitation is an estimate). The results suggest that 1 of the 3 lysine residues in this segment, out of a total of 38 lysines in 3Dpol (33, 34, 40, 41), is a phosphodiester recognition site in NTP binding. This site is not in the immediate vicinity of the highly conserved sequence (YGDD) found in many RNA polymerases (8, 9).

The binding of nucleotides to the poliovirus RNA polymerase occurs with sufficiently high affinity that chromatography on a commercial GTP-agarose column resulted in retention of the majority of the enzyme produced in E. coli harboring the plasmid pEXC-3D. Although total cell lysates appeared to contain too many competing proteins for efficient recovery of the polymerase, only minimal purification steps (high speed centrifugation and ammonium sulfate fractional precipitation) were required in order to utilize the affinity column for rapid isolation and marked purification. This was especially useful in analyzing a series of mutants that produced defective enzymes. Even though almost all of the altered enzymes had no polymerase activity, we were able to distinguish some that bound nucleotide quite efficiently, whereas other proteins had no detectable binding. The failure of any 3D peptides generated by V8 protease to bind to the affinity column suggests that the NTP binding site was disrupted by fragmentation of the protein structure. Furthermore, the fu-
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A variable fraction of 3DP produced in different batches of E. coli appeared to be unable to bind to the GTP-agarose column even upon re-chromatography of the original flow-through. This material was detected by immunoblot, but it had no enzymatic polymerase activity. It is likely that this material represents a population of denatured enzyme molecules that is present in the induced culture or that forms during subsequent cell breakage and manipulation.

The binding of ribonucleoside triphosphates to the poliovirus RNA polymerase observed in these studies occurs in the absence of template and primer. Addition of template does not noticeably alter NTP binding efficiency, suggesting that template need not bind to 3DP before NTP. All NTPs tested bound to the enzyme, and each competed with the binding of other nucleotides, suggesting that there is a single common binding site for all NTPs. GTP, however, protected the enzyme from heat denaturation to a greater extent than the other three NTPs, suggesting that its binding might be tighter. In fact, the $K_n$ for GTP is almost an order of magnitude lower than that for UTP (2.5 x 10^{-6} M for UTP; Ref. 14). However, the concentration required for maximum protection against heat denaturation (2 mM) far exceeds the $K_n$ for polymerization activity, and thus it is not clear what type(s) of binding confers protection against heat denaturation. Possible interpretations are that the enzyme assumes an altered conformation in the presence of high concentrations of nucleotides or that NTPs facilitate stabilization of an ordered conformation. Efforts to measure GTPase activity associated with the purified 3DP preparations yielded no detectable activity (data not shown).

The stability of nucleotide binding to the poliovirus RNA polymerase and the ability to covalently cross-link nucleotides to presumably specific residues in the enzyme will enable us to localize the binding site and to begin to analyze the biochemistry of substrate interaction with this prototypic RNA-dependent RNA polymerase.

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