The poliovirus RNA-dependent RNA polymerase (3D<sup>pol</sup>) has been shown to contain two NTP binding sites by chemical cross-linking of oxidized nucleotide to the intact protein. Only one site (Lys-61) was shown to be essential for RNA chain elongation activity by purified enzyme; however, a full-length viral RNA, coding for an altered lysine residue (K276L) in the second site, generated virus with a minute plaque phenotype that rapidly reverted to a wild-type phenotype with Arg-276 replacing Leu-276 in 3D. Viruses with lysine to leucine substitutions in other positions of the second binding site of their polymerase proteins grew with wild-type phenotype. To test the significance of the second binding site, poliovirus 3D<sup>pol</sup> was generated with lysine (wild-type), leucine, or arginine at residue 276 and tested for NTP cross-linking using [γ<sup>-</sup>32P] oxidized GTP. Analysis of cyano- gen bromide peptides of each 3D preparation showed that the second NTP binding site had severely reduced NTP binding in μ276(Leu) but not in the revertant μ276(Arg), despite the reported requirement for lysine in the cross-linking reaction. To eliminate the possibility that [γ<sup>-</sup>32P] oxidized GTP cross-linked to Arg at residue 276, a model system was designed with unmodified amino acid or acetylated (α-amino) amino acid and [γ<sup>-</sup>32P] oxidized GTP. Cross-linking to lysine, but not leucine or arginine, was observed thus eliminating the possibility that NTP could be cross-linked to residue 276 in 3D. We conclude that NTP binding at the second site in poliovirus 3D is at lysine residues at positions other than 276 (278 or 283), and nucleotide binding at these sites has no bearing on elongation activity or replication of the virus. Nucleotide binding only at the site including Lys-61 is essential for RNA replication.

Investigations of structure-function relationships have been conducted recently for a number of DNA- and RNA-directed polymerases (1–4). A unifying structural framework has been observed with three conserved sequence motifs for DNA-directed polymerases. Four conserved motifs are found in RNA-directed polymerases, two of which are shared with the DNA-directed polymerases (5). These conserved motifs contain several invariant amino acids, which appear in close proximity in three dimensional space and may have direct involvement in active site functions. Crystallographic studies demonstrate that DNA and RNA polymerases have marked structural similarities. The common structural features have been described as the "fingers", "palm", and "thumb" subdomains relating to a cupped right hand (2, 3, 6). A characteristic protein fold forms a nucleic acid binding cleft, and a trio of invariant acidic amino acids in the conserved motifs are clustered in the "palm", allowing binding of metal ions associated with the nucleotide to bring these substrates into the catalytic pocket. More recently T7 RNA polymerase, Taq polymerase, and the mammalian DNA polymerase β have been added to the list of polymerases studied, and all share these core structural features (4, 7–9).

Although these unified structural features are seen in DNA-directed DNA and RNA polymerases and in RNA-directed DNA polymerase, little has been reported for RNA-directed RNA polymerases. The poliovirus RNA-directed RNA polymerase, designated 3D<sup>pol</sup>, is the best-studied of this class of enzymes. As with the other enzymes, signature sequence motifs are present in the central portion of the protein sequence, and residues in the conserved YGDD sequence (residues 326 to 329) have been implicated in metal ion binding (10). The x-ray crystal structure has been solved, and structural alignments with other polymerases demonstrate similar fingers, palm, and thumb subdomains for this member of the superfamly as well.¹

To identify residues involved in the catalytic site of this enzyme, we performed chemical cross-linking studies to determine where NTP bound to the protein (12). Cross-linking to two different peptides was observed: one spanned amino acid residues 75 to 74, near the N terminus of the protein; the other was between residues 266 and 286, in the central portion of the molecule. It was unclear, however, whether NTP binding to both of these sites was required for polymerase function. Since the chemical cross-linking procedure used is reported to require lysine residues (13), we engineered mutations into the polymerase cDNA that changed each lysine individually to leucine (14). For the peptide near the N terminus, substitution of a specific lysine residue (Lys-61) abolished NTP binding measured by GTP-agarose affinity chromatography and abolished polymerase activity of the enzyme in vitro. Substitution of Lys-66, the only other lysine in this NTP binding peptide, had no effect on either activity. For the second cross-linked peptide, replacement of each of the three lysine residues (Lys-276, Lys-278, Lys-283) did not affect NTP binding and had no effect on polymerase activity. Thus, it initially appeared that NTP cross-linking to this portion of the polymerase protein was fortuitous and unrelated to any significant biological reaction. However, when the altered polymerase containing leucine at residue 276 was inserted into full-length viral RNA and used to transfect HeLa cells, an extremely slow growing virus emerged forming minute plaques, which quickly generated pseudorevertants with arginine replacing the leucine. Virus containing leucine residues at position 278 or 283 showed wild-type

¹ S. Schultz and J. Hansen, personal communication.
growth properties. Thus, the possibility remained that NTP binding at Lys-276 of the 3Dpol molecule was required for some step in the RNA replication reaction. Fig. 1 shows a schematic representation of the NTP binding sites and summarizes the properties of the enzymes containing different residues at position 276.

MATERIALS AND METHODS

Construction of Expression Vectors—For preparation of each 3D sequence altered at amino acid residue 276 (3Dpol276) a Blunted-SfiI (1200 base pair) fragment was excised from pTST3-3D, a plasmid expressing wild-type poliovirus polymerase (15), and exchanged with the corresponding fragments from pEXC-3Dpol276(Arg) or pEXC-3Dpol276(Leu), which were described previously (14), to generate plasmids pTST3Dpol276(Arg) and pTST3Dpol276(Leu). Mutations were confirmed by DNA sequencing.

Expression and Purification of 3D—Each plasmid was used to transform Escherichia coli BL21(DE3)pLysS for 3D expression. Expression was at room temperature in 2 x YT medium under ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) selection and were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside when the A600 reached 0.5; induction was continued for 20 h. Poliovirus RNA polymerase was purified from these cells, essentially as described previously (11, 16), including preparation of a soluble lysate, 0–40% saturation ammonium sulfate precipitation, phosphocellulose chromatography, and two MonoQ column purifications.

Analysis of Cyanogen Bromide-generated Peptides by HPLC—Cyanogen bromide digestion of polymerase-[32P]GTP complexes was done as described previously (12). Peptides were fractionated on a Vydac C4 (wild-type 3Dpol) or a Vydac C18 (3Dpol276(Leu) or 3Dpol276(Arg)) reverse phase column (4.6 x 250 mm) using Buffer A (0.1% trifluoroacetic acid in water) and Buffer B (0.1% trifluoroacetic acid in acetonitrile) in the following regimen: 15% Buffer B isocratic wash for 60 min, 15–50% Buffer B for 50 min, 50–100% Buffer B for 5 min, and maintain at 100% Buffer B for 5 min. Fractions (1 ml) were collected immediately after the isocratic wash and monitored by Cerenkov radiation in the scintillation counter.

Cross-linking Amino Acids to [32P]-oxidized GTP and Product Analysis by Thin Layer Chromatography—[32P]-GTP was oxidized with NaIO4 as described previously (12). The [32P]-oxidized GTP was purified by passage over Sephadex G10 (13). Cross-linking to amino acids was done in 50 mM HEPES, pH 8.5, 400 μM magnesium acetate with 200 μM [32P]-oxidized GTP, 2 mM amino acid (lysine, arginine, or leucine), and 1 mM NaCNBH3, at 0 °C, for 17 to 24 h. Products were analyzed by thin layer chromatography on cellulose polyethyleneimine (Baker-flex) sheets (J. T. Baker, Inc.) using ascending chromatography in a solvent of 0.8 M NH4HCO3. Plates were dried, and products were detected by autoradiography on Kodak BioMax MR film.

RESULTS AND DISCUSSION

NTP Binding to Poliovirus 3D with Different Amino Acids at Residue 276—To determine whether NTP binding to Lys-276 was essential for virus replication, enzyme containing lysine (wild-type), leucine (minute plaque), or arginine (revertant) at position 276 in 3Dpol was purified and analyzed for their abilities to cross-link to NTP. Fig. 2 shows the state of purity of each 3D preparation; each appears greater than 90% homogeneous by Coomassie Blue staining. [32P]-Oxidized GTP was cross-linked to 3D and stabilized by reduction with NaCNBH3, as described previously (12). The enzyme-[32P]GTP complex was digested with cyanogen bromide, and the resulting peptides were fractionated by reverse phase HPLC. Fig. 3 shows the elution patterns of [32P]labeled peptides recovered. Our previous work had identified the earliest eluting peak for wild-type enzyme (top panel) as a peptide consisting of residues 252 to 286 and containing lysine residues 276, 278, and 283. The second peak consisted of peptides containing residues 7 to 74, whereas the remaining material consisted of a mixture of multiple peptides including incomplete digestion products. Similar analysis of enzyme 3Dpol276(Leu) (middle panel) showed almost a complete absence of the earliest peak, indicating that protein with a leucine residue at position 276 failed to bind NTP to the central region of the protein sequence. (Total radioactivity in these samples is not directly comparable.) These data indicated either that NTP cross-linking occurred to Lys-276 in the wild-type protein or that contacts were at Lys-278 or Lys-283 but were prevented from forming due to structural alterations induced in the protein by the substitution at position 276. To distinguish between these possibilities, enzyme containing Arg-276 was purified and subjected to the same cross-linking and peptide analysis (bottom panel). This enzyme was fully functional for all essential activities, since the virus containing this coding sequence manifested wild-type growth properties (14). [32P]NTP cross-linking to the earliest peak was restored. If cross-linking to the oxidized nucleotide requires the ε-amino group of lysine for reactivity, then these data show that nucleotide cross-linking occurred at either Lys-278 or Lys-283. Elimination of either of these lysine residues had no effect on the properties of the enzymes or on the phenotype of virus harboring...
NTP Binding by Polio RNA Polymerase

Fig. 3. Elution profiles obtained by fractionation of cyano- gen bromide-digested [32P]GTP-3Dm complexes. Cyanogen bromide digests of 3Dm, previously cross-linked with [32P]GTP, were fractionated by reverse phase HPLC, and fractions were analyzed for 32P by Cerenkov irradiation in the scintillation counter. Wild-type poliovirus 3Dm-C18 column (top panel); 3Dm276(Leu), C18 column (middle panel); and 3Dm276(Leu), C18 column (bottom panel).

Fig. 4. Thin layer chromatogram of amino acids incubated with 32P-oxidized GTP and reduced with NaCNBH3. Arginine (lane 3), arginine (lane 4), and leucine (lane 5) were incubated with 32P-oxidized GTP, treated with NaCNBH3 at 0 °C, and then spotted on the thin layer sheet. Controls contain 32P-oxidized GTP alone (lanes 1 and 6), 32P-oxidized GTP incubated with NaCNBH3 (lane 2), and [32P]GTP alone (lane 7). The origin and solvent front are indicated on the left, and the positions to which [32P]GTP and 32P-oxidized GTP migrate are indicated on the right.

at pH 8.5; similar results were obtained at pH 11 by using the amino acid as the buffering agent (data not shown). Furthermore, cross-linking reactions were done with acetyl derivatives of each amino acid (Bachem Bioscience Inc.) in which the α-NH2 group was blocked, and the results were identical to those obtained with unblocked amino acids. We conclude that of these three amino acids, only lysine can be cross-linked to oxidized GTP under conditions used for cross-linking to poliovirus RNA polymerase (12). Thus by extrapolation to the polymerase, only wild-type enzyme could be cross-linked to oxidized GTP at residue 276. Since 3Dm276(Arg) shows essentially normal levels of nucleotide cross-linking to the 252–286 residue peptide, the observed cross-linking must occur at residue 278 or 283. Reduced cross-linking to GTP for 3Dm276(Leu) compared with 3Dm276(Arg) or 3Dwt276(Lys) is likely the result of a different conformational presentation at 278 or 283 to oxidized GTP in these polymerases. Thus nucleotide binding to this central region in poliovirus 3D appears to have no bearing on elongation activity or on any other essential steps in RNA replication.

The poliovirus RNA polymerase manifests approximately stoichiometric binding of NTP to each of two sites on the linear polypeptide chain, at Lys-61 and Lys-278 or -283. Binding at Lys-61 is implicated in catalytic activity in the polymerization reaction; the position of this residue in the three-dimensional structure of the enzyme has not been resolved. Binding at Lys-278 or Lys-283 appears not to be required for any essential activity. Although these residues are positioned toward the back of the fingers domain in the three-dimensional structure and might have been suggested as candidates for contribution to the active site, their efficient cross-linking appears to occur fortuitously due to site exposure and potential reactivity, and serves as a warning to the interpretation of cross-linking data.

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ing these mutations (Fig. 1); thus nucleotide binding to this portion of the 3D molecule is not relevant to any essential activity of the protein.

Elimination of Arginine at Residue 276 as a Possible Cross-linking Site—The above interpretation is dependent upon the presumption that the oxidized nucleotide cannot be cross-linked to arginine residues but shows stringent specificity for the lysine side chain. To confirm this conclusion, a model system was designed to test whether 32P-oxidized GTP could be cross-linked to arginine under conditions consistent with cross-linking to poliovirus polymerase. 32P-Oxidized GTP was reacted with lysine, arginine, or leucine residues present at position 276 in 3D for wild-type and mutant enzymes, and reaction mixes were analyzed by thin layer chromatography to look for an altered mobility of the labeled GTP. The results of cross-linking reactions are depicted in Fig. 4. [32P]GTP migrates with a Rp of 0.27 in this solvent system under our chromatography conditions (lane 7). 32P-Oxidized GTP remains at the origin (lanes 1 and 6); not all GTP is oxidized in this experiment. Reduction of the 32P-oxidized GTP with NaCNBH3 causes no change in the migration pattern of the oxidized nucleotide (lane 2). Lysine exhibits cross-linking to oxidized GTP (lane 3; Rp 0.38), whereas arginine (lane 4) and leucine (lane 5) do not. The experiment shown in Fig. 4 was performed...
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