A Conserved Tyr Residue Is Required for Sugar Selectivity in a Pol α DNA Polymerase[†]

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ABSTRACT: Many DNA polymerases select their natural substrates, deoxy- as opposed to ribonucleoside triphosphates, with a selectivity greater than 10 000-fold. The function of a highly conserved residue, Tyr416, in the palm domain of the parental enzyme, an exo$^-$ derivative of RB69 DNA polymerase (gp43), a member of the pol α DNA polymerase family, was examined for its role in helping the polymerase discriminate between ribo-, dideoxyribo-, and deoxyribonucleoside triphosphates. The parental enzyme selected dNTPs vs rNTPs with about the same preference as dNTPs vs ddNTPs. Pre-steady-state kinetic analysis was carried out with the parental enzyme and two mutants, Y416A and Y416F. The Y416A mutant incorporated ribonucleotide residues much more efficiently than the parental enzyme, whereas the Y416F mutant was more permissive toward ddNTP vs rNTP utilization than either the Y416A mutant or the parental enzyme. We also found that both dCDP and rCDP inhibited dCTP incorporation by the Y416A mutant, while only dCDP but not rCDP inhibited dCTP incorporation by the parental enzyme and the Y416F mutant. The parental enzyme and the Y416A and Y416F mutants were all able to add araCTP (1-β-D-arabinofuranosylcytosine-5′-triphosphate) to a primer but with reduced efficiency relative to dCTP. Based on our kinetic results, interpreted in the context of the crystal structure of the RB69 gp43 ternary complex, we suggest that sugar discrimination is provided mainly by the Tyr416 side chain which can sterically block the 2′-OH group of an incoming rNTP.

DNA polymerases have been grouped into six families according to sequence similarities (*1−4*). They share a common architecture and mechanism for the nucleotidyl transfer reaction which also extends to RNA polymerases (*5*, *6*) as well as to reverse transcriptases (*7*). It has been well established that replicative and repair DNA polymerases favor incorporation of dNTPs[1] over rNTPs by several orders of magnitude (*8*). Structural, mutagenic, and kinetic studies have provided insights into how DNA polymerases discriminate between NTP substrates with different sugar moieties. Among the examples that have been examined in detail, substitution of a single, highly conserved residue dramatically reduces the ability of DNA polymerases to discriminate between dNTPs and rNTPs. The conserved residue that plays a major role in sugar discrimination is located in motif A (*9*). In the pol α or B family, this residue is Tyr (*10−12*). In reverse transcriptases, it can be either Phe (*13*) or Tyr (*14*, *15*), but in the pol I or A family Glu performs this function (*16*, *17*). DNA-dependent RNA polymerases have Gly at the position corresponding to the conserved Tyr, Phe, or Glu residues which, in all known examples, is five residues downstream from a highly conserved Asp found in all polymerase families (*8*, *18*). This Asp is believed to be one of the residues essential for binding divalent metal ions required for catalysis (*5*).

Tyr416 in Motif A of RB69 gp43 is highly conserved (*12*), and we considered it to be the residue most likely to be involved in dNTP/rNTP discrimination based on sequence comparisons (*2*, *12*). As we predicted, substitution of Ala for Tyr416 in RB69 gp43 allowed rNTPs to be used efficiently to extend a primer using 13/20mer primer-templates (PT). We have used pre-steady-state kinetics to determine K_d and k_{pol} values for the parental and mutant enzymes in primer extension assays with dNTPs, rNTPs, and ddNTPs. This information should help us understand how RB69 gp43 selects dNTP over rNTP and ddNTP substrates. We have also shown that both the Y416A and Y416F mutants are more permissive for ddNTP incorporation than the parental enzyme. To probe sugar selection sites in greater detail, we have also attempted to determine pre-steady-state kinetic parameters for araCTP incorporation by the parental and mutant enzymes. We have tried to rationalize the behavior of the parental enzyme as well as the Y416F and Y416A mutants based on the structure of the RB69 gp43−PT−dNTP ternary complex. Our results are consistent with previous proposals that the conserved Tyr416 acts as a steric gate, but we are left with a puzzling observation relating to the kinetic behavior of the Y416A and Y416F mutants with ddNTP and araCTP that cannot be easily explained by modeling these two substrates with the Y416 mutants in the crystal structure of the RB69 gp43 ternary complex (*19*). Nevertheless, the kinetic results together with the structural information provide the basis for further experiments to

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[1] Abbreviations: PT, primer-template; dNTP, deoxyribonucleoside triphosphate; rNTP, ribonucleoside triphosphate; ddNTP, dideoxyribonucleoside triphosphate; araCTP, 1-β-D-arabinofuranosylcytosine-5′-triphosphate.

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Table 1: Oligonucleotides Used To Prepare Primer-Templates for Kinetic Studies

primer/template	nucleotide sequence[a]	length
13/20GT	5′-CCGACCAGCCTTG-3′	13mer
	3′-GGCTGGTCGGAAC**G**TTTTTT-5′	20mer
13R/20GT	5′-CCGACCAGCCTT$\mathbf{G_r}$[b]-3′	13mer
	3′-GGCTGGTCGGAAC**G**TTTTTT-5′	20mer

[a] The nucleotide residues in the template which are boldfaced and underlined are complementary to the incoming dNTPs. [b] The 3′-terminus of the 13mer primer is a ribosyl residue (G_r). It is boldfaced and underlined.

address these issues. Finally we provide data indicating that RB69 gp43 recognizes the sugar moiety of the incoming NTP before binding to its triphosphate tail.

EXPERIMENTAL PROCEDURES

Materials

T4 polynucleotide kinase was obtained from New England Biolabs; dCDP, rCDP from Sigma; araCTP from TriLink Biotechnologies; [γ-^{32}P]-ATP from Perkin-Elmer Life Sciences Inc.; dNTPs, ddNTPs, and rNTPs from Amersham/Pharmacia; electrophoresis reagents from American Bioanalytical Corp.; oligonucleotides were provided by W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). The sequences of the PT substrates for primer extension assays are given in Table 1. All other chemicals were analytical grade. The RB69 gp43 derivatives used in this study carry the D222A/D327A double mutation that eliminated the 3′−5′ exonuclease activity. Mutant derivatives of exo$^-$ RB69 gp43 were constructed and purified as described elsewhere (*20*).

Methods

Pre-Steady-State Burst and Single Turnover Experiments. Pre-steady-state rapid chemical quench experiments were performed with the KinTek Quench Flow Apparatus (model RQF-3, Kintek Corp., University Park, PA). For slower reactions, requiring sampling at time intervals of >20 s, aliquots were taken and quenched manually. Unless noted otherwise, all components of the reactions are reported as final concentrations after mixing. Burst assays were performed under the conditions in which the PT concentration was 3 times greater than that of the enzyme. Reactions were carried out at 25 °C by mixing equal volumes of 66 mM Tris-HCl, pH 7.5, containing the preincubated complex of 2 μM 5′-^{32}P-labeled PT and 600 nM parental exo$^-$ RB69 gp43 (or its mutants) with 66 mM Tris-HCl, pH 7.5, containing 20 mM $MgSO_4$ and 2 mM dCTP to give final concentrations of 1 μM PT, 300 nM enzyme, and 1 mM dCTP. The polymerization reaction was quenched with 0.5 M EDTA at defined time intervals. Products were analyzed by gel electrophoresis (20% polyacrylamide/50% urea) and quantified by gel scanning using a phosphorimager (Molecular Dynamics). Single turnover assays were performed in a manner similar to that described above except that enzyme (2 μM) was used in excess of the PT (300 nM), and concentrations of dCTP (or rCTP, ddCTP, araCTP) were varied to determine K_d and k_{pol} values.

Pre-Steady-State Inhibition Assays. Commercial dCDP and rCDP were purified by HiTrap Q column chromatography (Pharmacia) to remove the contaminating dCTP (or rCTP) and dCMP (or rCMP) as described elsewhere (*21*). Inhibition assays were performed in a manner similar to the single turnover assays except that various concentrations (0.1−2 mM) of dCDP or rCDP were used as inhibitors together with 10 μM dCTP.

Extension of a Primer Terminating in a Ribo- versus a Deoxyribonucleotide Residue. 13/20GT and 13R/20GT (where R stands for a ribonucleotide residue, Table 1) were used as PT in the assays. The final concentrations of the incoming dCTP or rCTP were both 1 mM. All the other concentrations and conditions were the same as used in the single turnover assays.

Data Analysis. Data from single turnover experiments were fit to a single exponential equation. The dissociation constants K_d for nucleotide substrates binding to the RB69 gp43−13/20mer complex were calculated by fitting the data to the equation: $k_{ob} = k_{pol}[S]/(K_d + [S])$, where k_{pol} is the maximum rate of nucleotide incorporation, [S] is the concentration of incoming nucleoside triphosphate, and K_d is the equilibrium dissociation constant for the interaction of incoming nucleoside triphosphate with the E−PT complex. The same analysis was used for dCDP and rCDP incorporation.

RESULTS

Pre-Steady-State Kinetic Parameters for Incorporation of dCTP by RB69 gp43 and the Y416A and Y416F Mutants. Pre-steady-state burst assays were used to determine the active site concentration of the parental enzyme as well as for the Y416A and Y416F mutants. A biphasic rate for product formation was observed in each case, and the concentration of the productively bound form of the E−PT complex was determined from the amplitude of the burst which was 75%, 80%, and 79% for the parental enzyme and the Y416A and Y416F mutants, respectively (data not shown). To determine the K_d for incoming dNTPs and the maximum rate, k_{pol}, for the nucleotidyl transfer reaction with the parental enzyme and the Y416A and -F mutants, we varied the dNTP concentrations using single turnover conditions for the assays. For the correctly matched dCTP, the Y416A mutant had an identical K_d but a lower k_{pol} compared to the parental enzyme, while the Y416F mutant showed an increase in K_d but a decrease in k_{pol} (Table 2).

Pre-Steady-State Kinetic Parameters for Incorporation of rCTP by the Parental Enzyme and Y416A and Y416F Mutants. The parental enzyme had a much higher K_d and a greatly reduced k_{pol} for rCTP relative to dCTP incorporation. The Y416F mutant had an almost identical K_d value, but an even lower k_{pol} for rCTP vs dCTP incorporation compared to the parental enzyme. As expected, the Y416A mutant had a 30-fold lower K_d value and a k_{pol} value more than 10-fold higher than the parental enzyme (Table 2). Thus, the parental enzyme selects dCTP vs rCTP by a factor of 6.4×10^4. The Y416F mutant discriminates against rCTP almost as well as the parental enzyme, but because rCTP has both a lower K_d and a higher k_{pol} with the Y416A mutant, it incorporates rCTP more efficiently than the parental enzyme (Table 2).

Pre-Steady-State Kinetic Parameters for Incorporation of ddCTP by the Parental Enzyme and the Y416A and Y416F Mutants. The parental enzyme had a much higher K_d and a

Table 2: Pre-Steady-State Kinetic Parameters for Utilization of dCTP, rCTP, ddCTP, and araCTP by the Parental RB69 gp43 and the Y416A and Y416F Mutants

	dCTP		rCTP			ddCTP			araCTP		
enzyme	K_d (μM)	k_{pol} (s^{-1})	K_d (μM)	k_{pol} (s^{-1})	selectivity[a]	K_d (μM)	k_{pol} (s^{-1})	selectivity	K_d (μM)	k_{pol} (s^{-1})	selectivity
parental	69 ± 16	200 ± 13	(1.6 ± 0.4) × 10^4	0.74 ± 0.2	6.4 × 10^4	(4.3 ± 0.8) × 10^3	0.17 ± 0.02	7.3 × 10^4	813 ± 200	118 ± 17	20
Y416A	70 ± 20	22 ± 2	520 ± 150	8.6 ± 0.9	19	33 ± 9	0.02 ± 0.002	520	NA[b]	NA	NA
Y416F	209 ± 60	36 ± 6	(1.8 ± 0.5) × 10^4	0.2 ± 0.07	1.6 × 10^4	170 ± 30	0.41 ± 0.04	71	NA	NA	NA

[a] The selectivity for dCTP over other NTPs is given by the ratio of (k_{pol}/K_d) for dCTP divided by (k_{pol}/K_d) for rCTP, ddCTP, and araCTP. [b] NA: the kinetic parameters could not be measured due either to the very high K_d (greater than 10 mM) or to the very low k_{pol} values (less than 0.01 s^{-1}).

greatly reduced k_{pol} for ddCTP vs dCTP incorporation, while both the Y416A and Y416F mutants had lower K_d values for ddCTP vs dCTP incorporation. Although the K_d values for Y416A, Y416F, and the parental enzyme, with respect to ddCTP incorporation, differed by more than 100-fold, the k_{pol} values varied by less than 20-fold. Thus, the selectivity factor for Y416F is only 71 whereas it is 520 for Y416A and 7.3 × 10^4 for the parental enzyme (Table 2).

Pre-Steady-State Kinetic Parameters for Incorporation of araCTP. To find out whether changing the orientation of the 2′-OH group on the ribose would allow nucleotides with an arabinosyl moiety to serve as a substrate for RB69 gp43, we determined pre-steady-state kinetic parameters for araCTP incorporation by the parental enzyme (Table 2). The K_d value for araCTP was ~10-fold higher than for dCTP incorporation, but was 20-fold lower than that observed for rCTP incorporation. The k_{pol} for araCTP was half of what was observed for dCTP. Although we could detect the addition of araCTP to the primer with both the Y416A and Y416F mutants, it was not possible to estimate kinetic parameters for araCTP because it was incorporated with such low efficiency. Contrary to expectation, it appears that a 2′-OH on the ribose can be tolerated by the parental enzyme if it points away from the Y416 side chain whereas the Y416A and Y416F mutants discriminate against araCTP incorporation even though they are more tolerant of other ribosyl moieties (Table 2).

Inhibition of the Pol Reaction by dCDP and rCDP. We have observed that dCTP incorporation by the parental enzyme could be inhibited by dCDP but not by rCDP, suggesting that the sugar moiety was recognized by the enzyme before the triphosphate moiety (*21*). Since the Y416A mutant was able to incorporate rNTPs more efficiently than the parental enzyme, we decided to see if rCDP would inhibit dCTP incorporation using the Y416A mutant. As shown in Figure 1B, both dCDP and rCDP inhibited dCTP incorporation but only with the Y416A mutant; rCDP had no inhibitory effect on dCTP incorporation by either the parental enzyme (Figure 1A) or the Y416F mutant (data not shown). These results provide additional support for our recent proposal regarding the order of NTP binding (*21*).

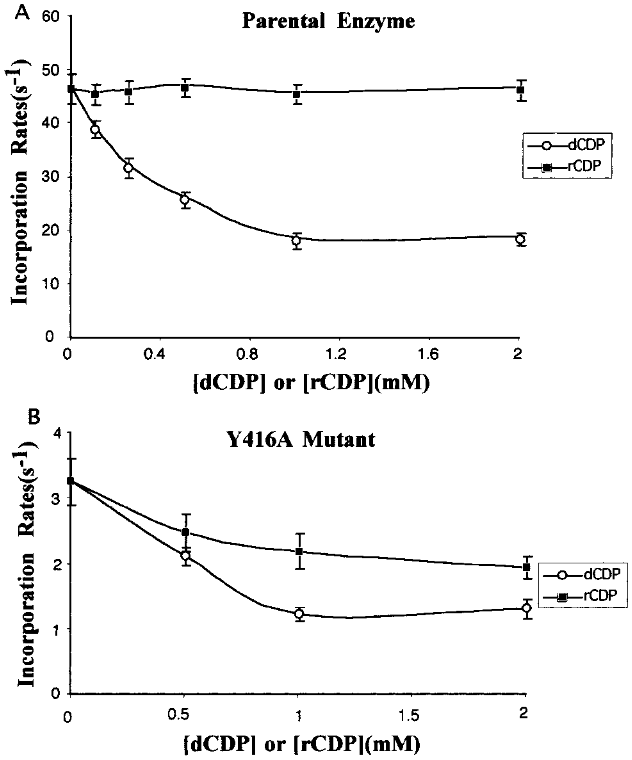

FIGURE 1: Inhibition of dCTP incorporation by dCDP or rCDP. (A) The rate of dCTP (10 μM) incorporation into a 13/20mer PT (300 nM) with 2 μM parental RB69 gp43 and increasing concentrations of dCDP (○) or rCDP (■). The plot of the rates vs dCDP concentration gave an estimated IC_{50} of 0.5 mM (*21*). (B) The rate of dCTP (10 μM) incorporation into a 13/20mer PT (300 nM) with 2 μM Y416A mutant and increasing concentrations of dCDP (○) or rCDP (■). The plot of the rates vs dCDP concentration also gave an estimated IC_{50} of 0.5 mM.

Table 3: Pre-Steady-State Kinetic Parameters for Utilization of dCDP and rCDP by the Parental RB69 gp43 and the Y416A Mutant

	dCDP		rCDP	
enzyme	K_d (μM)	k_{pol} (s^{-1})	K_d (μM)	k_{pol} (s^{-1})
parental	(1.2 ± 0.3) × 10^3	0.50 ± 0.07	ni[a]	ni
Y416A	(1.0 ± 0.5) × 10^3	0.14 ± 0.03	(6.8 ± 2.0) × 10^3	0.17 ± 0.05

[a] ni: no incorporation was observed.

Pre-Steady-State Kinetic Parameters for dNDP/rNDP Incorporation. Since we found that both dCDP and rCDP also could be incorporated by Y416A (Figure 1B), we decided to determine the relevant pre-steady-state kinetic parameters for dCDP and rCDP incorporation. The K_d and k_{pol} values for dCDP incorporation by the Y416A mutant were similar to those found for the parental enzyme (Table 3); however, the parental enzyme does not utilize rCDP, in contrast to the Y416A mutant which can utilize rCDP but less efficiently than dCDP (Table 3).

Extension of a 3′ Ribonucleotide-Terminated Primer with Ribo- vs Deoxyribonucleoside Triphosphates. Because the Y416A mutant can utilize rNTPs much more efficiently than either the parental enzyme or the Y416F mutant, we wanted to find out if the Y416A mutant would add an incoming

Table 4: Pre-Steady-State Reaction Rates for Incorporation of dCTP vs rCTP into Primers Terminating in either a Deoxy or a Ribonucleotide Residue[a]

	incorporation rate (s^{-1})			
	13/20GT		13R/20GT[b]	
enzyme	dCTP	rCTP	dCTP	rCTP
parental	110 ± 10	0.02 ± 0.003	0.6 ± 0.09	$(4.3 \pm 1.1) \times 10^{-3}$
Y416A	21 ± 1.3	4.9 ± 0.4	0.09 ± 0.02	0.02 ± 0.002
Y416F	35 ± 3	0.01 ± 0.001	0.03 ± 0.008	$<10^{-3}$

[a] All assays were done under single turnover conditions with 2 μM enzyme, 300 nM PT, and 1 mM dCTP or rCTP. [b] R represents a ribonucleotide residue.

NTP onto a primer terminating in a 3′ ribonucleotide residue with greater efficiency under single turnover conditions than the parental enzyme. We found that the reaction rate for dCTP or rCTP addition to 3′ ribo-terminated primer was much lower than for an all-DNA-PT. However the Y416A mutant added rCTP to the 3′ ribonucleotide-terminated primer at a rate 5-fold higher than the parental enzyme (Table 4).

DISCUSSION

dNTP vs rNTP Discrimination. A close-up view of the active center in the RB69 gp43−PT−dNTP ternary complex reveals that Y416 stacks underneath the deoxyribosyl moiety of the incoming dTTP (*19*). The distance between the sugar and the aromatic ring of Tyr416 (3.5 Å) clearly shows the potential for a steric clash if a 2′-OH group was present on the sugar ring (Figure 2A). This putative steric interference is consistent with both the large increase in K_d and the drastic reduction in k_{pol} for rCTP vs dCTP incorporation that was observed in single turnover assays with the parental enzyme (Table 2). Substitution of Y416 with Phe resulted in a mutant with similar pre-steady-state kinetic parameters for rCTP incorporation and similar dNTP/rNTP selectivity as the parental enzyme, suggesting that the volume occupied by the aromatic ring is one of the most important factors for discriminating between ribo- and deoxyribo-NTPs by RB69 gp43. Furthermore, the phenyl group in the Y416F mutant still confers sugar selectivity even though its rotomers are not quite as constrained as in the parental enzyme where the position of the aromatic ring is fixed because the Tyr OH group participates in a hydrogen bonding network that includes the peptide backbone of T622, T587, and F395 (Figure 2B). In addition, the aromatic ring packs against a number of highly conserved hydrophobic residues on the side opposite the ribose, which clearly would prevent very much movement of the side chain. It should be noted that fixing the position of the Y416 side chain results in a higher specific activity for dNTP incorporation when the parental enzyme is compared to the Y416F mutant (Table 2). Replacement of Y416 with Ala resulted in a 30-fold decrease in K_d, and a 10-fold increase in k_{pol} for rCTP incorporation compared to the parental enzyme which supports the idea, mentioned above, about the effect of the Y416 side chain volume on sugar selectivity (Table 2). The steric gate explanation for dNTP/rNTP discrimination in RB69 gp43 is bolstered by two other observations: (i) both dCDP and rCDP inhibit dCTP incorporation only with the Y416A mutant, but rCDP has no inhibitory effect on the Y416F mutant or the parental enzyme; (ii) araCTP has a K_d that is 12 times higher than dCTP, and its k_{pol} value is only half that of dCTP with the parental enzyme. These values indicate that the orientation of the 2′-OH in araCTP is such that there is only a minimal effect on binding and chemistry. On the other hand, it is a

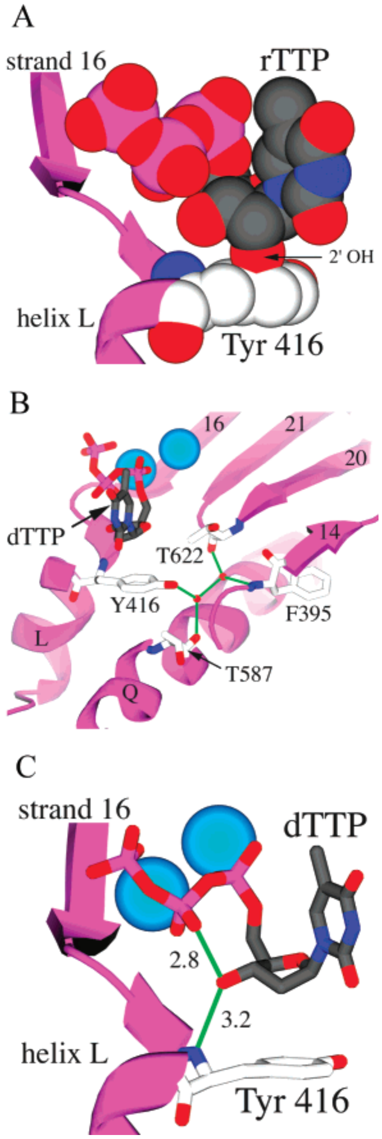

FIGURE 2: (A) Space-filling model indicating the steric clash that would occur between the side chain of Tyr416 and the 2′-OH group of an rNTP. The position of the 2′-OH in this rTTP model was determined by superimposing a C3′-*endo*-ribose onto the C3′-*endo*-deoxyribose actually present in the structure of the ternary complex of RB69 gp43, dTTP, and a primer-template DNA (*19*). All other atoms shown in the figure are unchanged from the ternary complex structure. (B) Hydrogen-bonding interactions between the phenyl OH of Tyr416 and other residues at the polymerase active site. Hydrogen bonds are shown as green lines, with water molecules involved in the hydrogen bonding network as red spheres. Selected polymerase residues are shown in stick form, with the remainder of the palm domain shown as a ribbon (helices L and Q, and β strands 14, 16, 20, and 21 are labeled). The structure shown is that of the closed ternary complex (*19*), with the bound dTTP in stick form, and the two catalytic metal ions shown as blue spheres. (C) Hydrogen bonds mediated by the 3′-OH of the bound dTTP. The dTTP and Tyr416 of the closed ternary complex are shown in stick form, with the two catalytic metal ions (blue spheres) behind the dTTP for orientation. The hydrogen bonds between the dTTP 3′-OH and a nonbridging oxygen on the β-phosphate, and between the 3′-OH and the backbone amide of Tyr416, are shown as green lines with distances indicated in angstroms.

very poor substrate with either the Y416A or the Y416F mutants for reasons that cannot be easily discerned from the structure. Nevertheless, compared to rCTP utilization by the parental enzyme, which is impaired by steric considerations, araCTP can be incorporated into the primer strand without much difficulty.

In the pol α family, two DNA polymerases, one from ϕ29 phage and the other (Vent polymerase) from a hyperthermophilic archaeon (*Thermococcus litoralis*), have been examined using steady-state kinetics with respect to sugar discrimination (*10*, *11*). Both of these polymerases have highly conserved residues with aromatic side chains in motif A which serve to select against rNTPs (*10*, *11*). For ϕ29 DNA polymerase, substitution of Tyr254 with Val reduced the selectivity between rNTPs and dNTPs by 10^3 (*11*). In Vent DNA polymerase, Tyr412 appears to act as a steric gate helping to discriminate against incorporation of rNTPs (*10*).

In the pol I family, studies with the Klenow fragment (KF) have shown that E710 prevents incorporation of rNTPs and that its replacement by Ala reduces the discrimination against rNTPs, allowing them to be utilized much more efficiently by the E710A mutant than by wild-type KF (*16*). In addition to E710, the aromatic ring of F762 in KF was shown to aid in sugar discrimination presumably by helping to correctly position the deoxyribosyl moiety of the incoming dNTPs for primer extension. When F762 was replaced by residues with smaller side chains, where fewer constraints are imposed on the incoming nucleoside triphosphate, E710 becomes less effective as a steric gate, thus allowing rNTPs to be utilized (*16*). With Klentaq I (the large fragment of *Thermus aquaticus* DNA polymerase), a Glu residue, E615, appears to be responsible for discrimination against rNTPs, but this restriction can be relaxed if the adjacent Ile614 is replaced by a residue with a smaller side chain (*22*). As a general rule in the pol I DNA polymerase family, Glu residues occupy the position corresponding to E710 in KF. In the structures of all the pol I family members studied to date, the conserved Glu residues are hydrogen bonded to OH groups of conserved Tyr residues (*17*, *23*, *24*). In addition, hydrophobic residues are found adjacent to the conserved Glu residues and presumably play the same role as Ile614 in Klentaq I, helping to exclude rNTPs from the active site (*22*).

Selection against rNTPs has also been reported for reverse transcriptases (RTs). For example, in Molony murine leukemia virus-reverse transcriptase (MMLV-RT), F155 functions in an analogous fashion to Y416 in RB69 gp43, since replacing F155 with Val, which has a smaller side chain than Phe, reduces the dNTP/rNTP discrimination by 330-fold (*13*). F155 of MMLV-RT is located in an analogous position to the highly conserved Tyr residues in motif A of the pol α family DNA polymerases and to the conserved Tyr or Phe residues in other reverse transcriptases (*13*). HIV-1 RT utilizes Y115 as its steric gate, providing selectivity that favors dNTPs over rNTPs by at least 10^5 (*14*).

RB69 gp43 Employs One Strategy To Select dNTPs over rNTPs and another To Select dNTPs over ddNTPs. The bias exhibited by the parental enzyme against rNTP incorporation also extends to ddNTPs. The selectivity exhibited by the parental enzyme in choosing dNTPs vs rNTPs and dNTPs vs ddNTPs is nearly the same ($\sim 7 \times 10^4$) (Table 2); however, the basis for the discrimination differs. Why is the affinity of the parental enzyme for ddNTPs so much lower than for dNTPs? When dNTP is used as a substrate, the 3′-OH of the nucleotide mediates hydrogen bonds that link the peptide backbone of the polymerase to the dNTP ribose and β-phosphate (Figure 2C), constraining the dNTP to a conformation suitable for catalysis. The absence of these hydrogen bonds with a ddNTP substrate affects nucleotide binding directly by loss of a hydrogen bond between nucleotide and polymerase, and indirectly because the triphosphate tail of the ddNTP is held less rigidly in the position necessary for catalysis. The interaction between the 3′-OH and the β-phosphate removes a good deal of the torsional flexibility of the bound nucleotide and favors a conformation that permits phosphoryl transfer. As other polymerase structures have shown, it is clearly possible to force a ddNTP to adopt this same conformation (*17*, *24*); however, the energy required to do this causes ddNTPs to be discriminated against.

How can we account for the observation that Y416F and Y416A mutants have greater affinity toward ddNTPs than the parental enzyme? While the missing hydrogen bond between the nucleotide and the protein backbone cannot be restored, these mutations may compensate for the altered conformation of a ddNTP by adjustments in the position of the steric gate residue. A Phe mutant will have lost the hydrogen bonding network mediated by the OH of Tyr 416 (Figure 2B), thus allowing a bit more freedom in the position of the side chain. Small movements of the aromatic ring of F416 could compensate for a slightly altered ribose position due to the different conformation of a ddNTP versus a dNTP. An alanine at position 416 will be even more accepting of different nucleotide conformations, explaining why the Y416A mutant has the lowest K_d for ddNTP of the three polymerases examined.

Other DNA Polymerases Have Aromatic Residues That Bias the Enzymes in Favor of dNTP vs ddNTP. Vent DNA polymerase is the only other example in the B family where the issue of dNTP vs ddNTP selectivity has been examined directly (*10*). In Vent, replacement of Y412 (equivalent to Y416 in RB69 gp43) with V or L had very little effect on dNTP vs ddNTP discrimination. Even though we replaced Y416 with A and F, our results are not consistent with Vent. There is a crystal structure for Tgo pol, a DNA polymerase from *Thermococcus gorgonarius* with 91% sequence similarity to Vent (*25*). Since the L and Q helices of Tgo pol superimpose very well with RB69 gp43, we cannot account for the discrepancies in the behavior between Vent DNA polymerase and RB69 gp43 with respect to ddNTP incorporation in terms of obvious structural differences. Additionally, a nonconserved residue, Ala488 in Vent DNA polymerase, also plays a role in sugar selectivity since its replacement by various amino acid residues relaxes the specificity with respect to ddNTP incorporation between 4- and 15-fold (*10*). When the corresponding residue in RB69 gp43, namely, N558, which is in helix P in the finger's domain, was replaced by Ala or Phe, the resulting mutants behaved like the parental enzyme with respect to their ability to select against ddNTPs (Yang et al., unpublished results).

In HIV-1 RT, substitution of Y115 (the "steric gate" equivalent residue in HIV-1 RT) with V increased the ability of HIV-1 RT to discriminate against ddNTPs (*15*). However, in another report (*14*), substitution of Y115 with V resulted in only a small decrease in discrimination between ddNTP or dNTP. Although these reports disagree each other, neither one is consistent with what we have found with RB69 gp43. To account for these differences, we would like to point out the following: (i) Even though the "sugargate" residue in both RB69 gp43 and HIV-1 RT is a tyrosine, there are a number of crucial differences between RB69 gp43 and HIV-1 RT. For example, in HIV-1 RT, the arrangement of the helices is not the same as in RB69 gp43. There is a helical turn from residue 113 to residue 117 in HIV-1 RT (*26*) instead of the two adjacent helices L and Q in RB69 gp43. (ii) Also in the case of HIV-1 RT, there is no equivalent hydrogen bonding network involving the residues corresponding to Y416. In HIV-1 RT, the 3′-OH of the incoming dNTP group extends into a space that is larger than the pocket in RB69 gp43 (*26*). Furthermore, this space is solvent-exposed in HIV-1 RT whereas it is enclosed in RB69 gp43 by residues from the fingers. For these reasons, it is difficult to draw conclusions from HIV-1 RT about ddNTP discrimination in RB69 gp43.

In the pol I family, an interesting situation exists with regard to the presence of Y or F residues (in positions corresponding to 762 in KF) on dNTP vs ddNTP selectivity. T7 DNA polymerase has a Y at position 526 and is quite permissive with respect to dNTP vs ddNTP incorporation. Replacement of Y526 with F increases the ability of T7 DNA polymerase to select dNTPs vs ddNTPs by over 2000-fold (*27*). In the crystal structure of the T7 DNA polymerase ternary complex, the 3′-OH group of the incoming dNTP and the phenolic OH group of Y526 are both within H-bonding distance of the β-*pro*-S_p oxygen of the incoming dNTP, and it has been suggested that the Tyr526 OH group might compensate for the missing 3′-OH in ddNTPs so that this pol I family DNA polymerase, with a Tyr rather than a Phe at this critical site, has reduced discrimination against ddNTPs (*23*, *27*). On the other hand, the presence of F at position 762 in KF (equivalent to 526 in T7 DNA polymerase) is responsible for a ∼3000-fold bias favoring dNTP over ddNTP (*28*). This preference disappears when F762 is replaced by Y.

These results pose some interesting questions about mechanisms used by various DNA polymerases to select against NTPs that lack a 3′-OH group. It should be noted that there is no evolutionary pressure to optimize ddNTP versus dNTP discrimination because DNA polymerases from various organisms do not normally encounter ddNTPs in their environment. Additional crystallographic studies on different DNA polymerase ternary complexes that include ddNTPs should clarify these issues.

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REFERENCES

1. Ito, J., and Braithwaite, D. K. (1991) *Nucleic Acids Res. 19*, 4045−4057.
2. Braithwaite, D. K., and Ito, J. (1993) *Nucleic Acids Res. 21*, 787−802.
3. Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) *Mol. Cells 8*, 7−8.
4. Cann, I. K., and Ishino, Y. (1999) *Genetics 152*, 1249−1267.
5. Steitz, T. A., Smerdon, S. J., Jager, J., and Joyce, C. M. (1994) *Science 266*, 2022−2025.
6. Steitz, T. A. (1998) *Nature 391*, 231−232.
7. Steitz, T. A. (1993) *Curr. Opin. Struct. Biol. 3*, 31−38.
8. Joyce, C. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1619−1622.
9. Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990) *Protein Eng. 3*, 461−467.
10. Gardner, A. F., and Jack, W. E. (1999) *Nucleic Acids Res. 27*, 2545−2553.
11. Bonnin, A., Lazaro, J. M., Blanco, L., and Salas, M. (1999) *J. Mol. Biol. 290*, 241−251.
12. Wang, J., Sattar, A. K., Wang, C. C., Karam, J. D., Konigsberg, W. H., and Steitz, T. A. (1997) *Cell 89*, 1087−1099.
13. Gao, G., Orlova, M., Georgiadis, M. M., Hendrickson, W. A., and Goff, S. P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 407−411.
14. Cases-Gonzalez, C. E., Gutierrez-Rivas, M., and Menendez-Arias, L. (2000) *J. Biol. Chem. 275*, 19759−19767.
15. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 3056−3061.
16. Astatke, M., Ng, K., Grindley, N. D., and Joyce, C. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3402−3407.
17. Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998) *Nature 391*, 251−258.
18. McAllister, W. T., and Raskin, C. A. (1993) *Mol. Microbiol. 10*, 1−6.
19. Franklin, M. C., Wang, J., and Steitz, T. A. (2001) *Cell 105*, 657−667.
20. Yang, G., Lin, T., Karam, J., and Konigsberg, W. H. (1999) *Biochemistry 38*, 8094−8101.
21. Yang, G., Franklin, M., Li, J., Lin, T.-C., and Konigsberg, W. (2002) *Biochemistry 41*, 2526−2534.
22. Patel, P. H., and Loeb, L. A. (2000) *J. Biol. Chem. 275*, 40266−40272.
23. Doublie, S., and Ellenberger, T. (1998) *Curr. Opin. Struct. Biol. 8*, 704−712.
24. Li, Y., Korolev, S., and Waksman, G. (1998) *EMBO J. 17*, 7514−7525.
25. Hopfner, K. P., Eichinger, A., Engh, R. A., Laue, F., Ankenbauer, W., Huber, R., and Angerer, B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3600−3605.
26. Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998) *Science 282*, 1669−1675.
27. Tabor, S., and Richardson, C. C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6339−6343.
28. Astatke, M., Grindley, N. D., and Joyce, C. M. (1998) *J. Mol. Biol. 278*, 147−165.

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