Significance of Nucleobase Shape Complementarity and Hydrogen Bonding in the Formation and Stability of the Closed Polymerase−DNA Complex†

Leonid Dzantiev,‡,⊥ Yuriy O. Alekseyev,‡ Juan C. Morales,§,|| Eric T. Kool,§ and Louis J. Romano*,‡

Department of Chemistry, Wayne State University, Detroit, Michigan 48202, and Department of Chemistry, Stanford University, Stanford, California 94305

Received November 7, 2000; Revised Manuscript Received January 11, 2001

ABSTRACT: DNA polymerases insert a dNTP by a multistep mechanism that involves a conformational rearrangement from an open to a closed ternary complex, a process that positions the incoming dNTP in the proper orientation for phosphodiester bond formation. In this work, the importance and relative contribution of hydrogen-bonding interactions and the geometric shape of the base pair that forms during this process were studied using *Escherichia coli* DNA polymerase I (Klenow fragment, 3′-exonuclease deficient) and natural dNTPs or non-hydrogen-bonding dNTP analogues. Both the geometric fit of the incoming nucleotide and its ability to form Watson−Crick hydrogen bonds with the template were found to contribute to the stability of the closed ternary complex. Although the formation of a closed complex in the presence of a non-hydrogen-bonding nucleotide analogue could be detected by limited proteolysis analysis, a comparison of the stabilities of the ternary complexes indicated that hydrogen-bonding interactions between the incoming dNTP and the template increase the stability of the complex by 6−20-fold. Any deviation from the Watson−Crick base pair geometry was shown to have a destabilizing effect on the closed complex. This degree of destabilization varied from 3- to 730-fold and was found to be correlated with the size of the mismatched base pair. Finally, a stable closed complex is not formed in the presence of a ddNTP or rNTP. These results are discussed in relation to the steric exclusion model for the nucleotide insertion.

Processive DNA synthesis by a DNA polymerase has been described by the repetitive steps of nucleotide binding, an induced conformational transition from the open to closed state, phosphodiester bond formation, a transition to an open conformation accompanied by the release of pyrophosphate, and, finally, movement along the DNA to the next position on the template. The conformational change from the open to closed complex is thought to participate in the mechanism that leads to the very high fidelity that these enzymes possess, since their high accuracy cannot be explained by the stability differences between correct and incorrect Watson−Crick (W/C)[1] base pairing (*1*−*3*). Despite the availability of crystal structures (reviewed in ref *4*) and kinetic and thermodynamic data (*5*−*8*) for the open and closed complexes of several DNA polymerases, the nature of the forces that produce this conformational change is still poorly understood.

The hydrogen bonding that occurs between the incoming dNTP and the template base has been shown to be not absolutely required for nucleotide insertion by the Klenow fragment of DNA polymerase I (KF) (*9*) and by other DNA polymerases (*10*). In these studies, it was shown that if the geometric shape of the resulting base pair closely resembles the Watson−Crick geometry, then efficient incorporation could occur. For example, KF is able to insert difluorotoluene deoxyribonucleoside 5′-triphosphate (dFTP) (Figure 1), a thymidine triphosphate shape analogue that lacks hydrogen-bonding ability, across from an A in the template almost as efficiently as dTTP is inserted (*11*). The importance of steric fit is even more clearly seen in experiments that show that a pyrene deoxyribonucleoside 5′-triphosphate (dPTP) can be efficiently incorporated across from an abasic site, apparently because the pyrene ring—roughly the size of a W/C base pair—can be sterically accommodated when its partner base is absent (*12*). A number of other non-hydrogen-bonding dNTP analogues have been developed that give similar results (*9*, *13*).

An unanswered question from these studies is whether these nucleotide analogues that lack the ability to form hydrogen bonds affect the stability and structure of the closed ternary complex that presumably must form prior to nucleotide incorporation. Using a gel retardation and tryptic digestion analysis, prior studies have shown that the structure of the nucleotide in the polymerase active site has a large effect on the stability of the closed complex and that even a minor change, such as the presence of a 2′-OH or the lack

†This investigation was supported by U.S. Public Health Service Grants CA40605 (L.J.R.) and GM 52956 (E.T.K.) awarded by the Department of Health and Human Services.

* To whom correspondence should be addressed. Tel: 313-577-2584. Fax: 313-577-8822. E-mail: LJR@chem.wayne.edu.

‡ Wayne State University.

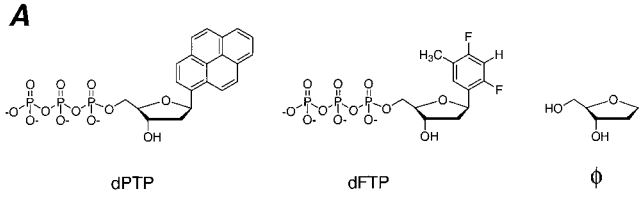
⊥ Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710.

§ Stanford University.

|| Present address: Puleva Biotech, Camino de Purchil 66, 18004 Granada, Spain.

[1] Abbreviations: KF, Klenow fragment; W/C, Watson−Crick; dFTP, difluorotoluene deoxynucleoside 5′-triphosphate; dPTP, pyrene deoxynucleoside 5′-triphosphate; K_d, equilibrium dissociation constant; dNTP, deoxynucleoside 5′-triphosphate.

10.1021/bi002569i CCC: $20.00
Published on Web 02/15/2001



B

5′-GACGAAAACGACGGCCACTTA-H [1]
3′-CTGC TTTTGCTGCCGGTGAATϕGTAGTG

5′-GACGAAAACGACGGCCACTTA-H [2]
3′-CTGC TTTTGCTGCCGGTGAATAGTAGTG

5′-GACGAAAACGACGGCCACTTAT-H [3]
3′-CTGC TTTTGCTGCCGGTGAATAGTAGTG

5′-TAATACGACTCACTATAGGGT-H [4]
3′-ATTATGCTGAGTGATATCCCACATGACT

5′-TAATACGACTCACTATAGGGTGT-H [5]
3′-ATTATGCTGAGTGATATCCCACATGACT

5′-TAATACGACTCACTATAGGGTGT-H [6]
3′-ATTATGCTGAGTGATATCCCACAFGACT

FIGURE 1: Panel A: Structures of the nucleotide analogues dFTP and dPTP and the tetrahydrofuran abasic nucleoside analogue ϕ. Panel B: Sequences of the primer-templates used in the study.

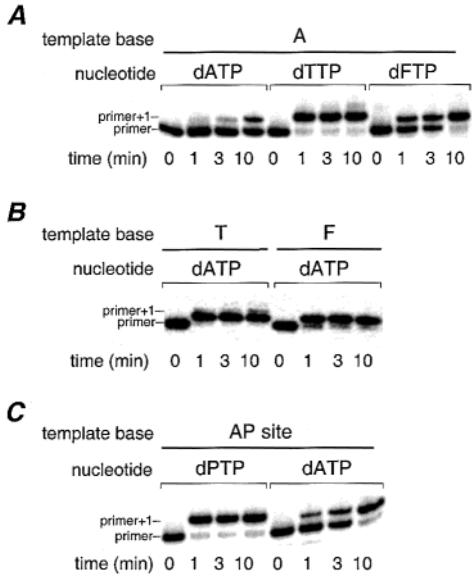


FIGURE 2: Primer extension analysis of nucleotide insertion by the Klenow fragment. ^{32}P-Labeled primer-templates were mixed with the dNTPs indicated at the top of the gels (1 μM), and KF (0.033 unit/μL) was added for the period of time indicated at the bottom of the gels. The reaction products were separated on the 20% denaturing polyacrylamide gel. Panel A: Primer-template **2** was used, making dA the next template base. The primer extension reactions were carried out in the presence of dATP, dTTP, and dFTP as indicated. Panel B: Primer-templates **5** and **6** were used, making dT or dF, respectively, the next template nucleoside. Primer extension was carried out in the presence of dATP. Panel C: Primer-template **1** was used, positioning the abasic site analogue in the polymerase active site. Primer extension reactions were carried out in the presence of dPTP and dATP as described in Materials and Methods.

of a 3′-OH, interferes with the formation of a stable ternary complex (*14*). Using this same tryptic digestion analysis, we show here that the hydrogen bonding between the incoming dNTP and the template base is not absolutely required for the formation of a stable closed complex. In addition, the relative contribution to the stability of the closed ternary complex by base−base hydrogen-bonding interactions and the geometric shape of the base pair formed in the active site was quantitatively determined by measuring the equilibrium dissociation constants (K_ds) for KF−DNA binding. Consistent with the previously proposed steric exclusion model for DNA replication (*13*), the most significant destabilization of the KF−DNA complex was observed when neither the shape nor the hydrogen-bonding requirement was satisfied, i.e., when the incoming nucleotide could not hydrogen bond to the template base and the nascent base pair significantly exceeded the available space in the active site. When the size of the incoming nucleotide is similar to or smaller than the space available for a normal base pair in the polymerase active site, the stability of the ternary complex was reduced compared to that found with a W/C base pair, but this reduction was less than that observed for a base pair that could not fit into the active site.

MATERIALS AND METHODS

Materials. The Klenow fragment of *Escherichia coli* DNA polymerase I (exonuclease deficient) was purchased from Amersham Pharmacia Biotech. The protein had been overproduced and purified from a strain carrying a double mutation D355A, E357A which results in about 10^5-fold reduction of endogenous 3′−5′ exonuclease activity (*15*). T4 polynucleotide kinase also was purchased from Amersham Pharmacia Biotech. Terminal deoxynucleotide transferase and trypsin were from Roche Molecular Biochemicals.

Unmodified oligonucleotides were obtained from Midland Certified Reagent or Operon Technologies. The 28-mer template containing an abasic site analogue (Figure 1A) was from Midland Certified Reagents. All dNTPs, rNTPs, and ddNTPs were ordered from Amersham Pharmacia Biotech. [γ-^{32}P]ATP was from ICN Biomedicals.

Synthesis of Nucleotide Analogues. Difluorotoluene deoxynucleoside 5′-triphosphate (dFTP) was synthesized as described in ref *11*. Synthesis of pyrene deoxynucleoside 5′-triphosphate (dPTP) is described in ref *12*. Structures of the nucleotide analogues are shown in Figure 1A.

Synthesis and Purification of Oligonucleotides. The sequences of the oligonucleotides that were used in this study to create the primer-templates are shown in Figure 1B. The template **6**, containing difluorotoluene deoxynucleoside (dF) at the 5 position, was synthesized as described in ref *16*. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. The primers lacking 3′-OH were obtained by extension of corresponding oligonucleotides with ddNMPs using terminal deoxynucleotide transferase as described (*14*).

Primer Extension Analysis. The activity of KF in the presence of non-hydrogen-bonding nucleoside analogues was analyzed by primer extension (Figure 2). ^{32}P-Labeled primer (2 nM) was annealed to an excess of the cold template oligonucleotide (15 nM) and incubated with dNTPs (1 μM) and 0.033 unit/μL KF in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin. At indicated time points after addition of the polymerase, 3 μL aliquots of the reaction mixture were taken, and the reaction was stopped with 15 μL of gel loading buffer containing 90% formamide and 5 mg/mL bromophenol blue and xylene cyanol. The samples were analyzed on a 20% denaturing polyacrylamide gel.

Determination of K_d for KF−DNA Interactions by a Gel Retardation Assay. Determination of the equilibrium dissociation constants for the interaction of the polymerase with primer-templates in the presence of a dNTP was carried out by the gel retardation assay as described previously (*6*). In this experiment DNA-binding reactions were performed in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, and 4% glycerol. The binding was carried out at 25 °C for 30 min in a 10 μL reaction containing 5−10 pM ^{32}P-labeled DNA heteroduplex, increasing amounts of KF (exo-) (typically 0−81 nM), and 0.4 mM dNTP (if present). The reaction mixtures were loaded on a running native 7% polyacrylamide gel preequilibrated with 0.4 × TB buffer (36 mM Tris−borate, pH 8.3). Gels were fixed with 7% acetic acid, dried, and scanned using a Molecular Dynamics phosphorimager. The amount of the polymerase−DNA complex formed at equilibrium (bound DNA) was calculated on the basis of the difference between the intensity of the dsDNA band without addition of polymerase and the intensities of the free primer-template band separated from the complex at each protein concentration. To obtain the K_d, the fraction of bound DNA was plotted against initial protein concentrations, and the data were analyzed using the program Ultrafit (BIOSOFT, Cambridge, U.K.) and fitted to the equation for single-site ligand binding. At least three independent experiments were carried out to determine each K_d value.

Tryptic Digestion Analysis. The conformation of KF bound to DNA primer-templates in the presence of dNTPs was probed by limited proteolysis as described before (*14*). The polymerase−DNA complexes were formed in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. The binding was carried out at room temperature for 15 min in a 12 μL reaction containing 0.6 μM annealed primer-template, 0.3 μM KF (exo-), and 10 mM dNTP (if present). Two microliters of trypsin solution in water (15 μg/mL final) was added to each reaction mixture, and the digestion was terminated after 6 s by addition of 6 μL of SDS sample buffer containing 0.125 M Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, and 10 μg/mL bromophenol blue. The samples were loaded on a 10% SDS gel, and the electrophoresis was performed according to the standard procedure (*17*). Gels were fixed and silver stained using the GELCODE Color Silver Stain (Pierce) according to the manufacturer's protocol. To further increase the sensitivity of silver staining and detect polypeptide bands containing less than a nanogram of the material, the gels were washed with water three times, and the staining procedure was repeated once or twice. Alternatively, the gels were stained by SYPRO Ruby protein gel stain (Molecular Probes) according to the manufacturer's protocol and scanned using a Molecular Dynamics Storm 860 system.

RESULTS

Several studies have shown that a major factor in determining polymerase fidelity is the shape of the nascent base pair in the active site rather than simply the ability of the incoming base to hydrogen bond with the template (*9*, *10*). Our current model proposes that the rigid active site of a DNA polymerase is able to accommodate only those base pairs that resemble the W/C configuration and that incorrect bases are sterically excluded. It has also been shown that the conformational change to the closed complex brings the incoming base into proper alignment with the template base, and it is thought that this movement is an important factor in the ability of the polymerase to discriminate between a correct vs an incorrect nucleotide (*1*, *2*). To address the question of whether W/C hydrogen bonding contributes to the formation and stability of the closed complex, we have begun to examine the polymerase−primer-template complex in the presence of non-hydrogen-bonding nucleotide analogues. In the present study we have used two derivatives, dFTP, the non-hydrogen-bonding analogue of dTTP, and dPTP, which, when positioned across from an abasic site in the template, is geometrically similar to a W/C base pair (Figure 1A).

Primer Extension in the Presence of Non-Hydrogen-Bonding Nucleotide Analogues. To ensure that the results presented here are consistent with prior studies (*11*, *12*, *18*) and not dependent on the template length and sequence, time course experiments for the incorporation of these analogues were carried out using the primer templates shown in Figure 1B. As was shown previously, dFTP is inserted across an A somewhat more slowly than dTTP, but much more rapidly than a mispaired dNTP (such as dATP) (Figure 2A). Also, consistent with the prior studies (*18*), the efficiency of insertion of dATP opposite dF is approximately the same as opposite dT (Figure 2B) and much higher than the efficiency of dATP insertion across either dA or an AP site (cf. Figure 2). Finally, the efficiency of dPTP insertion across from an AP site (Figure 2C) is almost indistinguishable from the formation of a natural W/C base pair (cf. Figure 2B,C) and much better than of dATP insertion across from an abasic site.

Detection of a Conformational Change in KF in the Presence of a Non-Hydrogen-Bonding Nucleotide Analogue. We have previously shown that the conformational change by KF from an open to closed ternary complex can be detected by a limited tryptic digestion method (*14*) and that, consistent with the steric exclusion model, this change is inhibited by various structural modifications in the incoming dNTP, DNA template, or primer terminus (*14*, *19*). In this study, the ability of KF to undergo this conformational rearrangement in the absence of hydrogen bonding between the incoming dNTP and the template base was tested. KF was bound to either primer-template **5** or **6** (Figure 1). These templates positioned either dT (**5**) or its non-hydrogen-bonding shape mimic dF (**6**) in the next position for incorporation in the template, thus making dATP the next correct dNTP. The conformation of the protein was probed by proteolysis in the absence or presence of each of the four natural dNTPs. As reported previously (*14*), limited proteolysis of KF bound to the 2′,3′-dideoxy-terminated DNA primer-template in the absence of dNTP (i.e., in the open conformation) produces as the major product a fragment with the molecular mass of about 64 kDa (Figure 3A, lane 1). In the presence of the dNTP complementary to the next template base, dATP in this case, proteolysis at this site is inhibited, indicative of closed complex formation (Figure 3A, lane 2). Proteolysis is not inhibited in the presence of any other dNTP (Figure 3A, lanes 3−5). When the template contains dF instead of dT in the template site, a similar albeit somewhat smaller inhibition of tryptic activity at this site is observed in the presence of dATP (Figure 3B, lane 2). The confor-

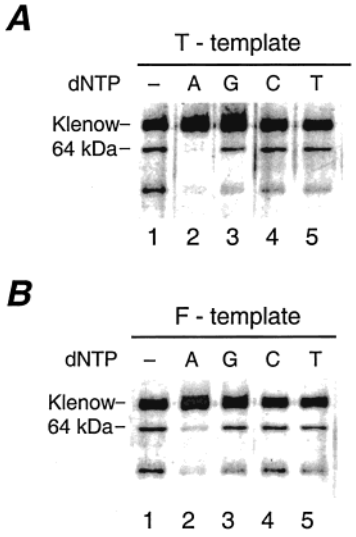


FIGURE 3: Polyacrylamide gel demonstrating the results of tryptic digestion of KF in the presence of the primer-template and dNTPs. KF (0.3 μM) was incubated with 0.6 μM primer-templates **5** and **6** and dNTPs (10 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described in Materials and Methods. Panel A: The primer-template **5** is present that contains dT as the template base. Panel B: The primer-template **6** is present that contains dF at the next template position for incorporation. Lanes 1−5: Tryptic digestion in the absence of dNTP (lane 1) and in the presence of dATP, dGTP, dCTP, and dTTP (lanes 2−5, respectively).

mational rearrangement is not observed on this template in the presence of any other dNTP. Taken together, these results show that shape complementarity and not hydrogen bonding is required for the conversion to the closed complex and suggests that the steric factors contribute to the high selectivity of closed complex formation. These results are consistent with previous steady-state kinetic studies of nucleotide insertion across from dF in the template (*18*) and provide further evidence that hydrogen-bonding interactions between the incoming dNTP and the template base are not absolutely required for the formation of the catalytically active closed ternary complex and consequently for nucleotide insertion.

Stability of the KF−DNA Complex in the Presence of dNTPs. Lack of detection of the closed complex in the presence of non-W/C nucleotides by the proteolytic approach described above does not ensure that the closed complex is not formed but rather suggests either that in these cases the level of conversion to the closed complex is too small to detect by this method or that once the conversion to the closed complex occurs, it is less stable, causing the polymerase to dissociate from the template. It is possible to envision that some of the protein−DNA interactions that are formed in the closed conformation in the presence of the W/C base may be lost when the base geometry is incorrect, thereby causing the closed complex to become perturbed and unstable. To support this idea and to quantify the degree of this destabilization, we have measured the thermodynamic stability of the closed complex in the presence of various combinations of the incoming dNTP and template base by a gel mobility shift assay. This method has been previously employed to measure stability of the complexes formed between the primer-template and either HIV-1 reverse transcriptase (*5*) or the Klenow fragment of DNA polymerase I (*6*). In the present study, the K_ds for the binding of KF to 2′,3′-dideoxy-terminated primer-templates were measured in the presence or absence of the natural or analogue dNTPs on six different primer-templates that positioning either dA, dG, dC, dT, dF, or an abasic site at the next template position for nucleotide incorporation (Table 1).

Table 1: Dissociation Constants (K_ds, in nM) for the Complexes of KF with Primer-Templates in the Presence or Absence of dNTPs[a]

primer-template	template base	nucleotide					
		−	dATP	dGTP	dCTP	dTTP	dFTP
1	ϕ[b]	0.26	0.70	1.9	0.32	0.49	0.30
2	A	0.25	6.1	4.0	1.1	0.03	0.62
3[c]	G	0.40	9.0	22	0.05	1.6	2.8
4	C	0.50	1.4	0.10	0.80	0.47	0.49
5	T	0.40	0.03	7.1	0.35	0.44	0.50
6	F	1.1	1.3	12	1.5	1.5	1.9

[a] Dissociation constants were determined using the gel retardation assay as described in Materials and Methods. The sequences of the primer-templates are shown in Figure 1B. The standard deviation error did not exceed 60% of the corresponding K_d value (omitted for clarity). [b] Abasic site. [c] K_d data for binding in the presence of the natural nucleotides for this sequence were taken from ref *6*.

Table 2: Dissociation Constants (K_ds, in nM) for the Complexes of KF with Primer-Templates in the Presence of dPTP

primer-template	template base	K_d[a]	K_d, normalized[b]
1	ϕ[c]	3.5	0.6
2	A	30	5
3	G	130	22
5	T	8.9	1.5
6	F	15	2.5

[a] Dissociation constants were determined using the gel retardation assay as described in Materials and Methods. The sequences of the primer-templates are shown in Figure 1B. The standard deviation error did not exceed 60% of the corresponding K_d value (omitted for clarity). [b] The K_d values measured for dPTP were normalized by dividing the experimentally obtained value by 6, reflecting nonspecific inhibition of protein−DNA binding observed for this compound (see text for details). [c] Abasic site.

Consistent with the prior results (*6*), the K_ds in the presence of the next correct W/C dNTP are in the range of 30−100 pM for all four natural primer-templates studied, indicating that the closed ternary complex is about an order of magnitude more stable than the open binary complex observed in the absence of dNTPs (Table 1). Also consistent with the previously published data (*6*) the KF−DNA interaction becomes weaker in the presence of noncomplementary dNTPs for all DNA primer-templates. Interestingly, there is a greater nucleotide-dependent destabilization of the complex when an attempt is made to accommodate a dNTP that is expected to cause a larger steric clash in the protein active site. For instance, the K_ds are in the range of 4−22 nM when a purine is being inserted across from a purine. This stronger destabilization is also observed when the bulky dPTP is inserted across from any template base (Table 2, discussed below). The destabilization is not as strong, however, when there is more space available in the protein active site for accommodating the incoming incorrect base. Thus, when a pyrimidine−purine mismatched pair is being created, the K_d for the polymerase−DNA interaction is in the range of 1−2 nM (with the exception of the dGTP−T mispair, which showed a somewhat stronger destabilization of the complex). When a pyrimidine is incorporated opposite a pyrimidine or when any of the dNTPs is inserted across

the AP site, i.e., when there is more space in the active site than necessary for accommodating of the dNTP, the complex is more stable, with K_ds in the range of 0.3−0.8 nM [again with one exception, the incorporation of dGTP across from an AP site (1.9 nM)]. These results are consistent with the steric exclusion model for a dNTP insertion that proposes that the geometric organization of the active site dictates which nucleotide should be incorporated.

Contribution of Hydrogen Bonding to the Closed Complex Stability. To study the dependence of complex stability on the formation of a hydrogen-bonded base pair, the stability of the presumed closed complex was measured in the presence of dFTP and compared with the stability of the complex in the presence of dTTP (Table 1, right two columns). For the template-primers where dTTP is not the next correct nucleotide (**1**, **3**, **4**, **5**, and **6**), dFTP caused similar effects on the complex as was found when dTTP was present, suggesting that this outcome is based predominantly on the shape difference of the base pair formed in the active site. However, when primer-template **2** is used—where dTTP is the next correct nucleotide—the complex is about 20-fold less stable in the presence of dFTP than in the presence of dTTP (Table 1). Since these dNTPs are almost identical in geometric shape (*20*), this difference in binding presumably reflects the contribution of the hydrogen-bonding groups on T to the overall stability of the ternary complex. The lower stability with F (which lacks hydrogen-bonding groups) may be the result of both the absent Watson−Crick pairing and the cost of desolvation of adenine opposite it (*13*).

The influence of proper hydrogen bonding on the ternary complex stability was investigated further by positioning a non-hydrogen-bonding analogue dF in the next template position for incorporation (primer-template **6**). The substitution of a T for an F in this template position (cf. **5** vs **6**) decreased the binding of the polymerase in the open conformation (without dNTP present) about 3-fold. A proportional decrease in binding (about 1.5−4.5-fold) is detected in the presence of all dNTPs except dATP. In this latter case, the binding to the F template was decreased about 40-fold compared with a T template, again apparently reflecting the contributions of hydrogen-bonding groups. This level of decreased stability is the same as was found in comparing dFTP vs dTTP incorporation (see above).

KF−DNA Complex Stability in the Presence of dPTP. Table 2 represents the results of the binding studies in the presence of dPTP on five primer-templates (**1**, **2**, **3**, **5**, and **6**). First, it must be noted that the much larger K_ds observed in the presence of this analogue are most likely due in part to nonspecific destabilization of the protein−DNA interactions caused by the dPTP. Control experiments that measured the binding of *E. coli* single-stranded DNA binding protein (*Eco* SSB) to ssDNA under conditions identical to the ones used for the KF−DNA binding studies demonstrated that dPTP, but not dFTP or the other dNTPs, destabilized the protein−DNA interaction by about 6-fold (data not shown). This fact makes it impossible to make a direct comparison of the K_d values for this nucleotide with the data obtained using the other dNTPs. Nevertheless, in the presence of dPTP the binding of KF to primer-template **1** containing the AP site is several times better than to the other templates (Table 2). Consistent with the steric exclusion model, these results suggest that the ternary complex is much more easily formed in this case due to lack of steric repulsion in the protein active site. Assuming that the binding of KF and *Eco* SSB to DNA is affected by dPTP to a similar degree, one can calculate the apparent K_d for KF binding to the AP-containing DNA to be about $3.5 \div 6 \approx 0.6$ nM. This value is approximately equal to the value obtained when dFTP is inserted across from an A and about 6−20-fold higher than when a W/C base pair is formed, again suggesting that proper hydrogen-bonding complementarity increases the stability of the complex.

Table 3: Dissociation Constants (K_ds, in nM) for Complexes of KF with Primer-Templates in the Presence of 2′-Deoxyribo-, 2′,3′-Dideoxyribo-, and Ribonucleotide 5′-Triphosphates[a]

template base	sugar	nucleotide base		
		guanine	adenine	cytosine
C	2′-deoxy	0.10	1.35	0.80
C	2′,3′-dideoxy	0.68	0.60	0.52
C	ribo	1.13	1.14	0.95
G	2′-deoxy	22	9.0	0.05
G	2′,3′-dideoxy	nd[b]	9.2	0.38
G	ribo	nd	7.8	2.5

[a] Dissociation constants were determined by the gel retardation assay as described in Materials and Methods and using the primer-templates shown in Figure 1B. The standard deviation error did not exceed 60% of the corresponding K_d value (omitted for clarity). [b] Not determined.

Stability of the Closed Complex in the Presence of Dideoxy- and rNTPs. To further investigate the impact of the substrate shape complementarity on the stability of the closed complex, a set of binding experiments were carried out in the presence of dideoxy- and ribonucleotide 5′-triphosphates. These compounds retain the ability of the dNTPs to form hydrogen bonds with the template but cannot be properly accommodated into the active site due to the altered size and geometry of the sugar. Also, the 3′-hydroxyl that is lacking in the ddNTPs has been proposed to be involved in the initiation of the conformational change that occurs following nucleotide binding with KF (*21*). Table 3 presents the dissociation constants measured for the KF complexes with two primer templates in the presence of dNTPs, ddNTPs, and rNTPs. First, it is evident that the complex is more stable in the presence of the W/C dNTP than in the presence of either the ddNTP or rNTP carrying the same base. Second, when the base is incorrect, ddNTP, rNTP, and dNTP cause a very similar destabilization of the complex.

DISCUSSION

The structural organization of the open and closed complexes has been recently determined for a number of DNA polymerases (reviewed in ref *3*). It is now clear that some of the interactions that occur between the amino acid residues in the active site that are present in the binary complexes are absent in the ternary complexes and vice versa (see ref *22* for one comparison). Therefore, it is likely that the open and closed complexes have different thermodynamic stabilities and that the formation and stability of the closed complex may be influenced by the nature of the dNTP being incorporated. In an effort to elucidate the mechanism of the replicative bypass of bulky DNA adducts, we have previously measured the dissociation constants for the interaction of KF with unmodified and adduct-modified primer-templates in

the presence of a dNTP (*6*). The results of this analysis revealed that the stability of these complexes containing unmodified primer-templates was significantly compromised by the presence of a non-W/C dNTP. We have previously suggested that this result is due to the incorrect geometry of the nascent base pair that may not allow the enzyme to complete the conformational rearrangement, thus perturbing the structure of the closed complex and making it less stable. This proposal is in good agreement with the well-supported steric exclusion model for the fidelity of DNA synthesis (*13*) that suggests that the efficient and selective incorporation of the correct W/C dNTP is controlled by the size and shape of the polymerase binding pocket and not by the formation of hydrogen bonding between the incoming dNTP and the template base.

In this study we have measured the binding affinity of KF to several DNA primer-templates in the presence of natural and analogue nucleotide substrates and find that for non-W/C base pairs there is in general an inverse correlation between the relative size of the base pair that is forming and the stability of the closed ternary polymerase−DNA complex. Thus, the most significant destabilization of the closed complex is observed when neither the shape nor the hydrogen-bonding complementarity is satisfied, i.e., when the size of the incoming non-hydrogen-bonding dNTP exceeds the available space in the active site (insertion of dPTP across any base or a purine−purine base pair and, to a lesser extent, a purine−pyrimidine mismatch). Any of these situations presumably leads to a steric clash in the polymerase active site (*9*), making the formation of the closed complex more difficult, increasing the rate of dissociation of the complex, and resulting in a relatively high K_d. When the size of the nascent base pair is similar or smaller than the available active site space, this steric clash is avoided, leading to a more stable complex than is found if the base pair is too large for the active site. Thus placing a T·C, T·T, C·C, C·F, or T·F base pair in the active site leads to ternary complexes of approximately the same stability ($K_d = 0.3-0.8$ nM) (Table 1). This relatively stable complex is also formed on the primer-template that contains an abasic site analogue in the presence of any dNTP, presumably because there is space available in the active site to accommodate any incoming dNTP. In contrast, positioning a bulky B[*a*]PDE adduct in the active site results in more instability of the complex in the presence of a dNTP (*23*), again supporting the idea that this destabilization is caused by the inability of the polymerase to form the ternary complex of the correct geometry because of the steric constraints. Most significant, if the nascent base pair fits in the active site and has the ability to form complementary hydrogen bonds with the template, then the stability of the ternary complex is greatly increased in all cases.

A close examination of the data presented in Table 1 shows that the KF−DNA complex is somewhat less stable in the presence of dGTP than in the presence of other dNTPs in most situations. This fact suggests that the polymerase may handle this nucleotide differently than it does the other dNTPs. Consistent with this possibility is a recent structural study of the Taq DNA polymerase (*24*) that showed that a closed ternary complex containing ddGTP had an additional interaction with the O-helix that was not found with similar structures containing the other dNTPs.

Since the steric exclusion model suggests that the hydrogen bonding between the incoming base and the template base is not a strict requirement for the synthesis of a given base pair, we studied the influence of the hydrogen bond formation on complex stability using the nucleotide analogues that have been shown to closely mimic the W/C geometric shape but lack the hydrogen-bonding ability. The relatively weak KF−DNA complexes formed when dFTP is offered opposite A or when dATP is opposite F are a clear demonstration of the results of eliminating hydrogen-bonding groups in a base pair. We surmise that formation of a stable complex with A·F and F·A pairs is opposed energetically by two factors: the lack of Watson−Crick bonds and the cost of desolvating adenine in the pair. Interestingly, these pairs are synthesized efficiently by the polymerase despite this destabilization. Two other factors that may contribute to this difference are discussed below.

The results of this work suggest that both geometric shape of the incoming dNTP and its ability to form hydrogen bonds with the template nucleotide are important for the maximum stability of the closed ternary complex but that only the shape requirement needs to be satisfied for successful insertion of a dNTP. When hydrogen bonds are formed, the complex is about 20-fold more stable than in the absence of them. This suggests a free energy difference of about 1.5 kcal/mol, a value that is reasonable for the loss of two hydrogen bonds or for desolvation of adenine. However, the results of primer extension kinetic experiments with these analogues indicate that stable closed complex formation found with a W/C base pair is not absolutely required for specific and fast nucleotide insertion. Presumably this is because (depending on concentrations) the polymerase−DNA dissociation rate in the presence of the analogues is still much slower than the rates of the conformational change and of the phosphodiester bond formation.

For the natural nucleobases we have observed a general correlation of KF−DNA binding propensity for a given terminal base pair with the efficiency of polymerase synthesis of that pair. However, with the non-hydrogen-bonding nucleoside analogues there are a number of situations in which polymerase−DNA binding does not correlate with nucleotide insertion activity. For example, the "A rule", in which adenine is preferentially inserted opposite an abasic site, is not reflected by differential binding in the presence of dATP. However, it should also be pointed out that the complex in which dATP is positioned across from an abasic site is more stable than any complex containing a mispaired dATP, which may mean that there is enough space to accommodate this nucleotide and that other factors are probably responsible for the selection of A over the other bases opposite this analogue. For example, although dATP cannot pair with an abasic site, it can bind in the active site at the end of the primer strand with higher stability because of its different stacking propensities. This would not be reflected in polymerase−DNA binding affinities (measured here) because the DNA is likely not sterically distorted either in the presence of dATP or in the presence of other nucleotides. In another case, KF does not bind DNA more tightly when dATP is present opposite F than with other nucleotides opposite F, despite the fact that dATP is inserted opposite F very efficiently. It seems clear that these differences reflect the fact that the polymerase can discriminate

between nucleotides in several ways: by differential DNA binding (as measured here), by differential rates in the conformational change and chemical bond-forming steps, and by differential nucleotide binding. The current study helps to pinpoint the physical sources of discrimination for different base pairing situations.

In light of this, it is possible that there are some minor but critical structural differences between positioning dNTPs of similar base sizes in the active site that translate into a lack of dNTP insertion. For example, when KF is bound to primer-template **2** (dA is the template base), the stability of the closed complex is similar in the presence of dFTP and dCTP, but the former is inserted into DNA much more efficiently. One obvious explanation for this observation is that even when the stability of the complex is not significantly decreased by an incorrect nucleotide, the lack of the W/C base pair geometry alters the way the phosphate residues of the dNTP are positioned in the active site, which, in turn, decreases the rate of the chemical step for its insertion.

Also consistent with the induced fit theory are the results obtained with nucleotide analogues containing modified sugars. Both ddNTPs and rNTPs are known to be inserted much more poorly than dNTPs by KF. It is clear that rNTPs are bulkier than dNTPs, and it has been proposed that the Glu-710 in KF sterically blocks the 2′-OH of the incoming rNTP (*25*). From the results of the pre-steady-state kinetic analysis it has been concluded that the unfavorable interaction with the 2′-OH must be manifested in the transition state and not in ground state binding. We have previously reported the lack of stable ternary complex formation with the rNTPs based upon the results of the limited proteolysis (*14*). The destabilization of the closed complex by the rNTP carrying the next correct base (Table 3) is entirely consistent with these prior results and indicates that KF is not able to position a ribonucleotide in the closed complex without a significant disruption of its structure.

The lower binding detected in the presence of the next correct ddNTP (Table 3) indicates that the polymerase may face a difficulty in positioning this nucleotide in the active site even though it is smaller than the native dNTP. There are two potential sources for this difficulty: inhibition of the initiation of the conformational transition by the lack of the 3′-OH or the inability of KF to form a closed complex having the correct geometry with a ddNTP due to the altered structure of the sugar. Consistent with the first possibility, the 3′-OH of the incoming nucleotide is thought to be required for the efficient conformational change (*21*). However, although it was shown that the conformational change occurs more slowly in the presence of a ddNTP vs a dNTP, the higher K_ds are probably not explained by the polymerase simply remaining in the open form under the conditions of the experiment because we observe a strong destabilization of the closed complex during the attempt to misinsert ddATP across from a template dG (Table 3). On the basis of our prior studies (*6*, *23*) and the results of this work, we believe that in this case a conformational change has taken place that leads to an unstable ternary complex caused by the improper geometry of the "incorrect" ddATP in the active site. It has been shown that the rate of this conformational change in the presence of the "next correct" ddNTP is much lower than with a dNTP (*21*) and comparable with the rate of dissociation of the enzyme from DNA. Therefore, it is possible that the somewhat higher K_d values detected in the case of a correctly paired ddNTP vs a dNTP can be explained by a higher probability of dissociation of the polymerase occurring during the conformational change, although a contribution from a geometric incompatibility might also play a role.

Overall, the results presented here support the steric exclusion model by demonstrating that the degree of destabilization of the closed complex can be directly correlated with the amount of space available to the mismatched pair in the polymerase active site. The results obtained with the non-hydrogen-bonding nucleotide analogues suggest that although the hydrogen-bonding groups of the nascent base pair contribute to the stability of the ternary complex, this additional stabilization is not required for the formation of the catalytically active closed conformation and consequently for the efficient nucleotide insertion.

REFERENCES

1. Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713.
2. Doublie, S., Sawaya, M. R., and Ellenberger, T. (1999) *Struct. Fold Des. 7*, R31−R35.
3. Kunkel, T. A., and Bebenek, K. (2000) *Annu. Rev. Biochem. 69*, 497−529.
4. Steitz, T. A. (1999) *J. Biol. Chem. 274*, 17395−17398.
5. Tong, W., Lu, C. D., Sharma, S. K., Matsuura, S., So, A. G., and Scott, W. A. (1997) *Biochemistry 36*, 5749−5757.
6. Dzantiev, L., and Romano, L. J. (1999) *J. Biol. Chem. 274*, 3279−3284.
7. Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) *J. Biol. Chem. 267*, 25988−25997.
8. Brandis, J. W., Edwards, S. G., and Johnson, K. A. (1996) *Biochemistry 35*, 2189−2200.
9. Kool, E. T., Morales, J. C., and Guckian, K. M. (2000) *Angew. Chem., Int. Ed. Engl. 39*, 990−1009.
10. Morales, J. C., and Kool, E. T. (2000) *J. Am. Chem. Soc. 122*, 1001−1007.
11. Moran, S., Ren, R. X., and Kool, E. T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10506−10511.
12. Matray, T. J., and Kool, E. T. (1999) *Nature 399*, 704−708.
13. Kool, E. T. (1998) *Biopolymers 48*, 3−17.
14. Dzantiev, L., and Romano, L. J. (2000) *Biochemistry 39*, 356−361.
15. Derbyshire, V., Freemont, P. S., Sanderson, M. R., Beese, L., Friedman, J. M., Joyce, C. M., and Steitz, T. A. (1988) *Science 240*, 199−201.
16. Morales, J. C., and Kool, E. T. (1998) *Nat. Struct. Biol. 5*, 950−954.
17. Ausubel, F. M. (1992) *Short Protocols In Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York.
18. Moran, S., Ren, R. X., Rumney, S., and Kool, E. T. (1997) *J. Am. Chem. Soc. 119*, 2056−2057.
19. Dzantiev, L., and Romano, L. J. (2000) *Biochemistry 39*, 5139−5145.
20. Guckian, K. M., Krugh, T. R., and Kool, E. T. (1998) *Nat. Struct. Biol. 5*, 954−959.
21. Astatke, M., Grindley, N. D., and Joyce, C. M. (1998) *J. Mol. Biol. 278*, 147−165.
22. Li, Y., Korolev, S., and Waksman, G. (1998) *EMBO J. 17*, 7514−7525.
23. Alekseyev, Y. O., Dzantiev, L., and Romano, L. J. (2001) *Biochemistry* (in press).
24. Li, Y., Mitaxov, V., and Waksman, G. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 9491−9496.
25. Astatke, M., Ng, K., Grindley, N. D., and Joyce, C. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3402−3407.

BI002569I