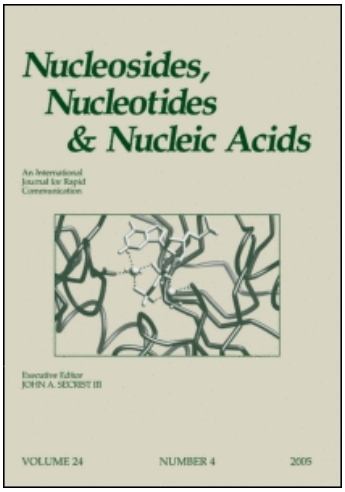

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Substrates of DNA polymerases with planar conformation of sugar: model of substrate transition state?

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Abstract: Several years ago, we published an hypothesis concerning conformation of the glycone moiety of different substrates in active centers of several DNA metabolizing enzymes (Nucleosides & Nucleotides 1993, 12, 649-670). This hypothesis prompted us to further study the subtle conformational changes on substrates of DNA polymerases. Data collected in our, as well as other laboratories, have been analyzed, and models of active centers of different DNA polymerases are discussed below. Based on the model of substrate requirements, we now can divide DNA polymerases into two distinguished classes.

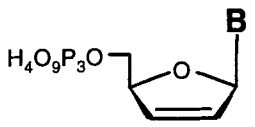
It is reasonable to assume there is a unified conformation in the active center of template-dependent enzymes, which are subsequently operating with a set of several substrates of different structures. DNA polymerases can be considered to belong to a group of such template-dependent enzymes. They catalyze the reaction of DNA chain elongation by adsorption and condensation of dATP, dGTP, dCTP or dTTP, one by one, onto the growing DNA which is attached to the polymerase. In every stage of DNA chain elongation, the substrate has to interact with elements of the active center of the enzyme in reciprocal orientation. Considering the high conformational flexibility of the 2'-deoxyribofuranose residue of a dNTP, it is quite probable that conformational contribution of dNTP is competent to form a reaction complex in the final stage, which would be quite important. Therefore, it is quite conceivable that a relatively common or single conformation of every dNTP substrate is required at the active center of DNA synthesizing enzymes for complex formation. It seems to be kinetically more favorable for DNA polymerases for both complex formation and catalytic action with substrate in a unified conformation than substrates with variable conformations.

During 1985-1986, distances between phosphates and all the protons in dNTP in the complex of [*E. Coli* DNA polymerase I x DNA template - primer] were measured using the NMR technique.[1,2] Based on these data, a model was constructed: the carbohydrate moiety in the model appears to be nearly planar. It should be noted, however, that the NMR method may not be perfect, as it requires a high concentration of complex, and the cation used was Mn^{2+} instead of natural Mg^{2+}. This ion displacement was found to cause marked increase in substrate misincorporations during the DNA elongation process, together with alteration of other reaction parameters. Consequently, it is difficult to assess how much this model reflects the natural situation for *E. coli* DNA polymerase I, as well as how much it corresponds to other DNA polymerases. This model also does not provide us conformational changes of the substrate during the catalytic process. More recent and detailed studies revealed that the carbohydrate conformation of dNTP in active centers of various DNA polymerases apparently deviates slightly in different enzymes.[3] Such deviations have been clearly observed in RNA and DNA dependent reverse transcriptases which are found to require the lowest substrate specificity.[4]

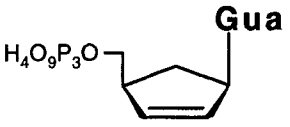
In 1987, it was demonstrated that 3'-deoxy-2',3'-didehydrothymidine 5'-triphosphate (**Ia**) acts as a terminating substrate for several DNA polymerases with high affinity to the corresponding DNA synthesizing complexes.[5] In the glycone, C1',C2',C3' and C4' atoms in the parent olefinic sugar nucleoside are planar. Only the ring oxygen O4' deviates by less than 0.1 A.[6,7] Later, several studies on the terminating activity of modified dNTP with restricted glycone conformation and planarity were published: 2',3'-olefin **Ib**,[8] carbocyclic olefin **II**,[9] acyclic olefins **III**,[10] 2',3'-lyxo-epoxide **IV** and episulfide **V**,[11] and ribo-epoxide **VI** and episulfide **VII**,[12] as well as other compounds.[13] All these compounds **I - VII** exhibited terminating substrate activity in DNA polymerases, including reverse transcriptases.

Affinity of **Ia** to DNA polymerases was investigated in detail. It was found that **Ia** has a high affinity to HIV reverse transcriptase (K_i = 0.082 - 0.32 μM in various testing systems; under these conditions K_m for dTTP falls between 5 and 6 μM)[14-17] and to human DNA polymerase γ (K_i = 0.0035 μM; for dTTP K_m = 0.63 μM).[8] Affinity constants of **Ia** to human and mammalian DNA polymerases β-type,[5,8,11,14] reverse transcriptase of avian myeloblastosis and Rous sarcoma virus,[5,11] murine leukemia virus[8] and mammalian terminal deoxynucleotidyl transferase[5,11] have also been determined. The K_i values, measured in polymerization reactions for modified nucleoside 5'-triphosphates,

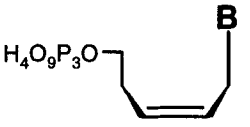
were comparable to the Michaelis constants (K_m) for the corresponding natural substrates. Only to human and mammalian DNA polymerase α, the affinity of **Ia** was found to be very low.[5,8,11,14] Compound **III** also exhibited terminating activity against DNA polymerases



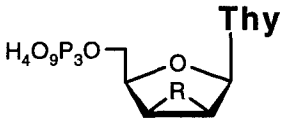
Ia B = Thy; **Ib** B = Cyt



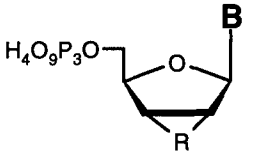
II



III B = Ade, Cyt, Thy



IV R = O, **VI** R = S

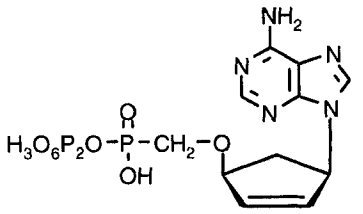


V R = O, B = Ade, **VII** R = S, B = Thy

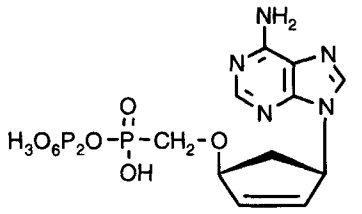
α and ε. The latter is the most selective among the DNA polymerases known. The affinity of **III** to these enzymes, however, was found to be relatively low.[10] The K_m values for compounds **IV, VI** and **VII** were two times higher than the values for the natural substrate, dTTP.[11].

In the case of the cyclopentene analogues of dATP of both D- and L-isomers (**VIII** and **IX**) they are found to have very high affinity to reverse transcriptases of HIV and avian myeloblastosis virus.[18] The K_i values of these compounds in one step DNA elongation in different conditions fall in limits of 0.004 - 0.06 μM, while K_m for dATP and ddATP are 0.03 - 1.72 and 0.19 - 0.21 μM, respectively. This finding is surprising, because in other cases, introduction of a 2',3'-double bond into modified dNTPs did not significantly increase the affinity as compared with the corresponding saturated counterparts.[9,19,20]

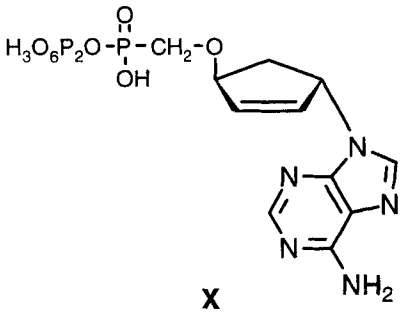
Compounds with a flattened glycone with the adenine and triphosphate residues in *trans* disposition are shown to be good substrates for terminal deoxynucleotidyl transferase



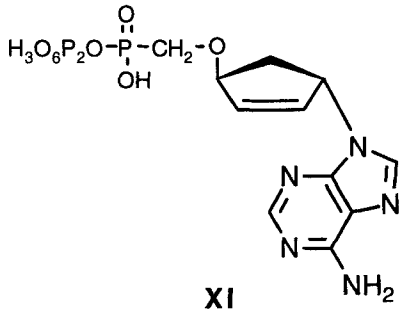
VIII



IX



X



XI

in both D- and L-series (**X** and **XI**).[18] Whenever α-D-dNTP is a very weak substrate for this enzyme, the corresponding α-L-dNTP is not a substrate at all (unpublished).

Conformational studies of the parent nucleotides of **IV - VII**, as well as others using X-ray and NMR analyses and molecular mechanics calculations, demonstrated that their glycone moieties are nearly planar, and this planarity is kept up to 65 °C.[21-23] The data obtained by NMR studies[1,2] and high affinity of modified dNTP with flattened glycone conformation allows us to propose that the planarity of glycone is the universal conformational feature competent for the formation of reaction complex. The reasons for such a conformational state may be as follows:

First, this state appears to be uniformly required for every substrate to be adsorbed by DNA synthesizing polymerase complex for its subsequent incorporation to the 3'-position of glowing DNA chain. In this transition, the distance between significant parts of the substrate molecules (nucleic base and triphosphate residues) and their reciprocal orientation become similar in every step of DNA elongation. Analyses of substrate specificity of modified dNTP principally support the idea that [DNA-polymerase + template] complex recognizes only the nucleic base (due to complementary base pairing) and the triphosphate residue.[24] X-Ray data of complexes of [DNA-polymerase + template-primer] support direct interaction of nucleic base of substrate dNTP with the corresponding

nucleotide in the template, and triphosphate residue with various amino acid residues particularly with Asp in the enzyme. Other data do not contradict this conclusion with the exception of direct interaction of the carbohydrate residue of the substrate. Unfortunately, these data are not adequate enough to resolute fine structure of dNTP in these complexes (in the best case it was 2.3A for polymerase β).[25-27]

Second, planar form of glycone decreases energetic barriers for rotation of the nucleic base of dNTP around the glycosyl bond, which makes base-pairing easier. It is not quite obvious that such partly planar substrate conformation must be realized at every position of growing DNA chain and for every DNA polymerase; some conformational deviation may be possible. It is possible that these deviations explain the difference in experimental data for modified dNTP with flattened glycone found in various publications.

As mentioned above, the reverse transcriptases of HIV and avian myeloblastosis virus as well as terminal deoxynucleotidyl transferase exhibit very similar affinity toward terminating dNTP substrates in both D- and L-series (**VIII** and **IX**).[18] Such properties are also demonstrated by the guanine analogues of **VIII** and **IX**.[28] Analogous results were obtained for other modified terminating substrates, 3'-thia-dCTP[29,30] and 5-fluoro-3'-thia-dCTP[31,32] in both D- and L-enantiomers. Thus, it appears that the area in the active center of these enzymes, where the glycone of dNTP is involved, lacks chirality fixing elements.

Analyses of the total set of data allowed us to conclude that dNTP glycone at the active center of DNA polymerases is required to become partially flattened, but does not interact with the enzyme in a specific manner. Thus, differences in substrate specificity of various enzymes toward modified dNTP depend only on topological restrictions as far as the glycone is concerned (Figure **1**). Namely, if a modified dNTP molecule finds no topological hindrance in the enzyme active center, it can be incorporated into the 3'-terminus of the growing DNA chain. The difference in substrate specificity of various DNA polymerases is, therefore, ruled mainly by topological hindrances which are formed by fine structural environments, *e.g.*, amino acid composition, sequence, *etc.*, around the active center of every DNA polymerase.

The known replicative DNA polymerases of human and mammalian origins, *i.e.*, α, ε and δ, are notable exceptions. In these enzymes at the active center, the 3'-OH group in dNTP appears to interact with surrounding amino acid(s) through H-bond(s) formation (Figure **2**), because replacement of the 3'-OH of dNTP by H results in loss of substrate activity. The replaced H cannot cause topological hindrance; nevertheless triphosphates of 2',3'-dideoxy nucleosides,[33,34] 2',3'-dideoxy-2',3'-didehydronucleosides[5,8] are devoid

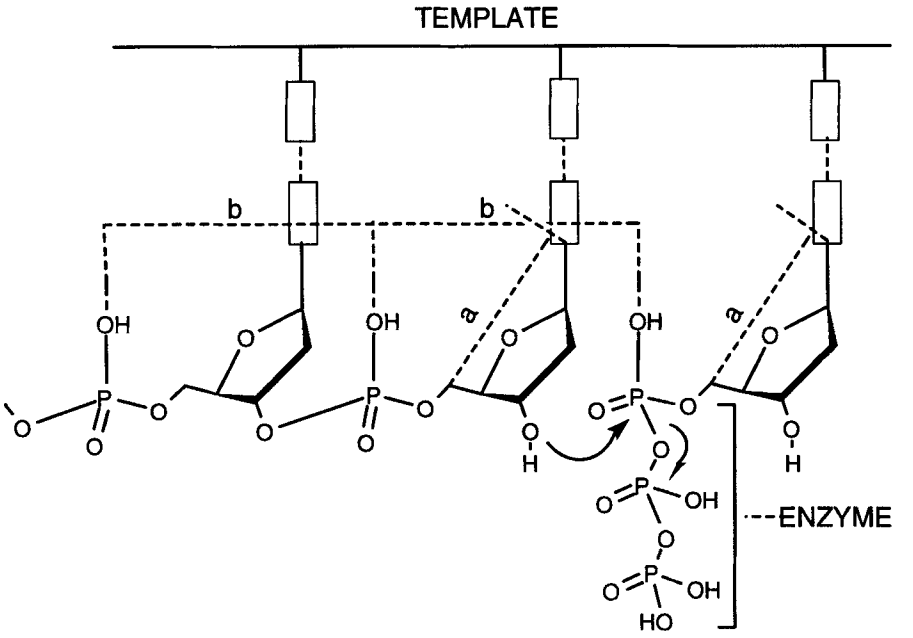


Figure 1. a - the distance between N1 (for pyrimidine) or N9 (for purine) and C5'
b - the distance between internucleoside phosphate groups

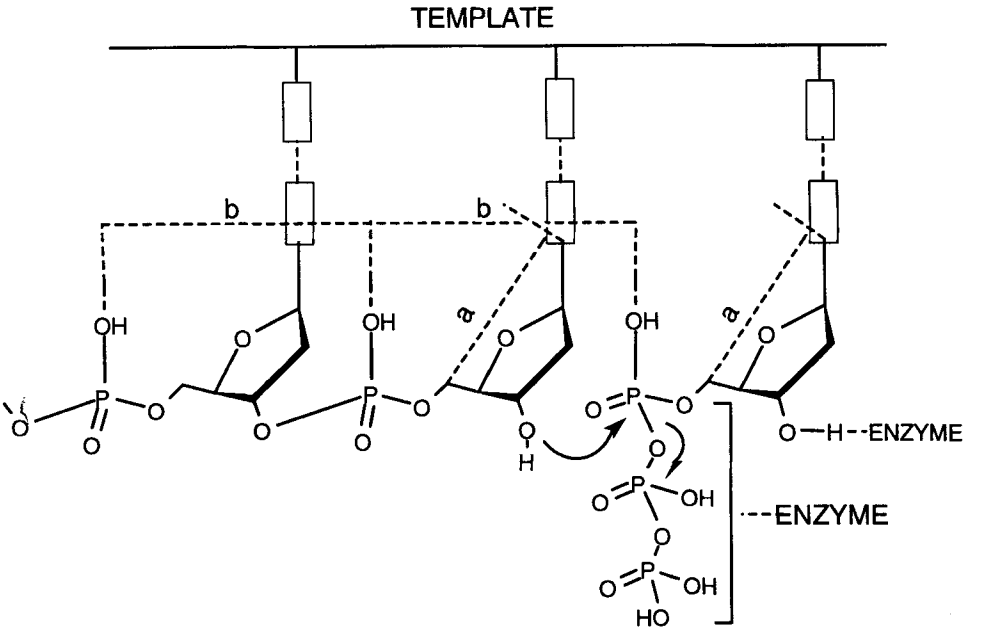


Figure 2. a - the distance between N1 (for pyrimidine) or N9 (for purine) and C5'
b - the distance between internucleoside phosphate groups

of substrate activity for these enzymes. Only substitution of 3'-OH with an amino group, that can participate in H-bond formation, keeps the effective substrate property of modified dNTP.[35,36]

It may now be possible to divide all DNA polymerases into two distinct classes. One class of polymerases (type 1) known thus far, are replication enzymes from mammalian origins, DNA polymerases α, ε and δ, which require involvement of 3'-OH group in binding in addition to the nucleic base (for complementary base pairing) and the presence of triphosphate function. The other class (type 2) includes the repair enzyme DNA polymerase β, mammalian mitochondrial DNA polymerase γ, terminal nucleotidyl transferase, reverse transcriptase of mammalian, retro virus and hepadna virus origins, DNA polymerases of DNA viruses, and *E. coli* DNA polymerase I. Only the nucleic base and the triphosphate residue of dNTP substrates bind to this class of enzymes. The substrate specificity of these enzymes is ruled only by topological hindrances around the active center caused by the surrounding peptides.[37]

Acknowledgment

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37. It may be argued that ACG-TP is not a substrate for HIV reverse transcriptase but is a substrate of HSV DNA polymerase. Thus, these enzymes should not belong to the same class. It should be noted that nucleosides that lack an hydroxyl group in the C3' position do not necessarily be the substrates of all the type 2 DNA polymerases. The type 1 polymerases do require 3'-OH, but all nucleosides with 3'-OH are not necessarily the substrates. Thus, some nucleosides are more selective to a particular polymerase than others of the same category.