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Review

Nucleotide analogues as probes for DNA polymerases

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Abstract. Transmission of the genetic information from the parental DNA strand to the offspring is crucial for the survival of any living species. In nature, all DNA synthesis in DNA replication, recombination and repair is catalyzed by DNA polymerases and depends on their ability to select the canonical nucleobase pair from a pool of structurally similar building blocks. Recently, a wealth of valuable new insights into DNA polymerase mechanisms have been gained through application of carefully designed synthetic nucleotides and oligonucleotides in functional enzyme studies. The applied analogues exhibit features that differ in certain aspects from their natural counterparts and, thus, allow investigation of the involvement and efficacy of a chosen particular aspect on the entire complex enzyme mechanism. This review will focus on a depiction of the efforts that have been undertaken towards the development of nucleotide analogues with carefully altered properties. The different approaches will be discussed in the context of the motivation and the problem under investigation.

Key words. DNA; DNA polymerase; DNA replication; nucleotide analogue; DNA replication fidelity.

Introduction

Transmission of the genetic information from the parental DNA strand to the offspring is crucial for the survival of any living species. In nature this process is catalyzed by the replication machinery in which DNA polymerases are essential for all DNA synthesis [1–7]. DNA polymerases catalyze proceeding DNA synthesis in a template-directed manner. Thus, all DNA synthesis required for DNA replication, recombination and repair depends on the ability of DNA polymerases to recognize the template and correctly insert the complementary nucleotide. DNA polymerases are presented with a pool of four structurally similar dNTPs from which the sole correct (i.e., Watson-Crick base paired) substrate must be selected for incorporation into the growing DNA strand. The mechanisms by which these remarkable enzymes achieve this tremendous task have been a matter of interest and intensive discussion since the discovery of the first DNA polymerase, *Escherichia coli* DNA polymerase I, by Kornberg about half a century ago. Enormous efforts from scientists in many disciplines have been undertaken to gain insights into the complex mechanisms and functions of these molecular machines.

Endeavors along this line are complicated through the fact that even relatively simple organisms have more than one DNA polymerase. Currently more than a dozen human DNA polymerases are known [8–11]. Some recently discovered enzymes exhibit features like high error propensity when copying undamaged DNA or the ability to bypass DNA lesions that block the replicative enzymes. DNA polymerases that are believed to be involved in DNA replication processes show low error rates (as low as only one error within one million synthesized nucleotide linkages) while certain enzymes that are competent to bypass DNA lesions (caused by, e.g., sun light) ex-

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hibit high error rates of up to one error within one to ten synthesized nucleotide linkages. Furthermore, mutations of DNA polymerases that alter their properties can be involved in the development of various cancers [12, 13].

Besides their decisive role in biological DNA synthesis, DNA polymerases are the workhorses in numerous important molecular biological core technologies like the ubiquitous polymerase chain reaction (PCR), cDNA cloning, genome sequencing, and detection of nucleotide variations within genes. Thus, DNA polymerases are essential tools in every molecular biology laboratory. Obviously, insights gained through biochemical studies of these enzymes make a direct impact on the development of improved or new methods for advanced biotechnological applications.

Recently, a wealth of valuable new insights into DNA polymerase mechanisms was gained through application of carefully designed synthetic nucleotides and oligonucleotides in functional enzyme studies [14, 15]. The applied analogues exhibit features that differ in certain aspects from their natural counterparts and, thus, allow investigation of the involvement and efficacy of the chosen particular aspect (e.g. hydrogen bonding, steric bulk) on the entire enzyme catalyzed reaction pathway. This brief survey includes some selected examples of the employment of nucleotide analogues in functional studies of DNA polymerases. The article covers aspects that include modifications of the phosphate, 2′-deoxyribose, and nucleobase residues. The respective nucleotide derivatives are discussed in the context of the biological task in question. Additionally, future potentials for biotechnological applications derived from the biochemical investigations using nucleotide analogues are discussed briefly.

Phosphate analogues

Several phosphate analogues of nucleotides and oligonucleotides have been developed in which one or more oxygen atoms of the phosphate group are replaced by other elements. They are all isoelectronic and isoster to the natural phosphates but small differences in size and major differences in polarity and charge are responsible for their different biological properties. In this survey we will focus on analogues that have been developed to study DNA polymerases or been tested as analogues for these enzymes.

Phosphorothioates

Phosphorothioates were the first reported phosphate analogues in studies of DNA polymerases [16] (fig. 1).

Replacing one of the non-bridging oxygens of the phosphate with sulfur leads to two diastereomeric phosphorothioate moieties (S_P and R_P) which were used for studies of the mechanism of DNA polymerase and other biological processes (fig. 2) [17, 18].

These studies showed that practically all DNA polymerases accept nucleoside triphosphate derivatives containing the phosphorothioate moiety in the α-position exclusively in S_P configuration and catalyze the polymerization reaction under inversion of the configuration at the phosphorus leading to phosphorothioate-containing oligonucleotides (S^--ODNs) with R_P configuration. Although the phosphorothioate-containing DNA has a negative charge within the phosphate-analogous bridge like natural DNA leading one to expect a similar geometry of the structure, the stability toward hydrolysis by nucleases is generally higher. However, the reaction rate of the hydrolysis of the phosphorothioates in relation to the natural phosphate diesters and the ratio of the reaction rate between the S_P and R_P diastereoisomers, varies for various nucleases [16, 19, 20].

Boranophosphates

Boranophosphates [21] are very similar analogues of the natural phosphodiesters because the borane group replacing one of the original non-bridging oxygens is only slightly larger and retains the negative charge. However, this group is unable to form hydrogen bonds and to coordinate metal ions. Boranophosphate-modified oligonu-

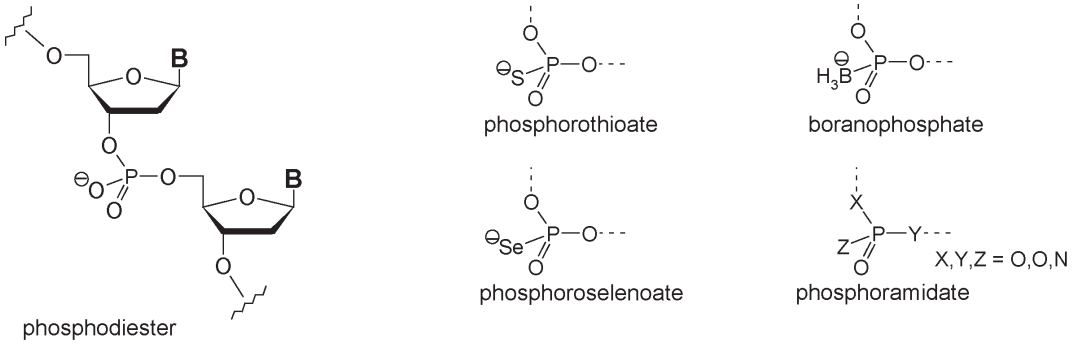

Figure 1. Structures of common phosphate analogues.

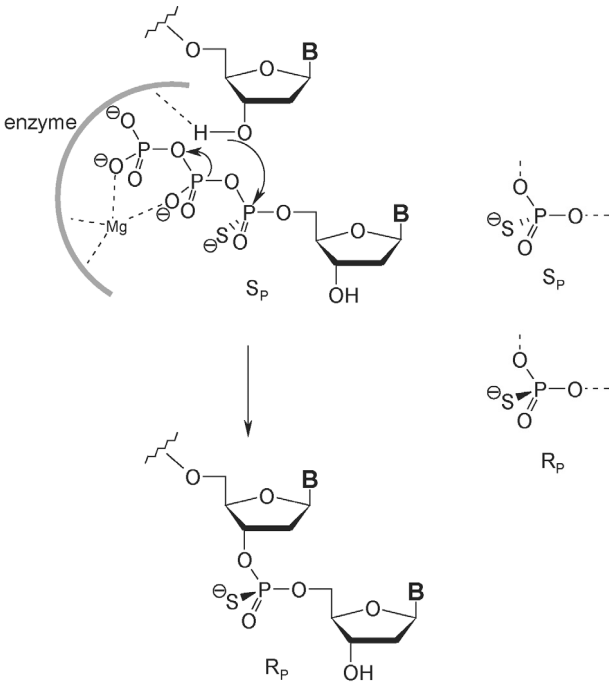

Figure 2. Stereochemistry of the mechanism of DNA polymerases with use of diastereomeric α-thio-substituted NTP analogues as substrates.

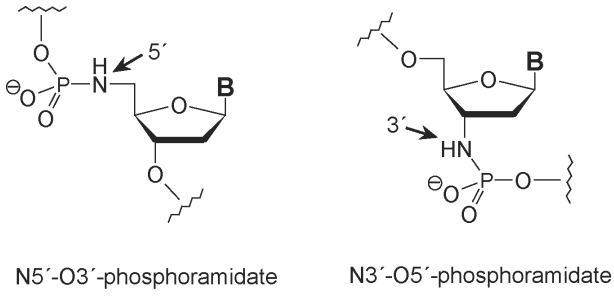

Figure 3. Structures of phosphate analogues phosphoramidates.

cleotides (BH_3^--ODNs) are able to penetrate the cell membrane more easily because of their increased hydrophobicity (18-fold for a dinucleotide) in comparison to natural oligonucleotides [22, 23]. Similar to the phosphorothioates, replacing one of the non-bridging oxygens by borane converts the phosphorus into a chiral center (fig. 1). The R_P-configurated diastereoisomers of the α-borane-substituted nucleotide triphosphates which are stereochemically equivalent to the S_P-configurated phosphorothioates are preferentially accepted by DNA polymerases [24]. BH_3^--ODNs are highly resistant toward hydrolysis by nucleases and are of interest as drugs for antiviral, antisense, and boron neutron capture therapies (BNCTs) [25, 26].

The synthesis and properties of some nucleotides and oligonucleotides containing double-modified phosphates, i.e. boranophosphothioate [27] and the uncharged boranomethylphosphonate [28], have also been reported.

Phosphoramidates

Phosphoramidates in which one non-bridging oxygen is replaced by nitrogen have been developed for various applications [29, 30] (fig. 3).

Depending on the substituent at the nitrogen they are uncharged or positively charged. Various oligonucleotides have been reported containing phosphate analogues with bridging oxygens replaced by other elements, i.e., N3′-O5′-phosphoramidates [31, 32] and N5′-O3′-phosphoramidates [33]. Recently, DNA polymerases have been shown to accept 5′-amino-2′,5′-dideoxy nucleoside analogues as substrate and synthesize DNA containing phosphoramidate bridges [34]. Based on these findings, new sequencing and strand cleavage protocols might be developed [35]. Phosphoramidate-modified oligonucleotides (NR_2-ODNs) contain little structural modification, have an increased resistance toward nucleases, and show only slight decreases in melting temperatures in hybridization studies. Because of these properties they have been developed for use as antisense therapeutics [36–38].

Phosphoroselenoates

In phosphoroselenoates, one of the non-bridging oxygens is replaced by selenium (fig. 1) [39]. Their chemical and biological properties are similar to those of the phosphorothioates. Oligonucleotides containing multiple phosphoroselenoates were stereospecifically synthesized by enzymatic synthesis with the pure R_P and S_P diastereomers of an α-selenium-substituted nucleoside triphosphate as substrates which were chemically prepared and separated by high-performance liquid chromatography (HPLC) [40]. Phosphoroselenoate-modified DNA is useful for biochemical investigations and structural analysis, especially for three-dimensional (3D) structure determination of DNA by X-ray crystallography [41].

Analogues with modified nucleobase residues

Nucleobase analogues with deleted single hydrogen bonds

Previously, Watson-Crick hydrogen bonding was presumed to be responsible for the selectivity of DNA polymerases. Many nucleotide analogues with modified bases have been synthesized and used for studies of further interactions in the minor groove of DNA.

To investigate the influence of single hydrogen bonds on the function of DNA polymerases, nucleobase analogues with deleted single hydrogen bonds were synthesized and used for these investigations (fig. 4).

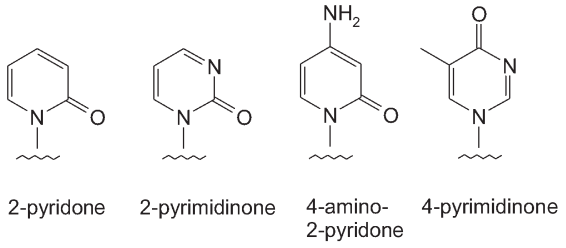

Figure 4. Nucleobase analogues for deleted-single-hydrogen-bond investigations.

Attempts to use oligonucleotides containing 2-pyridone as a nucleobase analogue, in which only one hydrogen bond acceptor is retained, as templates for the Klenow fragment of *E. coli* DNA polymerase I led to non-specific incorporation of nucleotides [42, 43]. Use of oligonucleotides containing 2-pyrimidinone which contains one hydrogen bond donor and one acceptor in the same experiment caused complete inhibition of replication. A surprising result was obtained for oligonucleotides containing 4-amino-2-pyridone as a nucleobase analogue in which one hydrogen bond acceptor is deleted in comparison to the corresponding natural nucleobase cytosine: they allow the incorporation of cytidine and of a lower amount of thymidine. The tautomeric form of 4-pyrimidinone probably allows hydrogen bonding with cytosine and thymine instead of the expected guanine [42, 43].

The 4-pyrimidinone derivative was found to be unsuitable as a nucleobase analogue, because it prefers to adopt unfavorable tautomeric forms [44, 45]. To overcome this problem, the use of C-nucleosides was suggested. The nucleoside triphosphates containing 3-methyl-2-pyridone and 2-aminopyridine as nucleobases were used as dTTP and dCTP analogues which have one hydrogen bond acceptor and one donor, but are lacking the 2-keto group as hydrogen bond acceptor. Neither was accepted as substrate for the Klenow fragment of *E. coli* DNA polymerase I [45]. This indicates that contacts with the 2-keto groups of incoming pyrimidine nucleosides are essential for processing nucleotide triphosphate substrates by at least this enzyme.

A similar compound, the 2′,3′-dideoxy nucleoside triphosphate derivative with 2-aminopyridine, as nucleobase analogue has been used for studies with HIV reverse transcriptase (fig. 5) [46]. The substrate is well accepted by this enzyme and leads to termination of nucleoside chain elongation because of the lacking 3′-OH group. The 2′,3′-dideoxy derivative is not accepted as a substrate by mammalian polymerases α, β, and γ. This remarkable selectivity was explained by different interactions of the O2-carbonyl group of the dCTP in the minor groove of the different enzymes and is of importance for the potential use of the compound as a potent antiviral drug with low toxicity.

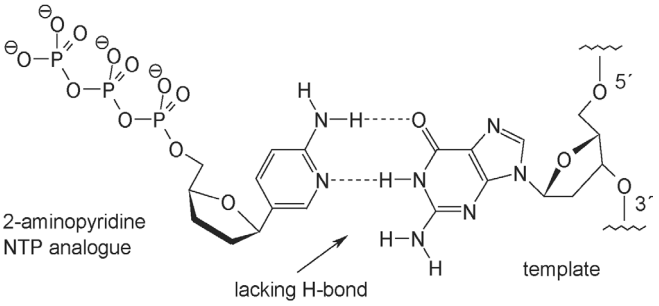

Figure 5. Hydrogen bonding pattern of an NTP analogue containing 2-aminopyridine.

Oligonucleotides containing 3-deazaguanine (fig. 6) which lacks one hydrogen bond acceptor have been used for mechanistic studies with the Klenow fragment of *E. coli* polymerase I [47].

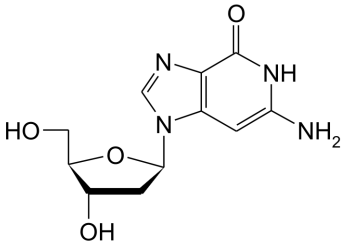

Figure 6. Structure of 3-deaza-2′-deoxyguanosine.

Experiments with primers in which the 3′-terminal guanosine is replaced by 3-deazaguanine indicate that a hydrogen bond exists between the Arg668 side chain of the DNA polymerase and the N3 of guanine in the minor groove of the primer (fig. 7). This hydrogen bond causes the orientation of the Arg side chain to enhance phosphodiester bond formation.

The nucleoside triphosphate analogue containing 3-deazaguanine was used to study the selectivity mechanisms of eukaryotic DNA polymerase η [48]. DNA polymerase η is a member of the recently discovered repair DNA polymerases and is proficient in bypass synthesis of several DNA lesions that block those enzymes that are involved in DNA replication [8–10]. Studies of high-fidelity DNA polymerases have indicated that interactions with the DNA minor groove acceptor and donor functionalities are crucial for the high fidelity of replicative DNA polymerases. DNA polymerase η exhibits remarkable error propensity when copying undamaged DNA. To gain insights into the mechanisms that govern the low fidelity of DNA polymerase η the authors investigated whether these kinds of interactions via the minor groove are at work as well [48]. They investigated DNA-polymerase-η-catalyzed nucleotide incorporation when 3-

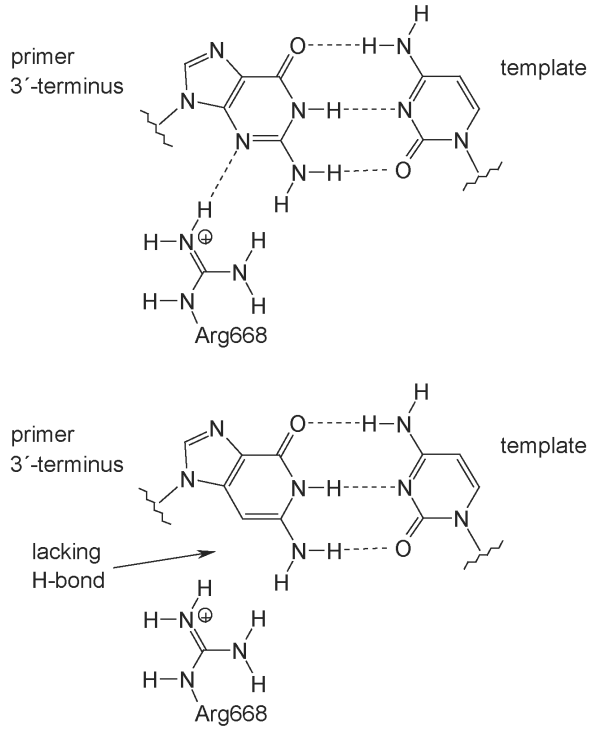

Figure 7. Comparison of the hydrogen bond patterns of natural guanosine and its 3-deaza analogue at the 3′-terminus of the primer.

deazaguanine is substituted for guanine at various positions in the DNA. 3-Deazaguanine is a base analogue that lacks the ability to form minor-groove hydrogen bonds with the protein when incorporated in double-stranded DNA. The results obtained suggest that DNA polymerase η makes only a single functional contact with the DNA minor groove at the position of the incoming dNTP. In this regard, this enzyme differs from high-fidelity DNA polymerases which might contribute to factors that determine the high error propensity of DNA polymerase η and its ability to bypass distorting DNA lesions.

Nucleotides with rearranged hydrogen-bonding patterns

Among several factors that govern the selectivity of pairing between two complementary oligonucleotides, the complementarity of interstrand hydrogen-bonding patterns certainly plays an important role. As noted by Benner, there are several alternative possible arrangements of mutually exclusive donor and acceptor functionalities in nucleobase pairs [49–52]. The bonding patterns of interstrand base pairs can readily be extended to six base pairs, provided that some bases are joined to the sugar via a carbon-carbon bond (fig. 8).

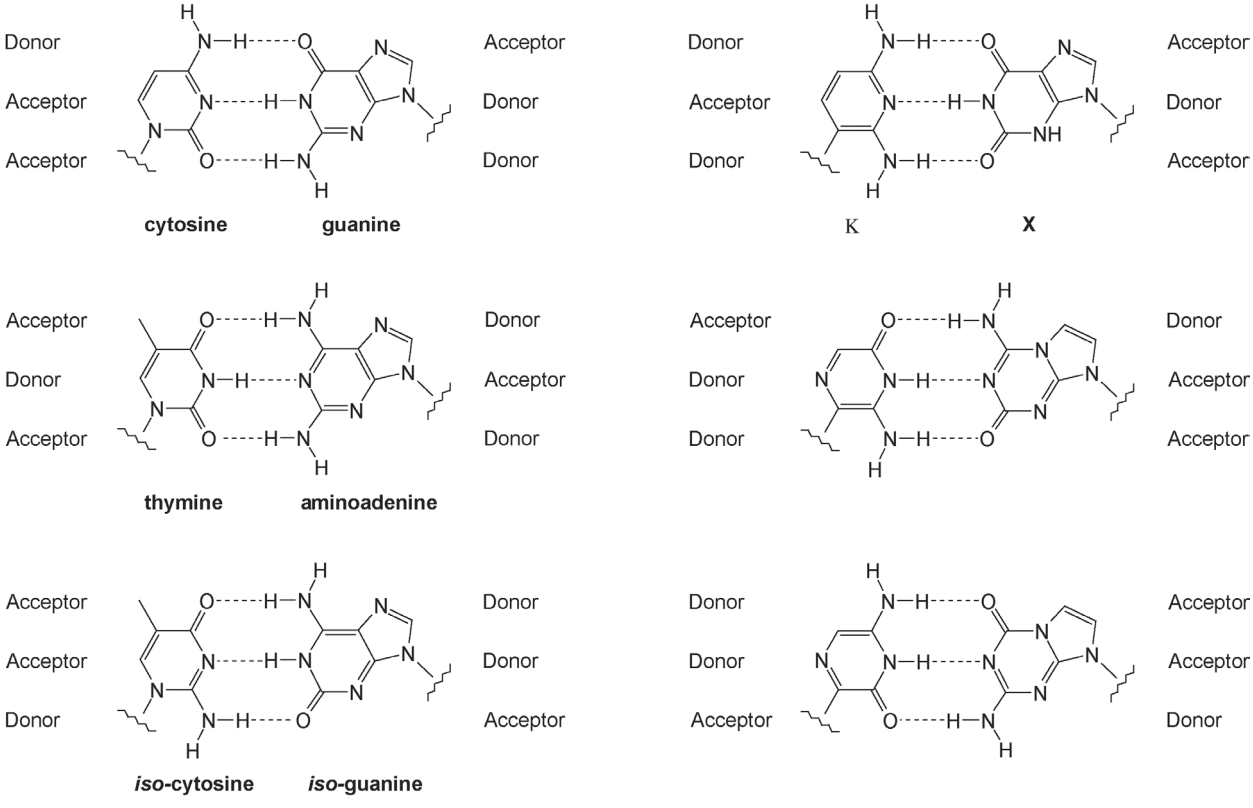

Figure 8. Possible arrangements of hydrogen-bonding patterns.

Only four of these are used by nature and, thus, if a model based on complementary internucleobase hydrogen bonding for DNA polymerase selectivity holds true, extension of the genetic alphabet through rearrangement of the donor-acceptor patterns should be possible. Along this line, the Benner laboratory has developed numerous nucleoside analogues with scrambled hydrogen-bonding patterns. First, they investigated the ability of DNA polymerases to form a iso-dC-iso-dG base pair and found that certain DNA polymerases will incorporate the analogues with shuffled hydrogen-bonding patterns into a growing DNA strand. However, the incorporation of iso-dG is somewhat less selective than that of dG, and iso-dC exhibits measurable instability in an aqueous environment. Subsequently, Benner et al. studied the properties of the base pair derived from diaminopyrimidine (κ) and xanthine (X) and found that both analogues are substrates for several DNA polymerases, albeit they are processed with lower selectivity and efficiency. Reduced fidelity is attributed to the presence of different minor tautomers that code for misincorporation of undesired nucleotides. New derivatives with a reduced tendency to tautomerize and increased stability were recently developed and might circumvent these problems [53]. However, despite these shortcomings, these new pairing systems have already been exploited in the development and application of new diagnostics methods [54].

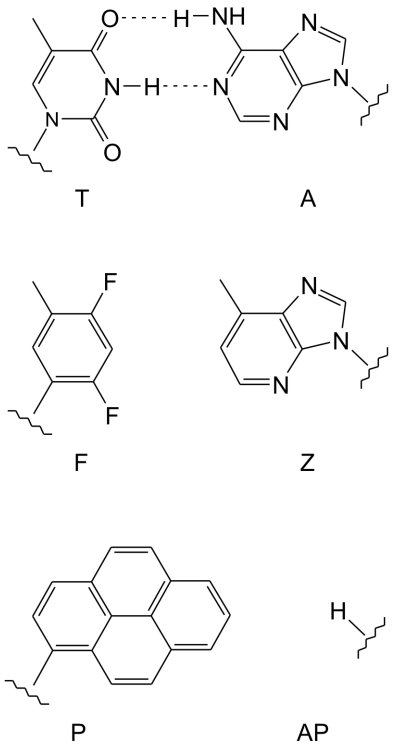

Figure 9. Comparison of the natural T-A base pair and the artificial isosteric F Z pair.

Non-polar isosters of natural nucleosides

Certain DNA polymerases achieve selective information transfer according to the Watson-Crick rule with error rates as low as one mistake within one million synthesized nucleotides. What are the underlying mechanisms accounting for this strikingly high selectivity? At first glance, the formation of distinct hydrogen bonding patterns between the nucleobases of the coding template strand and the incoming nucleoside triphosphate might be regarded as 'informational.' Thus, hydrogen bonding alone was commonly perceived to be solely responsible for the selectivity of DNA polymerases.

Nevertheless, thermal denaturating studies of matched and mismatched DNA complexes by Goodman and coworkers strongly suggest that these interactions alone are not sufficient to explain the degree of selectivity commonly observed for enzymatic DNA synthesis promoted by high-fidelity DNA polymerases [7]. Thus, additional factors must contribute to the observed high fidelity. Among these factors are exclusion of water from the enzyme active site, base stacking, solvation, minor-groove scanning, and steric constraints within the nucleotide-binding pocket resulting in geometric selection of the nucleobase pair with the right shape and size [1–7].

To evaluate the participation of hydrogen bonding in DNA replication selectivity mechanisms, Kool and coworkers described a seminal functional strategy based on chemically modified DNA polymerase substrates. They developed nucleotide analogues in which the polar natural DNA nucleobases are replaced by non-polar aromatic molecules, which closely mimic the shape and size of the natural nucleobases but have significantly diminished ability to form stable hydrogen bonds (fig. 9).

These hydrophilic nucleotide isosters were applied as functional probes to elucidate the impact of hydrogen bonding on DNA polymerase selectivity. In first experiments, Kool and coworkers studied the insertion of dNTPs opposite F, the isoster of thymidine (fig. 9), in the template strand [55, 56]. If hydrogen bonding alone drives selective nucleotide incorporation one would expect incorporation opposite F to be very inefficient and unselective. Interestingly, the contrary was observed when studying a $3' \rightarrow 5'$-exonuclease-deficient mutant of the Klenow fragment of *E. coli* DNA polymerase I. This enzyme was able to efficiently promote dATP insertion opposite F and with high selectivity [57]. Steady-state kinetic analysis revealed that the efficiency of artificial A-F pair formation was only fourfold lower than that of the natural pair. Additionally, they found that dFTP was inserted by the polymerase with highly efficiency, and that

the selectivity was as high as for the natural substrates. These results strongly suggest that, at least for the enzymes investigated, the observed selectivity cannot be ascribed to hydrogen bonding alone. This hypothesis was further supported by findings that a thoroughly artificial F-Z pair (fig. 9) can be efficiently processed by DNA polymerases accompanied by considerable selectivity [58]. Since these investigations clearly suggest that hydrogen bonding does not chiefly contribute to DNA polymerase selectivity, other factors like steric effects must play a significant role in DNA polymerase selectivity processes. In a steric model for DNA polymerase fidelity, the enzyme selects the right substrate (according to the Watson-Crick rule) through editing the shape and size of the nascent base pair. Further evidence for the steric model was derived from studying dPTP insertion opposite abasic AP sites in the template strand (fig. 9). The pyrene group in dPTP is nearly as large as a natural base pair and obviously has no significant hydrogen-bonding ability to the abasic site. The space occupied by the pyrene moiety fills in the blank of the removed base in the template strand. Remarkably, the Klenow fragment and T7 DNA polymerase are able to insert dPTP opposite abasic sites more efficiently than opposite natural bases or another pyrene-bearing moiety in the template [59].

In additional investigations, Kool and coworkers compared the action of several eukaryotic and prokaryotic DNA polymerases on non-polar nucleoside isosters [57]. When investigating interactions at the active site (modifications include the coding nucleotide and the nucleoside triphosphate), they found that several DNA polymerases differ significantly in their action on the isosteric analogues. T7 DNA polymerase, *Thermus aquaticus* DNA polymerase, and HIV-1 reverse transcriptase (RT) behave like the Klenow fragment of *E. coli* DNA polymerase I, suggesting related mechanisms that govern nucleotide processing. However, different results were obtained for calf thymus DNA polymerase α and avian myeloblastosis virus RT. The results obtained suggest the existence of an energetically important hydrogen-bonded interaction between the enzyme and the minor groove of the incipient base pair. A third group of DNA polymerases comprising human DNA polymerase β and Moloney murine leukemia virus RT failed to replicate the artificial F-Z base pair indicating that hydrogen bonds are needed at both the template and nucleoside triphosphate site.

As depicted above, several studies suggest the presence of hydrogen bonds between the DNA primer template complex and DNA polymerases that are important for enzyme function. Using the non-polar isosters, certain DNA polymerases like *T. aquaticus* DNA polymerase, HIV-1 RT and the Klenow fragment of *E. coli* DNA polymerase I were found to undergo important hydrogen-bonding interactions at the first extension site in the primer strand [60, 61]. Another group of enzymes comprising DNA polymerase α and β and T7 DNA polymerase fails to extend any of the non-H-bonding base pairs. Thus, these studies indicate that several enzymes need minor groove hydrogen-bonding interactions at both template and primer site or that they are especially sensitive to non-canonical DNA structure or stability.

Using the non-polar isoster F, eukaryotic DNA polymerase η was investigated [62]. As mentioned above, DNA polymerase η is an extraordinarily error-prone enzyme that is proficient in promoting bypass synthesis of several DNA lesions. This study revealed that the efficiency and accuracy of the incorporation of **F** by DNA polymerase η is severely impaired. Based on this finding, the authors suggest that Watson-Crick hydrogen bonding is required for DNA synthesis promoted by DNA polymerase η. Thus, this enzyme differs strikingly from high-fidelity DNA polymerases.

Taken together, the studies employing non-polar nucleotide isosters as probes strongly suggest different mechanisms employed by the various enzymes to achieve high selectivity. Studies on high-fidelity DNA polymerases (like T7 DNA polymerase, *T. aquaticus* DNA polymerase, and the Klenow fragment of *E. coli* DNA polymerase I) indicate that hydrogen bonding alone does not account for the origin of DNA polymerase selectivity. Thus, steric effects within the active site of the enzyme are believed to significantly, if not predominantly, contribute to the factors that drive selectivity of DNA polymerases, and canonical base pair formation relies on the complementarity of size and shape of the nascent base pair. Needless to say, this does not imply that hydrogen bonding according to the Watson-Crick rule does not contribute to the selectivity at all.

Analogues with modified sugar residues

Size-augmented nucleotides

DNA polymerases are assumed to form nucleotide-binding pockets that differ in properties like shape and tightness. Thus, high-fidelity DNA polymerases are believed to form more rigid binding pockets tolerating less geometric deviation, while low-fidelity enzymes exhibit more flexibility leading to decreased fidelity. However, this concept of active-site tightness needed to be tested experimentally. Recently, Summerer and Marx [63] introduced new functional means to investigate the effect of mainly steric constraints on the mechanism of DNA polymerase selectivity (fig. 10).

They developed steric probes by increasing the bulk of nucleoside triphosphates by substitution of the standard 4′-C-hydrogen of the 2′-deoxyribose with alkyl groups that gradually increase in steric demand. This size expansion is believed to cause less conformational flexibility

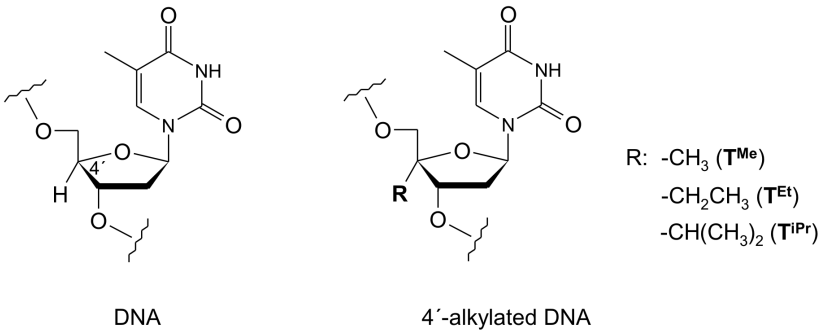

Figure 10. Structure of 4′-alkylated DNA.

within the DNA polymerase active site and, thus, the modifications should decrease the tolerance for geometrically altered conformations of nascent nucleotide pairs. If the steric model of DNA replication selectivity holds true this feature should result in an increase in nucleotide insertion selectivity. This was precisely what was found. Increasing the bulk of the nucleoside triphosphate substrate through employment of the probes $T^{Me}TP$ and $T^{Et}TP$ led to a marked increase in nucleotide insertion selectivity catalyzed by a proof-reading-deficient mutant of the Klenow fragment of *E. coli* DNA polymerase I [64–66]. Thus, these results support the model that steric constraints are at least one crucial determinant of DNA polymerase selectivity. As mentioned above, structural data suggest that less tight and more open enzyme active sites might be the origin of higher error propensity observed for some DNA polymerases. One would reckon that a more error-prone DNA polymerase (like HIV-1 RT) would process the bulkier thymidines more efficiently than the more selective enzyme Klenow fragment. Marx and colleagues assumed that if varied active-site tightness is indeed essential for differential nucleotide insertion selectivity this should be felt by the steric probes $T^{R}TP$. However, they observed that concerning 'correct' insertion of the different $T^{R}TP$s used, there is little difference between HIV-1 RT and the Klenow fragment [67, 68]. Analyzing misinsertion, the two enzymes behave differently. While 4′-C-methylation has little effect on the selectivity of HIV-1 RT, significant effects are observed for the Klenow fragment. Thus, based on the concept of active-site tightness, the results suggest that both enzymes must differ significantly when promoting misinsertion rather than insertion opposite canonical template bases. This might be the result of differential active-site conformations causing different steric constraints while promoting 'incorrect' nucleotide insertion.

In certain cases, amino acid substitutions in the active site are known to affect the selectivity of DNA polymerases. One such mutation in HIV-1 RT is M184V. Through M184V mutation, a β-methyl side chain present in valine is introduced that is believed to contact the sugar ring of the incoming triphosphate. This mutation has been shown to result in increased nucleotide insertion selectivity which is attributed to increased steric constraints within the active site. Employing the steric probe $T^{R}TP$, this size augmentation was monitored by the increased bulk and resulted in significantly lower misinsertion efficiency by the M184V mutant compared to the natural substrate.

In conclusion, these studies highlight the importance of tight fitting of the nucleotide substrate within the DNA polymerase active site. The presented data provide experimental evidence that minute changes in the overall shape and size of the substrate impose significant effects on nucleotide selection. Furthermore, these studies provide strong experimental evidence that variations of steric constraints within the nucleotide binding pocket of at least two DNA polymerases cause differences in nucleotide incorporation selectivity. First experiments by Marx and coworkers have indicated the applicability of the described probes in highly selective PCR amplification systems for DNA diagnostics [69–73].

Nucleotides with expanded or contracted 2′-deoxyribose residues

Recently, nucleotide analogues bearing either expanded or contracted 2′-deoxyribose moieties were studied for their interplay with DNA polymerases. These studies were in part intended to gain insight into which parts of the sugar moiety are necessary for recognition and incorporation by DNA polymerases and to reveal the importance of sugar conformation and conformational flexibility in these processes.

α-L-threose nucleic acids (TNAs, fig. 11) were suggested by Eschenmoser as a potential evolutionary progenitor of RNA and are composed of repeat units that are one atom shorter than those of DNA [74].

TNA is capable of antiparallel base pairing with complementary DNA, RNA, and TNA. To evaluate the potential of TNA in molecular evolution, its action on DNA polymerases was investigated. In a highly interesting set of

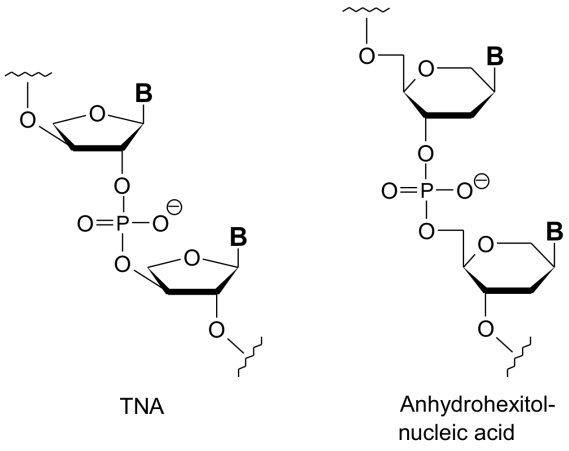

Figure 11. Nucleic acid analogues containing α-L-threose (TNA) and anhydrohexitols.

experiments, Chaput et al. [75] investigated the proficiency of several DNA polymerases to bypass stretches of TNA with natural nucleotides. They found that certain DNA polymerases are able, despite the significant differences in the sugar-phosphate backbone, to copy limited stretches of a TNA template. The authors suggest that due to the high activity of wild-type DNA polymerases, it might be possible to evolve a TNA-directed DNA-polymerase with improved reactivity. In subsequent studies, Chaput et al. [76] and Kempeneers et al. [77] investigated the recognition of α-L-threosyl nucleoside triphosphates by DNA and RNA polymerases. They found that some DNA polymerases are able to incorporate α-L-threose nucleotides into a growing polymer, albeit the enzymatic synthesis of TNA is blocked after incorporation of a limited number of α-L-threose moieties. These experiments suggest that certain polymerases might be a suitable starting point for directed evolution to generate TNA polymerases.

Along similar lines, Herdewijn and colleagues investigated several DNA polymerases for their interplay with anhydrohexitol nucleoside 5′-O-triphosphates (hNTPs), analogues that have conformational similarity to ribonucleoside 5′-O-triphosphates but bear a six-membered ring (fig. 11) opposed to the five-membered ring of ribose derivatives present in RNA and DNA [78, 79]. They were able to show that all DNA polymerases tested were able to insert the artificial hNTPs, although extension from inserted anhydrohexitol was somehow hampered. In comparison with the action of α-L-threose building blocks on the tested DNA polymerases Herdewijn and coworkers found that anhydrohexitol nucleotides are more easily accepted by DNA polymerases than the threose counterparts. Since the anhydrohexitol sugar moiety is a rigid six-membered sugar that mimics the natural nucleoside in its C3′-endo conformation and the threose moiety is a functional rather than a structural mimic of nucleotides, DNA polymerases seem to preferentially incorporate the structural analogues.

From DNA polymerase studies using nucleotide analogues to applications

As mentioned above, several studies that were motivated toward gaining mechanistic insights into the complex processes of DNA replication have influenced developments of new antiviral compounds or advanced applications in diagnostics.

Numerous nucleosides are widely applied and recognized as compounds with antiviral activity [80, 81]. However, to prevent side effects due to undesired action of the nucleoside analogues, one main aim of ongoing work is the development of more selective compounds with high efficacy. As discussed above, recent work by several research groups indicates that some mammalian DNA polymerases make important functional contacts to the minor groove functional groups of nucleobase residues in the primer and template strands. In addition the low incorporation efficiencies by mammalian DNA polymerases of nucleoside triphosphates that lack these functionalities were ascribed to similar origins. As depicted above, based on these findings, McLaughlin and coworkers designed a new nucleotide analogue that lacks minor-groove-binding capability and lacks a 3′-hydroxyl functionality, thus blocking further chain extension after incorporation of the analogue (fig. 5) [46]. Interestingly, this analogue exhibits remarkable selectivity toward HIV-1 RT, because it is processed by mammalian enzymes with lower efficacy. These results are a promising basis for further developments of this structural scaffold.

Antiviral compounds that act as chain terminators of viral polymerases were long thought to lack a 3′-hydroxy functionality. However, experiments using 4′-azido, 4′-acylated, or 4′-alkylated nucleotides have indicated that even nucleotides comprising a 3′-OH group can act as chain terminators (fig. 12) [61, 82–84].

Recently, a new class of highly promising antiviral nucleosides was developed based on 4′-ethynyl-modified sugar residues (fig. 12) [85]. They are potent agents against HIV-1, including several multidrug-resistant strains. Their mechanism of action on HIV-1 RT has only recently been investigated and they are believed to act as chain terminators after their incorporation despite possessing a 3′-OH group [86].

As mentioned above, 4′-alkylated nucleotides that were originally developed to study steric effects and their involvement in DNA polymerase selectivity could be employed to tune single-nucleotide discrimination in PCR when the modified building blocks are incorporated at the 3′ end of primer strands [67–71]. These results are promising for the development of PCR-based methods

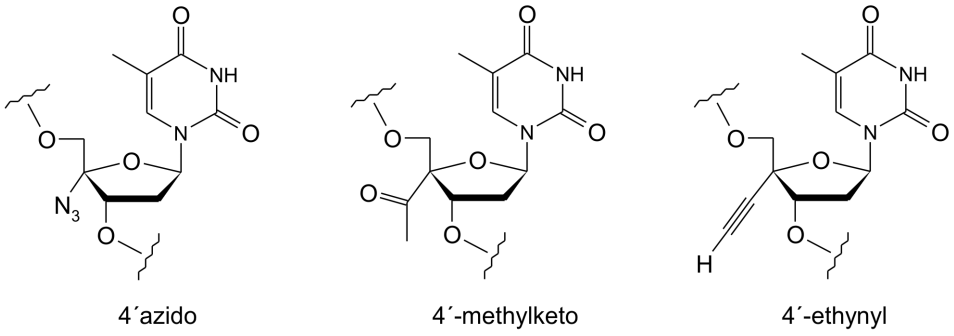

Figure 12. Structure of DNA containing 4′-modified nucleotides.

for diagnosis of single-nucleotide polymorphisms directly from genomic DNA.

Concluding remarks and future directions

As depicted above, numerous insights into the function of DNA polymerases have been gained through employment of chemically modified substrates. Nevertheless, we are far from a complete understanding of the complex mechanisms of these enzymes, which may be regarded as complex machines. Challenging our understanding even further, DNA polymerases make complex interactions with the primer-template strands during catalysis of DNA polymerization that reach far beyond the active site where the chemical step proceeds. The impact of these contacts on enzyme function is not well understood. Furthermore, several recently discovered DNA polymerases that exhibit remarkable and so far unprecedented features like the ability to bypass bulky DNA lesions that are known to be mutagenic remain to be mechanistically elucidated. We are convinced that new synthetic molecules with precisely designed new properties are needed to advance this exciting research field. Since DNA polymerases are important and essential tools in several biotechnological applications, these studies might pave the way for new or improved applications.

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