Recognition of base-pairing by DNA polymerases during nucleotide incorporation: the properties of the mutagenic nucleotide dPTPαS

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The highly mutagenic nucleoside dP (6-(2-deoxy-β-D-erythro-pentofuranosyl)-3,4-dihydro-6H,8H-pyrimido[4,5-c]-[1,2]oxazin-2-one) is a bicyclic analogue of N4-methoxy-2′-deoxycytidine. It exists as a mixture of its imino and amino tautomers in solution with a ratio of about 10 : 1 based on its tautomeric constant. The bicyclic nature of the heterocycle P restraints the amino substituent in an anti conformation and permits effective Watson–Crick base-pairing using either tautomer. The specificity of incorporation of dP by the 3′-5′-exonuclease-free Klenow fragment of DNA polymerase I (exo-free Klenow) has been studied using the 5′-(1-thio)triphosphate dPTPαS in combination with phosphoramidite-specific sequencing of the DNA products. The method provides a convenient qualitative assay for studying nucleotide incorporation and reveals for the first time a potential role for the minor tautomeric forms of the natural DNA bases in base misinsertion (substitution mutagenesis) during replication.

Introduction

The nucleoside analogues N4-hydroxy-, N4-alkoxy- and N4- amino-2′-deoxycytidine (1–3) are formed through the action of hydroxylamine and its derivatives on 2′-deoxycytidine. The resulting analogues all induce substitution mutations in vivo which arise from the incorporation of their 5′-triphosphates into DNA.1-4 All of these compounds display dramatically altered tautomeric equilibria relative to 2′-deoxycytidine. Thus tautomeric constants ([amino]/[imino]) vary from 0.02 to about 30,1,4 whilst for the natural nucleoside only about 1 in 108 molecules exist in the minor imino tautomer form.5

After consideration of the various base-pairing schemes available to the natural bases, Watson and Crick suggested that base misinsertion during DNA replication might arise as a consequence of the incorporation of the nucleotides with the bases in their minor tautomeric forms.6 These ideas were elaborated by Topal and Fresco7 as the “rare tautomer hypothesis” for substitution mutagenesis during DNA replication.

The template-directed incorporation of nucleotides catalysed by DNA polymerases involves a number of steps all of which contribute to varying extents toward fidelity.8,9 In prokaryotes DNA polymerases generally have an associated 3′-5′-exonuclease activity which is used to proof-read the newly synthesised base-pair and catalyse the removal of misincorporated nucleotides.10 There is now considerable evidence that the stereochemistry of the base-pair plays a critical role during fidelity.10,11 As pointed out by Topal and Fresco,11 the error rates of several DNA polymerases which lack an associated exonuclease activity is of the order of 1 in 105–107, which is similar to the estimated amounts of the minor tautomeric forms of the bases. However, the low concentrations of minor tautomeric forms of the natural bases in solution mean that figures for tautomeric constants are very approximate.

Some evidence for the rare tautomer hypothesis has been derived from studying the incorporation of the mutagenic nucleotide 5-hydroxy-dCTP (4) during DNA replication. This analogue shows a much higher insertion rate opposite adenine within a DNA template which is suggested to be directly related to the known increased level of the imino tautomer of this analogue in solution.12 In addition, for N4-amino- and N4-alkoxy-substituted 2′-deoxycytidines which display tautomeric constants much closer to unity, the incorporation of their corresponding triphosphates displays some relationship to the tautomeric bias in solution.13-16 In principle these compounds offer the potential for understanding the role of base-pair recognition in the fidelity of DNA replication. However, it is known that for the imino tautomers the syn conformation of the amino substituent is preferred which precludes effective Watson–Crick base-pairing.17,18 In contrast, the nucleoside analogue dP19 (6-(2-deoxy-β-D-erythro-pentofuranosyl)-3,4-dihydro-6H,8H-pyrimido[4,5-c]-[1,2]oxazin-2-one) (5) is a bicyclic analogue of N4-methoxy-2′-deoxycytidine in which the amino substituent is locked in an anti conformation. Consequently dP is able to form stable Watson–Crick base-pairs with either dA or dG using the appropriate tautomer (Fig. 1).20

We have been interested for some time in studying how mutagenic nucleotides such as dPTP, that display ambivalent base-pairing properties, are incorporated by DNA polymerases.
Recently we determined a value of 0.09 for the tautomeric constant of $\alpha$-methyl P. Consequently we envisaged that this analogue could be used to investigate the rare tautomer hypothesis without the complication of steric interference with base-pairing interactions. Here we describe a simple and efficient assay that is suitable for studying the incorporation of the modified nucleotide dPTP during DNA replication. This assay should also be of general use for studying the incorporation characteristics of other modified nucleotides.

**Results and discussion**

Conventional methods for DNA sequencing involve DNA replication in the presence of chain-terminating deoxyribonucleotides in which the sequence of interest provides the template for copying. Such methodology is clearly unsuitable for the location of mutagenic nucleotides within DNA sequences since the incorporation of the deoxyribonucleotides would be ambiguous in the same way as the initial incorporation of the mutagenic triphosphate. Consequently, we considered using a direct method to locate the presence of a modified nucleotide within DNA. Phosphorothioate-based DNA sequencing is an attractive methodology to achieve this aim as it can be readily applied to a range of analogues without the need to develop nucleobase-specific chemistries analogous to those used in Maxam–Gilbert sequencing. 5'-4-(1-thio)triphosphates (dNTPS) are readily incorporated into DNA by polymerases in place of their unmodified counterparts. The phosphorothioate linkage formed in this process is located using either chemical or enzymatic cleavage of the products and their subsequent fractionation by polyacrylamide gel electrophoresis (PAGE). Thus if a modified nucleotide is incorporated into DNA as its corresponding phosphorothioate, its location and extent of incorporation at a given position within the DNA sequence can be easily determined.

The synthesis of the 5'-(1-thio)triphosphate of dP (dPTPoS) (6) was based on the method of Frey and Arabshahi which involves the selective phosphorylation of the free nucleoside using thiophosphoryl chloride in the presence of 2,4,6-collidine as catalyst. The desired nucleotide dPTPoS was obtained in 50% yield as a mixture of Rp and Sp diastereoisomers following anion exchange chromatography. Only the Sp diastereoisomers of dNTPoS derivatives are substrates for DNA polymerases. Thus, the two diastereoisomers of dPTPoS were separated using reversed phase HPLC employing a tris-magnesium buffer. This mobile phase afforded a much better separation of the two diastereoisomers than can be achieved using triethylammonium bicarbonate (TEAB) as the buffer. The faster-eluting product was assumed to be the Sp diastereoisomer in accordance with the literature. The Sp diastereoisomer was then desalted using weak anion exchange sepharose and its purity was confirmed by phosphorus NMR. In accordance with the literature, the obtained product displayed a downfield phosphorus resonance relative to the crude mixture of the two (1-thio)triphosphate diastereoisomers.

The base specificity for the insertion of the nucleoside dP was investigated by studying the incorporation of dPTPoS (6) in competition with the four natural dNTPs. A 5'-fluorescein-labelled (FAM) primer was extended by 2',3'-exonuclease-free Klenow fragment of DNA polymerase I (exo-free Klenow) as shown in Fig. 2. In previous work we have noted that the insertion of a particular dNTPoS in the presence of all four natural dNTPs shows some variation in relation to the sequence context of the template (D. Williams, F. Eckstein, unpublished results). Since dPTP can potentially compete with both dCTP and dTTP, we performed control experiments, in which either dTPPoS or dCTPoS were incorporated in competition with the natural dNTPs. We anticipated that using dPTPoS in combination with all four natural dNTPs we could determine the template bases opposite which the analogue dP was incorporated.

Initially we investigated chemical cleavage methods for the identification of the phosphorothioate linkages within the product DNA. In previous studies, we have obtained better sensitivity and resolution following analysis by PAGE when using dilute aqueous hydrogen peroxide (D. Williams, F. Eckstein, unpublished results) rather than iodoethanol or epoxypropanol for the cleavage of the fluorescantly-labelled phosphorothioate-containing DNA. Thus the primer (Fig. 2) was extended in the presence of all four natural dNTPs and a dNTPoS (TTPoS, dCTPoS or dPTPoS) and the resulting phosphorothioate-containing DNA was separated by PAGE. However, the intensity of the bands on the gel were rather low (Fig. 3). Furthermore, phosphorothioate-specific chemical cleavage methods are known to produce two bands for each fragment (resulting from products terminated either in a 3'-phosphate or 3'-hydroxyl group) which could further complicate the analysis.

Consequently, we considered using enzymatic methods as an alternative for locating the phosphorothioate positions. The enzymatic method makes use of the dramatically increased resistance of phosphorothioate compared to phosphate diester linkages within DNA towards hydrolysis by exonucleaseIII (exoIII) generating 3'-hydroxy termini. ExoIII catalyses the sequential cleavage of phosphate diester linkages within DNA from the 3'-terminus. Since the method relies on selective inhibition by phosphorothioate linkages toward hydrolysis, we initially confirmed that exoIII hydrolysis was not inhibited per se by the modified nucleoside dP. Thus the primer (Fig. 2) was extended using exo-free Klenow in the presence of three natural

![Fig. 1](image1.png) Base-pairing of P-amino with G (left) and P-amino with A (right).

![Fig. 2](image2.png) Primer and template used for nucleotide incorporation experiments (all purines in the template are numbered sequentially).
dNTPs together with dTTP, dTTPαS, dCTPαS or dPTPαS followed by hydrolysis using exonIII. The products were then separated by PAGE (see Fig. 4). In reactions containing TTPαS, dCTPαS or dPTPαS, the cleavage of the full-length product by exonIII is clearly inhibited, whilst products synthesised using the 3dNTPs and dTTP (replacing either dTTP or dCTP) are rapidly hydrolysed by the enzyme within 30 seconds. In comparison to the chemical sequencing, we observed much stronger fluorescence and better resolution of the fragments using exonIII-based sequencing. Furthermore, exonIII digestion, unlike the chemical methods, results exclusively in products terminated by a 3'-hydroxyl group, hence only one band is seen in the gel for each fragment. Following PAGE and scanning densitometry of the gel, the amount of fluorescence and hence phosphorothioate incorporation associated with the respective incorporation of each of the three dNTPαS analogues was determined. A typical gel is shown in Fig. 5. In lane 5 of the gel, it can be seen that when dPTPαS replaces dCTP, additional bands are seen which correspond to those in lane 3 where dPTPαS replaces dCTP. Clearly the analogue is able to compete more effectively with dTTP than it can with dCTP. Lane 7 shows the competition of dPTPαS with all four dNTPs which reveals again that the analogue is inserted preferentially in place of dTTP.

The incorporation of a given dNTPαS opposite its cognate template base displays some sequence-specific variation when performed in competition with its natural triphosphate (D. Williams, F. Eckstein, unpublished results). For this reason we considered it necessary to obtain comparative data for the incorporation of dCTPαS and dTTPαS respectively in competition with the four natural dNTPs. Thus, the amount of dPTPαS incorporated opposite a given template base (right-hand lane Fig. 5) was compared with the amount of the natural dNTPαS that is incorporated at the same position (data not shown). This allows the determination of the efficiency of incorporation of dPTPαS opposite A versus G. The results of these three sets of experiments are displayed in Fig. 6 which presents the amount of fluorescence (determined following scanning densitometry) vs template base (see Fig. 2 for numbering). This information was used to compare the substrate properties of dTTPαS as TTP (i.e. insertion opposite template A) with dCTPαS as dCTP (i.e. insertion opposite template G) following normalisation using the corresponding data derived using dCTPαS and dTTPαS respectively. This was done by dividing the amount of dPTPαS incorporated at a given position by the amount of either dCTPαS or dTTPαS incorporated respectively at each position. From the resulting data, the averages of all values corresponding to dP opposite template dA and dP opposite template dG positions were calculated. This revealed a four-fold preference for the incorporation of dP opposite template dA rather than template dG, which is consistent with the preference for the P base to exist as its imino form in solution. The value determined by this methodology is clearly very similar to the 10 : 1 preference that would be expected on the basis of the analogue’s tautomeric constant. In earlier work, we have also found a preference for the incorporation of dP opposite template A by Tdp polymerase.28

![Fig. 3](image-url) Incorporation of dNTPαS analogues by exo-free Klenow determined following sequence-specific cleavage using hydrogen peroxide. Full-length products were generated using the primer/template shown in Fig. 2. The lanes from left-to-right contain; primer, full length product and dPTPαS replacing dTTP or dCTP or in equimolar concentration to the four natural dNTPs respectively. Samples were then heated in the presence of hydrogen peroxide (+) or in its absence (−). For full details of conditions see Experimental section.

![Fig. 4](image-url) PAGE following exonucleaseIII digests of DNA synthesised using dTTP or dPTPαS in place of dCTP or dTTP. Full length products were generated using the primer/template shown in Fig. 2, together with 3 natural dNTPs and the fourth dNTP indicated above the respective lanes of the gel. Samples were then digested with exonucleaseIII for the indicated times according to the procedure described in the experimental section. In both gels, lane 1 = primer (sequence shown in Fig. 2), lane 2 = full length product following DNA replication with natural dNTPs and primer/template (sequences shown in Fig. 2), lane 3 = full-length product following 30 minute digestion with exonIII. The remaining lanes comprise time courses for exonIII digests of full-length products prepared using 3dNTPs and the fourth dNTP as indicated. Time points in each case are 0, 0.5, 1, 2, 5, 10 and 30 minutes.
A and G.\textsuperscript{36,37} Tautomerisation of the P base is unlikely to arise within the active site of the polymerase due to the rigorous interactions between the base-pair and the protein and the absence of water within the active site.\textsuperscript{36,37} Taken together with overwhelming evidence for the requirement for Watson–Crick type geometry during DNA polymerisation, we envisage that only the imino form of dP can be incorporated opposite dA and only the amino form can be incorporated opposite dG. On this basis, the close correlation between the substrate specificities described for dPTP\textalpha{}S determined here and the known tautomeric constant for the analogue\textsuperscript{31} would be expected. Although there may not be significant differences between the thermodynamic stabilities of the Watson–Crick and various wobble-type base-pairs in which dP has been observed there is good evidence that the thermodynamic stability of a given base-pair in solution is not the main driving force during the fidelity process.\textsuperscript{38} In this context for example, the insertion of the mutagenic nucleotide O\textbeta{}-methyl-dGTP during DNA replication indicates a preference for base-pairing with thymine\textsuperscript{39} even though the modified nucleoside forms a more stable base-pair with cytosine.\textsuperscript{40} In the latter case a wobble base-pair is formed i.e. one which does not conform to the standard Watson–Crick geometry and which is discriminated against by the polymerase.

In conclusion we have developed a simple and convenient assay for examining the incorporation of modified nucleotides by DNA polymerases. Using this assay for the analogue dP we have provided experimental support for the rare tautomer hypothesis as a mechanism for mutagenesis. Furthermore, the study provides further evidence that the incorporation of a nucleotide by a DNA polymerase is directly related to the base-pairing properties of the nucleotide and supports the view that there is a strict stereochemical requirement placed upon the base-pair within the polymerase active site.\textsuperscript{30,31}

### Experimental

Chemicals and reagents were used as purchased or purified as follows: Anhydrous dimethylformamide (DMF) was purchased from Aldrich and stored over 3 Å molecular sieves. Trimethyl phosphate (Aldrich gold label) was stored over 3 Å molecular sieves. 2,4,6-Collidine was refluxed over calcium hydride and distilled into oven dried glassware. Thiophosphoryl chloride was distilled under vacuum into dried glassware immediately prior to use.\textsuperscript{31} P-NMR spectra (101.25 MHz) were obtained on a Bruker AC250 and are given in ppm relative to an external standard of 85% H\textsubscript{3}PO\textsubscript{4}. Bis(tributylammonium) pyrophosphate was prepared as a 0.5 M solution in anhydrous DMF according to Ludwig and Eckstein.\textsuperscript{27}

\[6-(2-Deoxy-\beta-D-erythropentofuranosyl)-3,4-dihydro-6H,8H-pyrimido[4,5-c][1,2]oxazin-2-one-5′-(1-thio)triphosphate (dPTP\textalpha{}S) \]

The nucleoside 5′-(1-thio)triphosphate of dP was synthesised from the nucleoside dP\textsuperscript{20} using a modification of literature methods.\textsuperscript{20} The nucleoside dP (270 mg, 1 mmol) was dried over P\textsubscript{2}O\textsubscript{5} at 70 °C overnight in a pear shaped flask then dissolved in anhydrous trimethylphosphate (2 ml), under argon, with gentle heating. To this solution, which had been cooled in an ice bath, was added freshly distilled thiophosphoryl trichloride (255 µl, 2.5 mmol), followed by 2,4,6-collidine (265 µl, 2 mmol) and the mixture stirred in an ice bath for 5 h. A well vortexed mixture of bis(tributylammonium) pyrophosphate (5 ml, 0.5 M in DMF), tributylamine (1 ml) and DMF (2 ml) was added quickly to the reaction mixture. The reaction mixture was then warmed slowly to room temp. After 30 min aqueous triethylammonium bicarbonate solution (TEAB) (20 ml, 0.05 M, pH 8) was added to quench the reaction. The triphosphate was then purified using DEAE sephadex A25 and a linear gradient of TEAB 0.05 M to 0.7 M, 2 L of each concentration. The

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**Fig. 5** Incorporation of dNTP\textalpha{}S analogues by exo-free Klenow determined following sequence-specific cleavage using hydrogen peroxide. Full-length products were generated using the primer/template shown in Fig. 2. The lanes from left-to-right contain: primer, full length product and dPTP\textalpha{}S replacing dTTP or dCTP or in equimolar concentration to the four natural dNTPs respectively. Samples were then subject to digestion with exoIII (+) or where no exoIII was added (−). The numbering on the right-hand side refers to the indicated template positions shown in Fig. 2. For full details of conditions see Experimental section.

**Fig. 6** Incorporation of dNTP\textalpha{}S analogues by exo-free Klenow using exoIII digestion for phosphorothioate-specific cleavage. The numbers refer to purine positions within the template (see Fig. 2). Data (in triplicate) were obtained following analysis of the polyacrylamide gel (Fig. 5) using fluorescent-scanning densitometry.

There is growing evidence that the fidelity of nucleotide incorporation by polymerases depends critically on the stereochemistry of a given base-pair during this process.\textsuperscript{36,37} Recent support derives from experiments by Kool and co-workers, in which the 5′-triphosphate of 2,4-diﬂuorotoluene, which lacks hydrogen-bonding potential but is isosteric with TTP, is preferentially incorporated opposite adenine within a DNA template.\textsuperscript{35} In considering the incorporation of dTTP it is noteworthy that the stabilities of the P : G and P : A pairs are comparable to those of the respective natural C : G and T : A pairs based on duplex melting studies in solution.\textsuperscript{30,31} NMR studies reveal normal Watson–Crick P : A and P : G base-pairs in which the respective imino and amino forms of the analogue are employed.\textsuperscript{34,35} However, the same studies revealed that the P : G base-pair is in slow exchange with a wobble base-pair, whilst X-ray crystallography of DNA duplexes have revealed that P may form both wobble and Watson–Crick base pairs with both
and length – diastereoisomer and 70 into two equal amounts and to one a premixed solution of 1 µL 37-fi was incubated at 37
exoIII; 660 mM Tris (HCl, pH 8.0, 60 mM MgCl2, 20 mM urea) at 1050 V for 1.5 h. Analysis was performed using a
Molecular Dynamics Fluoroimager.

The two diastereoisomers of dPTPαS were separated by reversed phase HPLC (Alltech Econosphere C18 10 µM, 250 × 22 mm column) using an isocratic gradient of 120 mM Tris-HCl, 20 mM MgCl2 at pH 7.65 ± 0.05 with a flow rate of 10 min mL⁻¹. Retention times were 55–60 min for the Sp diastereoisomer and 70–75 min for the Rp. The dPTPαS-containing fractions were diluted with 0.5 M tetrasodium EDTA (1 ml per 10 ml of eluant) and desalted using a sepharose weak anion exchange column (dimensions, diameter = 4 cm and length = 40 cm), using a flow rate of 15 ml min⁻¹, and the following mobile phase: t = 0 min, 0% B; t = 20 min, 0% B; t = 65 min 50% B; t = 95 min 50% B; t = 105 min, 65% B; t = 135 min, 65% B; t = 160 min, 100% B; buffer A = H2O containing 2% ethanol, buffer B = 0.5 M TEAB pH 8.5 containing 2% ethanol. The Sp diastereomer of dPTPαS was collected at approximately 0.4 M TEAB; (t, fl) 65 min 50% B; 60 min for the

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