Interactions of Carcinogen-Bound DNA with Individual DNA Polymerases

F. Peter Guengerich*

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

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Contents

* Address correspondence to Prof. F. Peter Guengerich, Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, 638 Robinson Research Building, 23rd and Pierce Avenues, Nashville, TN 37232-0146. Telephone: (615) 322-2261. FAX: (615) 322-3141. E-mail: f.guengerich@vanderbilt.edu.

1. Introduction and Significance

To appreciate the nature of this subject, it is useful to first consider the general topic of chemical carcinogenesis. In a sense the field began with the observations of two London physicians in the second half of the 18th century. Hill[1] associated nasal and oral tumors with the use of snuff tobacco, and subsequently Pott[2] associated scrotal tumors in chimney sweeps with the soots and tars these boys were exposed to. Later Rehn[3] linked bladder cancer with the high exposure of factory workers to aniline dyestuffs. In 1915 Yamagiwa and Ishikawa[4] reported the formation of tumors in the ears of rabbits treated with tars, and in 1933 Kennaway and his associates isolated benzo[*a*]pyrene as a carcinogenic component of coal tar.[5]

Thus, we have long had evidence that chemicals can cause cancer. Work by the Millers[6] and others, beginning in the 1940s, demonstrated that many chemicals must be converted to reactive forms in the body in order to cause cancer. These reactive forms of chemicals become attached to DNA and proteins, and the structures of many of these DNA−carcinogen adducts have now been characterized.[7−9] The concept that somatic cell mutations are involved in cancer goes back to the early 20th century[10] and was developed further in studies demonstrating the relationship of carcinogens, mutation, and cancer.[6,11,12] The general concept is that cells are initiated by damage resulting from a DNA-alkylating agent, yielding mutations that are fixed by subsequent rounds of replication. Most of the mutations are unlikely to yield detrimental effects because they do not produce changes in proteins or the changes are innocuous. However, some gene products may show a critical gain or loss of function and lead to loss of control of the functions of a cell, which can lead to a cancer state.

Today most researchers in the field of chemical carcinogenesis would agree that cancer results from many factors,

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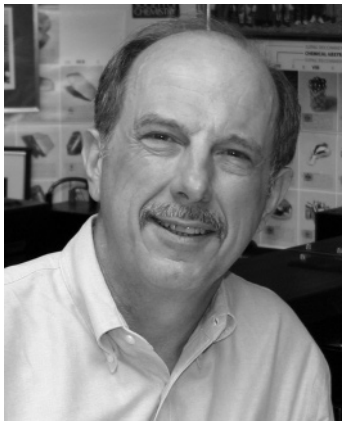


Dr. F. Peter Guengerich is a Professor of Biochemistry in the Vanderbilt University School of Medicine. He received his B S. from the University of Illinois in 1970 and then did his graduate work at Vanderbilt, receiving his Ph.D. in Biochemistry in 1973. After two years as a research fellow at the University of Michigan, he was hired as Assistant Professor of Biochemistry at Vanderbilt in 1975 and has been on the faculty since then, having attained the rank of Professor in 1983. Prof. Guengerich has been Director of the Center in Molecular Toxicology, an interdepartmental program, since 1981. His research laboratory deals with the chemical and biological mechanisms by which drugs and carcinogens are processed. One area of interest is the enzymology of cytochrome P450 enzymes, the major catalysts involved in the metabolism of drugs and toxicants. Other research involves the chemistry and biochemistry of modification of DNA, plus kinetic and structural studies of DNA polymerases with the goal of understanding the molecular basis of mutation and replication blockage in carcinogen-modified DNA.

not only the damage to DNA. For instance, modulation of receptors and cell signaling (without metabolic activation) is an issue in tumorigenesis (e.g. barbiturate and peroxisome proliferation responses), as is general cell proliferation.[13] What are some of the main pieces of evidence that DNA adducts have anything to do with cancer? At least four major pieces of evidence can be offered.

(i) The appearance of DNA adducts can be highly correlated with tumorigenesis in some experimental animal models. That is, treating animals with known carcinogens causes both DNA adducts and tumors. For instance, good correlation can be shown with aflatoxin B_1 (AFB_1) in trout and rats.[14]

(ii) Experimental causation in model systems provides strong evidence. A reactive product of a carcinogen can be added to a cell, and mutations and transformations result.[15] More directly, a reactive electrophilic metabolite (e.g. benzo[*a*]pyrene diol epoxide) can be administered to mice and shown to cause tumors.[16] With regard to DNA adducts, many of these have now been shown, using the technique of site-specific mutagenesis,[17] to produce mutations after transfection with the vectors.[18]

(iii) Mutation spectra show hot spots for different chemicals. This phenomenon has been recognized for some time.[19] With regard to human tumors, the tumor suppressor gene p53 shows mutations in many cancers. The patterns show good relationships to certain specific exposures, including sunlight and AFB_1[20] and possibly some tobacco components.[21] Some of these mutations have biological effects; they may be involved in the stepwise process of tumorigenesis. The mutation spectra can also be reproduced in experimental settings.[22]

(iv) Some genetic predispositions to cancer are now clearly recognized to be related to deficiencies in dealing with DNA adducts. For instance, xeroderma pigmentosum and other disease states can be traced to heritable changes in DNA repair enzymes and even DNA polymerases.[23,24]

Other lines of evidence could also be offered, but the above four, as a whole, argue that the processing of DNA−carcinogen adducts is an important area of research activity. DNA adducts are inert unless copied, and thus the roles of DNA polymerases are of paramount importance. In the last 5−7 years this field has expanded with new knowledge and the discovery of many new DNA polymerases. The interactions of DNA−carcinogen adducts with polymerases will be reviewed here. A PubMed search for "polymerases and carcinogens" (using the connector *and*) yielded over 3400 articles, and a similar Google search produced over 51 000 hits. Obviously not all papers can be covered in this review, and only a small fraction can be treated in much depth.

Before discussing some of the details, it is useful to briefly review the major methods in this field and some of the basic chemical and physical concepts underlying base recognition in general. Our current knowledge of the subject of mutagenesis indicates that major questions cannot be solved in the context of only studies done with DNA, in the absence of enzymes. However, the basic principles of thermodynamics are used (and tweaked) by DNA polymerases, and appreciation of the concepts and approaches is useful.

With this background several types of studies with DNA polymerases and carcinogen-modified DNA will be presented and discussed, along with some of the author's own interpretations about relevance. Although the interactions within cells are most important and many studies have been done, the focus of this review will be studies of individual, isolated DNA polymerases with oligonucleotides bearing chemically defined adducts at specific sites. The most detailed understanding of systems is possible at this level, albeit reductionist, particularly using kinetic analysis and X-ray crystallography to study structure and function. Some discussion of site-specific mutagenesis and mutation spectra will be included. The point can be made that reliance on only a single approach will never yield a particularly insightful understanding of mechanisms of chemically induced mutagenesis.

2. General Approaches

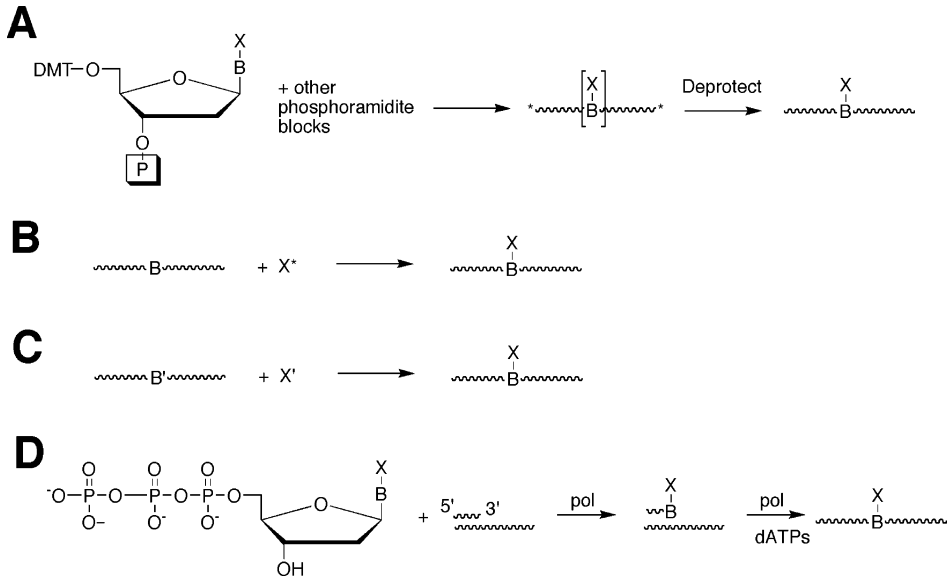
2.1. Synthesis of Modified Oligonucleotides

One of the problems in the research on the interactions of DNA polymerases with modified DNA is the need for highly defined reagents, specifically pieces of DNA with a single well-defined modification at an individual site. Thus, one cannot simply treat oligonucleotides or DNA with an alkylating agent and produce multiple modifications, as one might in a cellular mutagenesis assay. The reasons are twofold. First, almost all chemical and physical (e.g., radiation) agents produce multiple types of damage; that is, methylating agents produce some methyl adducts at most ring and exocyclic heteroatoms.[25] Knowing which modification is involved in the interaction with the DNA polymerase is important. The second issue is that of sequence-dependent variations, which will be discussed later. Therefore, the preparation of chemically modified oligonucleotides as reagents is an integral part of this research.

Four major approaches can be used to prepare modified oligonucleotides (Scheme 1).

(i) The modified deoxyribonucleoside can be prepared and incorporated using standard or modified oligonucleotide

Scheme 1. Methods for Synthesis of Oligonucleotides Containing DNA Adducts at Defined Positions[a]



[a] (A) Incorporation of a phosphoramidite reagent containing a modified base using conventional DNA synthesis. This is a generally useful procedure if the modified base is stable to the conditions of oligonucleotide synthesis and deprotection. (B) Postoligomerization strategy for modification of a canonical base with an electrophile. This approach can be useful if the electrophile is specific for reaction with a certain base. In practice, a single site contains that base or, if multiple copies are present, extensive separation is done after the reaction.[26] (C) Postoligomerization strategy involving a modified base. One base is modified such that, after reaction as shown, the relevant modified structure is generated. An example is the non-biomimetic reaction of a 2-fluorodeoxyinosine (in the oligonucleotide) with an amine to generate the products normally produced by the reaction of epoxides with dGuo.[27,28] (D) Enzymatic incorporation of a derivatized dNTP into DNA. This approach has been utilized but has three disadvantages: (i) only low scale reactions are feasible, (ii) modified dNTPs are usually poor substrates for polymerases, and (iii) purification and analysis are usually not trivial.

synthesis. The modified base must be compatible with the chemistry used for preparation of the deoxynucleoside phosphoramidite and subsequent deprotection.

In the early era of DNA synthesis, a number of different approaches were considered, but today the phosphoramidite approach has become quite standardized and is used with commercial solid-phase cassette methods. Further, the general protection methods used with the exocyclic groups have become more standardized today (in this laboratory we routinely use 4-*tert*-butylphenoxyacetyl (N) and 4-nitrophenylethyl or trimethylsilylethyl (O) protection). The variety of modified bases that have been incorporated using such approaches will not be elaborated here. In some cases, this work can be done by a commercial oligonucleotide supplier (e.g., with 7,8-dihydro-8-oxoguanine (8-oxoG) or O^6-MeG). The chemistry needed for deprotection must be compatible with the incorporated base. Most commercial oligonucleotide synthesis laboratories do not routinely accept synthesized nucleoside phosphoramidites from individual investigators, and such synthesis must be done independently. As a general rule, we find that 15−20 mg of a nucleoside phosphoramidite is a minimum for success. Sometimes removal of the cassette and manual coupling, with longer time, is useful for modified nucleosides.

Artifacts can be introduced during the deprotection chemistry.[29] These can be avoided with the addition of reductants in some cases, e.g. 8-oxoG.[30]

(ii) An oligonucleotide can be treated with a reagent to modify the desired residue. This strategy is useful when the chemistry is incompatible with phosphoramidite and deprotection chemistry (e.g. N^7-guanyl adducts). An inherent problem is that multiple modifications can occur. One strategy is to use only a single Gua or residue known to react, to minimize undesired reactions. In some cases positional isomers can be separated by chromatography (e.g. individual N^7-guanyl modifications).[26,31] This approach is still utilized in the preparation of unstable adducts such as the N^7-guanyl adduct generated by reaction with AFB_1 *exo*-8,9-epoxide.[32,33]

If this approach is used, the investigator bears the burden of rigorously documenting the identity and purity of the product. These standards hold in all cases, but particularly in this approach the potential for artifacts is very high.

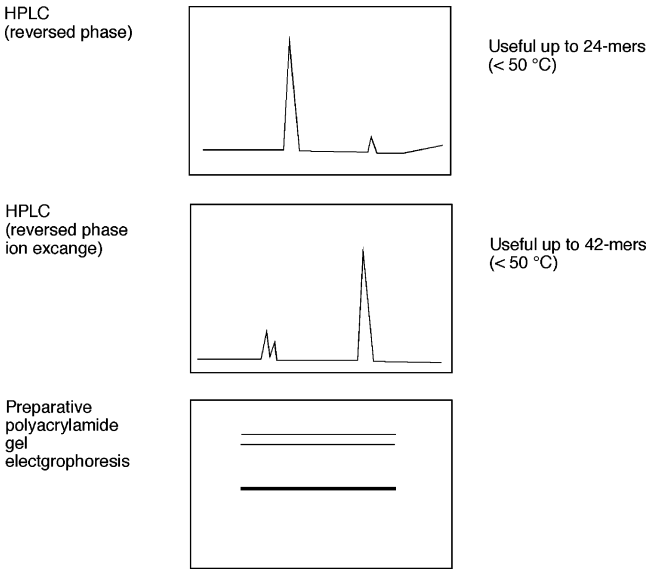
(iii) An approach can be used in which an appropriately modified base is incorporated into an oligonucleotide and then modified with a chemical to generate the final desired product. Such a "non-biomimetic" approach has been used to prepare a number of N^2-guanyl and N^6-adenyl adducts by Harris and his associates.[27,28] For instance, 2-fluorodeoxyinosine can be incorporated as the base and then reacted with an amine derivative of a (polycyclic aromatic hydrocarbon) PAH to generate the known biomimetic product.[34] This approach usually works best with a relatively short oligonucleotide and a bulky adduct, in that separation of unreacted oligonucleotide will be necessary.

(iv) An enzymatic approach can be used, although this is usually not feasible on a large scale. A modified dNTP is prepared and added to the end of a primer, in the presence of a polymerase (and template). The chemical preparation of dNTPs is not trivial, in that rigorously anhydrous conditions are needed.[35,36] Also, DNA polymerases often show poor incorporation of modified dNTPs.[36] The limited amount of oligonucleotides that can be produced may be sufficient for some biological studies, but the limitation may be an issue, even for characterization.

2.2. Purification and Characterization of Modified Oligonucleotides

Modified oligonucleotides are usually purified by HPLC or preparative gel electrophoresis (Scheme 2). Reversed-phase (C_{18}) HPLC can be used with short oligonucleotides

Scheme 2. Purification of Modified Oligonucleotides[a]



[a] The three most common techniques are shown. Reversed phase HPLC can be used to separate shorter oligomers, generally up to ~24-mers. Mixed-bed reversed-phase/ion-exchange systems are useful with longer oligomers. In our own experience oligomers as long as 42-mers and differing in one monomer length can be separated.[37] With both types of HPLC, resolution is improved with increasing temperature (up to a limit of ~50 °C), due to the attenuation of internal bonding interactions in the oligonucleotides. Preparative polyacrylamide gel electrophoresis is useful for purifying short or long oligonucleotides, although the recovery is often low (typically 50% or less). In all systems there is relatively limited separation of positional isomers of oligonucleotides. The degree of separation of a carcinogen-modified oligonucleotide from an unmodified analogue varies, depending upon the size of the adduct.

(up to ~20- to 24-mers), using increasing gradients of CH_3OH or CH_3CN in ammonium formate or acetate buffers (which allow for removal of the buffer by lyophilization). Reversed-phase HPLC is not effective for longer oligonucleotides, and a mixed resin HPLC approach is more useful (reversed-phase plus anion exchange). Elution is accomplished with an increasing NaCl gradient (in a buffer containing ~20% CH_3CN).[37] The salt must be removed by gel filtration. Increasing the temperature to 50 °C (maximum) improves resolution. Typically adequate resolution of (e.g.) a 41-mer and 42-mer can be accomplished, or alternatively 42-mers with and without an adduct present.[37]

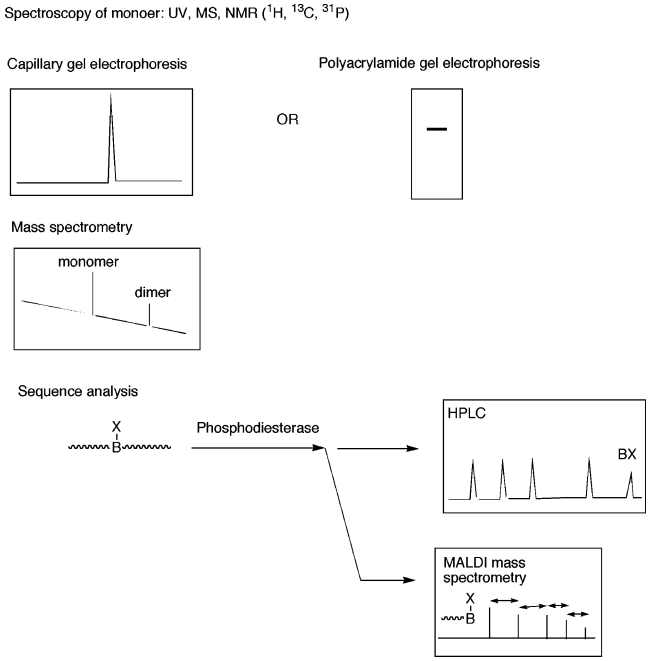
The major alternative is preparative gel electrophoresis (in the presence of 8 M urea). The experience in this laboratory has been that resolution is generally better than that with HPLC. Disadvantages are much lower recovery, the lower loading capacity, and, in some cases, the problem of sensitivity of the modifications to the UV light needed to visualize the oligonucleotide.

Standards for the demonstration of identity and purity of modified oligonucleotides are important and have not received sufficient attention, in general. Many biological experiments, particularly those involving mutation and other biological endpoints in cells, are extremely sensitive to impurities. In cells, the origin of observed mutations could be an impurity. Another common problem is the presence of an unmodified oligonucleotide in a modified oligonucleotide reagent. This is even a problem in noncellular *in vitro* experiments. For instance, the extent of elongation of a primer is often very low in polymerase extension studies, particularly with bulky adducts.[38] If even 1−2% unmodified oligonucleotide is present in an oligonucleotide with a bulky adduct, any apparent polymerase bypass past the adduct could be the result of the contamination rather than any inherent ability of the polymerase to copy past the bulky adduct.

In the author's opinion, all papers dealing with modified oligonucleotides must include appropriate evidence of purity and identity. With the implementation of electronic "Supporting Information", "Supplemental Data", etc. options by many journals to provide easy access to supplementary material, there is really no excuse for not providing such documentation.

The two most common methods used to evaluate purity of oligonucleotides are capillary gel electrophoresis and denaturing polyacrylamide gel electrophoresis (Scheme 3).

Scheme 3. Standards for Purity and Identity of Modified Oligonucleotides[a]

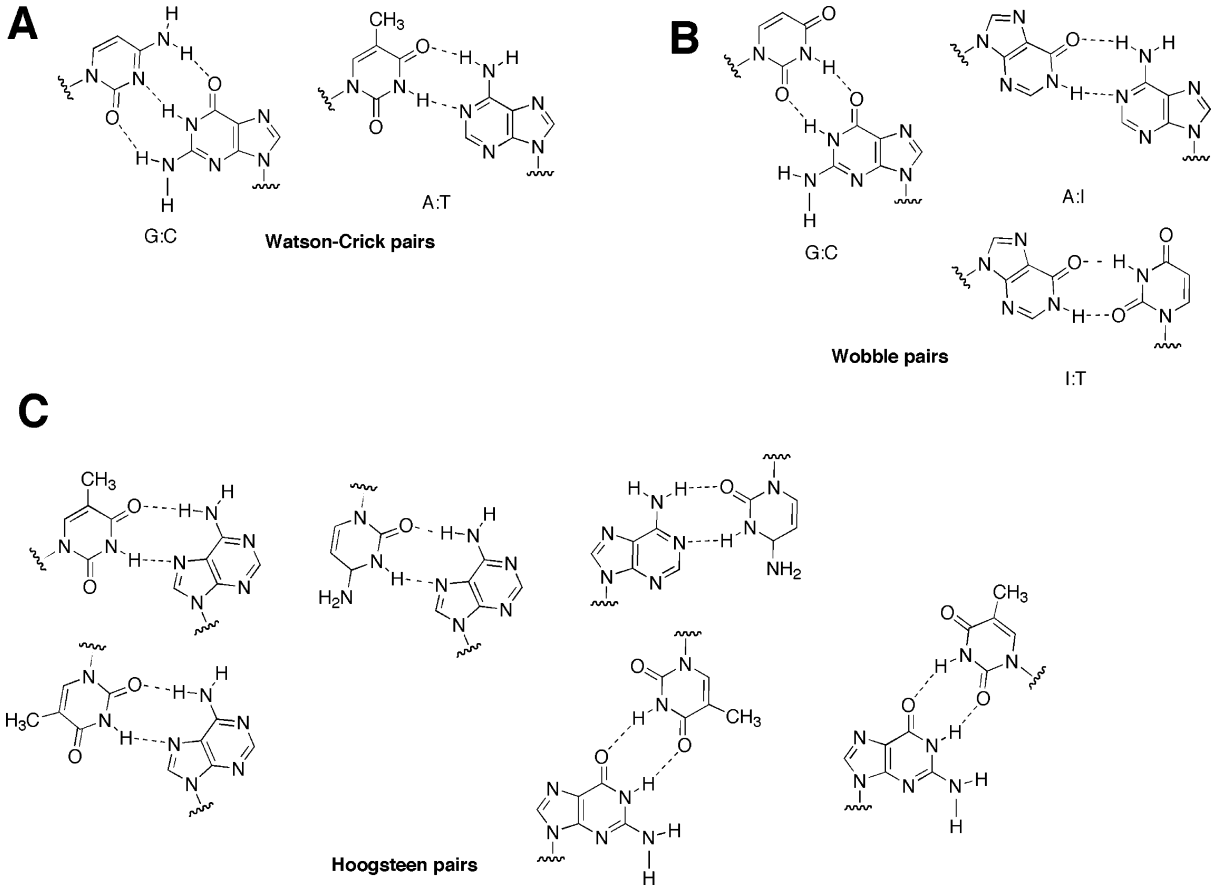


[a] The electrophoretic systems provide the highest resolution. Capillary gel electrophoresis is a sensitive and very convenient means of evaluating purity. Alternatively, polyacrylamide gel electrophoresis can be used. However, a ^{32}P or other label is needed to provide sensitivity. If preparative polyacrylamide gel electrophoresis has been used to purify the oligonucleotide, then analysis in the same system does not really provide a criterion of purity. MS is the most general method used today for establishing the identity of oligonucleotides. MALDI-TOF MS can readily be done with oligonucleotides 42 bases or less in length. Although some indication of purity is provided, MALDI-TOF signal intensity varies greatly and the absence of extra peaks should not be overinterpreted. Sequence analysis can be done by creating ladders of digestion products by collision-induced dissociation (CID)[39−42] or, more practically, with phosphodiesterase I or II,[43−47] cutting from the 3′ or 5′ end. The differences can be used to define the sequence of bases within the intact oligonucleotide.

The former has very high revolving power with oligonucleotides and uses only trace accounts of material.[48] Polyacrylamide gel electrophoresis is sensitive if an oligonucleotide is tagged with a radioactive or fluorescent label. There are some caveats about the use of the method. If preparative electrophoresis is used in the purification of an oligonucleotide, then using the same method to establish purity is of limited usefulness. In the past, we have found that even oligonucleotides purified by preparative electrophoresis can be resolved into multiple components using capillary gel electrophoresis.[49]

The issue of identity of an adduct is a separate one from that of purity. If a deoxyribonucleoside is synthesized and

Scheme 4. Watson−Crick, Wobble, and Hoogsteen Base Pairing Schemes[50] [a]



[a] The canonical Watson−Crick pairs can usually be observed using NMR spectroscopy.[51] The other binding modes are less common, and the most direct evidence for the existence of each comes from X-ray diffraction work.

then incorporated into an oligonucleotide, the deoxyribonucleoside should be characterized by spectroscopy as rigorously as possible before derivatization and incorporation. One approach to establishing identity is to digest the oligonucleotide with nucleases and separate the (dephosphorylated) nucleosides by HPLC. This approach is useful, although (i) a considerable amount of oligonucleotide is required if only UV detection is used and (ii) the method only provides a limited index of purity; that is, 5−10% error in the ratios of the nucleosides could be present. The sensitivity of the method could be improved with mass spectral detection.

The most common method of establishing identity today is probably mass spectrometry (MS), particularly matrix-linked laser desorption/time-of-flight (MALDI-TOF) MS. Intact oligonucleotides as long as 42-mers are readily handled, and the accuracy is better than 1 part in 10^3 to 10^4. The presence of the appropriate $[M + H]^+$ (or $[M - H]^-$) ion is usually a good documentation of identity in most cases. Further characterization can be done by sequence analysis. Typically an oligonucleotide is digested with a phosphodiesterase (3′ → 5′ or 5′ → 3′) to create "ladders", as observed in MS of the mixture (Scheme 3). The differences between *m*/*z* units of the peaks can be used to confirm the sequence, including the position at which the modified base is present.

3. Thermodynamics of Base Pairing

Before addressing the details of studies with polymerases, it is useful to consider the studies that have been done on the pairing of DNA bases in isolation from protein. These studies are instructive in terms of basic mechanisms and, despite their limitations, have played a major role in directing the thinking in the past. The considerations are almost exclusively dominated by thermodynamic considerations.

3.1. Monomers

Hydrogen bonding between the so-called "Watson−Crick" faces of purines and pyrimidines is a major factor involved in the interaction of the normal four DNA bases with each other (Scheme 4). However, the bonding of individual bases or nucleosides to each other (e.g. dGuo:dCyd) is not observed in aqueous solutions because of the hydrogen bonding to H_2O. The sugars can be modified with hydrophobic groups to render the nucleosides soluble in aprotic solvents such as $CHCl_3$, and hydrogen bonding is then observed. One manifestation of the bonding is the changes in NMR chemical shifts due to interaction.[52,53] This approach has been used in some studies but has had very limited use with carcinogen-adducted DNA bases.[54] A limitation is the quantitative description of the extent of binding.

3.2. UV and CD Measurements

Most studies with carcinogen-modified DNA have involved oligonucleotides of "medium" length, aside from some of the cellular experiments in which long vectors are necessary. The binding of two complementary strands of DNA (or RNA) is characterized by a hypochromic effect (decreased absorbance). Thus, a decrease in A_{260} is usually associated with base pairing, and conversely an increased A_{260} is associated with disruption of pairing.

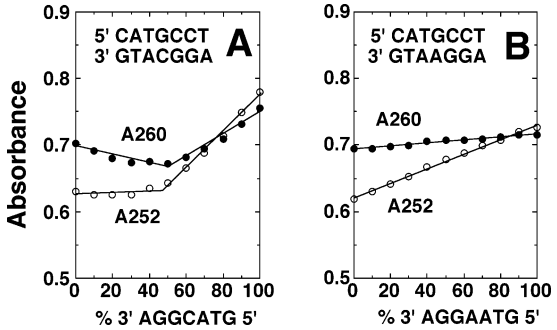


Figure 1. Mixing plot analysis of complementary oligonucleotides to demonstrate binding.[55] A complementary pair is shown in part A, and a mismatch, in part B. Absorbance was monitored at both 252 and 260 nm in this case to maximize for both purine and pyrimidines. The presence of an obvious minimum in such a Job plot (part A) indicates that the two oligonucleotides are hybridized, as opposed to the pair with a single mismatch in part B. (Reprinted with permission from ref 55. Copyright 1993 American Chemical Society.)

One way in which to use such phenomena is with mixing plots, sometimes referred to as Job plots[54,55] (Figure 1). As the percentages of the two strands are mixed, the A_{260} of the system will be at a minimum when maximum pairing occurs (Figure 1). Thus, pairing is achieved in the case shown in Figure 1A. If no break is observed, binding is weak or nonexistent.

CD spectroscopy provides another means of observing pairing of oligonucleotides. Normal B-type helixes are characterized by a positive band at 270 nm and a negative band at 250 nm. This pattern is diminished when the pairing is disrupted.[55]

3.3. T_m Measurements

Melting transitions are analyzed by any of a number of methods. Perhaps the most generally useful method is differential scanning colorimetry. The method is expensive in terms of the amount of sample required, but a single melting analysis can provide all relevant thermodynamic parameters, including $\Delta G°$, ΔH, and ΔS for the binding.[56,57]

An alternate approach is to use the spectral charges mentioned earlier to monitor the transition, as a function of increasing (or decreasing) temperature. The usual approach is to monitor A_{260} as a function of increasing temperature applied to a preformed complex of two DNA strands. The resulting sigmoidal plot is analyzed to determine the T_m, or midpoint for the change, usually with a derivative method. With appropriate equipment, work can be done with <1 mL of a solution with $A_{260} = 0.10$. The approach yields a T_m at a specific DNA concentration, but a careful consideration of the thermodynamics requires a van't Hoff analysis, i.e., plots of $1/T_m$ vs ln [DNA], and therefore multiple experiments at different DNA concentrations:[26]

$$1/T_m = (R/\Delta H°) \ln C + (\Delta S° - R \ln 2.356)/\Delta H°$$

The effects of a particular modification may appear different if only T_m is considered, compared to $\Delta H°$ and $\Delta G°$.[57,58]

The thermodynamics of DNA interaction is a complex subject and of considerable significance in the context of considerations of not only chemical carcinogens but also recombinant DNA and other biotechnology work.[59,60] One important aspect is DNA sequence effects, which are considered in modern predictive algorithms.[59,60] Pilch et al.[61] demonstrated the importance of sequence context on the conformational and thermodynamic properties of a cisplatin DNA intrastrand cross-link.

In addition to the sequence context, the nature of the chemical adduct and its stereochemistry can have major effects. The importance of adduct stereochemistry on thermodynamic (and biological) properties has been well documented in the case of the PAHs.[51,62] Also, even relatively small adducts can have major effects on DNA melting parameters. For instance, with 3,N^4-ϵ-Cyt the fluorescence and CD spectra indicate that only small changes occur in the structure and the DNA is still in the B form.[63] However, large changes are induced in T_m, $\Delta G°$, and duplex stability. Even with the simple adduct 8-oxoG, the results of spectroscopic and chemical experiments led Plum et al.[64] to conclude "...thermodynamic effects induced by the lesion 8-oxoG:C (or GG) can result in relatively large changes in enthalpy which are partially or wholly compensated entropically to produce relatively modest changes in free energy". The authors further suggest that "...preferential nucleotide insertion opposite 8-oxoG cannot be rationalized simply in terms of large thermodynamic differences", a point which is borne out in studies with individual DNA polymerases, which vary considerably in misincorporation frequency.[36,65,66]

3.4. NMR

With the availability of superconducting magnets it has been possible to determine the structures of short oligonucleotides and to establish the effects of bound carcinogens on these structures. A serious discussion of the analysis is beyond the scope of this section, and the reader is directed to a very comprehensive earlier review focused on the polycyclic.[67] See also a very comprehensive earlier review focused on the polycyclic aromatic hydrocarbons.[51] Suffice it to say that most studies are done on 11- to 13-mers at 500−600 MHz (^{1}H basis), combining ^{1}H and ^{13}C and sometimes ^{31}P measurements.[67] Two aspects are critical: (i) the through-space NOESY "walk" down the oligonucleotide chain to establish the resonances of all atoms and (ii) the downfield signals attributable to protons involved in hydrogen bonds.

3.5. X-ray Crystallography

Fewer structures of oligonucleotides have been established by X-ray crystallography than by NMR, particularly of carcinogen-modified oligonucleotides. Whether or not oligonucleotides will crystallize is highly dependent upon the sequence. The list of carcinogen-bound oligonucleotides consists of a few notable entities including 1,N^6-ϵ-Ade, 8-oxoG, and O^6-EtG.[68−70] The benefit of such structures is the high resolution.

3.6. Overall Considerations

A number of approaches can be used to examine the effects of bound carcinogens on the structure of DNA. This information is inherently useful in application to more complex problems, e.g. the interaction of modified DNA with polymerases and other proteins. A few general comments are in order.

(i) Establishing the 3-dimensional structure of a modified oligonucleotide is easier in these settings than within a polymerase.

(ii) One limitation is that almost all of these studies have the adduct "sealed" in the middle of two paired oligonucle-

otide strands, a situation that may not be relevant to that in which a polymerase senses an adduct at a replication fork. Thus, the analysis is done on a (polymerase-generated) product that may not be relevant to the substrate ground state and transition state.

(iii) The structural biology approaches and many of the others do not result in parameters that can be directly applied to compare the influences of modifications on binding, with the exception of the $T_m/\Delta G°$ approach, which still has caveats in the interpretation of effects of individual residues, depending upon the analysis.[59,60]

4. Polymerase Assays

As indicated earlier, thermodynamic approaches are interesting but all have limitations as to what information can be obtained. Another problem encountered in this and other laboratories has been that the same adduct in an oligonucleotide miscodes in different ways with different polymerases. This is not only a quantitative issue, i.e., varying extents of activity, but also one of varying preferences for the insertion of A, C, G, and T opposite a specific modified base.[49,71] Obviously these findings are not rationalized in the inherent thermodynamics of the DNA bases binding to each other, and the problem is a kinetic one. That is, the interactions of the catalyst (polymerase) play a major role in the course of the reaction.

Several approaches are commonly used in this type of research, and the salient features are treated briefly (Scheme 5).

4.1. Misincorporation Assays

In these assays, a primer template complex is mixed with a polymerase and a dNTP (Scheme 5A). The primer is end-labeled, usually with a ^{32}P-tag. The reaction is run for a fixed time (to keep the amount of product < ~20% of the substrate). A plot of reaction velocity (product formed/unit item) vs [dNTP] is usually hyperbolic. A simple comparison is with the k_{cat}/K_m (i.e. enzyme efficiency) for each of the four dNTPs. The proclivity for mutation is predicted by the ratio $(k_{cat}/K_m)_{wrong}/(k_{cat}/K_m)_{right}$, where "wrong" and "right" indicate incorporation of a base yielding a mutation and "right" denotes "correct" incorporation, i.e., the base normally pairing with the base that was modified (e.g. C with 1,N^2-ϵ-Gua).[72]

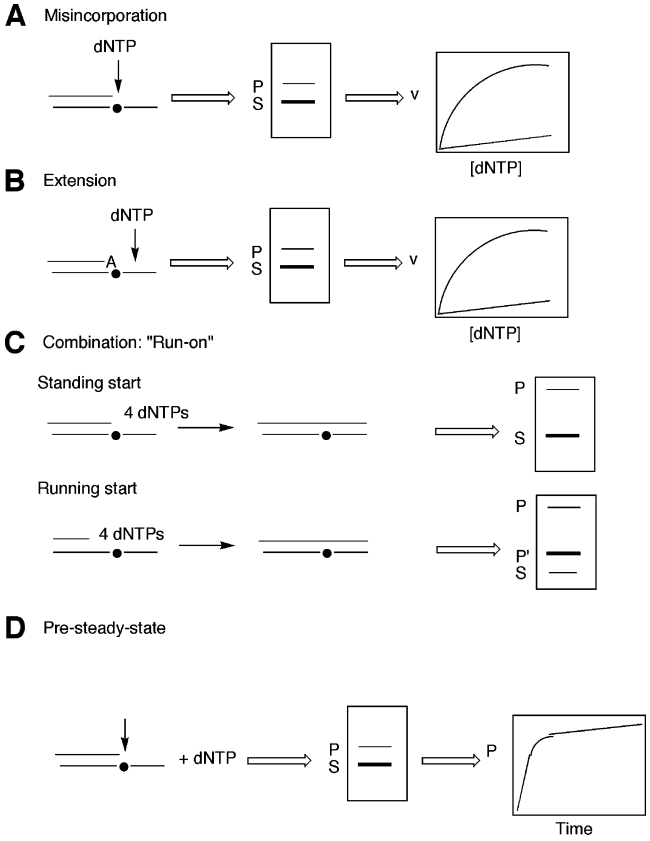
4.2. Extension

The ability of a polymerase to cause mutation is dependent not only on misincorporation *per se* (*vide supra*) but also on the tendency to extend the primer strand past the site of the incorrectly inserted base. Polymerases, or at least replicative DNA polymerases, have a strong tendency not to insert bases beyond a mispair.[73]

Experimentally the studies are done in a manner similar to those for misincorporation, except that a specific base is placed in the primer strand opposite the modified base (Figure 5b). The remainder of the analysis is similar.[73] A simple way to predict the tendency of a polymerase to produce a mutation is to expand the previous equation to give a parameter f':[74]

$$f' = [(k_{cat}/K_m)_{wrong}/(k_{cat}/K_m)_{right}]_{insertion} \times [(k_{cat}/K_m)_{wrong}/(k_{cat}/K_m)_{right}]_{extension}$$

Scheme 5. In Vitro Assays That Can Be Used with DNA Polymerases[a]



[a] The adduct is indicated as a solid circle. Parts A and B are steady-state measurements in which the rate (v) of conversion of the oligonucleotide substrate (S) to the product (P) (usually a 1-base extension) is measured at different dNTP concentrations. The plots usually fit to hyperbolae and yield the parameters k_{cat} and K_m. In this work it is appropriate to express v and k_{cat} in units of reciprocal time, i.e., pmol of product formed s^{-1} (pmol of DNA polymerase)$^{-1}$, or s^{-1} (as opposed to % product s^{-1}). The extent of conversion of S to P should be <20% to avoid problems with substrate depletion and product inhibition. In Part C, extension is done in the presence of all four dNTPs. The products usually include substrate, various lengths of extended product, full-length extended product, and sometimes full-length product to which a "blunt-end" addition has been made to yield a "+1" product. Although the approach has the advantage of making observations of insertion opposite the adduct, separation of rates of individual steps is difficult and the reactions can only be analyzed in the context of complex models. In pre-steady-state experiments (part D) rapid mixing equipment is used and the focus is on events that occur in the first catalytic cycle. If this first cycle is faster than events occurring after product formation, then the result is a kinetic "burst" and the first phase can be fit to a first-order plot and interpreted.

Another issue is determining what the product is. This is not a trivial exercise, and such experiments are usually not done. Historically, Maxam−Gilbert sequencing of the product has been done[75,76] but this approach is often ambiguous and very unsuited to analysis of mixtures.[77] As discussed later, MS provides a better approach.

4.3. Exonuclease Activity

DNA repair is obviously a major issue that influences the tendency of an adduct to produce a mutation in a biological setting.[78] More than 130 genes code for the enzymes that repair damaged DNA in humans.[79] However, before this action occurs, there is often a removal of damage within many polymerases. Many DNA polymerases have exonuclease domains within the protein. When a mispair is made, the polymerase can stall, as indicated above. This pausing

can allow time for the polymerase to release the DNA, and the damaged DNA can be sampled in the exonuclease site. If we consider some DNA polymerases that catalyze incorporation opposite bulky adducts, the turnover is approximately once per 30 min,[38] i.e., 0.0005 s^{-1}. However, a polymerase−DNA complex typically dissociates at a rate of ~1 s^{-1}. Exonuclease activity is relatively easy to analyze *in vitro*; one simply begins with a system such as that shown in Scheme 5B and measures rates of degradation, in the presence of Mg^{2+} and absence of dNTP. However, these measurements have generally been of less interest than polymerization, and exonuclease-deficient mutants are often used in studies with DNA polymerases to simplify the systems.[80] Some complex DNA polymerases (e.g., mammalian pol δ[81]) have not been prepared as exonuclease-deficient mutants and have been used with the exonuclease activity present.[81,82]

4.4. Combined Systems

One common system is the use of a primer−template pair with a DNA polymerase and all four normal dNTPs, in the presence of Mg^{2+} (Scheme 5C). This system is more representative of an *in vivo* setting and is simpler to set up. It also provides a rapid glimpse into the processivity of the polymerase, i.e., more "off" and "on" reactions yielding more intermediate-length bands instead of full-length product in the case of a "distributive" polymerase. These assays are often done prior to those depicted in Scheme 5A and B, as a function of increasing concentration of the polymerase.[49]

Although these assays are easier to run than the k_{cat}, K_m experiments (with the many individual concentrations of individual dNTPs required), quantitative analysis of the results of these experiments is not trivial. One rough parameter that can be used is the fraction of radioactivity appearing as total products under defined conditions and compared to other experiments. It is possible to fit results to a kinetic scheme with a "rise−fall" in the synthesis and decay of each product, with a set of appropriate equations.[80]

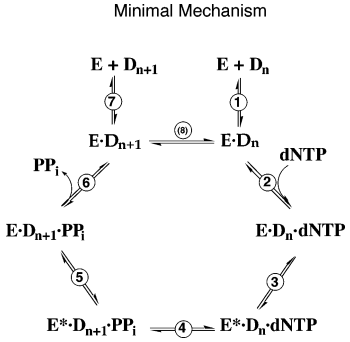
One point that deserves some comment here is the use of "running start" experimental designs (Scheme 5C), i.e., with a primer shorter than the part of the template leading to the adduct. The advantage of the former approach is that a block occurring *prior* to the adduct is observed in this setting. However, sometimes the running start model is viewed in an anthropomorphic manner, as if an enzyme can gain momentum and would be more likely to incorporate past an adduct in this setting. This is not the case; an enzyme must go through the cycle of Scheme 6 at each insertion step.

Another "mixed" case is the presence of two polymerases in a single experiment, one that favors the incorporation opposite the modified base and one which favors the extension.[87]

4.5. Pre-Steady-State Kinetic Experiments

One of the problems with doing steady-state experiments involving DNA polymerases and oligonucleotides is that the meaning of k_{cat} and K_m is generally obscure.[88] With the high affinity of DNA polymerases for DNA, the rate of release of the oligonucleotide is relatively slow and can even be considered rate-limiting in many settings. Thus, relatively little information may be revealed from the results of the steady-state kinetic experiments. The K_m value does not reflect the affinity of the DNA polymerase for a dNTP; indeed, the K_m has defied a simple interpretation in these systems.[89]

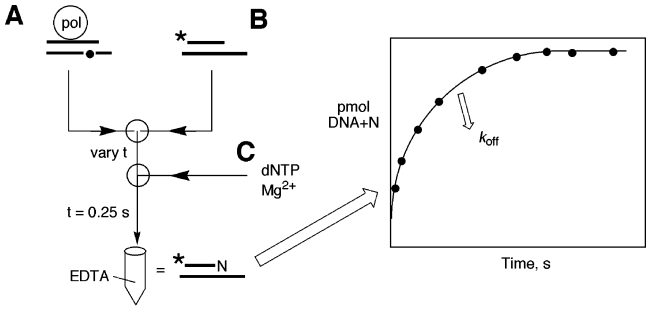
Scheme 6. Generalized Catalytic Mechanism for DNA Polymerases (Minimal Mechanism)[a]



[a] E is the DNA polymerase, D_n is the oligonucleotide substrate, dNTP is the deoxyribonucleotide triphosphate, E* is an "activated" conformational form of E, D_{n+1} is the oligonucleotide product, and PP_i is inorganic pyrophosphate. Step 8 occurs only in processive reaction, i.e., with movement of the polymerase along the oligonucleotide. For background into the evidence for the conformational change and discussion of what possible events may be involved, see ref 93. With some polymerase/DNA systems, strong evidence exists for additional intermediates in the cycle (see Scheme 11).[83−86]

One approach to gaining more insight into the reactions of DNA polymerases with DNA and also damaged DNA has been with the use of pre-steady-state kinetic approaches. The general concept is that the reaction is started and product formation sampled quickly (Scheme 7). With unmodified

Scheme 7. Rapid Chemical-Quench Apparatus and Use in the Estimation of k_{off} for a Carcinogen-Modified Oligonucleotide (k_7 of Scheme 6)[86,90,91] [a]



[a] Syringe A contains a DNA polymerase bound to an unmodified or modified oligonucleotide (the concentrations should be high enough to keep most of the oligonucleotide complexed). The contents of syringe A are rapidly mixed with a large molar excess of ^{32}P-labeled oligonucleotide. The DNA polymerase adducted oligonucleotide (denoted with a closed circle, ●) will dissociate and then bind to the ^{32}P labeled "trap" oligonucleotide. As the time in this segment of the experiment is increased, more dissociation and trapping will occur. An excess of dNTP·Mg^{2+} is introduced from syringe C and the polymerase reaction is allowed to proceed for a short, finite length of time to incorporate product into the ^{32}P-labeled oligonucleotide. The reaction is quenched (with EDTA), and the ^{32}P-labeled product is quantified using gel electrophoresis and phosphorimager analysis. The results can be fit to a first-order plot that yields an estimate of the rate of dissociation of the DNA polymerase−oligonucleotide complex. In principle, the system can be reversed to use ^{32}P with the modified oligonucleotide and an unlabeled trap oligonucleotide, but the design shown has advantages in that the unmodified oligonucleotide is a better substrate for DNA polymerase (i.e. yields more product).

oligonucleotides, most DNA polymerases yield what are termed "burst" kinetics. That is, the first cycle of the reaction (steps 1−6 of Scheme 6) is completed rapidly, followed by a slow product dissociation step (step 7 of Scheme 6).[89] The data will fit to a first-order exponential plus linear equation of the form[89]

$$y = A(1 - e^{-k_p t}) + k_{ss} t$$

The first-order rate constant is usually termed k_p or k_{pol} and describes the rate at which the first reaction cycle occurs, and k_{ss} is the "steady-state" rate. As discussed later, the limiting step is generally considered to be either a conformational event or the "chemistry" step itself (phosphodiester bond formation).[89,92,93] Exactly how carcinogen adducts influence the system will be discussed later.

These experiments have considerable advantages in the study of details of the interactions of DNA polymerases with DNA and have been a focus of investigation in the author's own laboratory.[36−38,73,81,82,85,86,94−97] Although the analysis may seem complicated to those inexperienced with kinetics, the purpose of doing pre-steady-state kinetics is to simplify the analysis.[98] The overall goal in kinetic analysis is to define rates of individual steps in reactions. With a multistep reaction mechanism such as that of a DNA polymerase (Scheme 6), the steady-state equations are very complex and, as mentioned earlier, defining the meaning of k_{cat} and K_m is a very complex task. However, pre-steady-state kinetic data, with the input of some results regarding binding etc., can be fit to a set of rate constants that can be used to describe the system reasonably well. For instance, the experimental design in Scheme 7 can be used to measure the polymerase−DNA dissociation constant.[86,90,91] It should be emphasized that kinetic analysis will seldom "prove" a mechanism. However, consideration of kinetics does quickly disprove some potential mechanisms and reduce interpretation to the point that only a limited number of mechanistic possibilities are feasible. Another general point to make is that kinetic analysis employs "minimal mechanisms" as much as possible. That is, in a DNA polymerase minimal mechanism (Scheme 6), additional events may be associated with some of the steps shown (e.g., more conformational changes in the protein) but these are included within the rate constants estimated for each step. Adding steps to a mechanism can be done, if necessary, to deal with fitting of kinetic data. However, such addition of steps must be justified.[83,85,86] Adding steps will always lead to better fitting but also increases the uncertainty about a system in that the number of unknowns in a set of equations is increased.

4.6. Site-Specific Mutagenesis

Site-specific mutagenesis refers to the process of introducing DNA modified at a specific site into a cellular system and then analyzing the mutations that occur.[17] The approach was pioneered by Essigmann and his associates[99,100] with O^6-MeG and has been subsequently applied to many other adducts. The term "site-specific mutagenesis", as defined here, distinguishes the process from "site-directed mutagenesis", the process of changing the primary structures of proteins for analysis in enzymology etc.[101]

Almost all of the work that has been done with this approach has been with extra-chromosomal DNA elements (vectors). The analysis often relies on initial screens with reporter genes, e.g. color formation in bacteria with the use of *lac* genes, survival in selective media, etc.[17,18,102] However, advances in hybridization technology permit the direct analysis of base-pair and some frameshift mutants directly in plates of bacterial colonies.[77,103,104] Advances in DNA sequence analysis have been considerable in the past two decades, and extensive analysis of replication products is now very feasible on a large scale. In this latter regard, such analysis has revealed the existence of numerous mutations at sites distant from the DNA modification itself,[105−107] and some possible explanations have been proposed. With "shuttle" vectors, it is also possible to place vectors in mammalian cell culture and then move them into bacterial systems to facilitate analysis.[108]

4.6.1. Polymerase Issues

The advantage of doing site-specific mutagenesis is that the biological effects of a defined lesion can be observed in a cellular environment (which is actually "*in vivo*" in the case of microorganisms). This provides a great advantage, at least in overall biological relevance. One of the problems inherent in experiments with isolated polymerases is the issue of what accessory proteins are really required. For instance, pol III, the replicative *Escherichia coli* polymerase, has about 10 subunits.[109] Mammalian pol δ apparently has four subunits,[110] plus proliferating cell nuclear antigen (PCNA); the enzyme will do some functions well with only two subunits,[81] but others may be useful in other settings.[110]

Moving to a more complex experimental setting has advantages in terms of addressing the relevance of a phenomenon but reduces the ability to understand details of a mechanism. With regard to DNA polymerases, a site-specific mutagenesis experiment does not inherently provide any information about which proteins are involved. *E. coli* has five polymerases.[111−113] Some insight can be gained by examining the need for the SOS response, but even then, three of the *E. coli* DNA polymerases are candidates.[112,113] Also, the SOS response can be leaky. Many older experiments with PAH adducts were done in the absence of SOS induction,[114] but in light of the current understanding of translesion polymerases, it would seem highly likely that the SOS-inducible polymerases are the principal actors in these systems.

The plethora of mammalian polymerases[115] makes assignment of the roles of individual polymerases particularly difficult. In contrast to the situation with the SOS response in bacteria, there is no simple method for preferentially activating some of the systems. Further, the number of translesion polymerases that could potentially be involved is far greater than that in bacteria.[116]

A considerable amount of literature has been generated about the ability of individual isolated polymerases to copy past individual adducts. Ultimately the information will have to be considered in the context of cellular systems. Two types of experiments can be applied. One approach is to use mutant cell lines devoid of particular DNA polymerases and examine the toxicity and mutagenicity of adducts, whether formed by added chemicals or with defined vectors transfected into the cells. This has already been done with human pol *K* and PAH adducts.[117] Further, transfection of pol *K* restored the phenotype to the wild type.[117] The other approach, which has not yet been reported to the author's knowledge, is to use siRNA methods to attenuate individual DNA polymerases in similar cell culture systems. Neither of these methods (nor mutants) will work in distinguishing a role for the major replicative DNA polymerases, though.

Another inherent issue in the cell-based studies is the use of extra-chromosomal DNA in almost all systems, with a few exceptions.[105,118,119] Although the assumption is generally made that the DNA polymerases involved in the replication of these vectors are identical to those used in copying the

same adducts in chromosomal systems, direct proof is lacking.

4.6.2. DNA Repair Background

Another issue to consider in cells is the DNA repair background. Obviously any conclusions must have caveats about repair, if comparisons are to be made. Studies with cells deficient in certain DNA repair activities have been done for some time already, both in bacteria and in mammalian cells.[120]

A related consideration is the choice of the vector used with the cell system, whether prokaryotic or eukaryotic. A major issue is whether to use a single-stranded or double-stranded DNA vector to introduce the DNA adduct and study it. Single-stranded vectors have the advantage of not being repaired by many of the known DNA repair systems (O^6-alkylguanine DNA alkyltransferase (AGT) is an exception[121]). In addition, any replication of the entity must proceed from the strand containing the adduct. Double-stranded vectors provide a model more relevant to the normal situation. However, they may be prone to DNA repair and DNA polymerases may use the opposite, unmodified strand to copy the information. For these reasons mutation frequencies are generally higher with systems employing single-stranded vectors compared to double-stranded vectors.

In a few cases, the introduction or overexpression of a DNA repair system leads to enhanced mutagenicity, e.g. AGT.[122−125] These systems appear to involve some type of cross-linking mechanism, and the AGT phenomenon will be discussed later (*vide infra*).

Sometimes conclusions are presented to the effect that a certain adduct is not mutagenic in bacteria but is mutagenic in mammalian cells, inviting general comparisons of these models for predicting risk. Before reaching conclusions, it is necessary to consider the nature of the vector systems, the DNA repair backgrounds of the cells, and aspects of the experiments that will influence which DNA polymerases would be utilized in replication.

5. Basic Issues Involved in Base Recognition

Before embarking on some of the specific issues involved in polymerase interactions, it is instructive to consider the general mechanisms proposed for coding and miscoding, in that these are also the forces under consideration with polymerases. Another article in this issue also deals with the general problem of polymerase fidelity, and the reader is referred to this.[126]

5.1. Hydrogen Bonds

The concept of the importance of the purine−pyrimidine hydrogen bonding in DNA structures was first advanced in the classic 1953 paper by Watson and Crick.[127] This classic pairing (Scheme 4A) is usually referred to as the "Watson−Crick geometry". The G:C pair has three hydrogen bonds, and the A:T pair has only two. These values are reflected in typical analyses of sequence effects in T_m values,[128] but base stacking is also a major factor (*vide infra*).[59,60]

Statements often appear that suggest that the fidelity of DNA replication is driven only by these hydrogen bonds. The point has been made many times, however, that the energy involved is not enough to explain the very high fidelity of DNA synthesis.[129,130] That is, the differences in the free energies of the "right" base pairs (G:C, A:T) are not sufficiently different from the case of the wrong pairs to explain the low error frequencies. Even without allowing for exonuclease activity (and DNA repair by other systems), systems must exist to amplify the small thermodynamic factors involved in the hydrogen bonding.[93,129] As indicated earlier, individual DNA polymerases can produce quite different misincorporation patterns.

Alternate bonding patterns are shown for the wobble and Hoogsteen systems (Scheme 4B and C), which also contain hydrogen bonds. The concept of alternate conformations imposed by DNA adducts is not new. For instance, Loechler[131] used the term "adduct-induced base shift" on the basis of studies done with molecular modeling. The concept is relatively simple: that adducts cause either base wobbles or base rotations that will lead to abnormal pairing due to unusual pairing mechanisms (e.g. Scheme 4B and C). Evidence for *anti* to *syn* changes (about the glycosidic bond) have been seen with a number of adducts, including the relatively simple 8-oxoG.[69] A general issue, more difficult to address, is whether these alternate conformations have much to do with miscoding. Evidence for some of these proposals has been developed in crystal structures of DNA polymerases, discussed later under the heading X-ray Structures of DNA Polymerases (item 7), including 8-oxoG[132,133] and 2-acetylaminofluorene (2-AAF).[134]

5.2. Watson–Crick Geometry

The concept has been advanced that an important factor in DNA polymerase coding is the overall size (and shape) of the two bases involved in the pairing scenario. That is, the issue is the maintenance of the "Watson−Crick geometry" for a DNA polymerase as a driving force in incorporation events.[135]

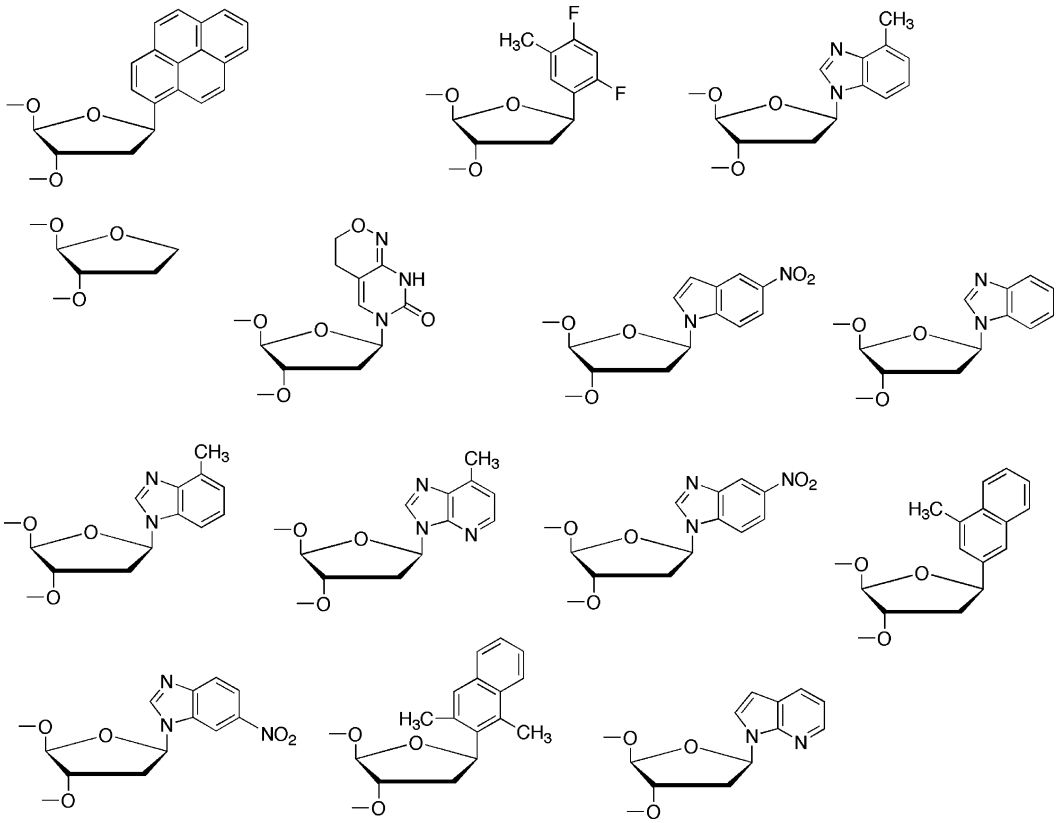
This view makes some sense if one considers the DNA polymerase to make a tight fit around the bases it samples and to only accommodate the geometry of the dimensions shown in Scheme 4A. One problem is that the existing DNA polymerase crystal structures are not so tight and, furthermore, would not necessarily have to be for all possible structures, even if these did not crystallize. Some motion is required to bring the DNA and dNTP in together, and one can probably not argue, from a single crystal structure, that this is the only possible near-attack conformer or intermediate approaching the transition state.

5.2.1. Experiments without Hydrogen Bonds

Evidence that hydrogen bonding is not an absolute requirement comes from studies by Kool's group and others, who have demonstrated selective and stable base recognition (in DNA T_m studies and in replication by DNA polymerases) with DNA base isosteres devoid of the capability for hydrogen bonding[136−138] (Scheme 8). That is, modified base pairs can be set up such that a deficiency in size in the base in an oligonucleotide can be compensated for by an increased size in the base of the incoming dNTP.[139,140,142,143]

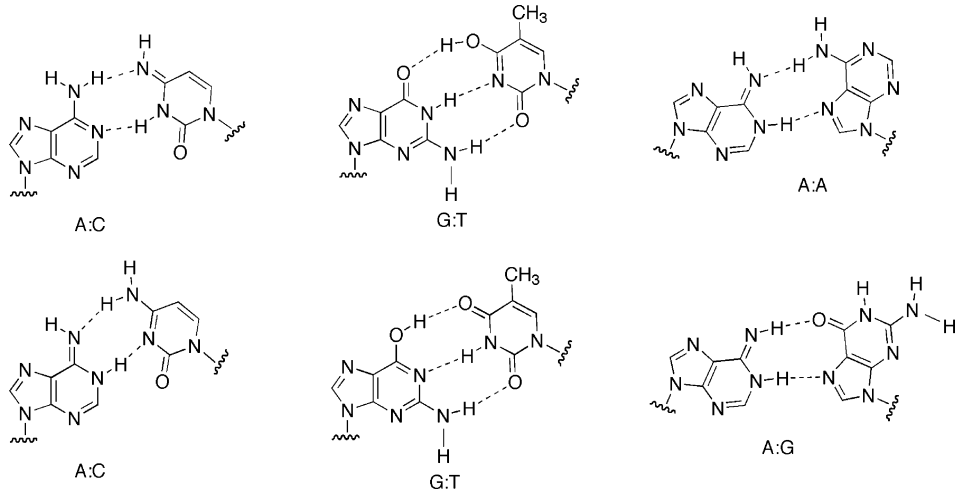
One of the other drivers in this work is base-stacking (*vide infra*). Although the results with the base isosteres are of interest, some caveats must be considered. One issue is that the catalytic efficiencies (k_{cat}/K_m) for the DNA polymerases using the isosteric non-natural base pairs are usually very low and often resemble carcinogen-modified bases.[139] One might not expect these systems to show the kinetic bursts typically seen in reactions for normal pairing (Scheme 5D), and indeed these are not seen except in one case.[141] Another

Scheme 8. Incorporation of "Isosteric" Base Analogues by DNA Polymerases[138−141] [a]



[a] Some have been inserted in the template while others have been used only as the dNTPs.

Scheme 9. Base Tautomers and Potential for Pairing[50]



issue is that some but not all polymerases will use these isosteres.[140,144]

5.2.2. Asymmetry

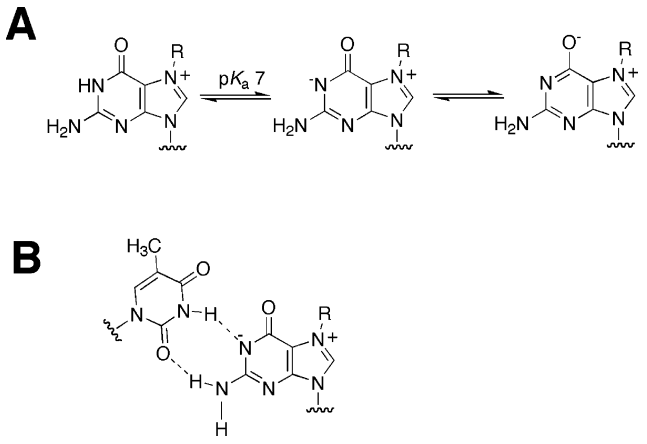
Another issue is that of symmetry of the base pairs. If the overall shape of the two-base pair were the only driver, then one might expect to see similar results for various coding/miscoding phenomena regardless of whether one of the pair was in the oligonucleotide or the dNTP. This is not the case, as shown in studies with 8-oxoG in the oligonucleotide vs 8-oxo dGTP.[36] These considerations also apply to other work on the significance of incorporation of modified dNTPs in mutagenesis; that is, the results of a miscoding study with a modified dNTP do not necessarily mean that the same mutagenic potential exists if the same modification is present in the DNA.[145]

5.3. Base Tautomers

The normal four DNA bases are usually shown in the form presented in Scheme 4, i.e., as the lactum tautomers. However, the bases can also be drawn in the enol tautomers. Such tautomers could participate in aberrant pairing schemes, as outlined in Scheme 9. The different tautomers are related by only the transfer of protons and bonds. The pK_a of dGuo is ~9, and therefore, a significant fraction should exist in the unfavored tautomer at neutral pH. The possibility that such pairing could contribute to and be a major factor in mispairing was first proposed by Watson and Crick in their

classic 1953 paper.[127] The possibility was presented again by Lawley and Brookes in 1961[146] in consideration as a mechanism for mutagenesis by methylating agents. At that time the only known methylation product was N^7-MeGua, and the authors reported that the pK_a for the loss of the only exchangeable proton (N^1-H) was 7. Thus, the anionic N1 atom could be involved in a pairing scheme (Scheme 10).

Scheme 10. (A) Ionization and Tautomerization of an N^7-Alkylguanine and (B) Possible Pairing of an N^7-alkylGua Adduct with T[a]



[a] See ref 26.

5.3.1. General Issues

The possibility of the contribution of base tautomers to miscoding was raised by Topal and Fresco in 1976.[147] Specifically, they proposed that tautomeric Hoogsteen *anti*−*syn* pairs were responsible for the formation of purine−purine pairs.[147] Several theoretical papers have suggested that the base pairing of rare tautomers is a real possibility.[148,149] Also, NMR studies with N^6-methoxyAdo and uridine derivatives, in $CHCl_3$, support the binding of tautomers.[150]

Morgan[151] has argued against the involvement of base tautomers in pairing and mispairing. The point is made that the current collection of X-ray and NMR structures of oligonucleotides has not revealed evidence for the existence of such entities, and the proposal that these tautomers are present in the events surrounding the transition state for base incorporation defies experimental tests.[151] In X-ray structures with G:T mismatches, the evidence supports wobble pairing and provides no evidence for tautomerism.[152] However, the possibility can be raised that only one form of the oligonucleotide crystallized. ^{1}H NMR experiments can detect hydrogen bonds, at downfield chemical shift values, but the resonances are not particularly good for distinguishing exactly what the individual hydrogen bonds correspond to.

More recently, Fresco's group has investigated the tautomerism of 5-hydroxy dCyd, a mutagenic product formed in the transition metal-mediated oxidative damage of dCyd.[153] Because of the 5-hydroxy group, the equilibrium is shifted considerably to the tautomeric enol form in solution. In this study, the approach involved the use of UV resonance Raman spectroscopy to detect the bands attributed to the minor anionic tautomer. Although the fraction of 5-hydroxy dCyd in this tautomer was only ~0.5% under physiological conditions, its presence could be detected by the distinct Raman band indicative of this form. Whether or not this tautomer exists in double-stranded DNA is unknown, and the sensitivity of the Raman spectroscopy approach may not be sufficient for this purpose. The authors note that the fraction of the nucleoside found as this tautomer (0.5%) is similar to the mutation frequency, although this congruence does not necessarily prove a causal relationship. Further, the authors propose that rare tautomers of the bases may be involved in miscoding events.

5.3.2. N^7-Guanyl Adducts

The issue of N^7-Gua adducts can be considered again. As mentioned earlier, in the initial methylation work, the Gua N^7-Me entity was considered to cause mutations by mispairing, to yield N^7-MeG:dThd pairs (Scheme 10).[146] With time, more methyl adducts were characterized and a series of studies and comparisons led to the view that the major mutagenic product resulting from methylating agents is O^6-MeG.[25] Support for this view comes from the ability of AGT to lower the number of mutations produced by an alkylating agent.[122] However, these studies do not necessarily indicate that an N^7-alkyl Gua cannot be mutagenic.

A number of studies have been done with *S*-[2-(N^7-guanyl)ethyl]GSH (GSH = glutathione) in this laboratory.[154] In particular, a high level of base pair mutations can be induced by treatment of *Salmonella* typhimurium cells with *S*-(2-chlorethyl)GSH.[155] The presence of traces of *S*-[2-(N^2-guanyl)ethyl]GSH and *S*-[2-(O^6-guanyl)ethyl]GSH can also be detected,[156] but comparisons of the number of measured adducts with mutations suggest that the major adduct, the N^7-alkylGua species, must account for some of the mutations.[155] However, in another bacterial system, expression of AGT did cause a partial attenuation of mutations and suggests that some of the mutations may be due to *S*-[2-(O^6-guanyl)ethyl]GSH (which is known to be a substrate for AGT).[122] Studies with nucleosides indicated that the formamidopyrimidine (FAPY) ring-opened product is not readily formed and should not be an issue.[156] Also, the mutation spectra generated with either ethylene dibromide itself[157] or *S*-(2-chloroethyl)GSH[156,158] in several systems consistently yield dominant G to A transitions. Depurination would be expected to produce predominantly G to T transversions.[159]

All three of the known GSH-ethyl derivatives of Gua block polymerases and can produce some miscoding, as judged by various parameters.[71] Of the ones examined, none showed a preference for insertion of T opposite the modified G, which would give rise to the G to A transitions.[71] Site-specific mutagenesis studies (in cells) have not been done, due to some technical issues with the adducts. The first comparison of the mutation spectra with sites of N^7-alkyl Gua damage did not yield evidence for similarity of the profiles.[156] However, in subsequent work in a yeast−human p53 system, it could be demonstrated that a substantial overlap of the *in vivo*-modified sites (N^7-alkyl Gua damage) overlapped the mutations (predominantly G to A) after a period of time, thus providing evidence (i) that the *S*-[2-(N^7-guanyl)ethyl]GSH adducts are producing G to A transitions and (ii) that rates of DNA repair are a major factor in the sequence specificity of mutation.[158]

Tautomeric-type base pairing may be a factor in these mutations. An oligonucleotide containing *N*-acetyl-*S*-[2-(N^7-guanyl)ethyl]Cys methyl ester, an analogue of GSH, was titrated (adduct opposite C) and yielded a pK_a of 8.2, as judged by UV measurements.[26] Thus, a significant fraction of the adduct could be deprotonated at neutral pH, and a possible mispairing scheme is shown in Scheme 10.

5.4. Stacking Interactions

Intercalation has long been recognized as a factor in increasing the interactions between DNA strands in some cases. For instance, the phenomenon has been described well for AFB_1 and some polycyclic aromatic hydrocarbons (PAHs), where T_m values are increased by the presence of these adducts.[62,160] In one of these cases,[62] the particular stereochemistry of the DNA adduct (*R* vs *S*) produced an increase or a decrease in T_m.

As indicated in the above discussion, hydrogen bonding is part of the explanation for driving base pairing, whether through classical Watson−Crick pairing or alternate pairs such as Hoogsteen, wobble, or minor tautomers. Work with entities not capable of hydrogen bonding indicates that alternate forces must also be involved.[138−141] One force appears to be base stacking. Base stacking is a major contributor to the physical chemistry of certain sequence selective phenomena,[59,60,161] and the free energy is significant, even though a canonical GC pair is intrinsically stronger than an AT pair. Thus, GC content only explains part of the T_m differences among nucleotides (*vide supra*).[59] Minetti et al.[161] consider the thermodynamic differences in adding the individual dNTPs in DNA polymerase reactions and compare enthalpies. They suggest a sequence-independent background of compensating enthalpic contributions to DNA synthesis, with discrimination expressed by differences in noncovalent interactions.

Base stacking was invoked to explain the stability of a pyrene:abasic site pair.[138] Reineks and Berdis[141] also considered a series of modified dNTPs incapable of hydrogen bonding, in the context of the ability of bacteriophage T4 DNA polymerase to pair these at an abasic site or a normal base. The size of the dNTP base was not a useful predictor of pairing. Some other polymerases (but not all) can also do pairing of analogues devoid of hydrogen bonding ability. Reineks and Berdis[141] attribute the phenomena to base stacking, particularly $\pi-\pi$ interactions with the base of the incoming dNTP. The variations of the polymerases in participating in these interactions are used as one argument that the stacking interactions are not only between neighboring bases in the DNA but also between the dNTP and aromatic residues of the polymerase.[141] The initial interaction may trigger the movement of other residues in the polymerase.

One criticism of many of the experiments done with pairs incapable of hydrogen bonding is that the catalytic efficiencies of the polymerases are extremely low with these systems.[139,141] These low efficiencies raise the question of how much the forces involved in these interactions reflect those involved in normal base pairing. However, Reineks and Berdis[141] found that one dNTP, 5-nitroindolyl-2′-deoxyriboside triphosphate (Scheme 8), was inserted with 10^3-fold greater efficiency than dATP with the polymerase gp43 exo$^-$. Further, burst kinetics were observed with a rate (k_{pol}) of 28 s^{-1}.[141] The polymerase would also insert the 5-nitroindolyl derivative opposite T and A, with k_{pol} values of 1 and 4 s^{-1}.

Other forces that can be considered here are related to desolvation in the active site.[141] Another view is that DNA polymerases achieve selectivity by negative discrimination, based on work of Reineks and Berdis[141] and Kuchta.[140] That is, polymerases are inclined to screen against matching of the incorrect components of the normal set of four bases, to avoid errors, but are not so well "trained" to recognize unusual types of bases.[140]

5.5. Bulk

Bulk might seem an obvious factor in blocking polymerases and causing miscoding. However, the situation is more complex. As mentioned earlier, some bulky residues intercalate between bases and produce *higher* T_m values.[160,162,163] However, these same bulky adducts can block polymerases. In work with GSH-ethyl adducts resulting from ethylene dihalides, the N^7-adduct was bypassed more readily than the O^6 or N^2 adduct.[71] Therefore, the position of the lesion is a major factor, in addition to the actual size of the adduct.

5.5.1. General Issues

Even lesions as small as O^6-MeG[164] and 8-oxoG[94,95] pose blocks to DNA polymerases and cause considerable miscoding, but some of the basis lies in the change in the hydrogen bonding and the *syn*−*anti* equilibrium of the nucleoside. In a study with O^6-MeG and O^6-benzyl (Bz) G (in the template strand), the replicative DNA polymerases bacteriophage pol T7 exo$^-$ and HIV-1 reverse transcriptase showed partial kinetic bursts.[86] The benzyl adduct showed poorer bypass and incorporation than the methyl adduct.

5.5.2. N^2-Guanyl Adducts

A systematic study of the effect of bulk at the N2 atom of Gua was done in this laboratory.[97] Previous work had shown that an N^2-ethyl group could miscode with *E. coli* polymerase I Klenow fragment.[165] With the replicative DNA polymerases T7 exo$^-$ and HIV-1 reverse transcriptase, a methyl group had a much greater effect here than at the O6 position, even though all atoms should have still been available. Strong blocking effects were seen for incorporation of C and extension, even with only a methyl group. The parameter k_{cat}/K_m for steady-state incorporation of C provides a quantitative means of comparing the effect of bulk (Figure 2). The effect increased markedly from no substitution to Me to Et and then was relatively constant up to a methylanthracenyl group (Figure 2). Similar decreases in the fast pre-steady-state kinetic phase were also seen.[97] It is of interest to note that the parameter k_{cat}/K_m for incorporation of A was relatively invariant with bulk and similar to the value for incorporation of C opposite the bulkier residues (Figure 2). Perhaps this latter phenomenon can be viewed several ways, but one view is that not much information is involved in the A incorporation or the C incorporation with the bulkier adducts. Most of the larger N^2-guanyl adducts in this series are not produced with real carcinogens.[97] However, the results obtained with PAHs and other relevant bulky adducts appear to be similar.[37,38]

For comparison, the results are very different for translesion polymerases.[166] Bulk has much less effect. For instance, when the study (and auxiliary experiments) was done with human pol η, the enzyme was able to incorporate dCTP opposite a guanine with an N^2-substituent as large as a naphthyl without decreasing the k_{cat}/K_m parameter[166] (an anthracenyl group did inhibit) (Figure 2). Further, pre-steady-state kinetic bursts could even be observed with a group as large as a naphthyl.[166] Similar results have been observed with this series of adducts and human pol ι (Choi, J.-Y., and Guengerich, F. P., unpublished results). The point should be made, though, that the difference (in the steady-state parameter k_{cat}/K_m) for incorporation of dCTP ("right") vs dATP ("wrong") is less with the translesion polymerases

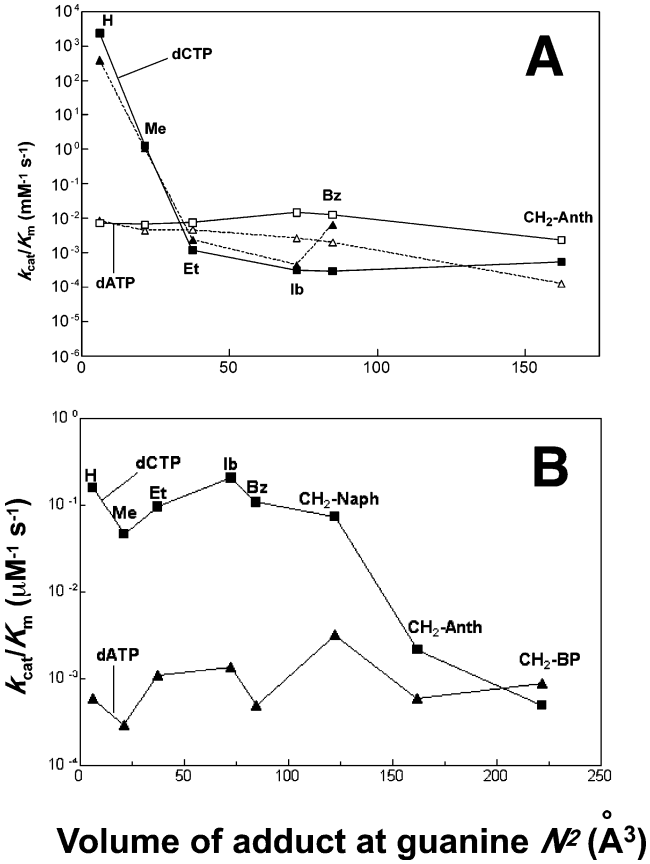


Figure 2. Catalytic efficiency (k_{cat}/K_m) of correct incorporation (dCTP) and misincorporation (dATP) opposite a series of N^2-guanyl DNA adducts with increasing bulk.[97] Correct incorporation (dCTP, solid symbols) and misincorporation (dATP, open symbols) are shown (Me = methyl, Et = ethyl, Ib = isobutyl, Bz = benzyl, CH_2−Naph = CH_2-(1-naphthyl), CH_2-Anth = −CH_2(9-anthracenyl), and CH_2−BP = CH_2-(6-benzo[*a*]pyrenyl)). (A) Pol T7 (▲, △) and HIV-1 reverse transcriptase (■, □). (Reprinted with permission from ref 97. Copyright 2004 American Society for Biochemistry and Molecular Biology.) (B) Human pol η. (Reprinted with permission from ref 166. Copyright 2005 Elsevier Publishing.)

(10^2−10^3) than with the replicative polymerases (~10^6, Figure 2). When the experiment of Figure 2 was done with recombinant pol δ (plus PCNA), that polymerase was intermediate (between pol T7$^-$/HIV-1 reverse transcriptase and human pol η) in its ability to accommodate bulky lesions.[166]

The results are rationalized by the available crystal structures of the DNA polymerases (Figure 3). The Dpo4 polymerase has a considerably looser fit due to less steric exclusion. Further, this view is reinforced by some of the structural results available for the translesion polymerases (*vide infra*). Thus, a paradigm is emerging that the highly accurate processive DNA polymerases fit tightly around DNA and have limited space for much more than the normal base pairs (A:T, G:C). The translesion polymerases have looser fits and more space for modified DNA, but use of these polymerases has a cost in terms of lower rates (*vide infra*) and, more seriously, less fidelity (in most cases, but not necessarily all, *vide infra*).

5.6. Interactions with Polymerases

Some insight can be gained into features of base interactions from studies with isolated oligonucleotides, in terms of hydrogen bonding, intercalation, and tautomers. However,

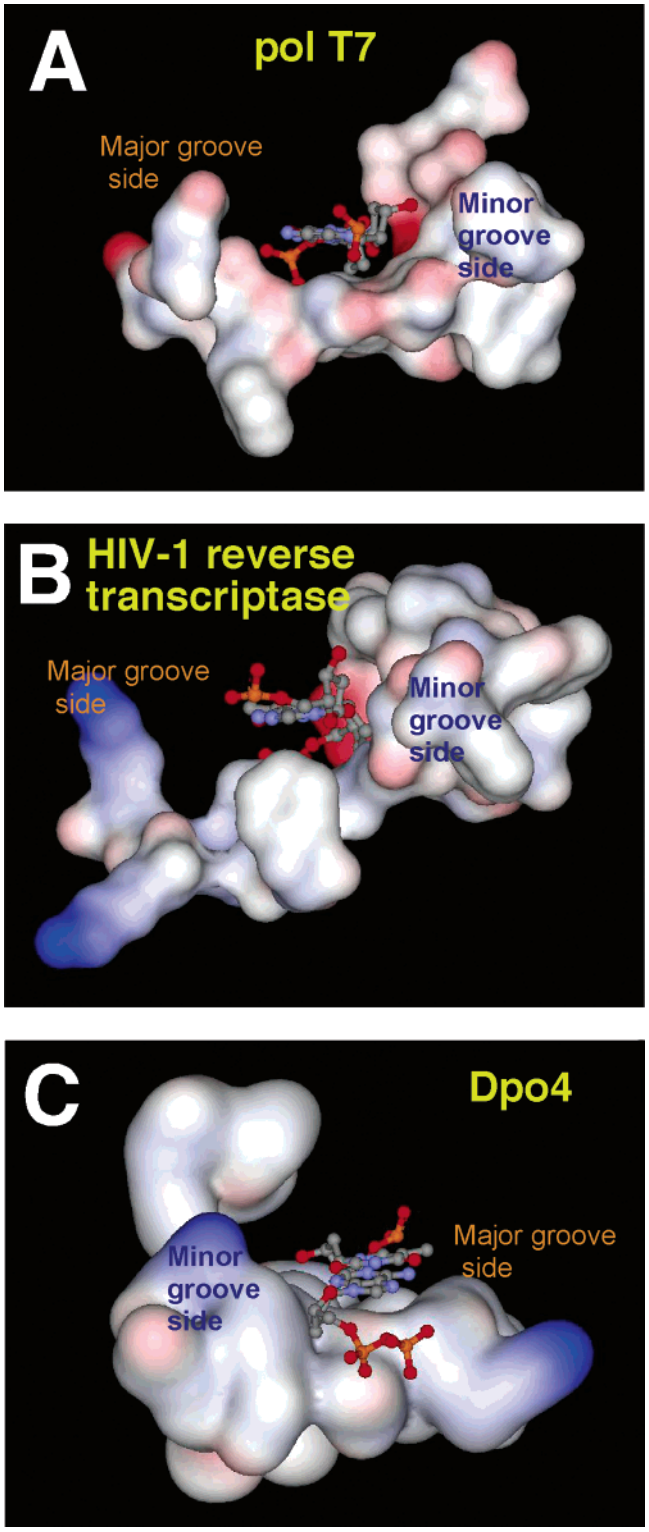


Figure 3. Structures of pol T7 (A) and HIV-1 reverse transcriptase (B) and Dpo4 (C) with DNA modeled in ref 167. The figure shows the extra space available for the DNA in the translesion polymerase Dpo4 compared to the replicative polymerase pol T7. (Reprinted with permission from ref 97. Copyright 2004 American Society for Biochemistry and Molecular Biology.)

as already pointed out, the interaction with the polymerases dominates the outcomes of catalytic events. Already mentioned is the case for a general selection of processive DNA polymerases for the overall geometrical boundaries of the size of A:T and G:C base pairs.[135] The other issue already mentioned is π−π base stacking of incoming dNTPs with

aromatic residues in the polymerases.[141] The case against symmetry of the bases in the template and the dNTP[36] must also be rationalized in terms of interactions of both entities with the polymerases.

A number of crystal structures of DNA polymerases are now available, and some will be discussed at further length below. Of course, many contact sites between the polymerases and the DNA strands have been identified. These change as the strand moves through the polymerase (or, from a different point of view, the polymerase moves along the DNA). The relevant interactions of dNTPs with a DNA polymerase are, in a sense, more difficult to identify in structural biology experiments. The four normal dNTPs must all bind to the polymerase and then move into appropriate intermediate states (or "near-attack conformers"[168,169]) for bond formation to occur and conformational changes of the polymerase to also occur (*vide infra*). Identifying which of the relevant steps a particular structure corresponds to may not be trivial. Nevertheless, structural information with the polymerases is certainly useful in answering questions about these polymerases, particularly regarding carcinogen-adducted DNA.

At this point, some mention should be made of the general issue of incorporation of carcinogen-modified dNTPs. A case has been made that much of mutagenesis could be attributed to this mechanism instead of miscoding opposite modified DNA. Some reactions with activated carcinogens do not readily occur with dNTPs, due to the need for intercalation etc.,[170] but in other cases reaction might be more facile due to the exposure of the atoms involved in hydrogen bonding. However, many of the reactions demonstrated with modified dNTPs are not very efficient. For instance, the reaction of 8-oxodGTP has a very unfavorable K_m.[36] (It is suspected that the lower K_m values reported by several groups are the result of trace contamination by dGTP, which even at a low level would explain the results. In our own work, the synthesis proceeded through a pathway that avoided contamination by dGTP.[36] Although the hydrolase MutT is generally considered to be an enzyme that exists to remove 8-oxo dGTP from cells, a search for 8-oxo dGTP in *E. coli* has provided negative results,[171] suggesting an alternate function.) Thus, limited evidence exists that major pools of modified dNTPs exist in cells and that incorporation is a major event contributing to mutation. As indicated earlier, experiments demonstrating incorporation of a particular modification of a dNTP should not necessarily be equated with similar miscoding due to that adduct in the DNA template.[36]

6. Kinetics

6.1. General Considerations of Relevance of Individual Polymerases

One of the issues is choosing a DNA polymerase to begin experimental work with. Many choices are available, although obtaining some of these is not trivial. Ideally, one would probably prefer a human polymerase that is easy to express, purify, and crystallize, has a high rate of activity, and has selectivity relaxed enough to permit some incorporation at noncanonical sites.

A list of the major DNA polymerases that have been studied is presented in Table 1. For simplicity, the "eukaryotic" classification is focused on mammals; some differences are seen with the yeast and Drosophila enzymes. "Bacterial" is focused on *E. coli*; some of the "model" polymerases are from *Archebacter* species.

Table 1. Major Polymerases Used in Kinetic and Structural Studies

Processive DNA Polymerases
bacterial
pol III (10 subunits)
eukaryotic
pol δ (2−4 subunits)
pol ϵ
models (viral and bacterial)
pol T4
pol T7 (exo$^-$)
HIV-1 reverse transcriptase
Bacillus stearothermophilus pol I fragment (BF)
RB69
Translesion DNA Polymerases
bacterial
pol II
pol IV (*din*)
pol V (*umu*)
eukaryotic
pol η
pol ι
pol κ
REV1
pol ζ
others (pol λ, pol μ)
model
Sulfolobus solfataricus Dpo4
Others
bacterial
E. coli pol I (Klenow fragment)
eukaryotic
pol α
pol β
other
pol X

First of all, the four polymerases listed under "others" (Table 1) are not particularly good choices for studying polymerization with carcinogen-modified DNA, even though several crystal structures are available. *E. coli* pol I (Klenow fragment, with or without exonuclease activity) is easy to express[94] and is commercially available, which facilitates its use. However, it is not particularly processive and is not well-behaved kinetically.[89]

Pol β is classified as a nucleotide transferase on the basis of sequence identity.[172] Its function is really in DNA repair, in the context of inserting a single base (dNTP) in a 1-base gapped primer. Such a primer strand would be the product of a glycosylase reaction on a modified base. Thus, pairing a dNTP opposite an adduct is a rather abnormal function for this enzyme, even if the protein is easy to work with. Pol β has some "extension" capability and, more, is observed with some mutants,[173] but this must be considered a model activity even if this is a mammalian enzyme.

Swine fever pol X is a small enzyme that binds DNA. Structures have been determined by NMR methods.[174,175] However, catalytic rates are extremely low and the function of this enzyme as a polymerase is unclear.

Leaving these polymerases aside for most studies, the first choice is whether to consider a processive polymerase or a translesion polymerase. The former have been known longer. The translesion polymerase literature developed quickly beginning about 1998, due to several reasons: (i) investigators had long desired to reconstitute the SOS response system (*umu*)[129] and were able to do so with the finding that the *din*

and *umu* gene products were polymerases, not merely accessory proteins.[176,177] (ii) Searches, particularly using homology, led to the identification of translesion polymerases in the newly available human genome.[178]

The processive DNA polymerases are the ones that first encounter DNA damage and thus have relevance even if they may be limited in their abilities to proceed. The translesion polymerases are able to copy past damage, although defining which lesions are copied by them is an empirical effort. The translesion polymerases are more distributive.

One might assume that the "bacterial" polymerases are simple and easy to purify relative to the eukaryotic DNA polymerases. This is not really the case. *E. coli* pol II is simple and easily expressed and purified.[94,179] However, *E. coli* pols III, IV, and V are very complex systems, with multiple subunits, clamps, clamp loaders, etc.[180]

Most of the mammalian translesion polymerases are not expressed well in bacteria (some success has been observed with human Rev1).[181] Some detailed kinetic work has been done with mammalian DNA polymerases purified from tissues (not recombinant) although there are difficulties in separating individual polymerases and with proteolysis.[81]

The "model" DNA polymerases listed in Table 1 have been popular. Those listed have been relatively easy to express and purify. Many of these have been crystallized. The processive polymerases in this group show good kinetic behavior, i.e., high rates and sharp bursts.

6.2. Normal Incorporation

The basic and generally accepted catalytic mechanism of DNA polymerases is presented in Scheme 6. For recent reviews of the action of DNA polymerases and some of the questions involved, see refs 93 and 182. The system is shown for a steady-state situation in which an oligonucleotide is used as the substrate and the product is released in each cycle, as would be the case when a single dNTP is used (and only one site in the template is able to pair with it). In a processive system with all four dNTPs available, step 8 would be operative and the DNA would not be released at every step.

The reaction begins with step 1, the binding of DNA and the polymerase. In enzymology, the interaction of two entities is treated as a second-order reaction between two spheres, in the absence of evidence for other steps.[183] The size of the two entities probably alters this rate somewhat, but the generally accepted estimate of a second-order "on" rate is in the range 10^6-10^8 M^{-1} s^{-1}.[183] The "off" rate, k_{-1}, will control K_d, the affinity ($K_d = k_{-1}/k_1$). In both steady-state and pre-steady-state kinetic experiments, one usually starts with the preformed polymerase−DNA complex.

The next step is the initial reaction with the dNTP, which is also assumed to be diffusion-limited and fast relative to other steps in the mechanism. However, this initial binding is not the whole story, in that the DNA polymerase binds all four dNTPs and has to sort through these to position the appropriate one for phosphodiester bond formation. Exactly how this happens is still unclear and is considered to be linked to the next step, the "conformational change," step 3. The K_d for dNTP binding, if measured in the presence of DNA, is not trivial to measure, even if phosphodiester bond formation is blocked, in that the reaction proceeds on and two forms of the enzyme may be involved, so the apparent K_d (e.g. measured fluorimetrically) is a mixture of constants, i.e., $K_d = (k_{-2}/k_2)/(k_{-3}/(k_3+k_{-2}))$.[37,88]

The next step, 3, and its rate k_3 (and of course k_{-3}) are perhaps the most elusive of the cycle. With the early crystal structures demonstrating the open and closed forms of DNA polymerases[92] came the view that this movement of the fingers domain constituted this step 3. However, this view has been questioned.[93]

How do we know that this step, whatever it is, really exists? Part of the argument is simple logic, that some repositioning of the appropriate dNTP is in order, as a part of an "induced fit" mechanism. However, this could be a very rapid process that would be kinetically silent. Another line of evidence is the long-standing observation, repeated with many systems, that substitution of a dNTP with an α-thio dNTP does not affect the rate of normal base incorporation but has a strong effect on the rate of misincorporation.[184] The assumption has been made that the only difference between a dNTP and an α-thio dNTP is the bond strength, akin to considerations of kinetic isotope effects.[185] This result, then, would mean that a step preceding phosphodiester bond formation in normal incorporation must be rate-limiting, in that the pre-steady-state kinetic analyses in which these effects are revealed measure only steps 2−4 in the burst phase.[88] However, the point has been raised that the effect seen in the experiments with α-thio dNTPs may reflect poor geometry in an intermediate rather than only bond strength.[186] Another line of evidence for the existence of a discrete step 3 comes from comparisons of results of pulse quench and pulse chase experiments.[80,93,187] The logic is that greater incorporation in a pulse chase experiment can only be rationalized by the existence of slow steps on both sides of the step in which the chemistry of product formation occurs.[93,187,188] Thus, we can conclude that a kinetically distinct conformation change step really does occur, even if it cannot be seen directly.

The next step, 4, with its rate k_4, is phosphodiester bond formation and is probably irreversible with most DNA polymerases, aside from movement of the oligonucleotide to an exonuclease site for proofreading. As mentioned earlier, steps 2−4 can be isolated in a typical pre-steady-state kinetic experiment in which product formation is measured, but unfortunately the analysis cannot clearly discern k_3 and k_4. Although some changes in fluorescence signals have been studied, none of these appear to have been clearly assigned to individual steps yet. Moreover, as pointed out by Joyce,[93] these seem to be rather variable among the polymerases.

The steps designated with the rate constants k_5 and k_6 are largely unstudied, except in a few "reverse" mode settings (e.g. instituted by PP_i addition to run the system backward).[189] These steps follow product formation and are kinetically invisible in the forward reaction. The steps are obvious even if silent, in that pyrophosphate must be released and, if a conformational change occurs in the first part of the cycle, then a relaxation must occur to restore the enzyme to the initial state.

The last step in the steady-state reaction is the release of the oligonucleotide product, unless all four dNTPs are present and processive synthesis occurs. In normal single nucleotide incorporation experiments with most polymerases, one observes a rapid "burst" phase and then a slower, linear phase (Scheme 5D). The results clearly indicate that a step following formation of the measured product is rate-limiting, and the "off" rate of the product is usually considered to be the main contributor to this. In principle, this rate should approximate k_{cat}. The k_{off} rate (k_7) has been measured by rapid

quench trapping experiments in several cases and found to be close to the k_{cat} or the value predicted by fitting data to kinetic models.[37,38,86,90,91,97]

The reader should note that steps 1 and 7 are not really different, except for the 1-base difference in chain length. Therefore models of the kinetics should have similar values for these parameters or explanations as to why differences should exist.

6.3. Checkpoints in the Catalytic Cycle

As an extension to the above discussion of the normal polymerase cycle, the steps can be considered at which the enzyme might pause to determine if all is well and the cycle should proceed, to be a bit anthropomorphic. The checkpoints have been reviewed recently by Joyce and Benkovic[93] and are related to the above discussion of the cycle.

One checkpoint is with a lesion so bulky that the DNA polymerase would not be able to admit it, e.g. large molecules cross-linked to DNA (*vide infra*). Another, and probably the key one, is whether a dNTP can be sensed to interact with the base to which it is supposed to pair (step 3). If an acceptable match is not sensed, then the enzyme does not position itself to try the reaction.

The coupling of steps 3 and 4 in the catalytic cycle is an issue. It could be proposed that step 4 is another checkpoint, if the proper geometry is not sensed. However, this may already be considered to a part of the step 3 sensing. One issue related to this is the possibility that the steps labeled 3 and 4 in Scheme 6 have an effective "reversal".[93] An alternative to the view that appropriate binding of a dNTP is sensed and generates a conformational change is that the (two) conformations are always in dynamic equilibrium and that proper dNTP binding to one entity distorts the equilibrium to the favored one.[93]

Another checkpoint occurs if a mismatch is made and leaves an unfavorable geometry for the next step. For all polymerases, extension of a mismatch among the canonical four bases greatly retards the following step. This phenomenon is actually used with some polymerases in strategies for the detection of genotypic variants. The situation can be altered with carcinogen-modified DNA bases, in that the pair with the "correct" base may not extend but the "wrong" base may give an appropriate geometry for extension. For instance, pol T7 readily extends an 8-oxoG:A pair but not an 8oxoG:C pair.[73]

When a DNA polymerase pauses at a checkpoint, there are three major possible outcomes: (i) The polymerase is halted but the DNA stays with the enzyme long enough to allow for replication to occur, but the observed rate is slower. (ii) If the DNA polymerase has an exonuclease domain, the DNA may be moved there for digestion. (iii) If the replication is blocked strongly enough, the polymerase−DNA complex will dissociate. Measured k_{off} values (k_7 of Scheme 6) of processive DNA polymerases are on the order of 0.1−1 s^{-1},[37,38,86,90,91,97] corresponding to a $t_{1/2}$ of ∼1−10 s. Thus, a polymerase has a limited amount of time to deal with an obstacle.

6.4. Alteration of Catalytic Steps by DNA Adducts

The topic of this review is DNA−carcinogen adducts, and their effects on DNA polymerases will be considered here.

Many steady-state studies have been done with various combinations of adducts and polymerases. A very general effect is that the K_m for the dNTP increases, usually more than the k_{cat} for the reaction decreases. Doing such experiments is not criticized, in that with many bulky adducts pre-steady-state experiments effectively become steady-state experiments anyway.[37,38,97] However, the interpretation of these parameters is very difficult.[88,98] The K_m for a certain dNTP should not be a measure of the affinity of the dNTP for the DNA polymerase. We do know that the fitting of the "correct" dNTP opposite a template base is impaired by adduction of the site, although isolating a parameter that measures this change is very difficult. Without more knowledge about the mechanism, one also does not know if k_{cat} is ∼k_7 or if k_{cat} is ∼k_3 or k_4. Thus, these parameters (k_{cat}, K_m) are of limited use except as primary screens of the tendency to misincorporate[72] or as preliminary data for other experiments.

One step postulated to be altered with modified DNA is that for k_{-1} and therefore $K_{d,DNA}$. That is, the affinity of some polymerases for DNA has been reported to be lowered after adduction.[190−193] In our own studies with both small[86,94,95] and bulky[37,38,97] adducts, we have not seen a pattern of this type. Several other studies have relied on gel shift experiments, which are rather unreliable for quantitative estimates of affinity in that a thermodynamically stable equilibrium is not established (the free concentrations of the components continue to change using the experiments). However, fluorescence titrations are simple and do proceed in an equilibrium situation. Kinetic estimates have a different basis but are also sound. Further, direct k_{off} measurements have not shown any major differences.[37,38,86,97,194]

The main steps of concern are those for k_3 and k_4, the rate constants of conformational change and phosphodiester bond formation. Relatively few pre-steady-state kinetic analyses of DNA polymerase action have been reported with carcinogen-modified DNA. Some of the first work was done by Lindsley and Fuchs[194] on 2-aminofluorene (2-AF) and 2-AAF C^8-G adducts, but the rates were very slow at the sites. Another early study by Tan et al.[195] involved O^6-MeG and the Klenow fragment and led to the conclusion that the changes were in either k_3 or k_4 of the catalytic cycle (Scheme 6).

Misincorporation and extension past 8-oxoG have been studied in this laboratory.[73,81,94,95] The results with several polymerases show that several steps are not affected and that the steps affected most by the substitution of 8-oxoG for G are steps 3 and 4, although distinguishing between these steps has only been done by thio effects and fitting to models. The conclusion was presented that the rate of extension of pol $T7^-$ (in the first reaction) past an 8-oxoG:A or 8-oxoG:C pair was limited by the rate of conformational change instead of phosphodiester bond formation, although this conclusion depends on the correct assignment of the observed fluorescence change.

With calf thymus pol δ, the difference between misincorporation at G and 8-oxoG appeared to be due to change in the $K_{d,dNTP}$ and the rate (k_4) of phosphodiester bond formation.[82] An interesting finding was that PCNA was a much more critical factor for replication of DNA containing 8-oxoG than for unmodified DNA.

A study of replication past O^6-MeG and O^6-BzG by pol $T7^-$ and HIV-1 reverse transcriptase was done.[86,96] There was a clear effect of bulk on the rates of insertion, which was due in part to the increased K_d for the dNTP. The presence of a dNTP also lowered the k_{off} for the oligonucleotide.[86]

The effect of adduct bulk was also noted in work with pol T7$^-$ and HIV-1 reverse transcriptase with N^2-Gua adducts.[97] The kinetic burst was reduced by a Me group and abolished by an Et or larger group. Thus, the effect of bulk is more severe at the N2 position of Gua than at O6, even though the former leaves the atoms involved in Watson−Crick pairing available (but does change the electronic distribution). With very bulky adducts such as PAHs or exocyclic additions that obscure the Watson−Crick binding face of Gua, no kinetic bursts are observed.[37,38,97] Thus, the rate of polymerization has been slowed to be less than the k_{off} rate (k_7 of Scheme 6). When the burst is lost, less information can be obtained about individual events within the catalytic cycle.

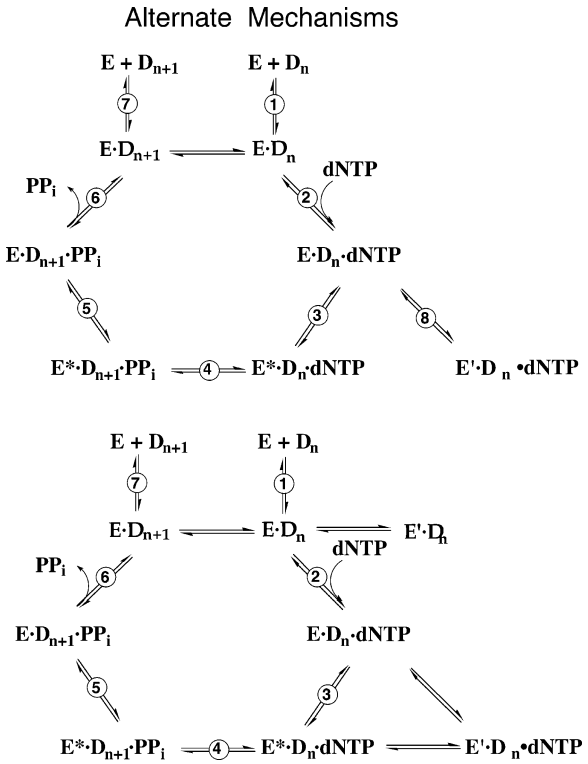
A number of studies by the Prakash group have examined various aspects of catalysis by some yeast and human translesion polymerases,[144,196−200] and in some cases pre-steady-state kinetics have been analyzed. With yeast pol η, incorporation of dNTPs into modified oligonucleotide systems is relatively slow compared to the case of the more processive polymerases (i.e. 1−2 s^{-1}, cf. 20−80 s^{-1}).[66,81,95,201] Also, the $K_{d,dNTP}$ values are much higher (27−100 μM, cf. 1−4 μM for the processive polymerases). An interesting result was that pol η inserted both bases (dATP) across from a T−T dimer as efficiently as for individual T bases.[201] However, with an abasic site no burst of incorporation was observed. On the basis of these results, the conclusion was made that both bases of the T−T dimer are present simultaneously in the active site. The analysis indicates that the rate-limiting step in copying either T of the T−T dimer is either step 3 or 4 of Scheme 6. Another interesting recent observation with yeast pol η is that the pre-steady-state k_{pol} and K_d are similar for incorporations of dCTP opposite G and 8-oxoG.[66]

Kinetic analysis of human pol ι has also been done.[200] The enzyme shows burst kinetics for normal incorporation but is not particularly inefficient ($k_{pol} = 0.6$ s^{-1}, $K_{d,dNTP} = 5$ μM). These studies argue that step 3 or 4 is rate limiting (Schemes 5 and 6). Bursts were not observed for misincorporation of the three nonmatched dNTPs. The authors concluded that opposite a template A the correct nucleotide (dTTP) is preferred because it is bound more tightly and incorporated more rapidly than the incorrect nucleotides. However, opposite a template T, both the correct (dATP) and incorrect nucleotides were incorporated at similar rates. The greater efficiency of dGTP incorporation relative to A was attributed to tighter binding of dGTP. Another conclusion was that the incipient base pair is accommodated differently in the active site of pol ι depending upon the template base; when T is the templating base, pol ι accommodates the wobble base pair better than the Watson−Crick base pair. This conclusion may be valid, although one general concern about the system is that dATP incorporation opposite template T is 30-fold slower than dTTP incorporation opposite template A[200] and other possible explanations may account for the difference.

6.5. Extra Steps in the Catalytic Cycle

In kinetic simulations, the general approach is to use a "minimal mechanism", that is the simplest one that can be used to describe the data (Scheme 6).[98] If extra steps are included, there is more uncertainty as to what the rate constants are. Many sets of polymerase kinetic data have been fit to the minimal mechanism (Scheme 6). However, several studies have proposed that the minimal mechanism does not adequately explain the altered kinetics observed with some unusual DNA substrates, e.g. DNA secondary structures,[83] DNA cisplatin and 8-oxoG adducts,[84,85] and natural "pause" sites due to hairpins.[202,203] Suo et al.[84] proposed an alternate polymerase−DNA binary complex that exists in equilibrium with the catalytically competent binary complex when either pol T7 or HIV-1 reverse transcriptase attempted to replicate DNA containing a cisplatin−DNA adduct (Scheme 11). Such an alternate conformation could explain

Scheme 11. DNA Polymerase Scheme Expanded To Include an Inactive Ternary Complex[85,97] [a]



[a] See Scheme 6 for a guide to symbols and the basic mechanism. (Reprinted with permission from ref 97. Copyright 2004 American Society for Biochemistry and Molecular Biology.)

the decreased affinity for dNTP seen with O^6-MeG.[96] However, an alternative explanation, used in our own work,[82,85,86,97] is that the alternative complex is a ternary one. Kinetic discrimination between the binary and ternary alternative complex models is not readily possible (see also discussion about whether dNTP binding is before or after the conformational change).[93] If the concept of a ternary alternate complex is accepted, discrimination of which other species would equilibrate is ambiguous.[85]

Several experimental situations have yielded substoichiometric bursts of product in pre-steady-state experiments, including the normal base incorporation by HIV-1 reverse transcriptase,[85] incorporation of C or A opposite 8-oxoG by HIV-1 reverse transcriptase and bovine pol δ,[82,95] incorporation of 8-oxo dGTP opposite template C,[36,85] incorporation of dCTP and dTTP opposite O^6-MeG and O^6-BzG by pol T7$^-$ and HIV-1 reverse transcriptase,[86,96] and incorporation of dCTP opposite N^2-MeG.[97] All of these sets of partial burst data were readily fit using the alternative complex model, with the ternary complex following step 2 (Scheme 11).

Some points should be mentioned. First, the concentration of polymerase must be known exactly. In work with HIV-1 reverse transcriptase, quantitative amino acid analysis was

used for this purpose.[85] However, even then the possibility can be considered that not all of the purified enzyme is active. In other studies with modified DNA, comparisons are made with the magnitude of the burst with unmodified DNA.[86,97] Also, in using the alternative model, only the rates of step 3 (conformational change) or step 4 (phosphodiester bond formation) and the step going to and from the alternative complex are allowed to change.[85,86]

Additional evidence for a reversible exchange with an alternate complex comes from single turnover experiments, which show biphasic kinetics. In this experiment (Figure 4),

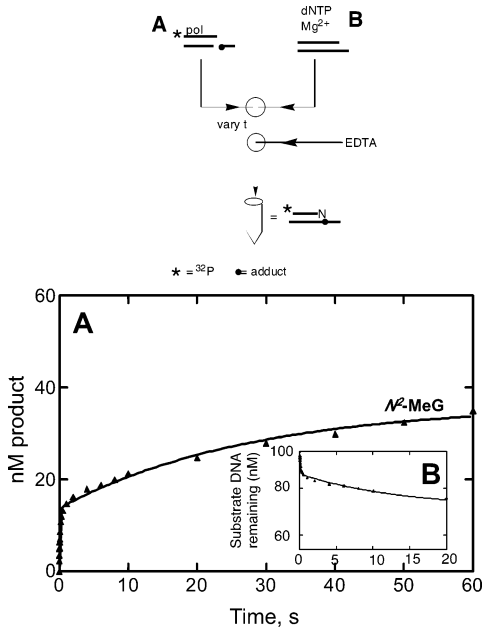


Figure 4. "Trap" experiment for restricting rapid kinetic analysis to the events occurring in the first catalytic cycle of Schemes 6 and 11.[83−86,97,202] The principle has some similarity to that presented in Scheme 7. A preformed complex of the DNA polymerase and a radiolabeled oligonucleotide is present in syringe A. The contents of syringe A are rapidly mixed with the contents of B, a dNTP−Mg^{2+} complex and an excess amount of an unlabeled "trap" oligonucleotide. The polymerization reaction is initiated by the (diffusion-limited) reaction of the polymerase−DNA complex with dNTP. When the catalytic cycle is completed, the radiolabeled DNA is dissociated and essentially only unlabeled DNA becomes bound to the polymerase; reactions occurring after the first cycle are invisible because no ^{32}P-label is present. (Reactions are quenched with excess EDTA.) With all radioactive products being generated before the second reaction cycle begins, the occurrence of multiphasic kinetics indicates that more than a single reaction cycle (Scheme 6) is involved. The phenomenon is rationalized by a pool of the DNA polymerase that is in equilibrium with the rest but converts slowly to the active form (Scheme 11). The biphasic nature of the data is clear in part A, and a semilogarithmic plot of the results is shown in the inset (part B). (Reprinted with permission from ref 97. Copyright 2004 American Society for Biochemistry and Molecular Biology.)

unlabeled trap DNA is added in the dNTP syringe so that only events occurring before the polymerase−oligonucleotide complex(es) persist are observed. The observations that two single-exponential steps occur in this reaction provides strong evidence that two forms of the polymerase complex are involved, and these experiments have been done in several cases.[83−86,97,202]

The kinetic analysis provides strong evidence for the existence of alternate polymerase−DNA complexes but does not indicate what the structures are, or even whether the conformational change is in the polymerase, the oligonucleotide, or both. Unfortunately none of the systems for which evidence for alternate conformations has been presented has been crystallized yet. However, some unusual pairing combinations have been observed thus far in the crystal structures that are available for other polymerase−oligonucleotide complexes. One possibility might be that the ternary complex consists of the dNTP bonded in a wobble or Hoogsteen pair which is in equilibrium with Watson−Crick pairing, which proceeds to yield product.

The existence of such alternate complexes is not excluded from other systems. If no kinetic burst is observed, then it is not possible to provide kinetic evidence for such an entity, even if it does exist and contributes to the reaction.

6.6. Polymerase Switching

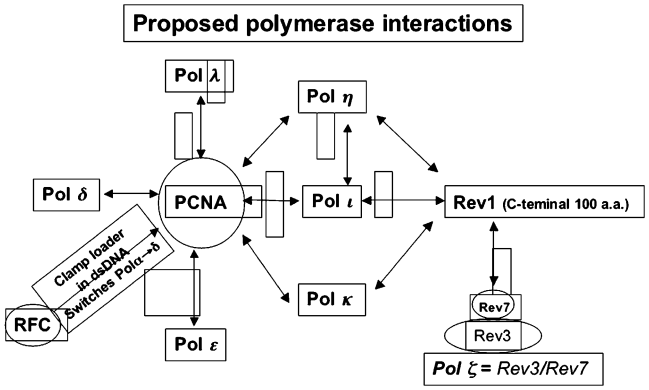
Some of the differences in the processive and translesion DNA polymerases have been discussed, in the sections on both general considerations of choices of polymerases to study and kinetic analysis. The resulting paradigm is this: a processive polymerase works along DNA until it comes to a lesion that blocks the polymerase because the rate of dNTP incorporation is slower than dissociation. One translesion polymerase now binds to the DNA and inserts a dNTP, or perhaps a few more before dissociating. The translesion polymerase should not operate for long on normal DNA because these polymerases tend to have high error rates when they copy normal DNA.[204,205] This sounds reasonable, but the question remains how. A 2002 review by Lehmann[206] outlines the problem: we do not know (i) if the replication machinery completely dissociates from the DNA at the site of damage or if it is temporarily displaced, (ii) how an appropriate polymerase is selected to carry out translesion synthesis (at a particular lesion), (iii) whether the replicative polymerase "hands over" to the translesion polymerase and the process is reversed immediately beyond the damage, or whether in some cases replication reinitiates beyond the damage, leaving a gap to be filled later by the translesion polymerase, (iv) whether lesions in the leading and lagging strands are handled differently, (v) what the functions of the ubiquitin-conjugating systems are, and (vi) how post-replication repair interacts with the cell cycle checkpoint mechanisms.

Some literature has appeared since that review. Fuchs and his associates have worked with *E. coli* pol IV and V and related the binding of these pols and pol III to DNA through the β-clamp.[180,207,208] Pol V is postulated to also interact with the tip of RecA. After a short stretch of synthesis by pol V, pol III is reassociated.[208]

Although many details still remain to be established in the *E. coli* model, mammalian DNA synthesis past damaged DNA is even more complicated and major questions remain (Scheme 12). Pols η and ι have both been shown to be localized to replication foci in cultured human XP-V cells.[215] However, the conclusion about pol η targeting pol ι to the replication fork does not resolve the issues raised by Lehmann.[206] That is, even if we imagine a large replication complex of all the processive and translesion polymerases together with the accessory factors at the DNA replication fork,[216] not all can be in the proper position at any one time.

Kunkel's laboratory has recently reported a series of experiments with *Saccharomyces cerevisiae* pols δ, ι, and η and T−T dimers.[87] These *in vitro* experiments appear to recapitulate the expected situation in terms of blocking,

Scheme 12. Proposed Interactions of Mammalian Polymerases with PCNA and Each Other[181,199,209−214] [a]



[a] The single-headed arrow for RFC (replication factor C) indicates its role in loading PCNA onto DNA (or long oligonucleotides). The double-headed arrows indicate postulated interactions among proteins.

incorporation by the translesion polymerase (pol η), and then extension by pol δ/ϵ. Apparently no PCNA was present in the system. PCNA is generally considered to act as a trimeric "clamp" around DNA to promote processivity.[217] A variety of effects have been seen with replicative and translesion polymerases, particularly with DNA damage.[81,82,199,212,218]

In principle, the experimental protocol of McCulloch et al.[87] could be extended into a system in which quantitative analysis is done (Scheme 13). A key question is what the "off" rates are for the individual polymerases, including the effects of individual DNA modifications. In principle, the kinetic issues could be addressed in basic *in vitro* systems, with and without DNA.

However, the system may be more complex *in vivo*, if other accessory factors are involved or if a known accessory factor is modified. Evidence exists that ubiquitin and SUMO modification have effects on PCNA that modulate the functions of yeast pols η and ζ.[215,219,220] The Lys164 residue of PCNA has been proposed as a site for both ubiquination and SUMO modification in a yeast system.[221] These studies were done in yeast cells. Unfortunately the preparation of specific ubiquitin- and SUMO-modified PCNA molecules is not trivial, at least in amounts sufficient to do sophisticated *in vitro* experiments with defined reagents.

Ultimately both *in vitro* and *in vivo* experiments will be needed to fully address the questions raised by Lehmann.[206]

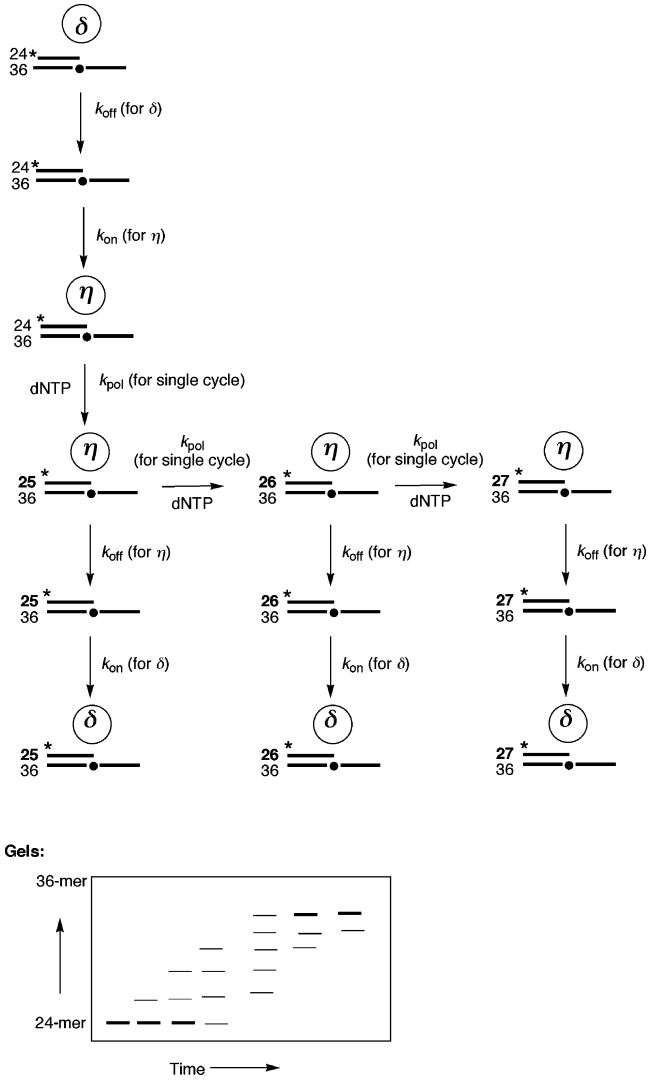
7. X-ray Crystal Structures of DNA Polymerases

7.1. General Features of DNA Polymerases

Until recently most of the work on the structures of DNA polymerases was done primarily with the Klenow fragment and mammalian pol β, plus some on replicative polymerases. Before consideration of structures of polymerases bound to modified DNA, it is useful to briefly consider the general features. For an earlier and much more extensive review, see ref 222.

Briefly, DNA polymerases have a well-characterized general structure that resembles a right-hand with fingers, palm, and thumb domains. The DNA sits in the palm and is contacted by the thumb. The polymerase has to be able to bind all four of the dNTPs, but this is a nonspecific initial reaction. When the correct dNTP is sensed at the template site, the "fingers" domain of the polymerase closes to convert the "open" form of the polymerase to a "closed" form.[92] This

Scheme 13. Strategy for Analysis of Rates of Association and Dissociation of Polymerases at Blocked Sites on Replication Forks[a]



[a] The experimental procedure involves incubating a 5′-end-labeled (*) primer−template complex (and all 4 dNTPs) with a mixture of pol δ and pol η in this case (± PCNA and any other factors), with analysis of the (radioactively labeled) products by gel electrophoresis/phosphor imaging. The time course of the formation and disappearance of individual products will be fit to a kinetic model with the rate constants k_{on}, k_{off}, and k_{pol} for pol δ and pol η (using DynaFit or other software programs[98,188]), and the parameters can be compared with those obtained in analyses using only single dNTP incorporations.

change has been sensed for many of the processible polymerases (but may not be part of the mechanism for translesion DNA polymerases, *vide infra*). This change had been associated with the induced fit or conformational change implicated in kinetic experiments, but today there is some controversy about whether this open/closed change is involved or some other step constitutes the conformational change.[93]

The DNA polymerases studied to date bind two metals, one of which appears to dissociate and reassociate in every catalytic cycle.[223] During catalysis under normal conditions, this metal is Mg^{2+}, but Ca^{2+} can be substituted in structural studies (to prevent catalysis).

Before considering the carcinogen DNA adducts, it is useful to briefly consider mismatch errors and the structural basis in polymerases. The chemical issues were considered

earlier in this review. Johnson and Beese[224] have used the thermophilic *B. stearothermophilus* DNA polymerase large fragment (termed "BF") and reported an extensive study of the structures of all possible 12 mismatches. The authors suggest four mechanisms that lead to stalling at the mismatches: (i) disruption of the template strand and preinsertion site (G:T, G:G, A:C, T:C), (ii) disruption of the primer strand and assembly of the catalytic site (T:T, C:T), (iii) disruption of the template and primer strands (A:G, T:G), and (iv) fraying of the DNA at the insertion site (A:A, G:A, C:C).[224] The pairing involves several components including wobble pair and *syn*−*anti* pairing. Three of the 12 mismatch structures were disordered at the site of the mismatch, suggesting the presence of multiple species and an equilibrium. Johnson and Beese also observed the effects of extending a mismatch up to 6 bp away from the primer terminus; long-range distortions in the DNA transmit the presence of the mismatch back to the active site. The authors conclude that while the binding interactions of equivalent correct base pairs are identical, as shown with this polymerase,[224] the various mismatches should all interact in unique ways with the polymerase. In light of this, we should expect a wide variety of modes of interactions of the myriad of DNA adducts with even a single polymerase, and the complexity will be even greater as more polymerases are considered. Nevertheless, the results of studies done over the next few years will probably be able to be organized into some common general modes.

7.2. Structures of Processive DNA Polymerases Bound to Carcinogen-Modified DNA

Characterizing structures of DNA polymerases in contact with DNA adducts has only been realized in the past few years, despite many earlier efforts. The choices of the right polymerase, DNA modifications, and oligonucleotide sequence are all interacting, and critical factors for success and are also empirical. Another point is that structural information is best understood when coupled with studies on the characterization of enzymatic events (i.e. sequence analysis of products) and kinetics.

Using the *B. stearothermophilus* BF replicative DNA polymerase, Beese and her associates have characterized structures with 8-oxoG and a PAH. The 8-oxoG results[132] indicate that an 8-oxoG:A pair is preferred and explain the kinetic proclivity for this outcome (which is relatively general among DNA polymerases, though to different extents).[36,94,95,225] As in the case of NMR and X-ray crystal structure work done in the absence of polymerases,[69] a favored mode involves a Hoogsteen pairing of 8-oxoG and dATP facilitated by adapting a *syn* conformation for 8-oxoG.[132] An *anti* conformation is used in pairing opposite C (dCTP), including template distortion in the DNA and the polymerase, which prevents the next template base from occupying the preinsertion site.

The same polymerase (BF) has been crystallized with a PAH derivative, the most common adduct derived from reaction of benzo[*a*]pyrene diol epoxide with DNA (an N^2-guanyl adduct).[226] In this structure the PAH adduct is paired with a C. This polycyclic adduct adopts a conformation that places it in the minor groove, leading to extensive disruptions between the DNA and the polymerase.[226]

Kisker and her colleagues have utilized a different replicative DNA polymerase and reported structures with two oligonucleotides, one containing an abasic site and one containing an 8-oxoG:dCTP pair.[65] The RB69 polymerase is a member of the B family. With a primed abasic site, a novel open and catalytically inactive conformation of the polymerase was observed, which may explain the blocking effects of these sites. With the 8-oxoG:dCTP pair, the anti conformation (of 8-oxoG) was observed and the ternary complex was reported to be almost identical to the normal G:dCTP system.[65] This latter result appears to contrast to the work with 8-oxoG and the BF polymerase,[132] but a direct comparison of the kinetics of the two polymerases and their tendencies to insert A vs C has not been done.

Ellenberger's laboratory[133] has also reported crystal structures of 8-oxoG-containing DNA with a DNA polymerase, pol T7 exo$^-$, for which considerable kinetic information is available.[36,73,95] With this enzyme, dCTP insertion is considerably preferred over dATP insertion.[95] Brieba et al.[133] were able to crystallize pol T7 with dCTP inserted opposite template 8-oxoG, but not dATP. In the structure, the O8 atom of 8-oxoG was tolerated due to the strong kinking of the DNA template. A model with dATP present predicts strong clashes that would attenuate (but not eliminate) A insertion. However, in this model, if A were inserted, it would be predicted to be paired with 8-oxoG in a Hoogsteen pair and the minor groove surface of the mismatch would mimic a normal G:C pair.[133] These results provide a reasonable explanation for the kinetic results.[95] In reviewing the results of the earlier pol T7 exo$^-$ kinetic results from our own laboratory,[95] it is important to note that the pre-steady-state results provide a better index of the discrimination between the insertion of dCTP > dATP than do the steady-state parameters. However, in most cases with DNA adducts, particularly bulkier ones, kinetic bursts are not seen and the assays effectively all become steady-state analyses.[37,38,97]

Ellenberger's group has also published structures of pol T7 with oligonucleotides modified with the bulky C^8-Gua adducts derived from 2-AF and 2-AAF. Previous studies with these adducts have indicated that the 2-AF adduct is blocking and causes mismatches while the 2-AAF adduct is very blocking and, when bypassed, causes frame shifts.[227] Similar results have been observed in attempts to do pre-steady-state kinetic analysis with pol T7 and these adducts.[194] The crystal structures (two with 2-AF, one with 2-AAF) show that the 2-AAF adduct adopts a *syn* conformation that leads to intercalation of the fluorene entity into the fingers domain and keeps the polymerase in an open configuration.[134] This result is proposed to be linked to blocking and frame shifts. The 2-AF crystals did not have well-defined electron density at the adduct, presumably reflecting considerable mobility.

As mentioned earlier, pol β is not a particularly good model for misincorporation opposite DNA adducts in that such events are rather unrelated to its function. However, a structure of pol β bound to a mispaired oligonucleotide has been published.[228] In the mispairs (both A:C and T:C), the two bases stack partially rather than engage in any kind of hydrogen bonding with each other. Instead of closing, as in normal incorporation, pol β adopts a partially open conformation that does not facilitate catalysis. In another structure of pol β with an 8-oxoG in the template,[229] the modified guanine residue is in the normal *anti* conformation and forms Watson−Crick hydrogen bonds with an incoming dCTP. To accommodate the oxygen at C8, the 5′-phosphate backbone of the templating dCTP is flipped 180°. The flexibility of the template sugar−phosphate backbone is one of the parameters that influences the *anti*−*syn* equilibrium of

8-oxoG.[229] In this work, a crystal was also obtained with damp opposite the template 8-oxoG, with the 8-oxoG also in an *anti* conformation.[229] The point should be made that with pol β there is always a sharp bend in the DNA as it sits in the polymerase active site, which may affect its behavior relative to other polymerases. (See note added in proof regarding Dpo4 structures with 8-oxoG.)

7.3. Crystal Structures of Translesion Polymerases

7.3.1. Dpo4

The translesion DNA polymerases quickly became popular targets for structural studies because of their ability to bind and process modified DNA. The archebacterial *S. solfataricus* polymerase Dpo4 is readily expressed, purified, and crystallized and has been used by several groups. The first structure was that of Ling et al.[167] and involved a ternary complex of Dpo4 with an oligonucleotide and ddATP. The structure resembles those of previously characterized DNA polymerases in having a palm/thumb/fingers structure, but there are two major differences: (i) the active site area has considerably more space for the DNA (Figure 3), and (ii) the structure contains an additional "little finger" domain.[167] Mutagenesis experiments have demonstrated that this little finger domain imparts some specificity to the different translesion polymerases.[230] Yang[231] has recently reviewed the available structures of Dpo4.

The Dpo4 structure was solved in the presence of a correct and an incorrect incoming dNTP (1.7 and 2.1 Å, respectively).[167] Dpo4 makes limited and rather nonspecific contacts with the replicating bases involved in the pair, thus limiting the base selectivity. The structures also reveal capture in the translocation of two template bases to the active site at once.[167] The so-called "type I" and "type II" structures exhibit different active site configurations. In the type I structure, only one template residue is accommodated in the active site pocket, coding for d(d)ATP (opposite T). The DNA minor groove faces the protein in the active site that is unusually accessible due to the small amino acid residues (Gly41, Ala42, Ala44, Ala57, Gly58). In type II structures, two adjacent template bases are admitted into the active site simultaneously. Thus, a T−T dimer could be accommodated (*vide infra*). To bypass such a lesion, Dpo4 may skip the first base and replicate only the second.

Dpo4 is inefficient in extending all mismatches. Trincao et al.[232] solved a structure of Dpo4 with a G:T mispair in the primer−template complex, in the presence of an incoming dNTP. A reverse wobble pair (Figure 5) deflects the 3′-hydroxyl of the primer strand away from the incoming dNTP.[232]

The first Dpo4 structure with an actual modified DNA lesion was that of a *cis*−*syn* cyclobutane thymidine dimer.[233] The two structures obtained can be considered in the context of the type I/type II discussion above. The 3′ T of the *cis*−*syn* T−T dimer forms a Watson−Crick base pair with the incoming ddATP, but the 5′ T of the lesion forms a Hoogsteen base pair with the ddATP in a *syn* conformation.[233]

Another Dpo4 adduct structure is that of a PAH adduct, a benzo[*a*]pyrene diol epoxide-N^6-adenyl derivative (Figure 6).[234] The ternary complexes have T opposite the modified A; attempts to place dATP, a preferred miscoding base, opposite this were unsuccessful. Two conformations were observed, one with the PAH moiety intercalated between base pairs and the other with the PAH extruded in the major groove and exposed to solvent.[234] In the former, the DNA is distorted. It is of interest to note that the rate of replication of the enzyme past the lesion was enhanced by the addition of organic solvent, which also appears to stabilize one of the conformations seen in the crystal structure.[234]

In another Dpo4 study, Ling et al.[235] addressed replication past abasic sites. The major outcome of bypass is a −1 frame shift. This phenomenon appears to be distinct from the pattern seen with the replicative polymerases (and *E. coli* SOS polymerases), which have a tendency to insert A in the mutational studies.[159] Ling et al.[235] solved a series of five structures, with different oligonucleotides, that present a story of the course of the polymerase copying. The group characterized structures corresponding to dNTP incorporation opposite the abasic site, a −1 frame shift, a +1 frame shift, and an "unproductive" complex. A major conclusion from this work is that the insertion step of translesion synthesis is highly template dependent, with the base 5′ to a lesion instructing dNTP incorporation, while the lesion itself is slipped out.[234] This pattern appears to apply with the adduct 1,N^2-ϵ-Gua (*vide infra*).

Another conclusion from the available work with Dpo4 is that this and other Y family polymerases do not show a discernible movement of the fingers domain upon binding of the correct dNTP. However, Suo has offered kinetic evidence that some type of induced fit process is operative with Dpo4.[188] The structural work shows that the little finger domain at the C-terminal undergoes rigid body movement, depending on the DNA substrate and the fit of the template base and incoming dNTP.[235] This movement (which may be linked to the "induced fit" process) is proposed to facilitate translesion synthesis and successive steps of nucleotide transfer. (See note added in proof.)

7.3.2. Dpo4 and 1,N^2-ϵ-Gua

Recent work in this laboratory has been done on the interaction of the model translesion polymerase Dpo4 with DNA containing 1,N^2-ϵ-Gua (Scheme 14).[42,105,236] 1,N^2-ϵ-Gua is one of the DNA adducts formed from oxidation products of vinyl chloride and other vinyl monomers.[237−240] This lesion is also formed from the oxidation products of fatty acids, resulting from lipid peroxidation.[241] In *E. coli* cells, the main outcome remains coding for dCTP insertion, and the main misincorporation is insertion of T (G to A transition), with A following (G to T transversion).[77] However, with several processive polymerases and *E. coli* pol I (Klenow fragment exo$^-$) and rat pol β, the residues C, G, T, and A were all inserted to some extent and −1 and −2 frame shifts were detected.[49] In a mammalian cell (chromosomal integration) study, a variety of mutations were seen including G to A transitions (insertion of T), plus some mutations away from the adduct site and rearrangements.[105]

1,N^2-ϵ-Gua has most of the Watson−Crick coding face covered with the added two carbons (Scheme 14). The results obtained with different systems are unusual and show some of the difficulties in studying these systems. In reactions with single dNTPs, Dpo4 inserts an A and then two more A's (Figure 7, Table 2). The last of these is opposite an A, and then the polymerase stops abruptly. However, if a mixture of all four dNTPs is used, a different result is seen (Schemes 15 and 16). The final product was a mixture, characterized using MS, and dependent upon the base 5′ of the adduct.[42] When a C was present 5′ of the 1,N^2-ϵ-Gua, two major

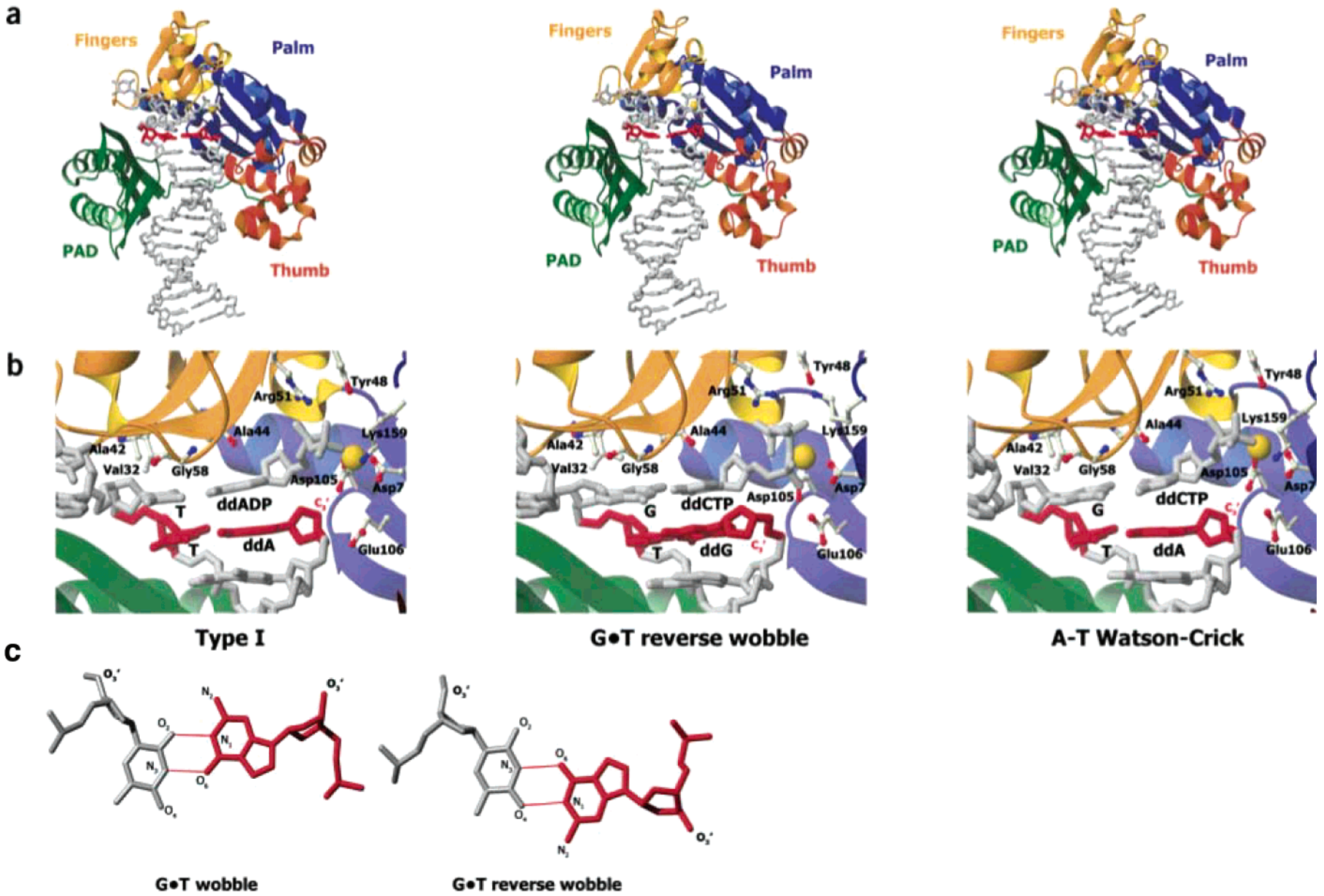


Figure 5. Dpo4 crystals with a G:T pair in a reverse wobble configuration.[232] Overall views of the different complexes are shown in part a. Close-up views are shown in part b. In part c, a comparison is shown for standard and reverse G:T wobble pairs (see Scheme 4). (Reprinted with permission from *Nature Structural & Molecular Biology* (http://www.nature.com), ref 232. Copyright 2004 Nature Publishing Group.)

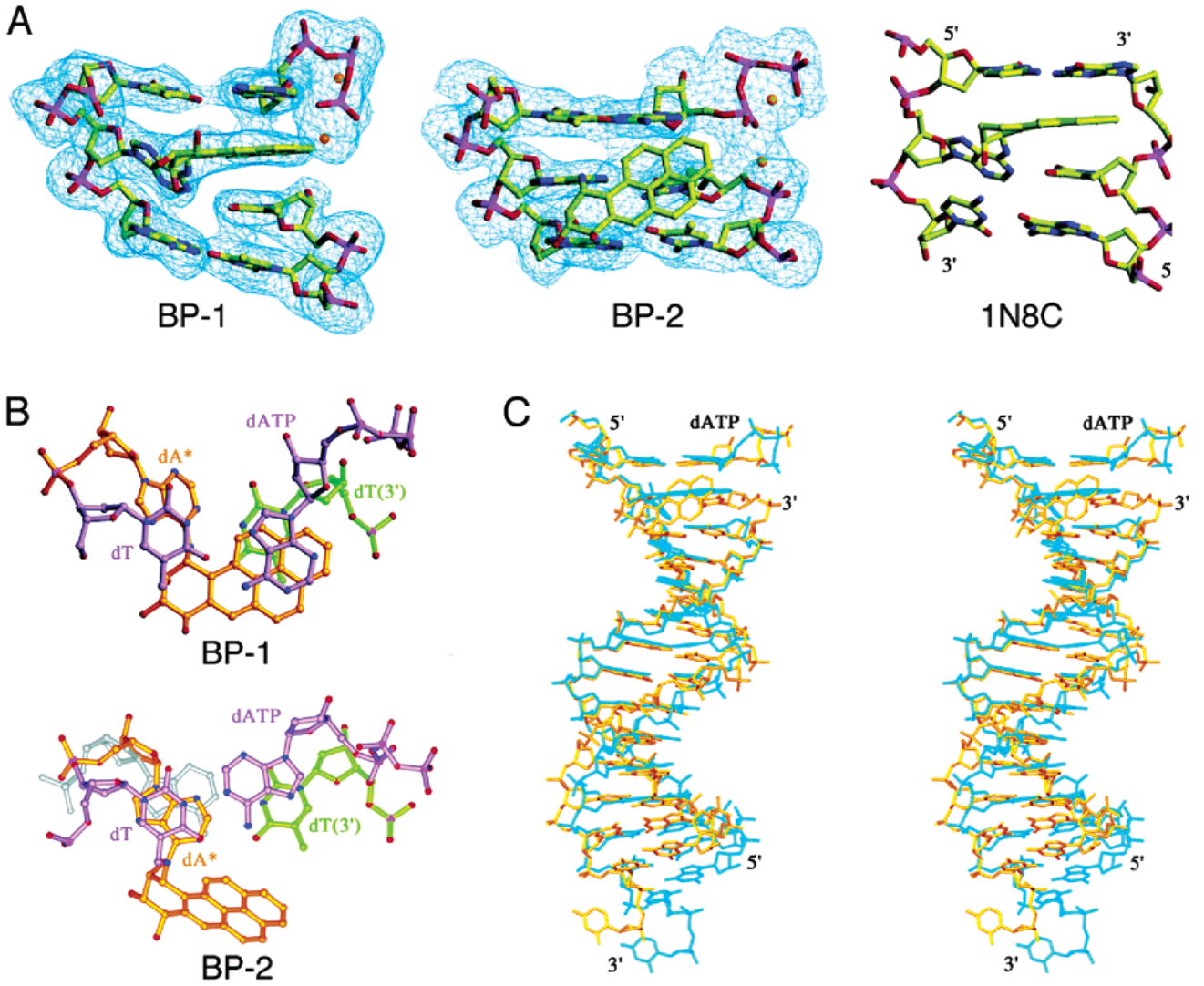
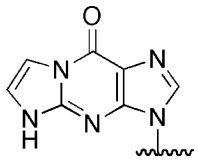


Figure 6. Dpo4 with an oligonucleotide with an N^6-Ade derivative obtained from (+)-(*7R,8S,9S,10R*)-benzo[*a*]pyrene diol epoxide.[234] (A) Comparison of the X-ray and NMR structures. The crystal structure is depicted with the electron density. The BP-1 and BP-2 forms are the two structures seen. (B) Hydrogen bond formation for the two structures. (C) Overlay of the BP-1 (blue) and BP-2 (gold) structures. See original reference for more description.[234] (Reprinted with permission from ref 234. Copyright 2004 National Academy of Sciences.)

Scheme 14. 1,N^2-ϵ-Gua[105,236,238] *a*



[a] This is one of the "etheno" bases, which can be derived from reactions of DNA with bis-functional electrophiles, particularly those generated from lipid peroxidation or from the oxidation of vinyl monomers (e.g., vinyl chloride, acrylonitrile). Other etheno adducts include 1,N^6-ϵ-Ade, 3,N^4-ϵ-Cyt, and N^2,3-ϵ-Gua.

products were obtained, as characterized by MS collision-induced dissociation (CID) analysis.[39] The minor product corresponds to a pairing of A opposite 1,N^2-ϵ-Gua, followed by correct insertion of the remaining bases opposite the template bases. The major product corresponds to a "skip" of the polymerase past the adduct and then accurate coding to yield a −1 frame shift (Scheme 15). However, when a T is 5′ of the adduct, then a mixture of four products was obtained (Scheme 16). The boxes in Schemes 15 and 16 indicate the intermediates for which X-ray structures were obtained.[42] See Figures 8 and 9 for structures of the Dpo4−DNA (1,N^2-ϵ-G)−dATP ternary complex shown in the boxes in Scheme 16A−C.

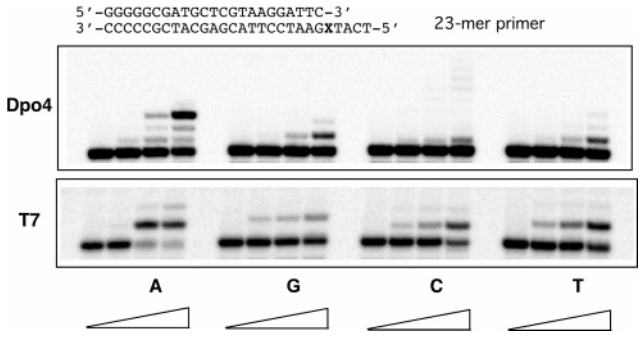
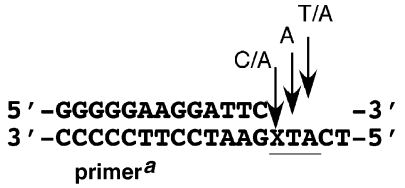


Figure 7. Insertion of bases opposite 1,N^2-ϵ-G by Dpo4 and pol T7$^-$. X = 1,N^2-ϵ-G. In each case, 100 nM primer−template (primer ^{32}P-end-labeled) was incubated with the indicated dNTP (A, G, C, or T; 250 μM) at 37 °C for 30 min with increasing concentrations of purified Dpo4 or pol T7$^-$ (thioredoxin) (0, 25, 50, or 100 nM), as depicted with gradient wedges from left to right. (Reprinted with permission from ref 42. Copyright 2005 American Society for Biochemistry and Molecular Biology.)

It should be emphasized that the approach of defining the product under these conditions is one that is not normally

Table 2. Incorporation of Nucleotides Opposite and Following 1,N^2-ϵ-G by Dpo4[42]



dNTP inserted	template	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m
dC	5′- 3′-**G**TA	4.7 ± 0.4	6.4 ± 0.13	1.4
dC	5′- 3′-**G***TA	96 ± 14	0.0057 ± 0.0002	0.6 × 10^{-4}
dA	5′- 3′-**G***TA	33 ± 5	0.027 ± 0.001	0.0008
dA	5′-A 3′-G***T**A	46 ± 6	0.15 ± 0.01	0.0033
dA	5′-A A 3′-G*T**A**	88 ± 11	0.29 ± 0.01	0.0033
dT	5′-A A 3′-G*T**A**	140 ± 22	2.0 ± 0.15	0.014

[a] The underlined section of the primer is shown in the table, with insertion opposite the bold.

done. In work with longer DNA, the product could be analyzed by fluorescence-coupled nuleotide sequencing. However, this approach has not been applicable to short oligonucleotides, unless they are ligated into longer pieces of DNA. Maxam−Gilbert sequencing can be used on short pieces of DNA and we have done this in the past,[49,77] but the results are not always clean and mixtures are a major problem. In work with longer DNA, the product could be analyzed by fluorescence nucleotide sequencing, but this is also difficult with mixtures. MS, used here, provides a much superior analysis of sequence.

Thus, the results obtained with single dNTP analyses appear to be misleading (Figure 7). Mechanisms to explain the observed results (with all four dNTPs) are presented in Schemes 15 and 16.[42] The proposed mechanisms are corroborated by the X-ray crystal structures.[42] Thus, the mechanisms involved include direct coding for A opposite the adduct (1,N^2-ϵ-Gua), what appears to be a "dNTP-stabilized misalignment",[242] an apparent variant of the latter that produces a 2-base deletion, and a more complex mode of the dNTP-stabilized misalignment that involves rearrangement of both the primer and the template (Scheme 16B). The intermediates in Scheme 16B are apparently stable; the two for which crystal structures have been obtained both contain the A:T pair with the 1,N^2-ϵ-Gua bypassed but not bulged out. The A:T pair has classical Watson−Crick pairing. This structure has some semblance to the Dpo4 work of Ling et al.,[167] suggesting that the active site of Dpo4 can accommodate two template bases. In the work with the T−T photo-cross-link, the entire dimer appears to occupy the active site and pairing to the "second" of the T's can occur in the absence of pairing to the first.[167,201]

These results demonstrate the difficulty of understanding the molecular details of events involved in catalysis. Thus, a mixture of kinetic and structural studies must be considered along with analysis of products. The methods applied here include steady-state and pre-steady-state kinetics, X-ray crystallography, and MS as well as the organic and analytical chemistry and enzymology needed to prepare the reagents.

Dpo4 has been a useful model for translesion polymerases and, because of the relative ease of purification and crystallography, of the interaction of carcinogen-modified DNA with DNA polymerases in general. Other Dpo4 crystals obtained, in collaboration with Prof. M. Egli, at the time of this writing include 8-oxoG and 8-hydroxypropano Gua. The MS CID analysis approach has also been applied to several adducts. 8-OxoG is essentially non-miscoding, even more so than pol η[66] or pol RB69.[65] O^6-MeG gave ~$^1/_3$ incoroporaiton of T with Dpo4. The C^8-guanyl adduct derived form the heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ) in the *Nar*I sequence[243] yielded a complex product, which involves multiple frame shift realignments (Zang, H., Stover, J., Rizzo, C. J., and Guengerich, F. P., unpublished results).

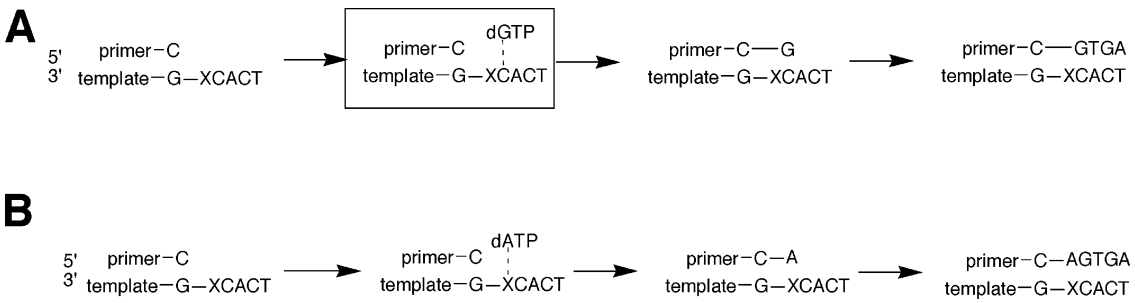
7.3.3. Other Translesion Polymerases

Three other translesion DNA polymerase structures are available, all coming from the Prakash/Agarwal groups. At the time of preparation of this review none of these polymerases has been crystallized with a DNA adduct.

A *S. cerevisiae* pol η structure has the classic right-handed palm, fingers, and thumb domains plus a C-terminal "polymerase-associated domain" (PAD), which corresponds to the little finger domain of Dpo4.[244] The residues involved in catalysis were identified. A feature of this structure is that the fingers and thumb domains are relatively "short and stubby."

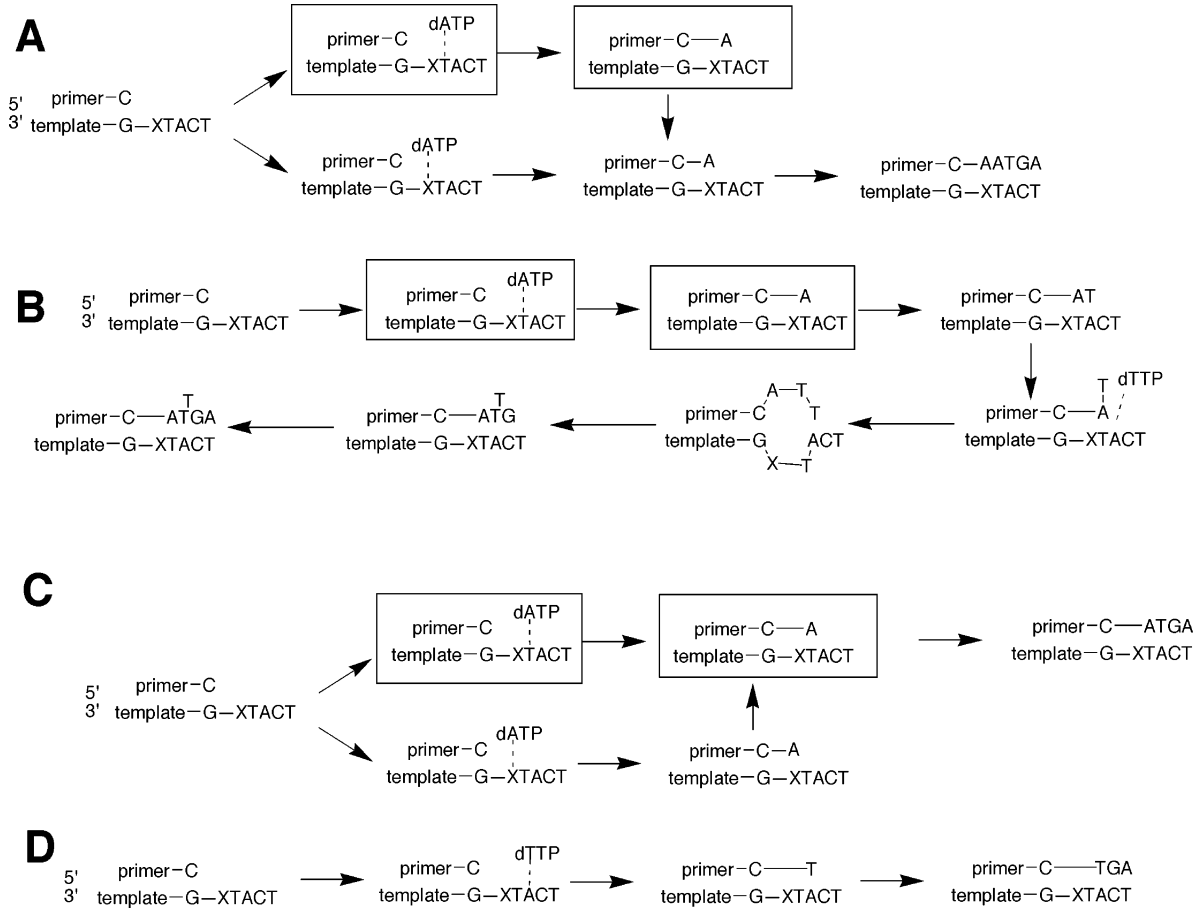
A human pol ι structure has been solved (2.1 Å).[245] The structure reveals two protein molecules bound per DNA, one at the blunt end of the oligonucleotide and the other at the replicative end. This latter active site has a template A paired

Scheme 15. Proposed Events in Incorporation of DNTPs into an Oligonucleotide Paired with 1,N^2-ϵ-Gua To Give the Characterized Products[a]



[a] In this setting, there is a C 5′ of the 1,N^2-ϵ-Gua. The MS analysis of the products indicated a 84:16 ratio of the products shown in parts A and B, respectively. An X-ray crystal structure has been solved for the intermediate shown in the box.[42] (Reprinted with permission from ref 42. Copyright 2005 American Society for Biochemistry and Molecular Biology.)

Scheme 16. Proposed Events in Incorporation of DNTPs into an Oligonucleotide Paired with 1,N^2-ϵ-Gua To Give the Characterized Products[a]



[a] In this setting, there is a T 5′ of the 1,N^2-ϵ-Gua. MS analysis indicated that the reactions shown in parts A, B, C, and D accounted for 31, 27, 24, and 18% of the products, respectively. X-ray crystal structures have been obtained for the intermediates shown in boxes.[42] (Reprinted with permission from ref 42. Copyright 2005 American Society for Biochemistry and Molecular Biology.)

to dTTP in a Hoogsteen mode.[245] This Hoogsteen pairing may explain the varying efficiencies and fidelities of pol ι at different residues. Nair et al.[245] propose that this is a mechanism for replication by this translesion DNA polymerase through minor groove adducts that are very blocking to other polymerases. Also Hoogsteen binding is a potential mechanism for "displacing" adducts that interfere with replication.[245]

A structure of the catalytic core of human pol κ has also been reported,[246] again without a DNA adduct. As with pol η, the fingers domain is "stubby". Other interesting features include the rather restrained nature of the fingers domain (regarding the fit of the DNA) and the presence of the polymerase-associated domain ("PAD") on the dorsal side of the palm domain. The suggestion is made that this polymerase has effective constraint during the incorporation/misincorporation event but less constraint after the insertion step.

8. Interaction with Cross-linked Elements

Knowledge about the interactions of cross-linked materials with DNA polymerases is relatively sparse. Some of the biological actions related to these involve more complex biological systems (e.g. recombination, which can lead to complex rearrangements and deletions).[125,247−249] The possibilities of processing of cross-links are still largely hypothetical. However, a few situations will be considered, and some systems bear consideration for study.

If two DNA strands are cross-linked together, it is difficult to understand how a polymerase would be able to copy past this, except a situation involving formation of a complex structure in which a triplex was formed with DNA folding back. One possibility is that a DNA−DNA cross-link could move into the active site of a polymerase and then decompose to a new entity that could be copied or could react with the polymerase itself. A hypothetical example is a N^7-guanyl:N^7-guanyl cross-link that could move into the active site of the polymerase, perhaps with some strain on the system, and then break the linkage to yield an abasic site or a FAPY adduct that could be copied. Presently this is a hypothetical situation. More viable possibilities are some of the systems studied by my own colleagues (Profs. Rizzo, Stone, Harris, Marnett) in which DNA adducts containing masked aldehydes can yield quasi-stable cross-links that could open again to yield reactive species.[248,250] For instance, the Gua malondialdehyde adduct (pyrimido[1,2-*a*]purin-10(3*H*)-one, termed "M_1G") is probably capable of such behavior.[125] In cultures of human embryonic kidney cells, mutations were dependent upon the presence of an active nucleotide excision repair system. This conclusion applied to base pair substitutions as well as the large deletions. DNA interstrand cross-links would not be expected, but conceivably the cross-link could rearrange to yield N^2-(3-oxopropyl)Gua or M_1G, the ring-closed form, which could then miscode. Another possibility is that the cross-link would decompose to a reactive aldehyde

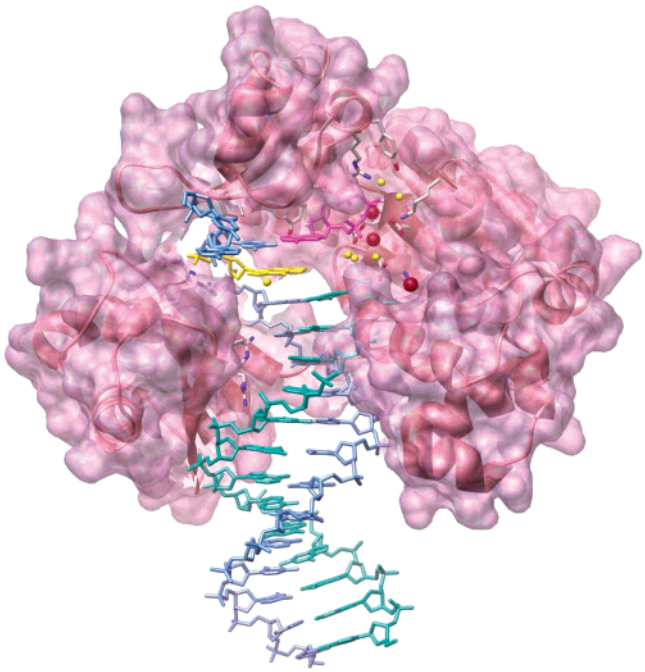


Figure 8. Dpo4−DNA (1,N^2-ϵ-G)−dATP ternary complex, to 2.1 Å. This is the first intermediate shown in the boxes in Scheme 16A−C. (Reprinted with permission from ref 42. Copyright 2005 American Society for Biochemistry and Molecular Biology.)

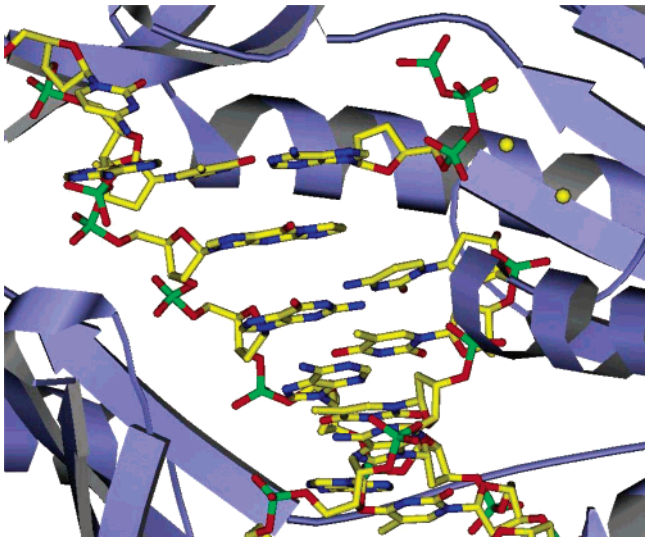


Figure 9. Expanded view of the Dpo4−DNA (1,N^2-ϵ-G)−dATP ternary complex. (Reprinted with permission from ref 42. Copyright 2005 American Society for Biochemistry and Molecular Biology.)

that would become covalently linked to the polymerase. In a model system, an oligonucleotide containing M_1G was mixed with the restriction endonuclease *Eco*RI and inhibited the enzyme and was covalently bound.[251] A similar result might be possible with a DNA polymerase.

Another example is one currently under investigation in this laboratory. Expression of AGT in bacteria greatly enhances the mutagenicity and toxicity of the potential bis-electrophile ethylene dibromide.[252] In collaboration with Prof. Pegg's group, we have shown that the phenomenon involves cross-linking of AGT to DNA.[122] However, there is a problem here in that a protein (AGT) of 25 kDa is cross-linked to the DNA and will be difficult to copy past. The mutations seen in bacteria are base pair mutations, so the mutagenic phenotype is probably not due to complex rearrangements.

How can cross-linking of a protein cause mutations? Part of the answer comes from an analysis of the tryptic peptides recovered after cross-linking to an oligonucleotide[123] (Scheme 17). Part of the fraction contained the active site Cys145 attached to an ethylene and then a Gua base.[123] This conjugate could be recovered following overnight heating at 37 °C (part of the typical trypsin procedure), a 60 min heat step (95 °C) at neutral pH, or hot piperidine treatment. The product results from an N^7-alkylation of Gua by the half-mustard generated from AGT reaction with ethylene dibromide (AGT−Cys^{145}−CH_2CH_2Br). Loss of the base due to destabilization of the glycosidic bond (due to N^7-alkylation) yields an abasic site. In an *E. coli* system, one would expect depurination to code for insertion of A and thus yield an increased fraction of G to T mutants in the mutation spectrum.[159] This was indeed the case when the mutation spectra were analyzed, compared with a control experiment devoid of AGT.[123] However, the explanation is not complete because (i) the G → T transversion increase amounts for less than half of the mutagenic events related to AGT expression, (ii) adducts with all of the four bases (A, C, G, T) can be formed, as indicated by the results of the cross-linking/gel shift experiments, and (iii) the recovered tryptic peptide−CH_2CH_2−Gua adduct accounts for less than half of the total radioactivity bound to DNA.[123] Further analyses are in order.

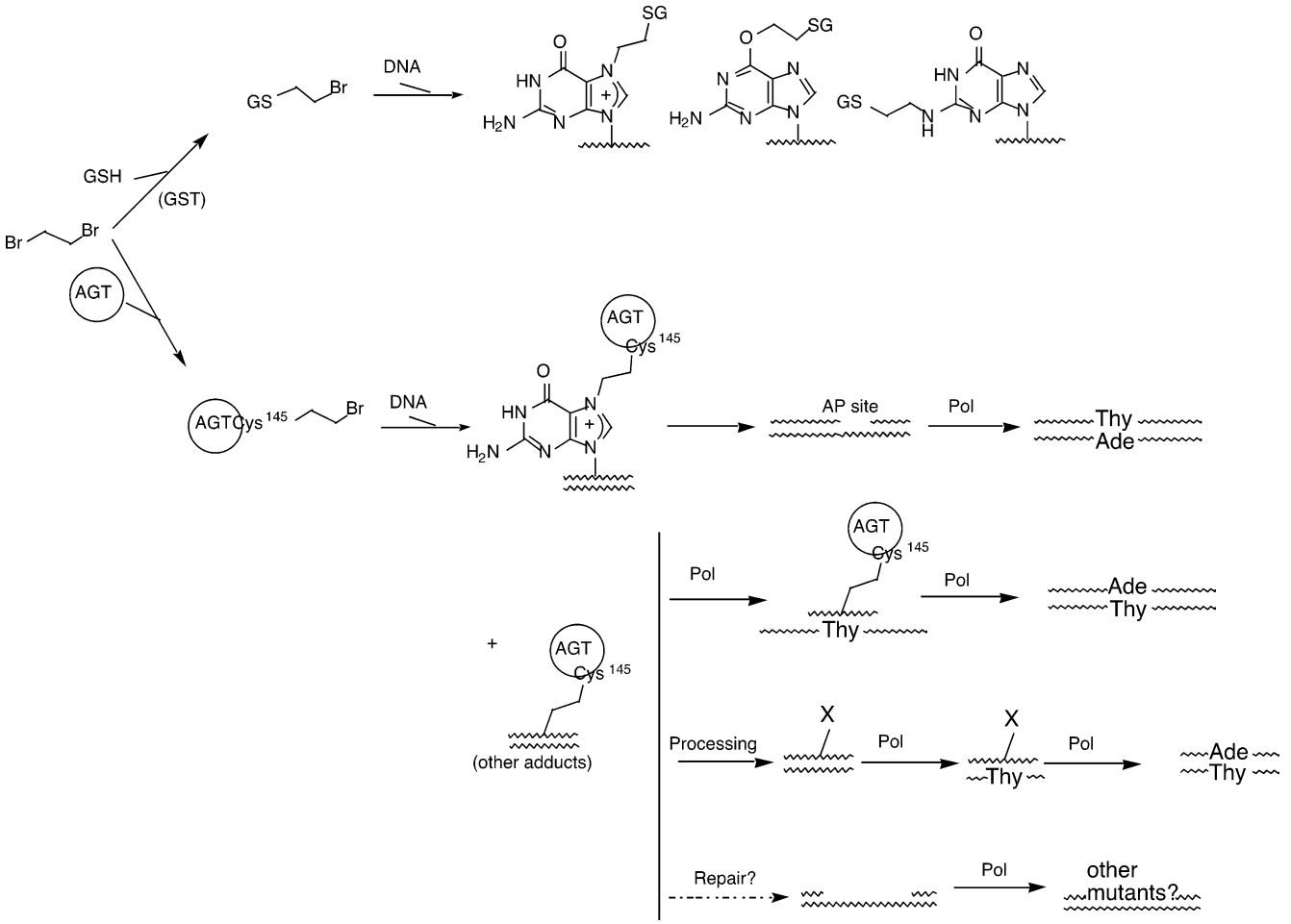
This same paradigm appears to apply to methylene bromide, diepoxybutane, and probably most other bifunctional electrophiles capable of producing cross-links in this manner.[124,157] AGT is involved in this phenomenon because of the high nucleophilicity of its active site Cys.[255] The possibility can be considered that other nucleophilic proteins are present near DNA and may also demonstrate this phenomenon. A search is in progress in this laboratory, in collaboration with Prof. D. Liebler.

The general question of how a bound protein on DNA can cause mutations is of interest, in that only some of the mutations can be attributed to the depurination mechanism.[123] The problem is that the dogma is that DNA is copied in a "double-stranded" manner.[256] However, only one of the strands of the original DNA is passing through the polymerase. However, even if a base were flipped out to allow for some type of Hoogsteen or other alternative pairing, the extra 25 kDa of AGT would presumably not be able to fit into the polymerase, even a translesion polymerase. The possibility has actually not been tested. Another possibility is that proteases digest the AGT in cells to leave only small peptides that enter the DNA polymerase and can cause mutations. That latter possibility has precedent in earlier ethylene dibromide work in this laboratory, in which GSH conjugates are mutagenic (Scheme 10). The protease hypothesis is experimentally testable.

9. Determinants of Mutation Spectra

Before concluding, it is of interest to consider the basis of mutation spectra, or "hot spots" for mutagenesis, in that one possible reason is the interactions of DNA polymerases with adducted DNA. The issue is of relevance in that many human tumors contain mutated genes, particularly p53.[20,257] Some of these mutations probably lie on the pathway of tumor initiation or development. Moreover, many efforts have been made to associate these mutational spectra with

Scheme 17. Events Proposed To Be Involved in the Activation of Ethylene Dibromide by GSH Transferases and AGT[123] [a]



[a] The GSH pathway involves the GSH half-mustard and an episulfonium ion intermediate[253] (not shown). The three guanine−ethylene−GSH conjugates all have the potential to block and miscode, at least with model polymerases,[71] although which of these adducts is most genotoxic in a cellular context is yet undetermined. The AGT pathway involves similar chemistry with the low pK_a Cys[145] group of AGT reacting with ethylene dibromide.[254,255] One of the sites of DNA conjugation is the N7 atom of guanine, and some of the mutations are explained by this modification and the resulting depurination (G to T transversion).[123] The sites of formation of the adducts to other bases and the mechanisms of mutagenesis, particularly the dominant G to A transition, are still under investigation. (Reprinted with permission from ref 123. Copyright 2004 American Society for Biochemistry and Molecular Biology.)

cancer etiology, particularly exposure to particular physical and chemical agents in the environment. Considerable evidence has been presented with UV light,[258−260] AFB_1,[261] vinyl chloride,[262] and tobacco.[21]

Topal[263] raised the prospect that the existence of mutation hot spots could be considered to be due to either selective chemical reactions by electrophiles or the "hiding" of adducts from DNA repair systems at certain sites. We have considered the molecular basis of mutation spectra in relationship to a particular chemical, ethylene dibromide, and refer the reader to the relevant discussion.[158] Five major phenomena can contribute to observed mutation spectra.

(i) The first issue is the binding selectivity of the mutagen. As indicated earlier,[158] this could be the result of electronic factors related to the sequence (e.g. electrostatic potential within runs of G's), effects of the sequence on the local structure in the DNA which in turn influence interaction with chemicals, and the structural influences of bound proteins. A relevant example may be the PAH-generated mutations in p53, which seem to be associated with enhanced adduction of CpG islands.[264]

(ii) Another possibility is a "secondary" change of an initial adduct. For instance, the conversion of an N^7-guanyl AFB_1 adduct to a FAPY derivative will change the biological properties,[265] as would depurination to yield an abasic site. The sequence location probably does affect these (biological properties) and other "postmodification" chemistry, although apparently the issue has not been addressed directly, to the author's knowledge. With some events, mapping techniques such as those we have used recently[158] could be useful.

(iii) Another possibility is the influence of the sequence context on rates of enzymatic DNA repair of an adduct. Evidence for sequence context effects exists with several DNA repair systems.[266] Cellular experiments have implicated such selective rates of DNA repair in the mutation spectra observed with UV light in mammalian cells[259] and for *S*-(2-chloroethyl)GSH in a yeast-based human p53 system.[158]

(iv) DNA polymerase activity context effects are relevant to the scope of this review. Several possibilities can be considered. There is already ample evidence that the course of action by a polymerase can be highly dependent upon the DNA sequence.[267,268] For instance, some sequences are inherently more prone to slippage and thus frame shifts.[269] The position at which a DNA adduct occurs can influence the blocking of a DNA polymerase, the rate and extent of misincorporation of a base(s) opposite the adduct (or frameshifts), and the proclivity of a polymerase to extend past the adducts. First of all, some aspects of the stacking interactions discussed under item 5.4 can be considered here as well. Different sequences confer different effects of

adducts on thermodynamic properties, e.g. of binding to complementary strands.[59,60,161] In one example, the extent of the perturbation of an O^6-MeG adduct was dependent on the sequence context.[270] Another example is the effect of a cisplatin intrastrand cross-link on the conformation and thermodynamic properties of DNA.[61] In this work the adduct could, in some contexts, convert the DNA from the B to an A form.

Another classic example for a sequence-dependent mutation effect comes from the work of Fuchs and his associates with 2-AAF−(C^8) guanyl adducts in the *Nar*I restriction site. Depending upon which of three closely located guanines is involved, the biological effects vary considerably.[227] With this bulky adduct, the bypass is very inhibited[194] and the major outcome (in bacterial systems) is deletion frame shifts.[227,271] Studies with chemical probes[272] and alternate sequences and T_m measurements[273] indicate a physical basis for some of the effects seen with adducts in this region. Further changes in the sequences (i.e., the neighboring bases in the *Nar*I site) also affect mutagenesis.[243]

Although such effects can be readily shown with isolated polymerase systems, the overall contribution to mutation spectra in cells is more difficult to ascertain. In principle this can be done using site-specific mutagenesis in cells with a null DNA repair phenotype associated with the adduct under consideration. For instance, if we consider C^8-guanyl-2-AAF adducts in the *Nar*I restriction site mentioned above, they produce well-characterized −1 and −2 frame shifts in SOS-induced *E. coli* but instead yield predominantly G to T transversion mutations in COS-7 cells.[271] Another consideration in all of this is what have been termed "action at a distance" mutations, which occur near but not opposite the DNA adduct.[105−107] The phenomenon is not well characterized but may contribute to the complexity of mutations.

(v) The last parameter affecting the mutation spectrum is the biology underlying the phenotype. In principle, one would like to eliminate this component in that it is independent of the exposure and confounds any etiological analysis of the system. However, this is probably an unrealistic goal with a complex protein such as p53, which continues to yield new biological functions. In experimental systems, one prefers to use targets in which many mutants show loss of function, e.g. *lacZ* or *rpoB*.[274,275]

These issues may not seem directly germane to the discussion of the basic mechanisms of interaction of DNA adducts with polymerases. However, this is an area of practical application and one of consideration of the significance of the basic events considered here.

10. Summary and Future Directions

What has been discussed here is the issue of DNA replication fidelity, discussed elsewhere[92,93,129,130] and in this series[126,276] as applied to considerations regarding carcinogen-modified DNA, in that this continues to be a major topic in the field of chemical carcinogenesis. A proper understanding of the interactions of DNA polymerases with carcinogens bound to DNA is an important component in understanding mutation spectra and their etiology, in making intelligent predictions about the proclivity of individual chemicals to cause cancer, and in the prediction of what genetic variations in particular DNA polymerases may mean in modifying risks of individuals to cancers.

As outlined here, the study of the interaction of DNA polymerases with carcinogen-modified DNA requires a battery of biological and chemical approaches. Some of the issues regarding the design of experiments have been presented to emphasize deficiencies in the field. The identity and purity of reagents are important considerations that do not always receive the attention they should. Also, an approach of publishing a research paper presenting data with only one adduct and one polymerase in the absence of a mechanistic explanation is no longer tenable. Work is needed in which (i) a single enzyme system is approached in a very mechanistic way and (ii) more general analyses of the roles of several individual DNA polymerases are done to define their contributions.

The physicochemical factors involved in base pairing have been reviewed again here, as well as in previous treatises.[130,135] These effects have been studied largely in the absence of catalysts (enzymes) and by themselves cannot explain the fidelity of DNA synthesis. However, DNA polymerases use and also amplify many of these forces in replicating DNA. In a review in 1997, Goodman stated "...the surface has barely been scratched in terms of understanding the interaction between polymerases and DNA that determines replication fidelity" and also "...precise molecular mechanisms governing mutagenic hot and cold spots remain obscure. Different polymerases copying the same primer−template DNA can exhibit markedly different mutation frequencies and spectra."[135] In the eight years since then,[135] significant progress has been made on some fronts, especially on the multiplicity of DNA polymerases and in the structural biology of relevant systems. Perhaps we can say that we have gone from a scratch to a dent (in the "surface"),[135] although we still have much to learn.

In recent years the interest in DNA polymerases has expanded. Among the areas of research are all aspects of the study of the translesion polymerases, several archebacterial DNA polymerases that have helped facilitate structural biology studies, detailed kinetic analyses of DNA polymerases interacting with modified DNA, site-directed mutagenesis studies, and determination of the structures of DNA polymerases bound to DNA. What has emerged thus far is some coupling of structural and functional studies, and this will improve in the future. Thus far we have seen several mechanism for coding and miscoding at DNA−carcinogen adducts. Some of these resemble the interactions with mispairs of the normal four bases,[224] but others do not.

What does the future hold and what are some of the major questions still to be answered? The author's opinion is that structural and functional studies will both continue to develop and that the best of these will be the ones that relate structure and function. In the relatively near future, more systems (DNA polymerases and adducts) will be characterized at a high level of sophistication. What will probably emerge is a set of categories into which the polymerase−DNA interactions can be classified. Beyond this, more studies on other adducts will be done but the work will be considered more "descriptive." However, many details of the interactions will continue to occupy basic scientists for a long time. We also need to consider the possibility that large gaps in our knowledge may be missing: 10 years ago the general concept of how the SOS system model worked was wrong[111] and, of course, translesion polymerases as such were unrecognized.

Following are a few of the author's thoughts about major questions that remain to be resolved in this field:

(i) How many general modes of DNA interaction are really involved in mispairing and correct pairing with adducts in

DNA polymerases?

(ii) Exactly what does control the conversion of a DNA polymerase−dNTP complex to an active form to yield phosphodiester bond formation?

(iii) We have a considerable amount of literature on DNA interactions in the absence of DNA polymerases, especially from NMR. The models and approaches are different (e.g. use of oligonucleotides with adducts "sealed" in the middle vs pairs overlapped to yield a primer−template complex resembling a replication fork). How predictive are the NMR results, in general?

(iv) How much do different translesion DNA polymerases compensate for each other in dealing with specific kinds of damage? (This approach requires that experiments with a single adduct be done with multiple polymerase considerations, either in cells or with isolated enzymes.)

(v) How does the switch of various polymerases at a site of damaged DNA (Scheme 12) really occur? Can a system be reconstituted *in vitro*, or are complex post-translational modifications of accessory proteins (e.g. PCNA) really required?

(vi) How do cross-linked species produce mutations?

(vii) What is the basis of "action at a distance" mutations?[105−107]

(viii) Will we be able to make good predictions of the outcomes for new adducts based on experience with other, unrelated adducts?

Progress should certainly be made toward some or many of these questions in the next few years. Some projects from this laboratory have been presented recently, along with conclusions about the role of bulk of Gua N2 and Ade N6 modifications on the functions of pol T7$^-$ and human pol η.[37,38,166] The effect of adduct bulk varies among DNA polymerases. Some of our own work with Dpo4[42] has been summarized here, and further work in this laboratory involves the used of these DNA polymerases to address some of the questions raised here.

11. Acknowledgments

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12. Note Added in Proof

Studies done since submission and revision of the manuscript have shown that Dpo4 inserts dCTP opposite template 8-oxoG more efficiently than opposite G, as judged by kpol/Kd measurements. The activation energy was 4 kcal mol^{-1} less with 8-oxoG than G. Crystal structures show the same (DNA) syn 8-oxoG:anti dATP binding as with other polymerases (*vide supra*). The bonding of the O_8 atom of 8-oxoG to Arg332 is hypothesized to be important in maintaining the template 8-oxoG in the anti configuration to produce the (anti-anti) 8-oxoG:dCTP interaction (Zang, H.; Irimia, A.; Choi, J-Y.; Angel, K. C.; Loukachevitch, L. V.; Egli, M.; Guengerich, F. P.; *J. Biol. Chem.* **2006**, *281*, in press).

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