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Techniques used to study the DNA polymerase reaction pathway

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Summary

A minimal reaction pathway for DNA polymerases was established over 20 years ago using chemical quench methods. Since that time there has been considerable interest in noncovalent steps in the reaction pathway, conformational changes involving the polymerase or its DNA substrate that may play a role in substrate specificity. Fluorescence-based assays have been devised in order to study these conformational transitions and the results obtained have added new detail to the reaction pathway.

Introduction

In the five decades since the discovery of the first DNA polymerase, substantial research effort has been devoted to understanding the workings of these sophisticated molecular machines. The ultimate goal of DNA polymerase enzymology is a complete description of the elementary steps in the reaction pathway, encompassing the structural transformations that take place and the influence of reaction rates on the flux of substrates through the pathway. X-ray crystallography has generated a wealth of cocrystal structures that almost certainly correspond to intermediates along the reaction pathway; the situation is analogous to having a wonderful collection of movie stills without a sure knowledge of the order in which they should be viewed or even if all are from the same movie.

A major focus in DNA polymerase research is the question of fidelity: how do these enzymes reconcile the opposing goals of efficiency and accuracy, the latter requiring them to select the dNTP substrate complementary to the DNA template from a sea of competing dNTPs and rNTPs? The fidelity problem has focused attention on the early steps of the reaction pathway since efficiency is best served if inappropriate substrates are rejected early in the pathway, before the polymerase is committed to the chemical step of nucleotide incorporation. Because these early checkpoints involve noncovalent transformations (conformational changes), their study has prompted the development of assays that interrogate more than just the conversion of substrate into product.

In this chapter, I will describe how the repertoire of techniques used to study DNA polymerase kinetics has been used to explore the complexities of the reaction pathway that results in nucleotide addition. In the interest of simplicity, I will focus on the processes that take place at the polymerase active site, and will omit discussion of accessory domains (e.g. exonuclease

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proofreading) or accessory subunits, such as sliding clamps, that are present in complex replication systems. The experimental approaches that were used initially with wild-type DNA polymerases are, of course, equally applicable to their mutant derivatives and this can provide valuable mechanistic insights.

Measurement of covalent changes

The simplest assays for DNA polymerase activity are based on measuring the rate of extension of a DNA primer. Once synthetic DNA oligonucleotides became widely available in the mid to late 1980s, DNA polymerase kinetics measurements almost invariably used defined sequence oligonucleotides (e.g. Fig. 1a), with the primer strand 5' labeled with ^{32}P . (Nowadays, fluorescent labeling and detection of the DNA provides a useful alternative strategy.) Adding a single complementary or mismatched nucleotide to a mixture of a DNA polymerase and its DNA substrate results in extension of the primer; substrate and product strands can be separated on a denaturing polyacrylamide gel and quantitated using a phosphorimager. The use of only a single nucleotide simplifies the product distribution (often to a single species) but also de-emphasizes the process of translocation, which is an important part of the reaction pathway in processive DNA polymerases. The readout in this assay is provided by the chemical step of nucleotide addition, but the rate information that can be extracted from the data depends on the way in which the experiment is set up, as illustrated in the schematics of Fig. 1 and described in the following sections.

Steady-state kinetics

Steady-state measurements of the DNA polymerase reaction have the advantage of being simple to carry out, and the disadvantage that the kinetic parameters obtained are frequently hard to interpret or provide information about relatively uninteresting steps in the reaction. The practical advantages stem from the steady-state requirement that the DNA substrate be in large excess over the polymerase; thus they conserve precious material and, because many enzyme turnovers are necessary to convert a substantial fraction of substrate into product, they do not require rapid kinetics instrumentation. The steady-state rate, k_{cat} , is dominated by the slowest step in the reaction cycle; for many wild-type DNA polymerases incorporating correctly paired nucleotides this is the dissociation of the Pol-DNA product complex (k_{off}) which must take place in order for each molecule of enzyme present in the reaction to process many DNA molecules. Because of the intervening steps between dNTP binding and the rate-limiting step, the steady-state K_{M} is a complex mix of elementary rate constants and often provides little or no information about the binding affinity of the Pol-DNA complex for the incoming dNTP [1].

Although the individual steady-state parameters, k_{cat} and K_{M} , are relatively uninformative, the ratio $k_{\text{cat}}/K_{\text{M}}$ is a useful measure of the specificity for competing substrates [1,2]. Thus, steady-state measurements can be used to compare efficiencies of incorporation of, for example, a series of mispairs or nucleotide analogues (see e.g. refs. [3–5]). The interpretation of steady-state data is clearer if a prechemistry step is slower than any of the post-chemistry steps, for example with some mutant polymerases or during misincorporation. In this situation, both single-turnover and steady-state measurements probe the same reaction steps and provide the same information.

Single-turnover kinetics

In single-turnover experiments, the DNA polymerase is present in excess over the DNA, and at a sufficient concentration to convert all the DNA to Pol-DNA binary complexes before the start of the reaction. Addition of nucleotide then results in extension of all the bound DNA molecules with no requirement for enzyme recycling because there is no unbound DNA. Thus

the post-chemistry steps become irrelevant and the observed rate (k_{pol}) reports the rate of the slowest step up to and including the phosphoryl transfer step in which the nucleotide becomes covalently attached to the primer terminus. Further experiments (discussed below) have been used to determine which of the prechemistry steps is rate-limiting. The hyperbolic dependence of k_{pol} on dNTP concentration gives K_D , which reflects the binding equilibria that precede the rate-limiting step. The ratio k_{pol}/K_D is the efficiency of the reaction and can be used to compare, for example, complementary versus mismatched dNTPs. In such a comparison, it is important to remember that the individual k_{pol} and K_D values do not apply to the same elementary steps of the reaction if the rate-limiting step changes for the different substrates.

For wild-type DNA polymerases incorporating correctly paired dNTPs, single-turnover rates are typically from around 10 s^{-1} [6,7] to several hundred s^{-1} [8,9], necessitating the use of a rapid-quench-flow instrument. Single-turnover measurements became more widespread in DNA polymerase studies following the introduction of the Kintek instrument [10]. An important feature of the Kintek rapid-quench instrument is the use of very small reagent volumes ($\approx 20 \text{ }\mu\text{l}$ per time point) so that experiments with purified DNA polymerases and defined synthetic oligonucleotides become feasible. Equally important, the instrument was an out-of-the-box solution in a field that did not have a strong tradition of building instrumentation from scratch.

Burst kinetics

A variation on the single-turnover experiment uses polymerase and DNA concentrations of similar magnitude, with the polymerase lower by about 2- or 3-fold [10]. In this case the first equivalent of enzyme, corresponding to the Pol-DNA complexes present at the start of the reaction, generates product at the single-turnover rate. Conversion of the remaining DNA to product requires enzyme dissociation and recycling and therefore takes place at the slower steady-state rate, limited by the product-dissociation step. Provided that the difference between the two rates is sufficient (≥ 20 -fold is ideal), the reaction is distinctly biphasic and the amplitude of the faster phase can be used to calculate the concentration of active enzyme (Fig. 1). The observation of burst kinetics when the experiment is set up in this way is diagnostic of a rate-limiting step after chemistry. Conversely, the failure to observe burst kinetics indicates that the slowest step in the reaction cycle is at or before the chemical step and therefore (as noted above) that both steady-state and pre-steady-state measurements will provide information on the same step of the reaction.

Identification of the single-turnover rate-limiting step

In a single-turnover chemical-quench experiment, the signal of product formation is the observation on a gel of a DNA molecule that has become longer than the starting material. The rate at which this product accumulates could be determined either by the chemical step itself or by a slower step that precedes it or by a combination of slow steps of similar rate. A variety of strategies have been used to investigate whether or not the chemical step is rate-limiting in this situation. Since all are subject to caveats, it is not surprising that the data obtained for a number of DNA polymerases do not show a consistent pattern of which step is rate-limiting [11]. Alternatively, the differences may be genuine, indicating that individual DNA polymerases share features of a common reaction pathway but differ in the relative stabilities of enzyme-bound intermediates and consequently in the rates of elementary steps of the reaction.

Sulfur elemental effect

If the phosphoryl transfer step in a DNA polymerase reaction is rate-limiting, the rate is predicted to be 4- to 10-fold slower when an α -thio-dNTP is used in place of the normal all-

oxygen substrate [12]. Because of the modest size of the predicted effect, distinguishing between full, partial or zero elemental effects can be a difficult judgment call. Moreover, because sulfur is larger than oxygen, steric effects may boost the elemental effect beyond the 4- to 10-fold expected from electronic effects, accounting for the more sizeable elemental effects seen in some situations [13–15] but making it a questionable diagnostic for the chemical step.

Comparison of pulse-chase and pulse-quench yields

This approach was first used in studies of Klenow fragment (Pol I(KF)) and provided evidence for a slow pre-chemistry rate-limiting step, a noncovalent step designated as E-DNA-dNTP forming E*-DNA-dNTP (Fig. 2) [16]. Using an α - ^{32}P dNTP, the key experimental observation was that the yield of labeled product DNA was lower when the reaction was quenched with acid, so as to instantly stop all interconversions, than when the reaction was chased with unlabeled dNTP to drive the E*-DNA-dNTP species towards product formation. This is evidence for a fast chemistry step flanked by slow steps both preceding and following, which allows accumulation of the E*-DNA-dNTP intermediate. Failure to observe a difference in the pulse-chase and pulse-quench yields does not necessarily rule out a slow step before chemistry; it could be caused by the lack of a slow step to provide a “bottleneck” after chemistry.

The pulse-chase/pulse-quench comparison has been used for several different DNA polymerases [11], but no consistent pattern for the relative rates of the individual chemistry and prechemistry steps has emerged. As pointed out by Johnson [17], a critical requirement in order for the E*-DNA-dNTP intermediate to accumulate is that the reverse of Step 3 (Fig. 2) must be slow, committing the intermediate to the forward reaction. This is more important than the forward rate of Step 3, as illustrated by a detailed analysis of the reaction pathway for incorporation of a correctly paired dNTP by T7 DNA polymerase [18], which revealed that k_4 (chemistry) is slower than k_3 , but the extremely slow reverse rate, k_{-3} , accounts for the lower pulse-quench yield.

Solvent deuterium isotope effect

Recent studies using solvent deuterium isotope effects [19,20] argue that the chemical step may be at least partially rate-limiting for 4 polymerases, serving as examples of all 4 classes of polymerases (DNA- and RNA-dependent, and with DNA and RNA products). The evidence supports a transition state in which two proton transfers take place, and is therefore consistent with the idea that the two-metal-ion mechanism for phosphoryl transfer [21] is assisted by a general acid and a general base derived from protein side chains. Because the hypothetical maximum isotope effect depends on the precise nature of the rate-limiting transition state, it is difficult to assess the extent to which the chemical step is rate-limiting. It is also unclear whether an isotope effect could be attributed to steps other than phosphoryl transfer, such as a conformational change in which proton transfers were associated with a rearrangement of hydrogen bonds or metal ligands.

Fluorescence assays for noncovalent steps

Because chemical-quench data will give information on noncovalent steps only if the noncovalent step is rate-limiting for product formation, some recent kinetic studies of DNA polymerases have complemented chemical-quench experiments with assays that will report directly on conformational transitions. Fluorescent probes, used in stopped-flow kinetic experiments, are ideally suited to report changes in their environment resulting from enzyme or substrate rearrangements. In our studies of DNA polymerases, two fluorescence strategies have been particularly useful in identifying early noncovalent transitions. We have used DNA substrates containing 2-aminopurine to track DNA rearrangements, and FRET probes as a

direct assay for the fingers-closing conformational change in the protein. It can be deceptively easy to find a fluorescence signal that changes during the polymerase reaction, but it is often extremely challenging to identify the corresponding structural transition and assign it to a step on the reaction pathway. A useful strategy is to compare the fluorescence signals obtained with an extendable DNA primer in the full polymerase reaction with those observed in situations where the chemical step cannot take place. Preventing phosphoryl transfer (by the use of nonextendable DNA substrates, non-cleavable dNTPs, or metal cofactors that do not support catalysis) limits observations to the prechemistry steps and thus simplifies the fluorescence traces. Additional fluorescence changes that are observed when chemistry takes place provide opportunities for investigations of phosphoryl transfer and later steps. For example, exchange-inert metal-dNTP complexes have been used to demonstrate that the two catalytic metal ions are required by DNA polymerase β at different steps of the reaction mechanism [22,23].

Studies using 2-aminopurine

2-aminopurine (2-AP, Fig. 3), a fluorescent analogue of adenine, can basepair with thymine or form mispairs (rather efficiently) with cytidine. Unlike other fluorescent base analogues, it does not disrupt normal helical DNA geometry. The fluorescence of 2-AP is quenched by stacking with its immediate 5' and 3' neighbors in the same DNA strand [24] so that the largest changes in fluorescence are seen when there is a substantial increase or decrease in stacking, as illustrated by the fluorescence of a series of model oligonucleotides (Fig. 3). Not surprisingly, the most informative probe positions are those that are close to the templating, or T(0), position; these have shown common features in the reaction pathways of several different DNA polymerases.

2-AP at the template (0) position

In most DNA polymerases that have been studied, addition of dTTP to a Pol-DNA complex containing a templating 2-AP results in a rapid fluorescence decrease, often occurring within the dead-time of the stopped-flow instrument (Fig. 4a) [7,23,25–28]. This is an example of a situation in which it is difficult to identify the structural changes responsible for the fluorescence change. In Pol I(KF), other conformational changes, including fingers-closing, take place after the step detected with the T(0) 2-AP probe [29]. Thus, the step in question takes place in the open complex and may correspond to little more than the initial binding of the incoming dTTP, causing some subtle repositioning of the T(0) 2-AP. It is difficult to assess whether the location of the templating base, within a pocket on the protein in the Pol-DNA binary complex [30], would correspond to a quenched or an unquenched environment (Fig. 5a).

2-AP at the template (+1) position

The T(+1) 2-AP probe, located 5' templating base, has been particularly informative in several DNA polymerase studies [26–28,31]. In every case, binding of a complementary dNTP to a nonextendable Pol-DNA complex with a T(+1) 2-AP-containing DNA resulted in a large fluorescence increase (Fig. 4b). A plausible structural explanation for this fluorescence signal is provided by DNA polymerase ternary complex structures, which show the T(+1) base flipped out from the template-primer helix (e.g. ref. [30]), suggesting that an intermediate with an unstacked T(+1) base could be responsible for the high fluorescence signal (Fig. 5b). With an extendable DNA primer, there is a subsequent fluorescence decrease which can be attributed to translocation following primer extension causing a further change in the environment of the 2-AP.

2-AP in studies of deletion errors

In addition to its use in the study of the incorporation of correctly-paired dNTPs by high-fidelity DNA polymerases, 2-AP has been valuable in investigating the formation of single-base deletion errors by lower fidelity polymerases [27,32]. The high fluorescence of an unstacked 2-AP can indicate whether an appropriately positioned 2-AP loses its base-pairing partner when a deletion error is made. In the experiment shown in Fig. 4c, the increased fluorescence of 2-AP at the T(-1) position (paired with the primer terminus) provides evidence for the indicated intermediate when the Y-family DNA polymerase Dbh skips over a template base and makes a single-base deletion [27].

FRET assays for fingers-closing

When the first cocrystal structures of Pol-DNA-dNTP ternary complexes revealed a novel conformational state distinct from that seen in the apo enzyme or the Pol-DNA binary complex [33], it was immediately obvious that the fingers-closing conformational change inferred from these structures (Fig. 6a) could play an important part in the reaction mechanism, potentially screening out inappropriate substrates in advance of the chemical step. Initially, it was assumed that fingers-closing corresponded to the rate-limiting conformational change (Step 3, see Fig. 2b) identified in the minimal mechanism of Benkovic and coworkers [34] but fluorescence experiments that measured the rate of fingers-closing directly have shown that it is too fast and must therefore precede Step 3 on the pathway [29,35].

Because the fingers-closing conformational change can be visualized from cocrystal structures (Fig. 6a), one can design a fluorescence assay capable of detecting this step, if indeed it is part of the reaction mechanism. (This is an entirely different strategy from that used in the 2-AP studies, where the structural changes responsible for the observed signals are less clear.) The fingers-closing transition involves relative movement and can therefore be detected using fluorescence resonance energy transfer (FRET), which reports the distance between two appropriately chosen fluorophores. One FRET probe is placed on the segment of the fingers subdomain whose position changes when going from the open to the closed conformation, and the partner probe is placed on a part of the complex whose position is largely unchanged during fingers-closing; in three studies the second probe was attached to the DNA duplex. Important controls established that complexes of labeled protein with unlabeled DNA, and vice versa, did not show a change in fluorescence on addition of the complementary dNTP; therefore the fluorescence change observed in the presence of both FRET probes could be attributed to the change in the interprobe distance when the fingers close. In separate studies of KlenTaq and Pol I(KF), measurement of the rate of change of the FRET signal by stopped-flow (Fig. 6b) indicated that the fingers-closing transition is faster than the dNTP incorporation rate measured by chemical quench [29,35], thus placing the fingers-closing ahead of the step that is rate-limiting for chemical incorporation. A third study, also of Pol I(KF), obtained a slower rate for fingers-closing, almost certainly due to interference in the reaction by the donor fluorophore which was attached close to the DNA primer terminus [36].

A more recent study of fingers subdomain movement in KlenTaq used a different strategy for placement of the FRET probes, with both fluorophores attached to the protein [37]. The results obtained were similar to those described above, but the labeling strategy, by avoiding the need to place a FRET probe on the DNA, allows greater flexibility for future studies.

Other single-fluorophore probes

In contrast to FRET-based experiments that, with the right controls, can relate a fluorescence change to a structural rearrangement within the enzyme-substrate complex, experiments using a single fluorescent reporter are inherently more ambiguous. In a study of T7 DNA polymerase,

Tsai and Johnson attached the coumarin fluorophore MDCC to a Cys side chain on the mobile part of the fingers subdomain and demonstrated that this probe reports a conformational transition that results from binding of a complementary dNTP to the Pol-DNA binary complex [18]. The reaction rates obtained for T7 DNA polymerase, viewed in relation to the fingers-closing rates for the slower Pol I(KF) and KlenTaq [29,35], suggest that the MDCC fluorophore is indeed reporting the fingers-closing conformational change. A second study of T7 DNA polymerase is more difficult to interpret. The fluorescent reporter, Cy3, was attached to the DNA template, 2 bases 5' to the templating base [38]. The location of the Cy3 relative to the protein structure is unclear; the attachment position on the DNA should be close to the fingers subdomain and yet the Cy3 fluorescence was influenced by the "Sequenase" deletion on the exonuclease domain. Moreover, the Cy3 probe reported a rate nearly 10-fold faster than that observed with the MDCC-labeled protein, indicating that the two fluorescence studies of T7 DNA polymerase are almost certainly targeting different steps of the reaction, further illustrating the difficulty of assigning fluorescence changes to known or hypothesized structural rearrangements.

A few studies of DNA polymerases have made use of the tryptophan fluorescence of the protein [31,39]. For obvious reasons, this approach is confined to polymerases that have only a single Trp residue (e.g. rat DNA polymerase β), or that lack Trp residues (e.g. *Sulfolobus solfataricus* Dpo4), allowing single Trp substitutions to be introduced at interesting locations. In general, it is difficult to establish a connection between structural changes and the Trp fluorescence signal, though in the DNA polymerase β study the Trp signal reported the same transitions as had been detected using 2-AP probes [31].

Despite the focus of fluorescence studies on the prechemistry steps of the DNA polymerase mechanism, the nature of the slow step that is rate-limiting for dNTP incorporation remains a mystery. In some cases, as described earlier for T7 DNA polymerase, it is possible that a more detailed analysis of the reaction pathway will reveal that the chemical step itself is rate-limiting [18]. In others, exemplified by Klenow fragment, the rate-limiting step may involve relatively subtle structural changes and consequently be fluorescently silent with the probes that have been used. A stopped-flow fluorescence experiment, in which Ca^{2+} was used instead of Mg^{2+} , suggests that the rate-limiting prechemistry step of Klenow fragment could involve the entry into the active site of the metal ion that activates the primer 3'OH [29]. Likewise, it has been suggested that entry of the second catalytic metal ion could take place immediately before chemistry in the DNA polymerase β mechanism [22,23].

Further applications of fluorescent probes

In addition to reporting directly the signal that results from a conformational change, fluorescence measurements are extremely versatile in that any process that can be linked to a fluorescence change can be harnessed to obtain useful information. For example, DNA binding or dissociation can be studied using any fluorescent reporter which gives a change in signal when going from the bound to the unbound state; useful probes are DNA substituted with 2-AP, or an appropriate pair of FRET probes attached to DNA and protein. The fluorescence signal can be used either in equilibrium measurements, to determine binding constants, or in kinetic measurements to determine on or off rates (Fig. 7). FRET probes also have potential for tracking the movement of DNA polymerases along a DNA substrate during processive synthesis, though they have not thus far been widely used for this purpose.

Analysis of kinetic data

Having generated single-turnover kinetic data, either by chemical quench or by the measurement of physical properties such as fluorescence, it is then necessary to extract rate

information. The first approach is to fit individual datasets to a suitable equation, usually one or more exponentials, and thus calculate the rate constant(s), k_{obs} . If rate measurements have been made at a series of dNTP concentrations, the k_{obs} values plotted as a function of dNTP concentration will give the maximal rate and apparent binding constant. This simple analysis is often sufficient to address the question at hand: comparing a misinsertion reaction with addition of the correct base, a mutant protein with wild-type, and so on. In an uncomplicated situation, where one step of the reaction is clearly rate-limiting and there is negligible contribution from the reverse reaction, the k_{pol} value obtained by this simple analysis is a reliable measure of the forward rate of the slowest step up to and including chemistry. However, it is important to be aware of the potential impact of other steps of the reaction on the rate that is being measured. In a chemical quench experiment, a slow step subsequent to product formation can cause the preceding steps to “back up” so that the reaction shows biphasic kinetics even under conditions expected to give a single-turnover. Biphasic kinetics or unexpected variations in reaction amplitudes are both good indicators of the need to consider the rates of other steps in the reaction pathway. An excellent illustration is provided by the incorporation of 8-oxo-dGTP opposite template C by the human mitochondrial DNA polymerase (Fig. 8). The single-turnover data are extremely biphasic, with the amplitude of the fast phase dependent on the nucleotide concentration [40]. This is accounted for by the extremely slow release of PP_i , which allows the chemical step to be in equilibrium with nucleotide binding (see reaction scheme in Fig. 8).

The analysis of kinetic data derived from stopped-flow fluorescence can be particularly challenging, in that one step may account for the observed rate constant but an entirely different step (or steps) may provide the fluorescence signal. For example, in our FRET-based assay of the Pol I(KF) fingers-closing, the pre-chemistry fluorescence signal was biphasic: about 80% of the fluorescence change had a fast rate, assigned as the fingers-closing step (Step 2.2 in Fig. 9), and the remaining 20% of the change took place at a rate similar to that of Step 3, the pre-chemistry conformational change that is rate-limiting for nucleotide addition. One possible interpretation is that both steps are associated with a fluorescence change, so that fingers-closing may take place in two stages. An alternative interpretation, which we prefer, is that the entire fluorescence change is associated with the Step 2.2 fingers-closing step, but the slower Step 3 allowed equilibration across the fingers-closing step, $\approx 4:1$ in favor of the forward reaction, so that conversion of the last $\approx 20\%$ of the complexes from open to closed was limited by the slow flux across Step 3.

Kinetic simulation is valuable for exploring the behavior of a complex reaction pathway. A variety of computer programs are available, ranging from simple interfaces derived from the original KinSim [41,42] (e.g. Tenua, available as freeware at <http://bililite.com/tenua/>) to the highly sophisticated and interactive KinTek Global Explorer [43]. Using simulation, one can evaluate the consequences of changes in substrate concentrations, changes in the rates of individual steps, or the addition of new steps, and one can determine which steps do or do not have a strong influence on the observed output signal. A useful reality-check is to simulate the output of a kinetics experiment, for example product formation as a function of time, and evaluate the extent to which a simple curve-fitting program succeeds in calculating the correct reaction rate.

Fitting kinetic data to a reaction pathway is a good strategy when it is apparent that several processes contribute to the observed reaction rate. It is tempting to include too many steps in the pathway, especially when one has independent evidence for a multi-step pathway, as in the DNA polymerase reaction. However, the only steps whose rates will be well-constrained by the fitting program are those that have a significant impact on the measured signal. Thus, in Fig. 8, a minimal reaction scheme that accounts for the observed kinetic behavior is shown. The mitochondrial DNA polymerase, like other DNA polymerases, has several pre-chemistry

steps but only the slowest step affects the rate measured in a chemical quench experiment. The rates of the faster steps can vary over a wide range while still fitting the experimental data, so that it is futile to include these steps in the mechanism used for fitting. A good understanding of the reaction pathway gained from kinetic simulations is therefore invaluable in deciding which steps to include.

Perspectives and future directions

Chemical quench experiments originally defined a minimal reaction scheme (Fig. 2) applicable to most, if not all, DNA polymerases. Subsequently, fluorescence assays targeting the noncovalent conformational rearrangements that are part of the DNA polymerase reaction pathway have contributed additional steps to the mechanism (Fig. 9). The focus has been predominantly on the prechemistry noncovalent steps since these will play a major role in the screening out of incorrect substrates, though one should not ignore the contributions of the reverse reactions or of postchemistry processes [17]. Examples of the use of kinetic measurements to define the DNA polymerase reaction pathway and to explore the effects of mispairs and nucleotide analogues, and the behavior of polymerase mutants, will be found in other chapters of this volume.

The investigation of misinsertion reactions is of interest to many groups involved in DNA polymerase research, and this topic illustrates a very important distinction between chemical observation of the polymerase reaction (by measuring the formation of product) and observations based on physical properties such as fluorescence. Chemical-quench experiments detect those molecules that successfully form product, regardless of how slow or inefficient the reaction may be, and therefore allow the measurement of kinetic parameters for misinsertion. Physical measurements such as fluorescence are a population average of the signal of all the molecules in a reaction. If only a few percent of those molecules are involved at any time in a misinsertion event, then the signal attributable to intermediates in that process will not be detectable. In order to study the physical properties of intermediates on the misinsertion pathway, it will be necessary to use single-molecule FRET techniques which allow the detection and study of interesting subpopulations of molecules [44]. Moreover, because bulk physical measurements on a population are unable to detect conformational fluctuations, it will be important to follow single-molecules as a function of time in order to appreciate fully the conformational dynamics that are the basis of the DNA polymerase reaction mechanism.

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Fig. 1. Overview of methods used in chemical quench studies of DNA polymerases

a. The 13/20mer template-primer used in many of the early DNA polymerase studies [34]. The primer strand is 5' labeled with ^{32}P .

b. Simplified pathway for the DNA polymerase reaction. Dashed arrows indicate processes comprising several steps. The single-turnover rate, k_{pol} , corresponds to the slowest step up to and including chemistry. The steady-state rate, k_{ss} , is the dissociation of the product DNA, required in order for the polymerase (E, outlined in blue) to carry out multiple turnovers.

c. The time course of product formation expected for three different experimental setups, calculated with k_{pol} set at 50 s^{-1} and k_{ss} at 0.2 s^{-1} . In each case, the vertical axis indicates the fraction of the DNA primer strand that has been converted to product by the polymerase, based on the equations shown. The important difference between the three experiments is the ratio of polymerase (E) to DNA (S_0). $[\text{E}]:[\text{S}_0]$ is 1:200 in the steady-state experiment, and 1:2 in the burst experiment. In the single-turnover experiment, the precise ratio is unimportant provided that the polymerase is in excess over the DNA and all the DNA is enzyme-bound.

Fig. 2.

The minimal reaction pathway for Pol I(KF) based on chemical-quench experiments [16,34]. When the reaction is quenched with acid, the species underlined in green are detected as product. When the reaction is chased, the yield of product is higher because the intermediate underlined in red is also converted to product.

Fig. 3. 2-aminopurine (2-AP, structure shown in **a**) as a fluorescence probe in DNA. **b.** Fluorescence emission spectra of model 2-AP substrates, using an excitation wavelength of 310 nm.. The model DNA substrates, where X indicates 2-AP on the listed sequences, were as follows. 1, 2-AP in fully duplex DNA; 2, 2-AP whose 5' neighbor lacks a base-pairing partner; 3, a template-primer duplex with 2-AP opposite the primer terminus; 4, 2-AP in single-stranded DNA; 5, 2-AP that lacks a base-pairing partner. The largest fluorescence signal was obtained from 2-AP lacking a base-pairing partner, illustrating the role of base-stacking in quenching 2-AP fluorescence. Adapted from ref. [27].

Fig. 4. Examples of the use of 2-AP-substituted DNA in the study of DNA polymerase reactions by stopped-flow fluorescence. The sequence around the primer terminus of the DNA substrate is shown for each set of experiments (X indicates 2-AP)

a. Addition of dTTP opposite 2-AP at the templating position, catalyzed by Pol I(KF), from ref. [26]. With both the nonextendable (dideoxy-terminated) and the extendable (deoxy-terminated) primer, a rapid fluorescence decrease occurs within the dead-time of the stopped-flow instrument ($\geq 1000 \text{ s}^{-1}$). With the extendable primer, there is a further fluorescence decrease whose rate is similar to that observed in chemical quench experiments, implying that the fluorescence and chemical quench assays are both rate-limited by the same slow prechemistry step.

b. An experiment similar to that in **a**, except that the 2-AP probe is in the position 5' to the templating base, from ref. [26]. The initial rapid fluorescence increase (200 to 500 s^{-1}) is followed, when the primer is extendable, by a fluorescence decrease at a rate similar to the chemical quench rate.

c. 2-AP fluorescence as a reporter of the base-skipping reaction catalyzed by the Y-family DNA polymerase, Dbh from *S. acidocaldarius* [27]. Fluorescence changes were observed by stopped-flow when dCTP (base-skipping) or dTTP (correct insertion) was added (final concentration 2 mM) to a binary complex of Dbh with DNA having 2-AP at the T(-1) position. The control trace corresponds to the addition of reaction buffer without nucleotide. The fluorescence increase on addition of dCTP, attributed to the intermediate shown to the right of the trace, required the complementary G at the T(+1) position and was not seen with a control DNA template lacking this G (data not shown).

Fig. 5.

The positions of the T(0) and T(+1) bases in binary (**a**, PDB file 1L3U) and ternary (**b**, PDB file 1LV5) complexes of the Pol I(KF) homologue from *B. stearothermophilus* [30]. In both panels, the protein is represented by the O and O₁ helices, and the highly conserved Tyr, Lys and Arg side chains on the O helix are shown. The primer terminal base pair is colored dark grey and the incoming dNTP in panel **b** is shown in CPK colors. The templating base, T(0), is colored green and its 5' neighbor, T(+1), is red. The T(+1) base is not present in the 1L3U data file, presumably because it was disordered in the crystals. This illustration was made using PyMOL (DeLano Scientific).

Fig. 6. A FRET-based assay for the fingers-closing conformational change [29]

a. The fingers-closing transition is illustrated using structural data from the Pol I(KF) homologue from *B. stearothermophilus* [30]. The backbone structure of the binary complex (PDB file 1L3U) is shown predominantly in beige. The chain trace of the ternary complex (PDB file 1LV5) is essentially identical to the binary complex except for the mobile portion of the fingers subdomain, shown in teal in the binary complex and dark blue in the ternary complex. The magenta sphere marks the beta carbon of the side chain (744 in Pol I(KF)) used for attachment of the FRET donor (IAEDANS). The DNA primer-template is shown predominantly in grey, with the template strand in a darker shade. The primer-terminal base pair is colored orange, and the green base at the T(-8) position marks the position of a dabcyI-dT quencher, serving as the FRET acceptor. The distance between donor and acceptor attachment points is 50.4 Å in the open conformation and 43.8 Å in the closed conformation (Förster distance $\approx$ 40 Å for IAEDANS-dabcyI). This illustration was made using PyMOL (DeLano Scientific).

b. Stopped-flow fluorescence study of the fingers-closing conformational change using the labeling scheme described in **a**. A binary complex of 744-AEDANS Pol I(KF) with a non-extendable dabcyI DNA was mixed in the stopped-flow instrument with the complementary nucleotide, dTTP, to give the final concentrations indicated. The rate of the fluorescence

decrease gave a fingers-closing rate of 140 s^{-1} . To improve the detection of rapid processes the data were collected and plotted using a logarithmic timescale.

Fig. 7.

Measurement of DNA dissociation using the FRET system described in Fig. 6. A binary complex of the fluorescent Pol I(KF) with the quencher-labeled (Q) DNA was mixed in the stopped-flow instrument with a large excess of unlabeled DNA. Dissociation of the quencher-DNA and its replacement with unlabeled DNA caused the fluorescence of the complex to increase (designated as * to **). The fluorescence traces gave a DNA dissociation rate of 2.4 s^{-1} from the binary complex and 0.5 s^{-1} from the ternary complex, formed by adding the complementary nucleotide, dTTP (to $50 \text{ }\mu\text{M}$). As in Fig. 6, a logarithmic timescale was used.

Fig. 8. The effect of a slow postchemistry step on chemical-quench data [40]

a. Chemical-quench data for the addition of 8-oxo-dGTP opposite a template C by the human mitochondrial DNA polymerase. Product formation was measured as a function of time at a series of nucleotide concentrations. Contrary to expectations, the reactions were extremely biphasic and the amplitude of the fast phase was dependent on nucleotide concentration.

b. Reaction mechanism that explains the rate data shown in **a**. The slow step after product formation causes the chemical step to come to equilibrium. Adapted from ref. [40].

Fig. 9.

A revised polymerase reaction pathway for Pol I(KF) from DNA binding up to the phosphoryl transfer step. The minimal pathway based on chemical-quench experiments [16,34] has been updated to reflect information derived from fluorescence studies. The numbering used by Dahlberg and Benkovic [16] has been retained and the new steps are designated 2.1 and 2.2. E_O and E_C represent the open and closed conformations of the polymerase. Formation of DNA^* in step 2.1 represents the DNA rearrangement that is detected with the T(+1)-2-AP probe. The order of steps 2.1 and 2.2 is based on experiments using ribonucleotides as substrate analogues [29]. Reprinted with permission from ref. [29]. Copyright 2008 American Chemical Society.