[2] Rapid Quench Kinetic Analysis of Polymerases, Adenosinetriphosphatases, and Enzyme Intermediates

By KENNETH A. JOHNSON

Introduction

Transient-state kinetic methods allow definition of the sequence of reactions occurring at the active site of an enzyme following substrate binding and leading up to product release. It is precisely these reaction steps that define the elementary steps accounting for the high fidelity of DNA polymerization or establishing the pathway for coupling of ATP hydrolysis to energy transduction by molecular motors. In each case, analysis of the reactions occurring at the active site of the enzyme by single turnover kinetic methods has allowed definition of the reaction sequence.¹⁻⁴ On the other hand, steady-state kinetic analysis only establishes the order of substrate binding and the order of product release; the steady-state kinetic parameters k_{cat} and k_{cat}/K_m only define the maximum rate of substrate binding, respectively. Steady-state kinetic analysis cannot address questions regarding the pathway of events occurring at the active site following substrate binding and prior to product release.¹

This chapter summarizes the methods of kinetic analysis used to define the sequence and rates of reactions catalyzed by DNA polymerases and by force-transducing adenosinetriphosphatases (ATPases) and gives a brief review of the detection of enzyme intermediates. The methods are of general utility in studying enzyme reaction mechanisms and have led to the discovery of several new enzyme intermediates.⁵⁻⁹ For a more detailed

- ¹ K. A. Johnson, *in* "The Enzymes" (P. D. Boyer, ed.), 4th Ed., Vol. 20, p. 1. Academic Press, New York, 1992.
- ² K. A. Johnson, Annu. Rev. Biochem. 62, 685 (1993).
- ³ K. A. Johnson, this series, Vol. 134, p. 677.
- ⁴ W. M. Kati, K. A. Johnson, L. F. Jerva, and K. S. Anderson, J. Biol. Chem. 267, 25988 (1992).
- ⁵ K. S. Anderson, J. A. Sikorski, and K. A. Johnson, Biochemistry 27, 7395 (1988).
- ⁶ K. S. Anderson and K. A. Johnson, Chem. Rev. 90, 1131 (1990).
- ⁷ K. S. Anderson, J. A. Sikorski, A. J. Benesi, and K. A. Johnson, J. Am. Chem. Soc. **110**, 6577 (1988).
- ⁸ K. S. Anderson, E. W. Miles, and K. A. Johnson, J. Biol. Chem. 266, 8020 (1991).
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analysis of transient kinetic methods in general, the reader is referred to [1] in this volume and to Johnson.¹

Principles of Transient-State Kinetic Analysis

The rapid quench transient kinetic methods described in this chapter involve the rapid mixing of enzyme with substrate to initiate the reaction. The reaction is then terminated after a short time interval by mixing with a quenching agent, such as a denaturant, and the amount of product formed is then quantified. By analyzing the time course of product formation the reaction can then be analyzed. If the experiment is done over a sufficiently short time interval and at a high enough enzyme concentration, then the kinetics of a single enzyme turnover can be measured.

Basically, two types of experiments can be performed. With substrate in excess over enzyme, one may observe a rapid "burst" of product formation, followed by steady-state turnover. Analysis of the rate and amplitude of the burst provides information to define the rate of the chemical reaction occurring at the active site, while the linear steady-state phase defines the rate-limiting steps leading to product release. However, a burst of product formation is only seen if the release of product, or some other step after chemistry, is slower than catalysis. If chemistry or a preceding step is rate limiting, then there is no burst and the initial rate would then define the rate of the chemical reaction. In this case, the absence of a burst could serve to establish that chemistry (or a step preceding chemistry) is rate limiting in the steady state.

A single enzyme turnover can be observed if the experiment is performed with enzyme in excess over substrate. If the enzyme is at sufficiently high concentration so that substrate binding is fast, then the kinetics measure the rate of the chemical conversion of substrate to product at the active site. Alternatively, at lower concentrations, analysis of the enzyme concentration dependence of the observed rate defines the kinetics of reactions involved in substrate binding and any steps leading to the observed chemical reaction. A single turnover experiment is most useful in attempts to define new enzyme intermediates because the 100% conversion of substrate to product within a single pass through the enzymebound species provides the most sensitivity to allow observation of any potential intermediates.^{1,5,6,8-12}

¹⁰ E. D. Brown, J. L. Marquardt, J. T. Lee, C. T. Walsh, and K. S. Anderson, *Biochemistry* in press (1994).

¹¹ E. D. Brown, J. L. Marquardt, C. T. Walsh, and K. S. Anderson, J. Am. Chem. Soc. **115**, 10398 (1993).

In the analysis of transient-state reaction kinetics, the enzyme is considered as a stoichiometric reactant rather than a trace catalyst. Therefore, the reactions occurring at the active site of the enzyme can be observed and quantified directly. Moreover, because the data define the kinetics of the reaction in terms of absolute concentration units that can be related to enzyme active site concentrations, the pathway of events occurring at the active site can be established without the indirect inference typical of steady-state kinetic methods. It is important to stress the value of information provided by the amplitude of a chemical reaction in absolute concentration units. Such information is often just as valuable as measuring the rate of a reaction in order to establish the pathway.^{1,5,6}

In this chapter, I summarize the logic and the methods of analysis used to establish the reaction pathways of polymerases and force-transducing ATPases. Previous studies on these two systems provide a recipe for kinetic analysis in terms of the order and type of kinetic experiments to perform to examine other enzymes in these classes. In addition, I briefly summarize the key elements of kinetic analysis leading to the identification of new enzyme intermediates.

Chemical Quench-Flow Methods

Rapid chemical quench-flow methods are based on the rapid mixing of substrate with enzyme to initiate the reaction, followed at a specific time interval by the mixing with a quenching agent to terminate the reaction. The product formed in this time interval can then be quantified. In principle these experiments are done by driving syringes first to mix enzyme with substrate such that a reaction occurs while the mixture is flowing through a reaction loop of tubing (see Fig. 1). The reaction is then terminated when the enzyme-substrate mixture is combined with the quenching agent from a third syringe. The time of reaction is determined by the time it takes the enzyme-substrate mixture to flow from the point of mixing, through the reaction loop, and to the point of quench. The reaction time can be varied by changing the length of the reaction loop and the rate of flow. In practice there are limits on the rate of flow between the maximum rate that can be achieved at reasonable drive pressures and the minimum flow rate which is necessary to maintain efficient mixing. Within a factor of 2 or 3 in flow rate, a given length of tubing can be used to obtain a range of reaction times.

¹² H. Cho, R. Krishnaraj, E. Kitas, W. Bannwarth, C. T. Walsh, and K. S. Anderson, J. Am. Chem. Soc. 114, 7296 (1992).



FIG. 1. Schematic of a pulsed-quench-flow apparatus. (Courtesy of KinTek Corporation, State College, PA.)

The slowest rate of flow is limited by the conditions to maintain turbulence. Turbulent flow through a tube can be predicted by calculation of a Reynolds number, which is a dimensionless parameter dependent on the flow rate, V, the viscosity, v, and the diameter of the tube, d: R = Vd/v. Turbulent flow occurs when the Reynolds number exceeds 2000.¹³ Thus, for a 0.08 cm diameter tubing, and a viscosity of 0.01 poise (g/cmsec), a linear flow rate of 2.5 m/sec (1.25 ml/sec) must be maintained. Although there has been some debate as to the validity of this calculation, in practice an instrument designed within these limits succeeds in measuring the rate of known chemical reactions,^{3,14,15} and the failures of both private and commercial ventures in the manufacture of quench-flow instruments can be traced to a flow rate too slow to maintain turbulence by this criterion.

41

¹³ H. K. Wiskind, in "Rapid Mixing and Sampling Techniques in Biochemistry" (B. Chance, R. H. Eisenhardt, Q. H. Gibson, and K. K. Lonberg-Holm, eds.), p. 355. Academic Press, New York, 1964.

¹⁴ R. W. Lymn and E. W. Taylor, Biochemistry 9, 2975 (1970).

¹⁵ J. P. Froehlich, J. V. Sullivan, and R. L. Berger, Anal. Biochem. 73, 331 (1976).

Quench-Flow Design

Most quench-flow instruments utilize a design based on loading enzyme and substrate into the drive syringes. This design is extremely wasteful of the precious reagents, however, because at the end of each drive the reaction loop still contains the two reactants. This solution, often 200 μ l in volume, must be discarded in order to flush the system prior to collecting the next time point. An alternative design is shown in Fig. 1 (KinTek Corporation, State College, PA). In this instrument, the reactants are loaded into small sample loops, and then buffer in the drive syringes is used to force the reactants together, through the reaction loop to the point of mixing with the quenching agent, and then out into a collection tube. With this design, sample volumes as low as 15 μ l can be obtained with nearly 100% sample recovery, making these experiments feasible for almost any enzyme system. An eight-way valve is used to select various reaction loop volumes, and a computer-controlled stepper motor is used to drive the reactants at a precise speed. By using eight different reaction loop volumes and with variation of the flow rate over a factor of about 2, reaction times ranging from 2 to 100 msec can be obtained. Longer reaction times are obtained in a push-pause-push mode, where the reactants are forced together, held in the reaction loop for a defined period of time, and then forced out to mix with the quench solution. Using this mode, reaction times from 100 msec to 100 sec (or longer) can be obtained.

Choice of Quenching Agent

Most commonly employed quenching agents are 1 N HCl and 1 N NaOH (final concentrations). In some experiments with 5-enolpyruvoylshikimate 3-phosphate synthase (EPSP synthase; 3-phosphoshikimate 1-carboxyvinyltransferase), 1 N trifluoroacetic acid gave better results than HCl. After stopping the reaction with acid, the reaction mixtures can be neutralized by addition of a small volume of a high concentration of buffer and base (i.e., 4 M Tris, 2 M NaOH). In some cases it is necessary to vortex the acid-quenched solutions with chloroform prior to neutralization to prevent renaturation of the enzyme;⁵ that is, the enzyme must be killed twice to keep it from coming back to life. To first isolate the tetrahedral intermediate in EPSP synthase, neat triethylamine is used as a quenching agent, which when mixed 1:2 with sample gives an aqueous layer with approximately pH 12.⁷ In experiments with DNA polymerases, 0.3 M EDTA has often been used to chelate the Mg²⁺ to prevent further reaction without the complications involved with the use of acids.

Regardless of the reagent used to quench the reaction, it is important to perform the appropriate control experiments involving mixing enzyme with quenching agent prior to adding substrate. This sample provides the most appropriate "blank" for measurement of background in the assay for product in addition to providing a necessary control for stopping the reaction.

Analysis of Reaction Products

Ultimately, the quality of the data is limited most by the methods used to separate substrate from product and quantify the amount of product formed. Methods have ranged from ion-exchange high-performance liquid chromatography (HPLC)⁵ to the use of charcoal columns with batch elution¹⁶ to isolate [³²P]phosphate from [γ -³²P]ATP. For studies on ATPase reactions, the best results are obtained using $[\alpha^{-32}P]ATP$ and resolving ADP from ATP by thin-layer chromatography (TLC) on poly(ethylenimine) (PEI) cellulose F plates (EM Separations, Gibbstown, NJ). Small aliquots $(1-2 \mu l)$ are applied to the TLC plates. The plates can be washed with methanol to remove salts and then dried and developed with 0.6 MKH₂PO₄ at pH 3.4. ADP and ATP standards run in the outside lanes can be visualized with a hand-held fluorescent lamp so that appropriate areas of each lane of the plate can be cut and quantified by scintillation counting. Alternatively, the TLC plate can be quantified directly using a two-dimensional radioactivity detector, such as a Betascope (Betagen, Waltham, MA).

The kinetics of DNA polymerization can be followed on a sequencing gel by first labeling the DNA primer on the 5' end with ³²P using T4 polynucleotide kinase. The products of reaction are then separated by electrophoresis of a 16% (w/v) polyacrylamide gel containing 8 M urea. The gel can then be quantified using a Betascope. Alternatively, by first exposing and developing a sheet of X-ray film, the gel can be placed on the X-ray film and the approriate bands cut with a razor blade and quantified by scintillation counting.

Regardless of the method, it is important to have an internal standard for the recovery of sample from the rapid quench-flow instrument. For HPLC, TLC, or gel analysis, the total radioactivity in a given sample or lane is used to normalize the data such that the analysis is a dependent on determining the fraction of product formed relative to starting reactant concentration. For example, in the case of gel analysis for DNA polymerization, the ratio of the elongated DNA (i.e., 26-mer) product relative to total labeled DNA (25-mer plus 26-mer) establishes the fraction of the reaction that has occurred. This fraction times the concentration of DNA in the original reaction mixture gives the concentration of product formed.

¹⁶ K. A. Johnson, J. Biol. Chem. 258, 13825 (1983).



FIG. 2. Pathway of DNA polymerization. (Redrawn from Patel et al.¹⁷)

Note on Utility of Concentrations Rather than Moles of Product

Reaction rates are governed by the concentrations of reactants present during the reaction. Accordingly, to interpret any experiment properly, one needs to know the concentrations of enzyme present and the concentrations of products formed as a function of time. In most laboratories performing enzyme kinetics, it appears to be the convention to graph moles (nanomoles or picomoles) of product formed as a function of time and to provide details as to the number of moles of enzyme present in the reaction mixture. However, to interpret these results, the reader must determine the total volume of the reaction (if it is even given) and then calculate the concentration of product formed. It is much more straightforward to always operate in concentration units, referring to the concentrations of product formed during the reaction. Quantitative analysis of the data is greatly facilitated by maintaining this convention.^{1,5,6}

Kinetics of DNA Polymerization

The pathway of DNA polymerization is shown in Fig. 2. The pathway was derived in studies on T7 DNA polymerase¹⁷⁻¹⁹ and defines the steps in the pathway catalyzed by reverse transcriptase⁴ except for the absence of a proofreading exonuclease. Enzyme first binds DNA tightly, and then processive synthesis involves multiple rounds of polymerization without dissociation of the DNA. The binding of a correct deoxynucleoside triphosphate (dNTP) leads to a rate-limiting conformational change from an open to a closed state ($E_1 \cdot DNA_n \cdot dNTP \rightarrow E_2 \cdot DNA_n \cdot dNTP$) which is then

¹⁷ S. S. Patel, I. Wong, and K. A. Johnson, *Biochemistry* 30, 511 (1991).

¹⁸ I. Wong, S. S. Patel, and K. A. Johnson, *Biochemistry* 30, 526 (1991).

¹⁹ M. J. Donlin, S. S. Patel, and K. A. Johnson, *Biochemistry* 30, 538 (1991).

followed by a fast chemical reaction. Relaxation of the closed state is then followed by release of pyrophosphate and translocation, all of which are much faster than the rate-limiting conformational change.

The important questions to address in studying polymerases center on understanding the extraordinarily high fidelity of DNA replication in terms of the elementary steps of the reaction sequence. The selectivity of the correct dNTP is a function of the steady-state kinetic parameter k_{cat}/K_m . However, measurement of k_{cat} and K_m does not define the mechanistic basis for fidelity; it only quantifies the problem. In fact, most often steadystate kinetic analysis of DNA polymerases is complicated by the nature of the processive synthesis whereby a single encounter of the polymerase with the DNA template results in multiple rounds of synthesis. Once the polymerase has filled in the template to which it is bound, the dissociation of the enzyme from the DNA is several orders of magnitude slower than polymerization. Accordingly, steady-state measurements of incorporation are a poorly defined average of the rate of synthesis and the rate of dissociation of the DNA template.

Steady-state reaction measurements do provide an important check on the validity of measurement of partial reactions along the pathway by transient kinetic methods. For example, "processivity" is defined as the number of bases incorporated per encounter of the enzyme with the DNA. Therefore, processivity is simply the ratio of the rate of polymerization divided by the rate of dissociation of the enzyme–DNA complex. For T7 DNA polymerase, a maximum rate of polymerization of 300–500 sec⁻¹ and a dissociation rate of 0.2 sec⁻¹ predicts a processivity of 1500–2500, a value observed by bulk solution measurements.

To define the reactions leading to the high fidelity of replication, one needs to identify the rate-limiting step for incorporation and determine the contributions to fidelity for each step in the reaction sequence. This can be accomplished by analyzing the kinetics of single nucleotide incorporation using synthetic oligonucleotides.

Kinetics of Single Nucleotide Incorporation

Experiments can be performed using synthetic oligonucleotides to examine the kinetics of addition of a single nucleotide after the addition of only one dNTP to the reaction mixture. For example, the following 25/45-mer has a 25 base primer complexed to a 45 base template such that the addition of dATP results in the elongation by one residue only:

5'-GCCTCGCAGCCGTCCAACCAACTCA |||||||||||||||||||||||| 3'-CGGAGCGTCGGCAGGTTGGTTGAGTTCCAGCTAGGTTACGGCAGG-5' The kinetics of elongation can be examined by mixing 50-100 nM enzyme with 200-300 nM DNA. The reaction shows a stoichiometric burst of product formation (26-mer) followed by slow steady-state turnover (see Fig. 3), which is limited by the release of the DNA product from the enzyme.⁴ The reaction time course can then be analyzed by the burst equation, $Y = A \exp(-k_2 t) + k_{ss} t$. The concentration of E-DNA active sites is obtained from the amplitude, A, and the rate of product formation from the exponential rate constant, k_2 . The steady-state rate, k_{ss} , divided by the amplitude, A, defines the rate constant for DNA release, k_6 (Fig. 2).

The analysis of the amplitude in such simple terms is dependent on the assumption that the rate of the reaction, k_2 , is at least 20-fold faster than the rate of product release, k_3 , for a simple three-step pathway.¹ In general, the amplitude of the burst is reduced owing to more rapid product release according to $A = [k_2/(k_2 + k_3)]^2$. In either case, the data can be fit to the burst equation and then interpreted according to the mechanism. It is not generally useful to include the equation for the amplitude in the



FIG. 3. Kinetics of a burst of DNA polymerization. The kinetics of single nucleotide incorporation were examined by reacting 40 nM reverse transcriptase (active site concentration) with 200 nM DNA (25/45-mer synthetic oligonucleotide) for the times indicated. The reaction was initiated by the addition of Mg^{2+} (10 mM) and dATP (25 μ M) and terminated by the addition of 0.3 M EDTA. Products were analyzed on a sequencing gel to quantify the formation of the product 26/45-mer. The data were fitted to a burst equation with rate constants of 20 and 0.18 sec⁻¹ for the rate of incorporation and release of DNA, respectively. (Reprinted with permission from Kati *et al.*⁴)

fitting process because the amplitude may often be lower than that predicted because of dead enzyme or an internal equilibrium between substrate and product at the active site. Ultimately, data fitting based on computer simulation provides the most complete analysis to relate the reaction pathway directly to the observed kinetics (see Data Analysis).

Active Site Titration and Determination of K_d for DNA Binding of Polymerase Site

The rate of dissociation of the $E \cdot DNA$ complex is slower than polymerization; therefore, when polymerization is measured on a fast time scale, only those enzyme molecules with DNA bound at the polymerase site will react to form product. Accordingly, the amplitude of the fast polymerization reaction provides a direct measurement of the concentration of $E \cdot DNA$ complexes at the start of the reaction. By measuring the variation in the burst amplitude with increasing concentration of DNA, one can establish the concentration of active enzyme sites and measure the dissociation constant for the binding of DNA. It is important to stress that this experiment provides a K_d for DNA binding in a productive mode at the active site of the enzyme because it is based on the ability of the DNA to be elongated rapidly.

Typically, the K_d values for DNA are in the range of 5–20 nM.^{2,4,17} The experiment can then be performed at 50 nM enzyme and concentrations of DNA ranging from 10 to 250 nM, if the data are analyzed by fitting to the quadratic equation (see Data Analysis). The hyperbolic equation is derived based on the assumption that substrate is always in excess of the enzyme concentration, and it is therefore not valid for this experiment.

Deoxynucleoside Triphosphate Concentration Dependence

Analysis of the dNTP concentration dependence of the observed rate of the burst of incorporation provides an estimate of the K_d for ground state nucleotide binding for formation of the productive complex poised for catalysis. The reaction kinetics follow a two-step sequence:

$$E \cdot DNA_n + dNTP \rightleftharpoons E \cdot DNA_n \cdot dNTP \rightleftharpoons E \cdot DNA_{n+1} \cdot PP_i$$

Although the reaction pathway contains additional steps, the rate of incorporation is governed solely by the nucleotide binding followed by a ratelimiting step leading to elongation of the DNA.^{4,17} As illustrated by the data in Fig. 4A, the rate increases with increasing concentration of dNTP, reaching a maximum.⁴ As shown in Fig. 4B, the fit of the concentration dependence of the rate to a hyperbola yields the maximum rate k_{pol} and the dissociation constant, K_d .



FIG. 4. Nucleotide concentration dependence of the rate of incorporation. (A) A solution of reverse transcriptase (80 nM) was incubated with 25/45-mer DNA (200 nM) and then mixed with Mg²⁺ (10 mM) and varying concentrations of dATP to initiate the reaction. The following dATP concentrations were used: $0.75 \ \mu M$ (\odot); $1.5 \ \mu M$ (\Box); $3.0 \ \mu M$ (Δ); $9.0 \ \mu M$ (\bigcirc); $15 \ \mu M$ (\Box); and $24 \ \mu M$ (Δ). The solid lines represent the best global fit to the data. (B) At each dATP concentration, the rate of polymerization was obtained by fit to a single exponential. The dATP concentration dependence was then fit to a hyperbola (solid line) to obtain a K_d of $4.0 \pm 0.4 \ \mu M$ and a maximum rate of $33 \pm 1.2 \ \text{sec}^{-1}$. (Reprinted with permission from Kati *et al.*⁴)

The fit to a hyperbola is dependent on two assumptions. First, the enzyme concentration must be much less than the K_d , so that the dNTP concentration added can be assumed to equal the free dNTP concentration. Second, the binding of dNTP must be a rapid equilibrium; that is, k_{-1} (the dNTP dissociation rate) must be much greater than k_{pol} . It is not always easy to prove this experimentally. The rapid equilibrium assumption is supported by the observation that the polymerization reaction is a single exponential at all dNTP concentrations. A two-step irreversible mechanism predicts a lag phase clearly observable at low dNTP concentrations, most prominent at the half-maximal rate. In addition, pulse-chase type experiments can be used to examine the kinetics of formation of a tight nucleotide complex.^{17,20}

Kinetics of Processive Synthesis

It is important to establish that the rate of the first enzyme turnover is not different than subsequent turnovers. Most often this can be accomplished by careful comparison of the steady-state parameters with the rates and mechanism determined by transient kinetic methods. In the case of DNA polymerases, one can perform an experiment to measure the kinetics of processive synthesis involving the incorporation of a small number of nucleotides. For example, using the synthetic oligonucleotide described above, the addition of dATP, dCTP, and dTTP (leaving out dGTP) results in the elongation of the DNA by 5 base pairs (see Fig. 5). The kinetics of processive elongation can then be examined by following the polymerization reaction on a sequencing gel and quantifying the formation and decay of each intermediate from $DNA_{25} \rightarrow DNA_{26} \rightarrow DNA_{27} \rightarrow$ $DNA_{28} \rightarrow DNA_{29} \rightarrow DNA_{30}$. The reaction kinetics are complex and require analysis by numerical integration.^{4,17,21,22} Nonetheless, using this analysis, the rates of polymerization for each step of elongation can be obtained.⁴

Pyrophosphorolysis

The kinetics of the reverse of polymerization, pyrophosphorolysis, can be analyzed by following the shortening of DNA on a sequencing gel if there is no exonuclease in the reaction mixture. If an exonuclease is present, then the kinetics of hydrolysis to produce dNMP can be distinguished from the pyrophosphorolysis to produce dNTP by labeling the 3-terminal base. It is necessary to run the reaction in the presence of

²⁰ J.C. Hsieh, S. Zinnen, and P. Modrich, J. Biol. Chem. 268, 24607 (1993).

²¹ B. A. Barshop, R. F. Wrenn, and C. Frieden, Anal. Biochem. 130, 134 (1983).

²² C. T. Zimmerlie and C. Frieden, Biochem. J. 258, 381 (1989).



FIG. 5. Kinetics of processive polymerization. A solution of reverse transcriptase (40 nM) was preincubated with 25/45-mer DNA (100 nM) and then reacted with dATP, dCTP, and dTTP (20 μM each) with 10 mM Mg²⁺ for the times indicated before quenching with 0.3 M EDTA. The data show the formation of 26-mer (\bullet), 27-mer (\blacksquare), 28-mer (\blacktriangle), 29-mer (O), and 30-mer (D). The solid lines represent the best fit to the data obtained by computer simulation using rates of 18, 31, 26, 9, and 13 sec⁻¹, respectively, for the five consecutive nucleotide incorporation reactions and DNA dissociation rates of 1.8, 3.4, 3.5, 1.2, and 1.2 sec⁻¹. (Reprinted with permission from Kati *et al.*⁴)

excess unlabeled dNTP to trap any radiolabeled dNTP. Analysis of the kinetics of production of dNMP and dNTP can give the relative rates of pyrophosphorolysis and exonuclease hydrolysis. However, the time dependence of the reaction is a composite of both reactions such that the rate of pyrophosphorolysis, obtained as a fit to a single exponential, will be the sum of the rates of hydrolysis and pyrophosphorolysis even if care is taken to monitor the production of dNTP only.¹⁷

Another complication in measuring the kinetics of pyrophosphorolysis is due to the precipitation of magnesium pyrophosphate at concentrations required to saturate the rate of the reaction (above 2 mM). The problem is only partially overcome by initiating the reaction with Mg^{2+} in one syringe and pyrophosphate in the other. Precipitation begins almost immediately on mixing.

Exonuclease Kinetics

The kinetics of exonuclease hydrolysis can be easily measured by monitoring the shortening of DNA as observed on a sequencing gel with 5'-labeled DNA. Although multiple products will be observed owing to multiple rounds of hydrolysis, the quantification is based on the kinetics of disappearance of the starting material. In the case of T7 polymerase, different experimental designs have given different results that have been interpreted mechanistically. Single-stranded DNA is hydrolyzed quite rapidly (700–900 sec⁻¹).¹⁹ In contrast, duplex DNA is hydrolyzed at rates of 0.2 and 2.3 sec⁻¹ for correctly and incorrectly base-paired 3' termini, respectively.¹⁹ These results have been taken to imply that the movement of the duplex DNA from the polymerase site to the exonuclease site is rate limiting and that the hydrolysis of single-stranded DNA represents the rate of hydrolysis of the DNA once it occupies the exonuclease site. In each case, the DNA is first incubated with the polymerase in the absence of Mg²⁺, and the reaction is initiated by the addition of Mg²⁺; the data can be fit to a single exponential (see Data Analysis).

Biphasic kinetics have been observed when the reaction is initiated by mixing duplex DNA containing a mismatch with DNA.¹⁹ There is a fast initial hydrolysis of 50% of the DNA (with enzyme in excess), whereas the remainder of the DNA is hydrolyzed at a rate of 2.3 sec^{-1} . These data have been interpreted in terms of a model whereby the DNA first binds to the exonuclease site and then partitions between hydrolysis and sliding into the polymerase site during the fast phase of the reaction. During the slow phase, the rate is limited by the sliding of the DNA from the polymerase site to the exonuclease site.

Complete Polymerase Pathway

The complete reaction pathway can be obtained by combining information from each experiment measuring the kinetics in the forward and reverse directions including estimates of the overall equilibrium constant for the reaction to constrain the fitting process. In the final fitting process, computer simulation can be used to model all of the data to a single model and a single set of rate constants.^{1,4,17,21,22}

Adenosinetriphosphatase Mechanisms

In general, a mechanism for coupling ATP hydrolysis to do useful work can be analyzed in terms of the pathway shown in Fig. 6A. The enzyme exists in two states, E_1 and E_2 , and the interconversion of the two forms is driven by the cycle of ATP hydrolysis coupled to do work. The pathway of coupling ATP hydrolysis to work is established by specifying the route through the maze shown in Fig. 6A by determining the kinetics of ATP binding, hydrolysis, and product release and the kinetics of the interconversion of the two enzyme forms. In the two examples





FIG. 6. Pathways of ATPase coupling. Pathways for ATPase coupling in general (A) and for actomyosin (B) and microtubule—kinesin (C) are shown. A represents actin; M, myosin; K, kinesin; Mt, microtubules.

given, actomyosin (Fig. 6B) and the microtubule-kinesin ATPase (Fig. 6C), the E_1 states are bound to the filament ($A \cdot M$ is actomyosin, $Mt \cdot K$ is microtubule-kinesin), and the E_2 states are dissociated from the filament (M is myosin, K is kinesin). Force is produced during conformational changes coupled to the changes in state while rebinding to the filament.

For actomyosin, ATP binding leads to rapid release of myosin from the actin (2000 sec⁻¹), which is followed by hydrolysis of the ATP on the free myosin molecule.^{23,24} The myosin-products complex ($M \cdot ADP \cdot P_i$) rebinds to the actin, and the release of products (ADP and P_i) is coupled to force production. The dissociation of products from $A \cdot M \cdot ADP \cdot P_i$ is the rate limiting step (~20 sec⁻¹) for the maximum actin-activated ATPase.

²³ R. W. Lymn and E. W. Taylor, *Biochemistry* 10, 4617 (1971).

²⁴ K. A. Johnson and E. W. Taylor, *Biochemistry* 17, 3432 (1978).

A similar pathway has been observed for the dynein ATPase except phosphate release precedes rebinding to the microtubule.^{16,25}

In contrast, in the kinesin pathway, ATP hydrolysis precedes the dissociation of the kinesin from the microtubule.²⁶ Phosphate release occurs from the free kinesin molecule, and the pathway is completed by the binding of the kinesin–ADP species (K · ADP) to the microtubule. Microtubules accelerate the release of ADP greater than 2000-fold. The release of kinesin from the microtubule following ATP hydrolysis is the ratelimiting step (20 sec⁻¹).

In these two examples, the pathways are similar and the maximum rates of the fully activated ATPase are similar. In each case, however, a different step is rate limiting. For actomyosin, the extremely fast dissociation of the motor from the filament leads to a pathway where the motor spends very little of the duty cycle attached to the filament. In contrast, kinesin spends most of the time attached to the microtubules. These observations are in keeping with the biological roles of these motors wherein myosin operates in ordered arrays, whereas kinesin works to transport vesicles along tracks of individual microtubules through the cytoplasm.

The experiments described below allow the pathway of coupling to be established by direct measurement of partial reactions.

Kinetics of ATP Hydrolysis

The kinetics of ATP hydrolysis can be measured in a rapid-quench experiment by first mixing enzyme with ATP and then quenching with 1 N HCl. Because the acid liberates any products bound at the enzyme active site, the amount of ADP observed is given by the sum $[ADP]_{obs} = [E \cdot ADP \cdot P_i] + [E \cdot ADP] + [ADP]$. Accordingly, the time dependence of the formation of ADP follows burst kinetics. The burst phase provides a measurement of the rate of formation and amount of $E \cdot ADP \cdot P_i + E \cdot ADP$ at the active site.

To achieve a maximal burst rate and amplitude so as to measure the rate of hydrolysis at the active site, a high concentration of ATP must be mixed with enzyme to initiate the reaction such that ATP binding is not rate limiting. The experiment is difficult because the K_m for ATP is typically much larger than the concentrations of enzyme that can be obtained, and the signal is limited by the background of hydrolyzed ATP (1%) in the most purified preparations. An ATP concentration equal to 5 times the

²⁵ K. A. Johnson, Annu. Rev. Biophys. Biophys. Chem. 14, 161 (1985).

²⁶ S. P. Gilbert, M. R. Webb, M. Brune, and K. A. Johnson *Nature (London)* submitted (1994).



FIG. 7. Design a pulse-chase experiment.

 $K_{\rm m}$ provides only a lower limit on the concentration of ATP that may be required to achieve a binding rate faster than catalysis. The steady-state $K_{\rm m}$ only defines the concentrations required for the binding rate to equal the steady-state turnover rate. Because hydrolysis may be an order of magnitude faster than net turnover, a correspondingly higher concentration of ATP is required.

To satisfy the most stringent requirements for optimal signal-to-noise ratios (S/N), we have found that the kinetics of ATP hydrolysis can be monitored most accurately using $[\alpha^{-32}P]ATP$ and quantifying the appearance of $[\alpha^{-32}P]ADP$ following separation by TLC on PEI cellulose F plates.²⁷ The concentration of ADP formed is then calculated from the counts per minute in the ADP spot (cpm_{ADP}) relative to counts in the ATP spot (cpm_{ATP}):

$$[ADP] = [ATP]_0 cpm_{ADP} / (cpm_{ADP} + cpm_{ATP})$$

The data can be fit to the burst equation (see Data Analysis) to obtain estimates of the rate and amplitude of the hydrolysis reaction. However, if the kinetics of ATP binding are known and the rate is less than 10-fold faster than hydrolysis, then the hydrolysis kinetics will exhibit a lag phase which is a function of ATP binding. Accordingly, the rates of hydrolysis will be underestimated unless the lag phase predicted by the binding kinetics is included in the fitting process.¹⁶ This is most easily accomplished by computer simulation (see Data Analysis).

Kinetics of ATP Binding: Pulse-Chase Experiments

The kinetics ATP binding can be measured using a pulse-chase protocol outlined in Fig. 7. The reaction is initiated by mixing with $[\alpha^{-32}P]ATP$

²⁷ S. P. Gilbert and K. A. Johnson, *Biochemistry* 33, 1951 (1994).

and then, at various times, an excess of unlabeled ATP is added. The reaction is allowed to continue for 5-8 half-lives of the hydrolysis reaction, a time sufficient to convert all tightly bound ATP to ADP. The reaction is then terminated by the addition of acid. The amount of ADP formed is equal to the sum $[ADP]_{obs} = \theta[E \cdot ATP] + [E \cdot ADP \cdot P_i] + [E \cdot ADP] + [ADP]$, where θ is the fraction of bound ATP that is hydrolyzed before it is released from the enzyme. As described above, the data can be fit to a burst equation to a first approximation, and then fit more completely by computer simulation to the complete mechanism.

It is important that the chase period be as short as possible to prevent the accumulation of product owing to the binding and hydrolysis of the diluted radiolabeled ATP. A control experiment must be performed by mixing the enzyme with the diluted ATP for the time of the chase to ensure that the rate of dilution of the label is sufficient for the time required.

Kinetics of Phosphate Release

The kinetics of phosphate release from the enzyme can be measured by stopped-flow using a method newly developed by Webb using fluorescently modified phosphate-binding protein.²⁸ The details of this method, however, are beyond the scope of this review. Care must be taken to scrub the system of contaminating phosphate using a phosphate "mop." With appropriate attention to details given in the original manuscript, a burst of phosphate release of 50 nM can easily be observed at a concentration of 1 mM ATP.

Kinetics of ADP Release

The rate of ADP release can be measured by several methods. The easiest method relies on the use of fluorescently labeled ADP, mant-ADP.²⁹ On excitation at 350 nm, mant-ADP emits light at 440 nm. Mant-ADP fluorescence is quenched by solvent, and therefore there is a decrease in fluorescence on dissociation of the mant-ADP from the enzyme. When kinesin-ADP is mixed with microtubules, a single exponential decrease in fluorescence is observed, providing a direct measurement of the rate of ADP release from the microtubule-kinesin-ADP complex.

Kinetics of Motor Dissociation and Rebinding to Filament

The kinetics of the dissociation and rebinding of the motor to the filament can be measured by stopped-flow light-scattering measurements.

²⁸ M. Brune, J. L. Hunter, J. E. T. Corrie, and M. R. Webb, *Biochemistry* 33, 8262 (1994).

²⁹ A. Sadhu and E. W. Taylor, J. Biol. Chem. 267, 11352 (1994).

For example, the mixing of ATP with actomyosin or microtubule-kinesin leads to a reduction in light-scattering intensity that can be monitored in a stopped-flow apparatus. The method is relatively sensitive so that concentrations of 0.1 μM protein can be used and any concentration of ATP from 1 μM to 5000 mM can be employed. The data fit a single exponential. Plotting rate versus ATP concentration in the case of actomyosin yields a straight line, with the rate approaching 2000 sec⁻¹.²⁴ In contrast, the ATP concentration dependence observed for the microtubulekinesin complex follows a hyperbola.²⁶

Putting It All Together

The pathway of ATPase coupling is finally obtained by combining information from all of the partial reactions. For example, for kinesin, the rate of ATP hydrolysis is faster than the rate of dissociation of the kinesin from the microtubule, thereby establishing that ATP hydrolysis precedes kinesin-microtubule dissociation. Phosphate release occurs at a rate equal to the rate of kinesin-microtubule dissociation; therefore, one reaction must be much faster and follow the other. To complete the cycle without proposing additional intermediates, the most simple pathway involves the fast release of phosphate from kinesin after its dissociation (as $K \cdot ADP \cdot P_i$) from the microtubule. This conclusion is then supported by measurements of the rate of binding of kinesin-ADP (K · ADP) to the microtubules. Finally, measurement of the rate of ADP release from the microtubule-kinesin-ADP complex (Mt · K · ADP) shows that ADP release is fast, supporting the identification of the single rate-limiting step involving the dissociation of the kinesin from the microtubule. The overall pathway can then be modeled by computer simulation to confirm that it accounts for all of the observed reactions.

Detection of Enzyme Intermediates

The search for enzyme intermediates is often a frustrating and difficult task because when one begins the investigation one may not know the properties of the intermediate or the time and substrate concentrations required for its formation, and it may be formed in low amounts. Experiments based on single turnover kinetics overcome many of the difficulties and have led to the identification and isolation of several new enzyme intermediates.⁵⁻¹²

We consider a single intermediate on the pathway from substrate to product:

 $E + S \rightleftharpoons E \cdot S \rightleftharpoons E \cdot I \rightleftharpoons E \cdot P \rightleftharpoons E + P$

To prove the existence of the intermediate, I, it must be shown to be kinetically competent, and, if possible, it must be isolated to prove its structure. Kinetic competence is most precisely addressed by showing that the intermediate is formed and is broken down at rates sufficient to account for net conversion of substrate to product. A single turnover experiment with enzyme in excess over the substrate, S, provides the most sensitive means to look for intermediates and allows the kinetic competence of the intermediate to be established directly.^{5,6,30}

The kinetics of a single turnover of EPSP synthase is shown in Fig. 8A.⁵ The reaction involves the combination of shikimate 3-phosphate (S3P) with phosphoenolpyruvate (PEP) to form a tetrahedral intermediate, which decays by elimination of phosphate to yield the product enolpyruvoylshikimate 3-phosphate (EPSP). The reaction shown in Fig. 8A was initiated with 10 μM enzyme in excess over limiting [¹⁴C]PEP (3.5 μM) in the presence of excess S3P (100 μ M). The key to the success of the experiment lies in the use of enzyme excess over the limiting, radiolabeled substrate. This allowed the direct observation of the intermediate although it formed on only one-third of the enzyme sites. The kinetics of the reverse reaction are shown in Fig. 8B, again showing the formation of the intermediate from EPSP. The kinetics of the reactions could be fitted to the reaction sequence to obtain a unique set of rate constants describing the rates of formation and decay of the tetrahedral intermediate in each direction.⁵ The kinetic analysis led directly to defining conditions under which the intermediate could be synthesized in quantities sufficient for structure determination.⁷

Data Analysis

Kinetic data can be fit to one of several simple equations involving either one or two exponential terms. The observed rates and amplitudes can then be interpreted in terms of the pathway of the reaction. As the pathway becomes more complex, then assumptions must be made to allow the use of the simplified equations. In general, there should be one exponential term for each step of the reaction,¹ except for steps in rapid equilibrium. Although fitting data to analytical solutions of the rate equations is useful and is often a necessary first step, it is usually desirable to fit the data directly to the complete mechanism by numerical integration. Computer simulation allows all of the kinetic data to be fit directly to the enzyme reaction pathway by global analysis without the need for simplifying assumptions. This section first describes the fitting of data to

³⁰ K. S. Anderson and K. A. Johnson, J. Biol. Chem. 265, 5567 (1990).



FIG. 8. Kinetics of a single turnover of EPSP synthase. (A) A single turnover in the forward reaction was observed by mixing a solution containing enzyme $(10 \ \mu M)$ and S3P $(100 \ \mu M)$ with [¹⁴C]PEP (3.5 μM). The reaction was terminated by the addition of 1 N HCl, and then the solution was vortexed with chloroform and neutralized. Products were analyzed by ion-exchange HPLC. (B) Kinetics of the reverse reaction. The time course is shown for formation and disappearance of PEP (\blacksquare), EPSP (\bigcirc), and pyruvate (\triangle), a breakdown product of the intermediate. The curves were calculated by numerical integration according to the complete solution of the reaction pathway. (Reprinted with permission from Anderson *et al.*⁵ Copyright 1988 American Chemical Society.)

standard solutions of simplified rate equations, then discusses the methods of global analysis.

Equilibrium Binding Measurements

It is often necessary and useful as part of the kinetic analysis to examine the equilibrium binding of substrates to the enzyme:

$$E + S \rightleftharpoons E \cdot S$$

If the binding is weak relative to the concentration of enzyme required to measure the binding, then the data can be fit to a hyperbola:

$$[\mathbf{E} \cdot \mathbf{S}] = [\mathbf{E}]_0 [\mathbf{S}]_0 / ([\mathbf{S}]_0 + K_d)$$

where $[E]_0$ and $[S]_0$ are the total enzyme and substrate concentrations, respectively. Data should be fit by nonlinear regression, including $[E]_0$, the starting enzyme concentration, as an unknown, if possible. If the data are in the form of an optical signal with an unknown extinction coefficient, *a*, then the signal must be fit including these additional unknowns, and the enzyme concentration must be assumed to be known:

$$Y = a[E \cdot S] + c = a[E]_0[S]_0/([S]_0 + K_d) + c$$

Several graphics programs containing nonlinear regression routines are available for personal computers (GraFit, Erithacus Software, Ltd., Staines, U.K.; KaleidaGraph, Synergy Software, Reading, PA, USA). After entering the approriate equation and initial estimates of the parameters, the program will find the best fit by minimizing the sum of squares error between the calculated and observed curves. A failure to get the program to converge to a solution is most often due to errors in the initial estimates. Take note of the form of the equation and the values of y at $x = \infty$ to estimate the parameters from the graph.

If the binding is tight relative to the enzyme concentration, then the data must be fit to a quadratic equation:

 $[E \cdot S] = \{(K_d + [E]_0 + [S]_0) - SQRT[(K_d + [E]_0 + [S]_0)^2 - 4[E]_0[S]_0]\}/2$

Again, care should be taken in considering the relationship between any optical signal and the concentration of $E \cdot S$ and in making initial estimates of the unknown parameters.

Single Exponential

Most kinetic experiments can be fit to a single exponential of the general form

$$Y = A \, \exp(-kt) + C$$

where A is the amplitude, k is the rate, and C is the end point. All three parameters should be taken as unknowns in fitting by nonlinear regression. In making initial estimates, note that a reaction with a signal increasing with time has a negative amplitude. At t = 0, Y = A + C, where as $t \rightarrow \infty$, Y = C.

Double Exponential

The equation for a double exponential follows the general form

$$Y = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C$$

which is analogous to a single exponential except that there are two amplitude and rate terms. This equation is used whenever the reaction shows two phases, such as a fast and slow phase or a lag preceding a reaction.

Burst Kinetics

The kinetics of a pre-steady-state burst followed by linear steady-state turnover can be fit to the standard burst equation:

$$Y = A \exp(-k_1 t) + k_2 t + C$$

where A is the amplitude of the burst and k_1 is the rate of the burst (in units of sec⁻¹), whereas k_2 is the steady-state rate (in units of concentration/sec). Divide k_2 by the enzyme concentration or amplitude of the burst to convert it to a first-order rate constant defining product release rates.

Global Analysis

Ultimately the goal of kinetic analysis is to derive a single mechanism to account completely for the results without introducing steps or intermediates not required by the data. It is now possible to fit all of the kinetic experiments directly to the mechanism by nonlinear regression based on numerical integration of the rate equations.^{21,22} Computer simulation is also a useful learning tool that can aid in the design of experiments to optimize conditions to test opposing models.

In fitting data by numerical integration, no simplifying assumptions are needed, but it is necessary to be close to the best fit before beginning the nonlinear regression. In addition, it is necessary to allow only those constants to float which are sensitive parameters pertaining to each experiment. For example, if an experiment is done at high substrate concentration so the binding rate is fast and the observed reaction rate is a function of the chemistry step, then the binding rate must be fixed at a known value while floating the rate of the chemical reaction to obtain the best fit. Alternatively, for a series of experiments done as a function of substrate concentration, one may float the substrate binding rate while maintaining a fixed value for the rate of the chemical reaction. In the final analysis, the overall pathway can then be fit to all of the data and including in the fitting process estimates of rate constants even though they may pertain to different parts of the mechanism that are not tested by a given experiment. In this way, even subtle effects on the kinetics can be taken into consideration in the fitting process to achieve a global best fit.

Using these methods, the pathway of enzyme-catalyzed reactions can be established by direct measurement of individual steps of the reaction. By comprehensive analysis of the reaction kinetics, the partial reactions can be assembled to provide a simple, complete reaction pathway.

[3] Analysis of Enzyme Progress Curves by Nonlinear Regression

By RONALD G. DUGGLEBY

Introduction

The usual way in which an enzyme-catalyzed reaction is monitored is by measuring the *amount* of reactant remaining or product formed at one or more times. By contrast, most kinetic models are formulated in terms of *rates* of reaction. There is, therefore, a fundamental incompatibility between the data and the underlying kinetic model. This incompatibility can be resolved in two ways. Either the model can be integrated to give a description of the time course of the reaction, or the data can be differentiated to determine rates.

Traditionally, enzyme kinetic studies have employed the second approach and have focused on determining rates, especially initial rates, by measuring tangents to the reaction progress curves. There are several reasons for this preference for initial rates. (1) The substrate concentration is exactly equal to that which is added. (2) There is a vanishingly small concentration of potentially inhibitory reaction products present, unless these are added deliberately. (3) There is no opportunity for the enzyme to have undergone partial inactivation.

There are, however, several advantages in studying progress curves, and, ironically, the first two of these are restatements of what were mentioned above as reasons for preferring initial rates. (1) The substrate con-

[3]