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Review

The identification of chemical intermediates in enzyme catalysis by the rapid quench-flow technique

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Abstract. Traditionally, enzyme transient kinetics have been studied by the stopped-flow and rapid quench-flow (QF) methods. Whereas stopped-flow is the more convenient, it suffers from two weaknesses: optically silent systems cannot be studied, and when there is a signal it cannot always be assigned to a particular step in the reaction pathway. QF is a chemical sampling method; reaction mixtures are aged for a few milliseconds or longer, 'stopped' by a quenching agent and the product or the intermediate is measured by a specific analytical method. Here we show that by exploiting the array of current analytical methods and different quenching agents, the QF method is a key technique for identifying, and for characterising kinetically, intermediates in enzyme reaction pathways and for determining the order by which bonds are formed or cleaved by enzymes acting on polymer substrates such as DNA.

Keywords. Enzyme mechanism, protein dynamics, rapid-reaction technique, kinetics, ATPase, DNA-protein interaction.

Introduction

The study of transient kinetics of enzyme reactions necessitates the observation of intermediate and product formation during the short time course from mixing enzyme and substrate until the steady state or equilibrium is reached. There are two complementary approaches to the application of transient kinetics to the exploration of mechanisms. What might be called its grammar requires the algebraic resolution of the exponentials (time constants and amplitudes) describing the time course of the transient concentration changes. The principles of various methods pertinent to this approach have been described in a recent book [1]. The second part of the study of transient kinetics is the optical and/or chemical identification of the intermediates that occur on the timescales of transients. It is worthwhile re-emphasising the complementarity of the two approaches. Here, we review the contributions made by the quench-flow (QF) technique to the identification and determination of lifetimes of chemical intermediates during transients.

QF first received limited attention 70 years ago from F. J. W. Roughton, the father of rapid-flow techniques for the study of reactions of CO_2 [2]. Its time resolution has since been improved by an order of magnitude to about 3 ms [3]. The relatively limited use of QF, despite its improvements and application to enzyme-catalysed reactions, is due to a number of factors. First and foremost, biochemists have become used to applying methods that give a continuous record of the reaction, regardless of whether they are studying steady-state or transient kinetics. The QF method entails the individual analysis of each time sample and, therefore, requires more time and effort. However, steady improvements have been made to QF equipment in recent years, to economise in reactant volumes and to simplify sampling. Furthermore, modern analytical techniques

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have vastly extended the range and ease of applications for sample analysis. Gibson [4], reviewing rapid reactions in biochemistry, thought that 'QF developed by Barman & Gutfreund [3] was an interesting method but not likely to find wide application'. This was written at a time (1966) when its only achievements were the measurements of rates of cleavage steps in the reactions of trypsin, chymotrypsin and alkaline phosphatase [5, 6]. In subsequent years, many fruitful applications, largely though by no means entirely with reactions involving phosphate compounds, have demonstrated the strength of the approach in identifying spectroscopically silent intermediates and their chemistry [7]. This has made the technique sufficiently popular to justify the production of four commercial instruments (www.tgkscientific.com, www.bio-logic.info, www.Rintek-corp.com, www.photophysics.com).

More surprising is the frequent underestimation of the capacity of the technique for development or adjustments, making it possible to use QF to dissect a wide range of enzymatic reactions. A typical example is the statement that QF is only useful for following product release [8]. This last step is the one that is the most difficult to identify unambiguously by QF because quenching techniques are likely to disrupt the non-covalent enzyme-product complexes that precede the final dissociation of the end product. This is connected to the fact that in many enzyme reactions, unlike classical Michaelis-Menten models, the chemistry is not rate limiting. Protein conformation changes controlling product release are often the slowest step [9] and result in significant concentrations of intermediates accumulating during turnover that can be identified chemically by QF. For example, we will describe below how 'on enzyme equilibria' (i.e. equilibria between enzyme-substrate and enzyme-product complexes) have been characterised in ATPases by this technique.

In this review, we use selected examples to demonstrate the successes of the QF technique in identifying the chemistry of what Britton Chance called kinetically competent intermediates and placing them in a sequence on the pathway of enzyme-catalysed reactions. We do not intend to provide a comprehensive description of all the work done by this method. The examples discussed have been selected to highlight either specific technical applications or seminal contributions to biochemical mechanisms. We hope to demonstrate that the more laborious procedure of following the progress of an enzymatic reaction by repeating the mixing of the reactants many times, and stopping the progress of the reaction after well-defined time intervals, allows the accurate chemical identification of spectroscopically silent intermediates.

Experimental

All flow techniques depend on efficient mixing of reactants. Apart from some very elaborate procedures, mixing times of 1 ms have not been significantly improved since the first application of flow methods 80 years ago. The QF technique depends on a second mixing event, the reaction mixture with a quenching reagent, at variable – but defined – time intervals after the first.

The equipment

The QF technique is a chemical sampling method. This is a standard method in enzymology: enzyme and substrate are mixed, the reaction mixture allowed to age whilst samples are removed, quenched in acid or some other reagent and assayed for product. Traditionally, such experiments are carried out manually, over time scales of tens of seconds to minutes. They are thus usually limited

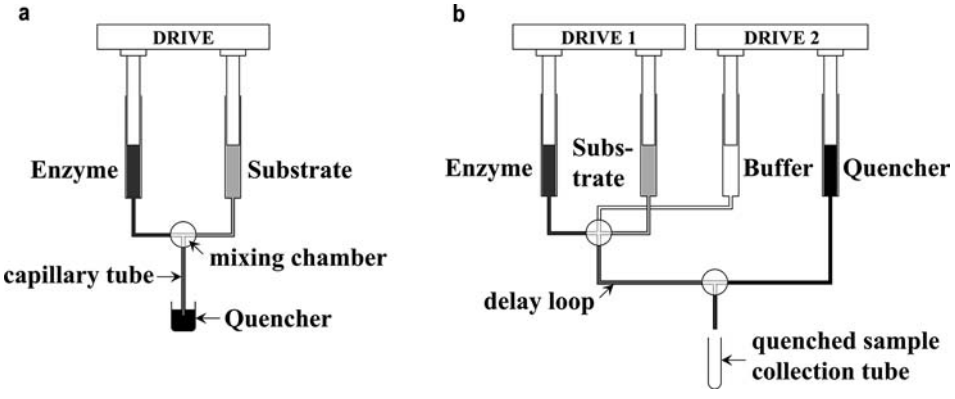


Figure 1. Principle of QF in continuous (*a*) and time-delay (*b*) modes. (a) In this apparatus, the reaction mixture ages during flow through the capillary tube. The reaction time is t = V/S where V is the volume of the capillary and S the rate of flow of the reaction mixture down the tube. By varying V and S, times in the range 3–300 ms were obtained. (*b*) There are two drives, each with two syringes: first drive, enzyme and substrate; second drive, buffer and quencher. The first drive is activated: enzyme and substrate are mixed; the mixture fills a delay loop and then stops. The reaction mixture is allowed to age for a predetermined time when the second drive is activated: the reaction mixture is expelled from the delay loop by buffer, mixed with quencher in a second mixer, and the now-quenched mixture collected and analysed. In this mode, reaction times from 150 ms to minutes are possible, with reaction mixture volumes less than 50 µl.

to obtaining overall time courses of product formation at low enzyme concentrations. To detect intermediates, chemical sampling has to be carried out at high enzyme concentrations, in the millisecond time range, and therefore requires fast reaction equipment such as QF.

Early QF apparatuses consisted simply of two syringes that contained the reagents, connected to a mixer and a capillary tube dipped in the quenching solution, as in Figure 1a. Typically, the syringes were driven by a variable speed motor via a clutch and brake unit operated by switches [3]. This simple method has two limitations. First, experiments are limited to a maximum time of about 300 ms. For longer times, slow flow rates result in poor mixing. Second, it is wasteful because it requires large volumes of reaction mixture – typically 0.5–1 ml per push. To overcome these limitations, two important technical improvements have been made.

First, to reduce the large volumes needed, Eccleston et al. [10] constructed a low-volume QF apparatus in which the reactants are loaded into loops between the mixing chamber and drive syringes which contain buffer only. For an experiment, the driving syringes are activated, the buffer pushes the reagents into the mixing chamber and the 'slug' of reaction mixture is quenched in a second mixer and then analysed. With this type of apparatus, reaction mixtures can be reduced to 50 µl or even less.

Second, to allow for reaction times > 300 ms, Fersht and Jakes [11] constructed a time-delay (or pulse-flow) QF apparatus. The principle of this is illustrated in Figure 1b.

In commercial QF equipment, such as those from TgK Scientific, Bio-Logic or KinTek, the reaction mixtures are less than 100 µl and the continuous-flow and time-delay modes are combined. Further, the syringes are driven by stepping motors that are controlled by computers, features that facilitate greatly the operators' work. Alternatively, Applied Photophysics provide a QF adaptor for their stopped-flow apparatus.

Experimental strategies

The following aspects of experimental design must be considered. First, what is to be assayed? Ideally, one would like to obtain the concentration of each intermediate on a reaction pathway, but in actual practice this is usually reduced to measuring a product. For example, when reaction mixtures are quenched in strong acid, it is assumed that any enzyme intermediate with bound product decomposes, so the measurement reports *total* rather than just free product. Thus, when product release is slow, one can obtain the kinetics of formation of enzyme product intermediates as well as those of the release of products.

Information on different intermediates can be obtained by changing the quenching agent: indeed, an important feature of the QF method is that one can 'stop' reactions in different quenching media. For example, when reactions of myosin with [γ-^{32}P]ATP are 'quenched' with unlabelled ATP, the kinetics of the ATP-binding process are obtained specifically, whereas when they are quenched in acid, the kinetics of ATP cleavage and P_i release are measured (see below). In Table 1, we summarise examples of different quenching agents that have been used for QF experiments.

How can intermediates or products be measured? The QF technique is a point-by-point-method. To obtain a convincing time course, multiple reaction mixtures, quenched at different times, are needed and each of these has to be analysed. In the early days, this was hard work but with the advent of automated high-performance liquid chromatography (HPLC) equipment coupled with the array of analytical methods now available, this problem has to a large extent been resolved. As illustrated below, the use of [γ-^{32}P]ATP has facilitated greatly studies on the ATPase and kinase family of enzymes.

Three further experimental strategies must be considered. One can work at different enzyme to substrate ratios, i.e. [E] < [S] (multi-turnover) or [E] > [S] (single turnover), or on different time scales. Finally, a way to obtain mechanistic information is to experiment over a large temperature range [12]. Myosin ATPase is used here as an example to illustrate these strategies.

Examples

A large proportion of QF studies have examined enzymes that use ATP as a substrate. This is partly because of their biological importance. They are also relatively convenient to study, since in these systems the time course can often be obtained merely by measuring the concentration of P_i formed during the reaction or by the quenching process.

Myosin ATPase is an excellent model to illustrate the versatility of the QF method. Information about the different steps on the reaction pathway of the enzyme was obtained by varying the myosin to ATP ratio (to give either multiple- or single-turnover reactions), the time scale of the experiment and the quenching agents. These different strategies are summarized in Table 2.

Myosin ATPase

The QF method was of crucial importance in the initial elucidation of the reaction pathway for myosin ATPase. In their pioneering work, Lymn and Taylor [13] studied the kinetics of P_i formation during the hydrolysis of ATP by myosin. Reaction mixtures aged from 5 ms upwards were quenched in acid and the total P_i measured (i.e. enzyme bound as well as free P_i). The time course was biphasic, a rapid rise or 'P_i burst', followed by a steady-

Table 1. Examples of quenching agents other than acid [see also ref. 7].

Agent	Observations	Examples
Chemical		
Strong alkali	used when intermediates of interest are unstable in acid	nucleoside diphosphate kinase [33]; phosphoglucomutase [60]
SDS	protein denaturation without brutal change in pH	certain enzymes acting on DNA; kinetics of interaction of myosin with EDC-activated actin [61]
EDTA	stops divalent-metal-dependent reactions	certain enzymes acting on DNA; ATP sulphurylase [62]
Excess unlabelled substrate: substrate chase	stops binding of radioactive substrate under mild conditions	substrate = [γ-^{32}P]ATP:myosin ATPase; mitochondrial ATPase [63]; substrate = [^{3}H]farnesyl diphosphate:protein farnesyl transferase [64]
N-ethylmaleimide in acetic acid	blocks remaining enzyme cysteines in reaction mixture	ribonucleoside diphosphate reductase [65]
Physical		
Filtration	limited to particulate, low-turnover systems	Ca^{2+} binding to membrane Ca^{2+}-ATPase [66]; GTP hydrolysis by transducin [67]
Rapid freezing	reaction mixtures squirted into organic solvent at less than –140 °C and studied by e.g. EPR	xanthine oxidase [68]; nitric oxide synthase [69]
Rapid evaporation	spray of reaction mixtures subjected to high-voltage field, ions analysed by mass spectroscopy	5-enolpyruvoyl-shikimate-3-phosphate synthase [70]; tryptic hydrolysis of a specific ester substrate [71]

Table 2. Myosin ATPase: information obtained from different types of experiment using the QF method.

Type of experiment	Reaction mixture	Time scale range	Quenching agent	Information obtained
P_i burst (multi-turnover)	myosin + ATP	ms to s	acid	ATP cleavage kinetics
P_i burst (single turnover)	myosin + ATP	s ($1/k_{cat}$)	acid	equilibrium constant of ATP cleavage; k_{cat}
Cold ATP chase	myosin + ATP	ms to s	unlabelled ATP, then acid	ATP-binding kinetics and active-site titration
ADP displacement	myosin·ADP + ATP	s ($1/k_{cat}$)	acid	ADP release kinetics

In all the experiments, [γ-^{32}P]ATP was the substrate and [^{32}P]P_i was determined in the quenched reaction mixtures.

state phase (as illustrated below; Figure 2). Lymn and Taylor [13] proposed that a ternary myosin·ADP·P_i complex accumulates in the steady state. In support of their proposal, Taylor and colleagues [14] subjected myosin + ATP reaction mixtures to gel filtration and, by a rapid separation procedure, confirmed that both ADP and P_i are associated with the myosin. Using QF and ^{18}O exchange methods, Bagshaw et al. [15] and Webb and Trentham [16] studied the cleavage step of the myosin ATPase reaction and showed that it is freely reversible, i.e. that the binary enzyme-substrate complex, M·ATP, is freely interconvertible with the enzyme-products complex, M·ADP·P_i. Taken together, these studies are summarized by the Lymn-Taylor scheme:

$$M + ATP \overset{1}{\rightleftharpoons} M{\cdot}ATP \overset{2}{\rightleftharpoons} M{\cdot}ADP{\cdot}P_i \overset{3}{\longrightarrow} M + ADP + P_i \quad \text{(Scheme 1)}$$

where step 2 is reversible and step 3 is rate limiting, since the M·ADP·P_i complex accumulates in the steady state.

ATP binding. Further QF studies were directed towards the details of the ATP-binding process: is this merely diffusion controlled (as implied in Scheme 1) or does it also involve a protein isomerisation step (i.e. an 'induced fit' mechanism [17]), as in Scheme 2?

$$M + ATP \rightleftharpoons M{\cdot}ATP \rightleftharpoons M^*{\cdot}ATP \quad \text{(Scheme 2)}$$

where * indicates an ATP-induced conformational change of the myosin.

The kinetics of the binding of ATP to myosin were examined by the 'ATP chase' method [18, 19]. Myosin plus [γ-^{32}P]ATP reaction mixtures were aged in a QF apparatus and quenched in a large molar excess of unlabelled ATP. After incubation for 2 min, this mixture was then

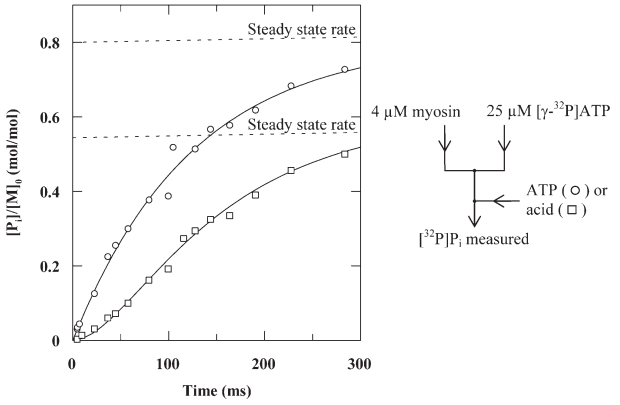


Figure 2. ATP chase (○) and P_i burst (□) time courses with myosin under multi-turnover conditions at 15 °C. Steady-state rates were measured from reactions over longer time scales (seconds upwards) than those shown here. The buffer was 50 mM Tris-acetate pH 8.0, 150 mM KCl and 2 mM Mg acetate. In the flow diagram, the concentrations refer to the reaction mixtures at t = 0, $[M]_0$ and $[ATP]_0$. [Shown, with permission, from ref. 20. © the Biochemical Society].

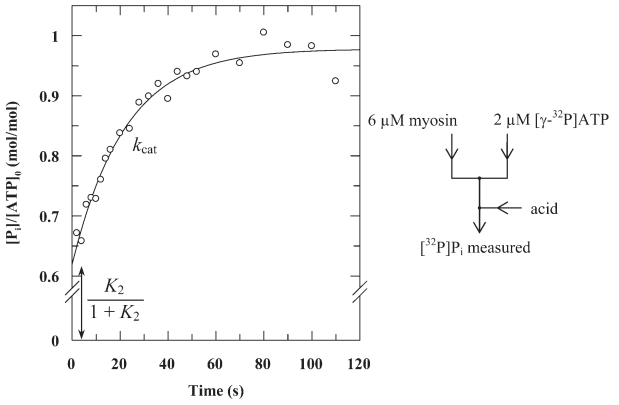


Figure 3. P_i burst experiment with myosin under single-turnover conditions at 4 °C. $[ATP]_0$ is the ATP concentration in the reaction mixture at t = 0. The buffer was 50 mM Tris-acetate, pH 7.4, 100 mM potassium acetate and 5 mM KCl. [Reprinted with permission from ref. 24. Copyright (1992) American Chemical Society].

quenched in acid and the concentration of $^{32}P_i$ was determined (Fig. 2). The application of this method led to the following conclusions [19]: (i) ATP binds essentially irreversibly, confirming previous work [20]; (ii) the binding is a two-step process, as proposed previously [21]; (iii) the ATP chase can be used to titrate the kinetically competent sites in myosin.

The ATP chase experiment shown in Figure 2 reveals a rapid exponential rise in P_i followed by the steady-state phase of ATP hydrolysis (k_{ss}). The amplitude of the rise shows that the myosin used titrated 0.8 equivalent active site per mole myosin. Thus, $k_{cat} = k_{ss}/0.8$, where k_{cat} is expressed as mole ATP hydrolysed per second per equivalent *active site* and k_{ss} as mole ATP hydrolyzed per second per *mole myosin*. The rate constant of the exponential rise increased hyperbolically with the ATP concentration, which is evidence that ATP binds in two steps [1].

ATP cleavage. In P_i burst experiments, reaction mixtures are quenched directly in acid and $^{32}P_i$ determined. A P_i burst experiment, carried out under the same conditions as the ATP chase, is also illustrated in Figure 2. The time course consists of three phases: an initial lag, a transient burst and finally a steady-state phase. The lag phase is a reflection of the ATP binding process and the burst phase the ATP cleavage step. On *increasing* the ATP concentration, the lag phase diminishes and the rate of the burst phase increases to a limiting plateau, as the kinetics of the ATP cleavage step become rate limiting (step 2 in Scheme 1). On *decreasing* the ATP concentration, the rate of the burst phase becomes limited by the ATP-binding kinetics, so the lag phase again diminishes.

P_i burst experiments have also been carried out under single-turnover conditions, i.e. with myosin in excess of the ATP [21–23]. A typical experiment is illustrated in Figure 3: it reveals a very rapid transient burst phase of P_i production (too fast to measure on the time scale used here) followed by an exponential rise to the complete hydrolysis of the ATP. The two parameters obtained (amplitude of the transient burst and kinetics of the slower rise) are independent of the ATP and myosin concentrations. The amplitude corresponds to $K_2/(1 + K_2)$ where K_2 is the equilibrium constant of the cleavage step (step 2 in Scheme 1), and the rate of the slow phase corresponds to k_{cat}.

Release of products. From the single-turnover P_i burst experiments, it appears that P_i release directs the steady-state rate under multi-turnover conditions: is ADP released at the same time (from M·ADP·P_i) or is it released from an M·ADP complex as in Scheme 3?

$$\mathrm{M{\cdot}ADP{\cdot}P_i} \overset{\mathrm{P_i}}{\rightleftharpoons} \mathrm{M{\cdot}ADP} \longrightarrow \mathrm{M + ADP} \qquad \text{(Scheme 3)}$$

Two types of experiments were carried out to answer this question.

(1) *ADP displacement with [γ-^{32}P]ATP: measurement of ADP release kinetics.* In this approach, which is based on a stopped-flow method [24], [γ-^{32}P]ATP is added to a solution of myosin and ADP (i.e. to an M·ADP complex). After ageing, the reaction mixtures are quenched in acid and $[^{32}P]P_i$ is determined. The rationale is that the ATP can only bind to myosin after the ADP has been released from the M·ADP complex.

A typical experiment is illustrated in Figure 4 together with controls. In the absence of ADP, there was a rapid burst phase (kinetics too fast to measure on the time scale used here) followed by the steady-state phase. When ADP and [γ-^{32}P]ATP were added together to the myosin, there

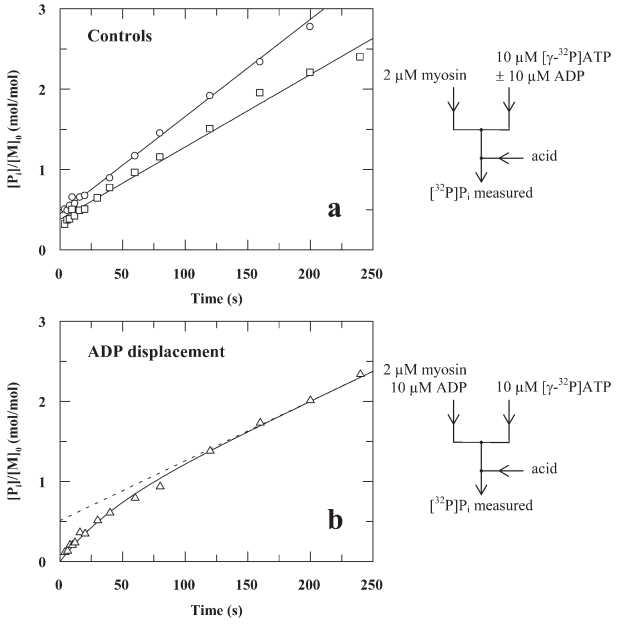


Figure 4. Time courses for P_i bursts under multi-turnover conditions at 4 °C: effect of ADP. No ADP (○) or ADP as competitive inhibitor (□) (*a*); ADP in M·ADP displaced by ATP (△) (*b*). The buffer composition was as in Figure 3 [our unpublished data].

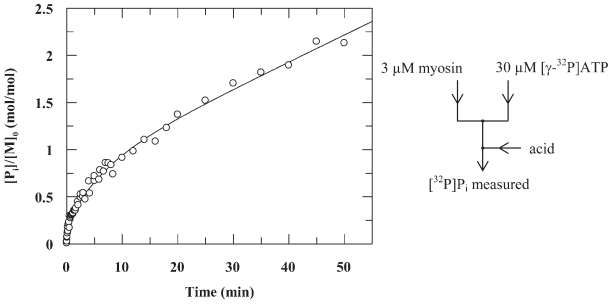


Figure 5. Progress curve for P_i burst with myosin under multi-turnover conditions at –15 °C, with 40% ethylene glycol as antifreeze. The buffer was as in Figure 3. [Reprinted with permission from ref. 28. Copyright (1999) American Chemical Society.]

was only a small effect on these parameters (Fig. 4a). However, when [γ-^{32}P]ATP was added to the myosin with ADP (i.e. to M·ADP), the rate of the burst phase was significantly slowed (Fig. 4b). The kinetics of this phase are a function of the rate of release of the products [25], and they show that the kinetics of ADP release are faster than those of P_i.

(2) *Multi-turnover P_i burst under cryoenzymic conditions.* A powerful way to obtain information on an enzyme reaction pathway is to work under cryoenzymic conditions. This technique involves two perturbants: temperature and an antifreeze. It may permit the accumulation of reaction intermediates that cannot be observed under normal conditions, by slowing down the kinetics of their formation, by a change in a rate-limiting step, or by shifts in equilibria [12].

With myosin ATPase, there is a change in the rate-limiting step as the temperature is decreased: P_i release above 0 °C, ADP release below 0 °C [26]. The effect of this 'switch' on the time course of a P_i burst experiment at –15 °C is illustrated in Figure 5. The progress curve is triphasic: a biphasic transient phase followed by a slow steady-state phase. The initial fast transient is a manifestation of the kinetics of the formation of M·ADP·P_i, the second transient the P_i release kinetics, and the final steady-state phase, the rate-limiting release of ADP. Thus, a single P_i burst experiment at –15 °C gave kinetic information on three steps on the myosin ATPase reaction pathway [27].

Kinases

A strategy to study the transient kinetics of the kinases is to quench reaction mixtures in acid or alkali and then to measure directly or indirectly phosphorylated product as P_i. For example, when creatine and 3-phosphoglycerate kinase (PGK) reactions are quenched in strong acid, the phosphorylated products are hydrolyzed to give P_i, which can then be measured [28–31]. cAMP-dependent protein kinase, which serves as a model for the protein kinase family, has also been studied by QF. Reaction mixtures with a peptide substrate were quenched in acid and the concentration of phosphopeptide measured directly [32]. Nucleoside diphosphate kinase is an example of an enzyme that catalyzes phosphoryl transfer by a 'ping-pong' mechanism involving the phosphorylation of a histidine residue in the enzyme. Reaction mixtures (enzyme + ATP) were quenched in 0.15 N NaOH and the phosphoprotein intermediate measured as phosphohistidine [33].

The PGK system is discussed here in more detail because it illustrates another strategy: 'equilibrium perturbation'. Further, this enzyme serves as a model for the application of the QF method to a reversible system. It catalyses the reaction ATP + 3-phosphoglycerate (PG) ↔ ADP + 1,3-bisphosphoglycerate (bPG). Although the forward reaction is unfavourable, PGK is usually studied in this direction because of the instability of bPG.

Two types of experiment were carried out with yeast PGK, using [γ-^{32}P]ATP. In both, reaction mixtures were quenched in acid and [1-^{32}P]bPG determined as [^{32}P]P_i [31]. In the first type, the time course of bPG formation was obtained: it consisted of a transient burst of enzyme-bound bPG followed by a steady-state phase until the final equilibrium was reached. The kinetics of the transient gave information on the putative hinge-bending motion of PGK [34]. In the second type, reaction mixtures at equilibrium were perturbed by the injection of ADP, the new reaction mixtures aged for different times before finally quenching in acid and determining the bPG concentration. As shown in Figure 6, there was a rapid decrease in bPG. This was interpreted by the added ADP react-

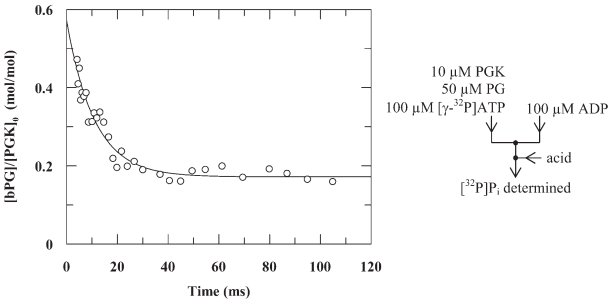


Figure 6. Perturbation of a PGK reaction at equilibrium by ADP at 4 °C. The concentrations given are those immediately after mixing. At equilibrium (>100 ms), the total bPG concentration was 5.7 μM. The buffer was 20 mM triethanolamine pH 7.5, 100 mM K-acetate, 1 mM free Mg acetate and 30% (v/v) methanol. [Reprinted with permission from [36]. Copyright (2005) American Chemical Society.]

ing with a PGK·bPG complex in the equilibrium mixture to give rise first to a PGK·bPG·ADP complex, then to PGK·PG·ATP and finally to free ATP.

The use of these strategies led to the following conclusions [35]. First, a binary PGK·bPG complex is an important intermediate on the PGK reaction pathway. This implies that ADP is released before bPG. Second, by perturbing reaction mixtures with ADP, the PGK reaction can be studied in the physiologically important direction without having to handle the unstable bPG. Finally, by the use of a global fitting procedure [ref. 35 and references therein], estimates of the kinetic constants of a seven-step pathway for PGK were obtained. Essentially, in this procedure, the experimental data obtained at different concentrations of ATP (bPG bursts) or of ADP (ADP perturbation) were fitted simultaneously assuming the pathway, using Scientist (version 2.0, MicroMath Research). The differential equations describing the time-dependent change in concentration for all the species were entered and time courses were derived by numerical integration.

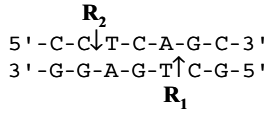
Enzymes acting on DNA

The QF method has been applied widely to reactions of proteins on DNA. Examples include the replication and proof-reading activities of DNA polymerases, strand separation by helicases, ATP hydrolysis by topoisomerases, transcription by RNA polymerase and its regulation by transcription factors, DNA cleavage by nucleases and DNA modification by methyltransferases [see refs. 36–48 and references therein]. There are at least two reasons why QF has been used so often on these systems.

First, most reactions on DNA are rate-limited by the dissociation of the enzyme from the final product. Moreover, the polymerases, the helicases and some other enzymes [36, 37, 39, 44, 46, 48] act processively and catalyse many consecutive reactions on a single DNA before dissociating. In these situations, very little information about the reaction pathway can be deduced from the steady-state turnover of the enzyme. This information can only be obtained by monitoring the individual steps directly.

Second, with few exceptions, reactions on DNA are optically silent. They seldom produce any change in either UV or visible absorption, or fluorescence, so they cannot be observed in a stopped-flow spectrophotometer. The majority of DNA reactions are therefore carried out by incubating the protein and the DNA for the requisite time, and then quenching the sample to stop any further reaction. Many enzymes that act on DNA need Mg^{2+} and these can often [40], but not always [37], be stopped by using EDTA to chelate the Mg^{2+}. Alternatively, reactions on DNA can be quenched with acid or alkali, or with a reagent that denatures the protein but not the DNA, e.g. phenol or SDS. Finally, the DNA substrate is separated from the reaction products, usually by electrophoresis, and the concentrations of each determined.

The BbvCI restriction endonuclease provides an example of this strategy [47]. The orthodox restriction enzymes such as EcoRI or EcoRV are dimers of identical subunits that cleave DNA at sites with the same 5′-3′ sequence in both strands [40, 42]. BbvCI differs from the orthodox by cleaving DNA at a site with different sequences in each strand, and by having two different subunits, R_1 and R_2, each specific for a particular strand:



The substrate for these experiments was a 4-kb plasmid with one recognition site for BbvCI. Cutting one strand of this DNA converts it from its native supercoiled (SC) state to the relaxed open-circle (OC) form, while cutting both strands at the same site yields its linear (LIN) form (Fig. 7a). The three forms can be separated from each other by electrophoresis (Fig. 7b). In reactions with BbvCI at a lower concentration than the DNA, when only a small fraction of the DNA is enzyme bound, the initial product liberated from the enzyme is LIN DNA rather than nicked OC DNA [47]. The steady-state reactions thus reveal only the rate of dissociation from the product cut in both strands and fail to provide any information about the rates at which either the R_1 or the R_2 subunits cut their respective strands. In contrast, by using the QF apparatus to examine single-turnover reactions with BbvCI in excess of the DNA, the transient formation and decay of the nicked OC DNA was observed as an enzyme-bound intermediate (Fig. 7c).

The data in Figure 7c were fitted to the kinetic equations [1] for the reaction scheme, SC → OC → LIN, to obtain apparent rate constants for cutting first one and then the other strand, k_a and k_b, respectively. The best fit was obtained with a value for k_a about four times larger than

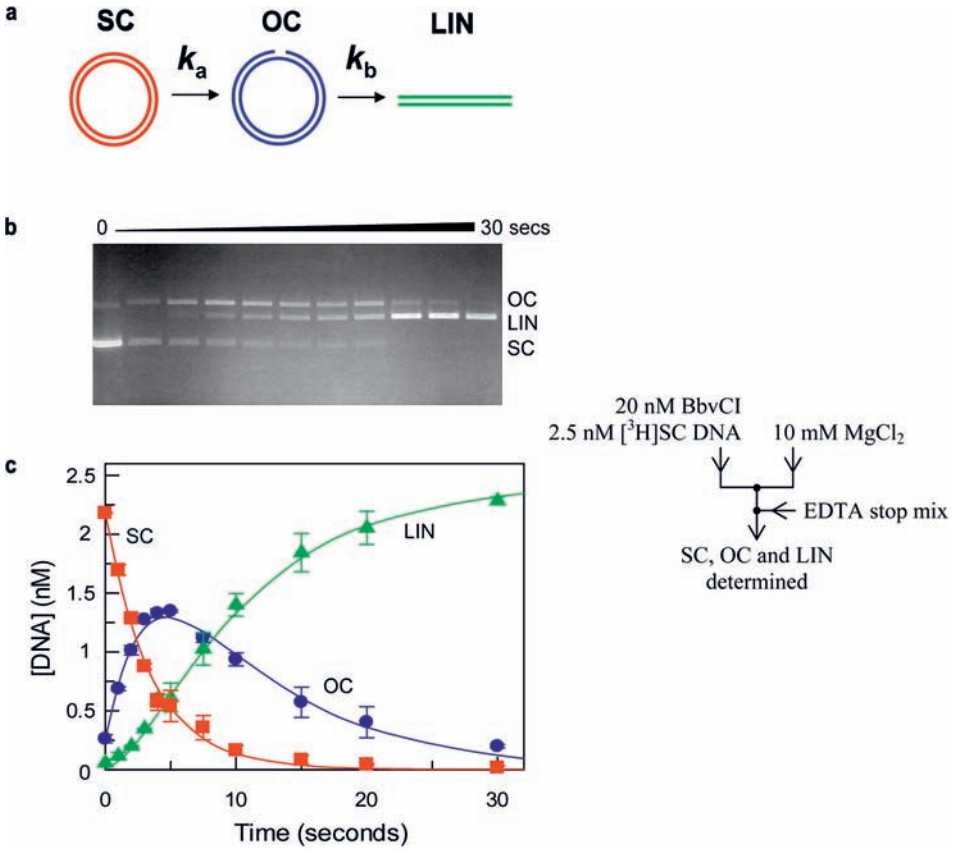


Figure 7. QF analysis of DNA cleavage by BbvCI [48]. (*a*) Cleavage of one strand of a covalently closed circle of DNA, with one recognition site for BbvCI, converts the supercoiled (SC) form of the DNA into the nicked open-circle (OC) form; cleavage of the remaining intact strand at the site gives the linear (LIN) product: k_a and k_b are the apparent rate constants for each step. (*b*) The QF apparatus was used to mix a solution of BbvCI endonuclease and a plasmid with a single BbvCI site with another containing $MgCl_2$ (final concentrations indicated) at 37 °C in a standard buffer at pH 7.5. After various times (0–30 s), the reactions were quenched with EDTA and the DNA analysed by electrophoresis through agarose. The time intervals are indicated above the gel and the mobilities of the SC, OC and LIN forms on the right. (*c*) The amounts of each species present during the reaction in (*b*) were measured. The amounts were fitted to the model in (*a*) and the optimal fit is shown. [With permission of Elsevier.]

that for k_b. This differs markedly from the homodimeric restriction enzymes like EcoRV and EcoRI, which cleave the two (identical) strands at their sites at equal rates [40, 42]. However, BbvCI is a heterodimer, so the different rates with this enzyme might reflect distinct activities by its R_1 and R_2 subunits. To examine this possibility, the reaction was carried out repeatedly in the QF, to collect multiple samples of the time point when the OC form was at its maximum (2 s; Fig. 7c). The OC DNA was then analysed to determine which strand had been cleaved. About 80% had been cleaved in the strand cut by the R_1 subunit, and 20% in the strand cut by R_2. Hence, the relatively large rate constant, k_a, is due mainly to the R_1 subunit acting on its strand and the smaller rate constant, k_b, due mainly to R_2 on the other strand [47].

The reaction in Figure 7 was set up by adding $MgCl_2$ to a solution containing both the BbvCI enzyme and the DNA. The same results were obtained when BbvCI and $MgCl_2$ in one solution were mixed with DNA in another. Hence, at these concentrations, the rate of DNA cleavage is not limited by the rate at which the enzyme binds to its recognition site in the DNA.

In other systems, additional information has been obtained by 'order of mixing' experiments. For example, on adding Mg^{2+} to a solution containing the EcoRV nuclease and a plasmid substrate, DNA cleavage started immediately. But when EcoRV was added to a solution of plasmid and Mg^{2+}, DNA cleavage started only after a lag phase [40]. The lag phase reflects the time taken by EcoRV to bind to its target in the plasmid. Since the lag showed second-order kinetics with respect to the enzyme, the rate for binding to the recognition site must be limited by the initial bimolecular association of the enzyme in free solution to any site on the plasmid: the subsequent transfer of the enzyme from its initial random site to its final specific site is too rapid to limit the overall rate. The transfer occurs mainly by multiple dissociation/reassociations within the same DNA rather than by sliding [49].

Similar strategies to those illustrated here with restriction enzymes have been applied to many other reactions

on DNA. For example, both the polymerase and the 3′-5′ proofreading exonuclease activities of several DNA polymerases have been elegantly dissected by QF [37, 46]. The polymerase substrates usually consist of a primer strand of n (typically 13) bases annealed to a longer template. Following the reaction, the samples are analysed by electrophoresis, to separate the primer from the products of the polymerase reaction (of length n + 1, n + 2… bases) and, under appropriate conditions, the exonuclease products (n – 1 bases).

In addition to monitoring the progress of a reaction on DNA, QF can answer other questions about DNA-protein interactions. In particular, where is the protein located on the DNA and when does it take up that location? One method for determining the equilibrium position of a protein on DNA is *footprinting*: an end-labelled DNA is subjected to non-specific cleavages throughout its length, by DNase I or by hydroxy radicals, under 'single-hit' conditions where each DNA molecule is cleaved just once. This generates a series of fragments extending from the label to every possible position along the DNA. But if a section of the DNA is covered by a protein, that section is protected from cleavage, so the series of fragments lack representatives from this section. By modifying this approach for QF, the kinetics of proteins binding to specific DNA sites can be measured on the millisecond time scale [50].

In the example shown (Fig. 8), the Lac repressor was first mixed in a QF device with *lac* operator DNA [41]. After various times, this solution was mixed with a third containing a high concentration of DNase I. The resultant mixture then flowed into a solution of EDTA, to stop the DNase I. The DNase I reaction is thus allowed to proceed for as long as it takes the solution to flow from the second mixing chamber to the EDTA, but this was sufficient for 'single-hits' [50]. The resultant gel (Fig. 8a) shows that the section of the DNA encompassing the *lac* operator becomes progressively better protected as the time interval before adding the DNase I is increased [41]. The association rate constant of the protein for its DNA site was then evaluated (Fig. 8b).

The ability to monitor the position of a protein on DNA is particularly significant for the mechanism of transcription by RNA polymerase [48]. RNA polymerase forms a succession of complexes at its promoter sites before initiating RNA synthesis. It then leaves the promoter and moves along the DNA to extend the chain in the elongation phase of its reaction [36]. By using a rapid QF mixing device coupled to a novel ultra-fast footprinting procedure, the precise position of the RNA polymerase on the DNA has been mapped at every stage of its reaction [48].

Not all reactions on DNA are optically silent. Some can be monitored from the hypochromicity of DNA, the fact that single-stranded DNA has a higher extinction at 260 nm than double-stranded DNA [51]. Others have been examined by attaching a fluorescent label to the 5′ end of the DNA [52], or by incorporating a fluorescent base such as 2-aminopurine into the DNA [43, 53]. In most of these cases, the optical signal reports on either the association/dissociation of the enzyme from the DNA or a structural

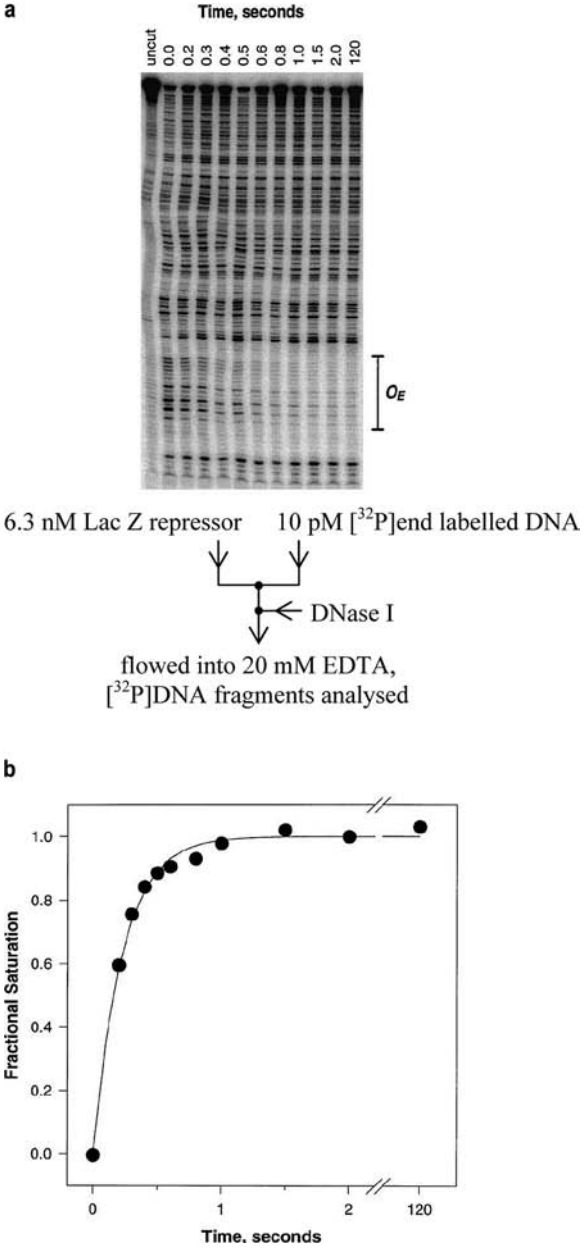


Figure 8. QF analysis of Lac repressor binding to its Operator [42]. (*a*) The autoradiogram shows samples from a QF footprinting experiment measuring the kinetics of Lac repressor binding to a 185-bp DNA fragment with a *lac* operator site (O_E). The time intervals between the initial mixing of the repressor protein with the operator DNA and the subsequent addition of the DNase I are noted above each lane. The section of the DNA carrying the operator site is as marked on the right. (*b*) The autoradiogram in (*a*) was analysed to determine the degree of protection of the O_E site at each time point, to give the progress curve shown. The solid line is the best fit to a single exponential. [Shown with permission of the American Society for Biochemistry and Molecular Biology.].

Table 3. Further examples of systems that have been studied by QF.

Enzyme	Reaction	Procedure	Information obtained	Reference
Aspartate transcarbamylase	L-aspartate + carbamyl phosphate = carbamyl aspartate + P_i	reaction mixtures ± regulatory subunit quenched in acid and carbamyl aspartate measured	in the absence of regulatory subunit, no transient phase; in its presence, transient lag phase: kinetics of an allosteric transition	[72]
ATP sulphurylase	1. ATP + SO_4 = adenosine 5′-phosphosulphate (APS) + PP_i 2. GTP ↔ GDP + P_i	enzyme pre-incubated with AMP, PP_i and Mg^{2+}, mixed with GTP, quenched in EDTA and P_i and GDP determined	reactions 1 and 2 are coupled tightly to drive APS formation: five-step mechanism of GTP hydrolysis by E·AMP·PP_i complex	[62]
Ribonuclease (RNase)	cytidine 2′:3′-cyclic phosphate (C > p) = 3′-CMP (hydrolysis) or cytidylyl-3′:5′-cytidine 2′:3′-cyclic phosphate (CpC > p) (synthesis)	quenching problem: RNase stable, C > p and CpC > p unstable in acid; reaction mixtures quenched in pH 2 buffer with pepsin which 'stops' and then inactivates RNase irreversibly by cleaving Phe120-Asp121 bond	profiles of product progress curves suggest connection between synthesis and hydrolysis	[73]
CTP synthetase	ATP + UTP + glutamine = ADP + CTP + glutamate + P_i	reaction mixtures ± GTP quenched in acid and P_i determined	without GTP, time course had a P_i burst; with GTP, no transient: mechanism for CTP formation involves phosphorylation before NH_3 attack, rather than the reverse as in [74].	[75]
Phenylalanine-tRNA ligase	ATP + L-Phe + $tRNA^{Phe}$ = AMP + PP_i + Phe-$tRNA^{Phe}$	enzyme pre-incubated with tyrosine and ATP, mixed with $tRNA^{Phe}$, quenched in acid, and Tyr-$tRNA^{Phe}$ measured	time course of Tyr-$tRNA^{Phe}$ production: rapid rise then decay in 10-ms time range as Tyr-$tRNA^{Phe}$ *hydrolyzed*; shows fidelity of overall process for Phe-$tRNA^{Phe}$ production	[76]

rearrangement of the protein-DNA complex, rather than the chemical step of the reaction pathway. Consequently, even when an optical signal is available, additional information can be obtained by carrying out parallel experiments in the QF to observe the chemical step.

For example, the cleavage of a 12-bp DNA into 6-bp products yields an increase in A_{260} if the reaction is carried out at a temperature below the T_m (melting point) of the substrate but above that for the products: the substrate is then double-stranded while the products melt to single strands [51]. When EcoRV reactions on a 12-bp substrate were examined by this method and by QF, the increase in A_{260} was not coincident with DNA cleavage, as measured by QF, but occurred after it, upon dissociation of the cleaved DNA [54]. Presumably, the 6-bp products remain double stranded while they remain in the DNA-binding cleft of the protein and only melt to single strands in free solution.

Reactions on the nucleotide bases in DNA are often monitored using 2-aminopurine as a reporter, as it has a low fluorescence when inside the double helix but a high fluorescence when exposed to solvent [43, 53]. In many of these cases, the enzyme 'flips' a base out of the DNA into its active site where it carries out a reaction on that base. In these cases, the fluorescence from 2-aminopurine reveals the base-flipping step in the reaction pathway [53] but quench methods are then needed to observe the subsequent reaction on the exposed base [38].

Further QF studies

We have selected five examples, from the large number of QF studies in the literature, that are of particular interest because of the use of a special procedure or the results obtained. These five examples are summarised in Table 3.

To conclude, multienzyme systems – even whole cells – can be studied by QF, provided that control experiments are carried out and, if necessary, precautions taken. Thus, the fragile fatty acid synthetase complex was studied successfully by QF after appropriate measures had been taken [55]. Myofibrils are fibrous structures (typically 1 μm diameter and 30 μm length) that appear not to be damaged when mixed with buffer in a QF apparatus [56] and several studies have been carried out on their ATPases (ref. 57 and references cited therein]. The sugar uptake of whole blood cells has been studied by QF [58].

Conclusions

Here our intention was to show the importance of the QF method in the study of enzyme mechanisms. With the array of analytical methods currently available and

by the use of different quenching agents, it is a highly versatile method. Thus, unambiguous information on the chemical and kinetic properties of the intermediates that make up enzyme reaction pathways can be obtained: it can not only report on product release as suggested by Fisher [9], but also on essentially all of the preceding steps in the pathway. We have illustrated its importance in the study of the mechanisms of ATP-handling enzymes such as myosin ATPase and phosphoglycerate kinase and in determining the mode of action of enzymes acting on DNA, for example the restriction endonucleases. Finally, the availability of computer programmes, including those for global fitting, has facilitated greatly the treatment of the data obtained in QF experiments [see refs. 35, 59].

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