A beginner's guide to flow kinetics

Clive R. Bagshaw
(University of California Santa Cruz, USA)

For a liquid flowing through a tube at constant velocity, the distance from the point of origin can provide a measure of the reaction time. This concept of continuous flow, first applied over a century ago to follow biochemical reactions by absorption spectroscopy, is now being used in conjunction with high-resolution structural methods such as X-ray crystallography and cryo-electron microscopy. The resultant kinetic information is crucial to understanding the mechanism.

The 'Beginner's Guide' series covers key techniques and offers the scientifically literate, but not necessarily expert audience a background briefing on the underlying science of a technique that is (or will be) widely used in molecular bioscience. The series covers a mixture of techniques, including some that are well established amongst a subset of our readership but not necessarily familiar to those in different specialisms. This Beginner's Guide introduces flow kinetics.

Barely a week goes by without an article appearing in *Nature* with a title of the form 'A structural-based mechanism of ...', that presents a functional model of some biochemical mechanism, but contains no time-dependent measurements. In effect, structures are placed in a sequence that seems to make sense, but this does not constitute evidence that the assumed sequence is correct. This emphasis on structure is not a new phenomenon. Over 20 years ago, Cornish-Bowden complained: "And for all the current and rather silly emphasis on structural biology, understanding enzymes means understanding catalysis and catalysis is concerned with kinetics, not structure: as Jeremy Knowles aptly remarked, studying the photograph of a racehorse cannot tell you how fast it can run." No doubt, the over-the-top wording was chosen to attract attention, in an attempt to redress the balance of research. However, kinetics requires a signal, and if the signal comprises detailed structural information, so much the better. Kineticists should rejoice when novel detection methods are developed because it opens new avenues for time-resolved measurements and reinstates kinetic methodology at the cutting edge of research where it belongs.

The evolution of continuous flow

Recent examples of structural measurements that encompass the fourth dimension include time-resolved crystallography based on the continuous-flow concept, first developed in the 1920s to study the kinetics of oxygenation of haemoglobin (Figure 1a,b). Both assays faced the problem that the measurement process (recording the X-ray diffraction pattern or the visible absorption spectrum, respectively) took longer than the event under investigation. By flowing the reactants at a constant known velocity past the detector, the distance of recording point from the initiation point, d, provides a measure of reaction time on the milli- to microseconds timescale. The original continuous-flow method (Figure 1a) made extravagant use of materials, requiring litres of reactants. This led to development of stopped flow in the 1940s, where flow was used to achieve rapid mixing and the reaction was monitored in real time after bringing the newly mixed reactants to a halt within about 1 millisecond. This advance was only possible when optical detectors were fast enough to capture the signal in real time. Stopped-flow instruments with optical detection have been the mainstay for analysis of transient kinetics of enzymes and other macromolecules in the last half century. For reactions without an optical signal that require offline product analysis, the continuous-flow method was extended into the quenched-flow technique. Here, the reaction is brought to a halt by mixing with a third component (e.g. acid or another denaturant) that stops the reaction at a known time (determined by the length of the ageing tube and flow velocity) and the products are analysed subsequently. A variant of this method is to rapidly freeze the reaction mix after it flows through a calibrated ageing tube. This approach was particularly useful for electron paramagnetic (spin) resonance spectroscopy (EPR/ESR) because many seconds were needed to run the spectrum. More recently, the method has been applied to electron microscopy (Figure 1c), which has made a huge impact over the last decade

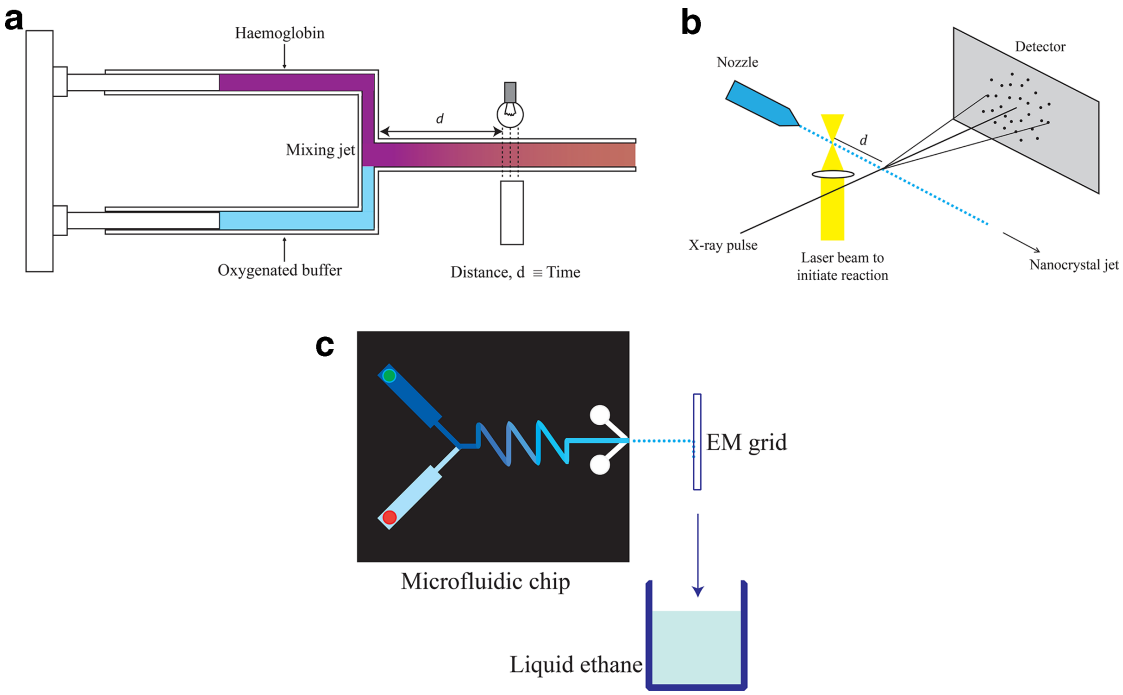

Figure 1. The evolution of continuous-flow methods. **a**) The original design used to study the oxygenation of haemoglobin. A hand-held spectroscope was used to monitor the colour change along the length of the ageing tube. **b)** A jet of nanocrystals is interrogated by X-rays, following the initiation of a photochemical reaction using a laser beam. **c)** A microfluidic device for time-resolved cryo-electron microscopy. Reactants are introduced via the red and green ports and pressurized gas is introduced via the white ports to spray the sample onto the grid. The latter is mounted on a plunger that quickly transfers the grid into liquid ethane. In the case of reactions involving a colour or fluorescence change, the microfluidic channel can be imaged to determine the time course along its length.

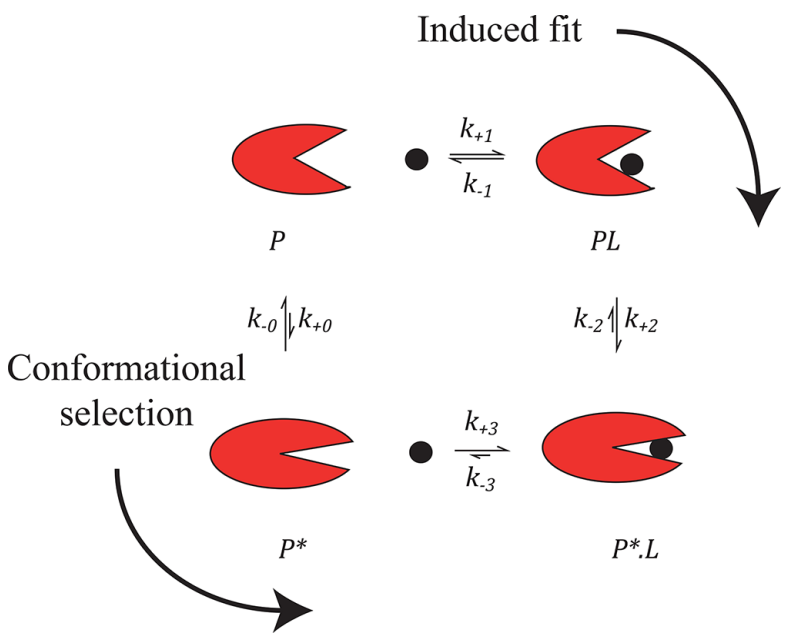

Figure 2. Conformational selection. A ligand (black circle) binding to a protein is often accompanied by closure of the binding site to make more favourable interactions. The closure may occur before or after binding. In real life, elements of both mechanisms are likely to apply, but one step (k_{+0} or k_{+2}) may limit the observed kinetics.

as structure determination has reached near-atomic resolution.

Cryo-electron microscopy has the potential advantage over X-ray crystallography in resolving heterogeneous class structures, which may suggest a dynamic system. However, the inadequacy of structural information alone to determine the mechanism is well illustrated by the long-standing discussions of induced-fit versus conformational-selection models for ligand binding (Figure 2). Conformational selection requires that the apo state of the binding partner is present in two or more conformations, one of which resembles the structure in the ligand-bound state and preferentially binds the ligand compared with the other conformation(s). However, this thermodynamic property does not translate directly to the kinetic pathway. Finding a bound-like conformation of the apo state amongst multiple conformations does not, in itself, prove conformational selection is the dominant pathway. Conversely, failure to find such a conformation does not rule out conformational selection because such a state may be below the detection limit. Only kinetic information can resolve this conundrum.

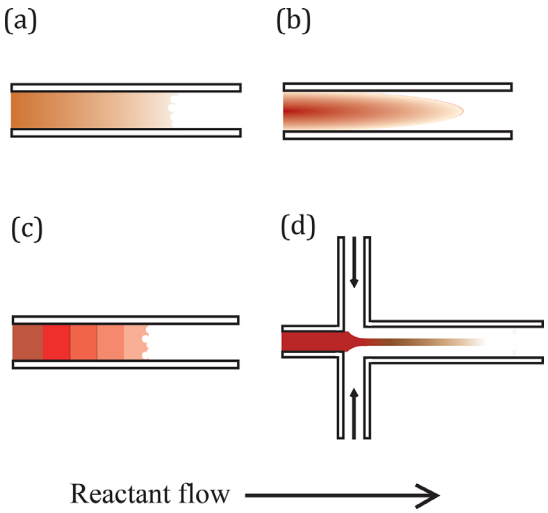

Figure 3. Flow characteristics in narrow-bore tubes.
a) Turbulent flow, **b)** laminar flow, **c)** pulsed turbulent flow and **d)** hydrodynamic focusing.

Some characteristics of flow

Given the renewed interest in applying flow methods in structural biology, it is pertinent to review some critical aspects of flow. In order for distance of fluid flow along a conventional ageing tube to be a proxy for reaction time, it is important that the flow is turbulent (Figure 3a). If the flow is too slow, it becomes laminar and the velocity of the liquid in contact with the side walls becomes zero (Figure 3b). Consequently, the age of the reaction mix at any point along the tube becomes ill-defined, even though the net flow is constant. Turbulence is also required during the mixing of reagents to bring the reactants into close proximity. The Reynold's number, R_e, defines the minimum flow velocity required through a tube to maintain turbulent flow.

$$R_e = \frac{\rho D v}{\eta}$$

where ρ is the solvent density, D is the diameter of the tube, v is the velocity of the fluid and η is the viscosity of the liquid. R_e is a dimensionless number, but care is required in using self-consistent units (e.g. kg, m and second) for the parameters in the above formula. An R_e value of <2000 will result in laminar flow, while >4000 is likely to be turbulent. It is evident that as instruments are scaled down in size (reduced D) to accommodate the limited availability of many biomaterials, so it becomes harder to achieve turbulent flow. Increasing v helps compensate, but this will shift the accessible time range to shorter values and may lead to excessive back pressure. One solution is to use pulsed flow where repetitive short but high-velocity pulses are applied to drive the solution through the ageing tube with a net slower velocity (Figure 3c).

In the case of microfluidic devices, where tube diameters are a few hundred micrometres or less, the turbulence criterion is more difficult to meet. The dispersion in timing due to laminar flow may be ignored, or at least tolerated, in cases where an approximate sampling time is sufficient. For example, in the device of Lu and colleagues used for rapid freezing of samples for cryo-electron microscopy, a mean reaction time of about 9 milliseconds was achieved using a 40 × 100 × 6000 µm channel with a flow rate of 6 µL /second^{-1}. The overall reaction time comprised 0.5 millisecond mixing time, about 4 millisecond of microfluidic flow, 5 millisecond spray time to the grid and <1 millisecond freezing time. For longer microfluidic flow channels, the dispersion of flow times, and hence reaction times, becomes more significant. This dispersion may be acceptable when characterizing the dominant species within a reaction mixture, but care is required in the interpretation of the kinetics of minor species. Laminar flow can be reduced by introducing one or more U- or Z-bends in the flow channel (Figure 1c).

The effect of laminar flow can also be minimized using hydrodynamic focusing (Figure 3d). For two-dimensional hydrodynamic focusing, buffer is introduced on either side of the new mixed reactants to give a thin ribbon of reactant that is largely clear of the side walls and proceeds along the centre of the tube linearly with time. In the case of three-dimensional hydrodynamic focusing, the reactants are introduced via the centre channel of concentric delivery tubes, which can focus the reactants into a core with a diameter of the order of 30 nm. Such an apparatus has been used to follow macromolecule refolding reactions, where the small molecule denaturant diffuses out of the core on the microsecond timescale, allowing a slowly diffusing macromolecule to refold as it progresses along the central core. The problem of side wall interactions with laminar flow can be avoided altogether by firing the reactant jet as microdroplets into air at a defined constant velocity and using the time of flight before sampling or detection as a measure of reaction time (Figure 4).

The initiation of the reaction also needs consideration. Usually, this is achieved by mixing two solutions together in a jet, so that the boundaries between the solutions are reduced to a few micrometres and diffusion completes the mixing process on the timescale a few hundred microseconds. Again, reducing the dimensions of the apparatus can assist in this process and some microfluidic devices can mix solutions within tens of microseconds. Photosensitive reactions can be initiated on an even shorter timescale with a brief light pulse.

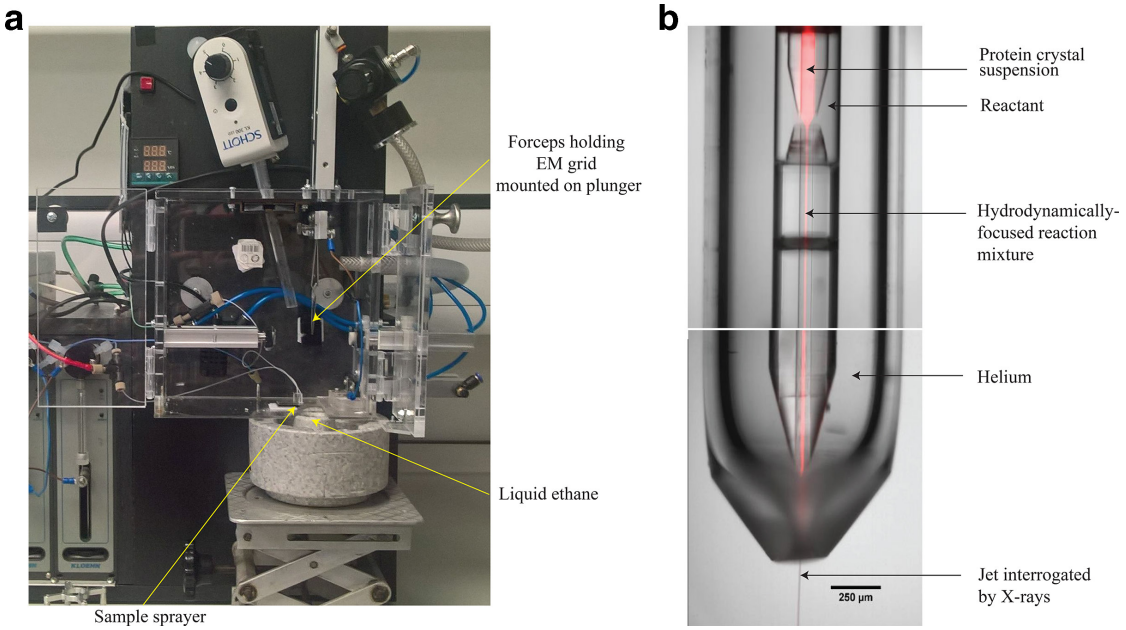

Figure 4. a) Time-resolved cryo-EM apparatus of Kontziampasis et al. (2019) (reproduced under Creative Commons License). In this apparatus, the spray is controlled by application of a high voltage rather than gas pressure as in Figure 1c. **b)** Mixing jet for time-resolved X-ray crystallography developed by Calvey et al. (2016) (reproduced under Creative Commons License), where a small molecule reactant is brought in close proximity to a suspension of crystals by hydrodynamic focusing and then fired into the X-ray beam using helium gas.

Data analysis

Data analysis is another important step in determining a mechanism. For example, consider a hypothetical reaction monitored by time-resolved cryo-electron microscopy, which is initiated by mixing an excess ligand (L) to a macromolecule (A). Excess ligand concentration ensures the binding reaction occurs under pseudo-first-order conditions with $k_1 = k_{bind}.[L]$, where k_{bind} is the second-order rate constant. Supposing the study identifies a novel transient structure formed with a half-time of about 0.07 second ($k_{obs} = \ln 2/t_{1/2} \approx 10$ second^{-1}) whose population decays to zero with a half-time of about 0.7 second ($k_{obs} \approx 1$ second^{-1}). The data are most simply analysed by a two-step mechanism:

$$A \xrightarrow[L]{k_1} B \xrightarrow{k_2} C$$

Equation 1

where A represents the apo state and B represents the novel intermediate structure. It might be tempting to assign the rate constant, $k_1 \approx 10$ second^{-1} to the binding step and $k_2 \approx 1$ second^{-1} to the second step. However, this assignment is ambiguous in the absence of amplitude information. Supposing it is observed that the transient conformation B only reaches a maximum of 8% of the total number of molecules. In this case, it is more likely that the assignment order is reversed with $k_1 \approx 1$ second^{-1} while $k_2 \approx 10$ second^{-1} (Figure 5). To distinguish these scenarios, we need to follow the kinetics of disappearance of the initial A state. If A disappears with an observed rate constant of 1 second^{-1}, then the latter assignment is favoured. Alternatively, it may be found that some A remains at the end of the reaction, indicating the presence of inactive molecules. If 90% remains as A, then the 10% active fraction of A would display a $k_1 = 10$ second^{-1} and a $k_2 = 1$ second^{-1}. The reaction profile of B could also arise if the first step is reversible. Any combination of rate constants that satisfy the conditions $k_1 + k_{-1} + k_2 = 11$ second^{-1} and $k_1 . k_2 = 10$ second^{-2} with active A being between 10% and 100% can explain the profile of B (e.g. $k_1 = 3.3$ second^{-1}, $k_{-1} = 4.7$ second^{-1}, $k_2 = 3$ second^{-1} with the initial A being 30% active). Changing the ligand concentration to modulate k_1 would help to remove this ambiguity. Note $k_{-2} \approx 0$ because B approaches 0 at the end point of the reaction.

There are also technical problems that could affect determination of the populations of the different states. The analysis of electron micrographs attempts to assign each molecular image to a structural class, but some views may be ambiguous as to their assignment. Also, for longer times in a microfluidic continuous-flow device, laminar flow becomes more problematic, so that the peak in B may be broadened and the extended tail would bias the analysis towards slower kinetics. Three important conclusions can be drawn from this example:

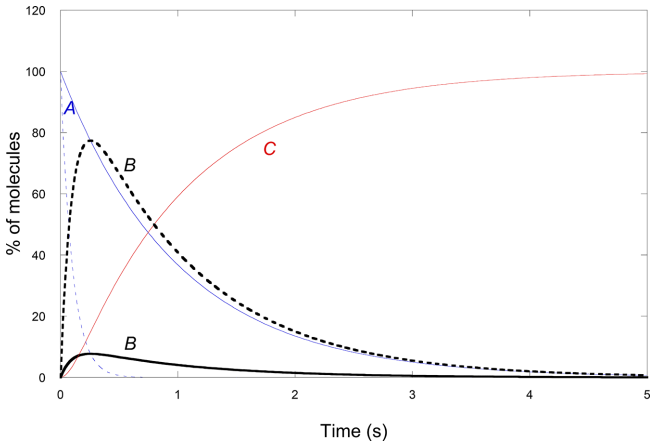

Figure 5. Reaction time course for a sequential reaction shown in Equation 1. Solid lines are for the condition k_1=1 second^{-1} and k_2=10 second^{-1}, while dashed lines correspond to k_1=10 second^{-1} and k_2=1 second^{-1}. Note the formation of *C* is independent of the assignment order, while the profiles for *B* only differ in amplitude and the half-times for the rising and falling phases are independent of the order. For *A* the amplitudes are the same, but the decay times depend on the assignment order.

(i) amplitude information is equally as important as the observed decay time in analysing kinetics; (ii) it is useful to explore the kinetics of reactions by two or more independent methods to check for instrumental or other technical contributions and (iii) assignment of an observed rate constant (i.e. one derived by fitting a time course to an exponential function) to a specific transition in a reaction can lead to erroneous conclusions. The latter often arises when the measured k_{on} and k_{off} values are assigned to the binding and dissociation steps of a mechanism, respectively. The observed k_{on} generally reflects the sum of several rate constants ($k_{+1}[L]+ k_{-1}$ at a minimum) and may be dominated by a first-order term (e.g. the reverse rate constant, k_{-1}). Also, when k_{off} is measured by displacement or washout, it is important to establish that k_{off} is limited by the dissociation step by varying the concentration of the displacing agent or the flow rate.

The recent advances in technology for time-resolved structural analysis described above have involved developments by specialized and multidisciplined teams and might seem an inappropriate topic for a 'Beginner's guide'. However, the concepts behind the analysis most likely will involve some fairly basic kinetic principles whose understanding is important for researchers, reviewers and literature readers alike, regardless of the level of instrumental sophistication. ■

Further reading

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Clive R. Bagshaw is an Emeritus Professor of Physical Biochemistry at the University of Leicester and currently a Research Associate at the University of California at Santa Cruz. He spent most of his research career investigating the kinetics of myosin ATPases, but more recently has been involved in a number of collaborative projects involving telomerases, clock proteins and MHC–peptide interactions. He recently published a textbook on Biomolecular Kinetics, which covered both experimental and theoretical aspects. Email: cbagshaw@ucsc.edu

