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The techniques described in this book provide an alternative method to understanding cellular biological structures and processes. Molecular genetic methods mainly analyse single molecules or very small cellular parts, which does not necessarily enhance our understanding of the fundamental cellular processes. However, using techniques such as atomic force, infrared microscopy, neutron reflection, stopped-flow, laser, or biosensoric-based electronics it is possible to study cells or biological systems in their entirety. Physiological processes of cells, such as movement, development, plasticity, regeneration and communication, can be visualized using the high precision biophysical techniques described here.

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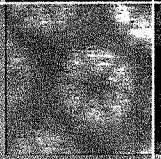

Modern Optics, Electronics, and High Precision Techniques in Cell Biology

Springer

Principles and Practice

CHAPTER 8

The Study of Fast Reactions by the Stopped Flow Method

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Contents

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8.1 Summary

The mechanisms involved in protein reactions may be elucidated by investigating the reaction kinetics. There are two investigative approaches in ascertaining the combination of elementary steps which constitute these mechanisms: (1) steady state kinetics permits analysis of the overall reaction in which protein substrates are converted into products without examining the protein molecule itself; and (2) transient kinetics allows the direct measurement of each component in the overall reaction. In this latter case, attention is focused on changes occurring in the molecule upon binding to another protein whereas the study of the equilibrium (steady state) reaction does not examine the protein molecule directly. Equilibrium studies have wider applicability since they usually require only a small amount of protein and do not involve the use of special equipment. Necessarily, the information obtained is indirect and often ambiguous. Although transient kinetics requires special techniques for measuring the rates of fast reactions in solutions, it provides information which is far more direct and useful for elucidating complicated mechanisms of reactions. Thus, the two approaches are complementary and both are indispensable for the study of protein reactions.

Advances in measurement techniques (AFM, NMR, FTIR, FRAP; see various chapters in this book) have permitted the interpretation of protein structures at atomic resolution, enabling an examination of the reaction mechanism. Concurrently with such studies, developments have been made in techniques for kinetic studies of rapid protein reactions. The stopped flow method is now commercially available and commonly used in studies on the transient kinetics of protein reactions.

Steady state kinetic analysis has been systematized and widely used for multisubstrate reactions, and several studies have been published on this subject (Gutfreund 1972, 1995; Hiromi 1979; and general references). On the other hand, the study of transient kinetics of protein reactions is still an emerging field (Eccleston 1987; Goldmann and Geeves 1991). The objective of this chapter is to present the basic procedures used in transient kinetics with the goal of motivating the use of this technique in the field of cell and molecular biology.

8.2 Introduction to Protein Kinetics

This chapter concentrates on transient kinetics by the stopped flow method. The principles of steady state kinetics and the differences and significance of the two approaches will also be discussed.

Steady state kinetics is a powerful tool for distinguishing mechanisms, especially for multiprotein systems, as it may be used to demonstrate the preferred protein-protein binding sequence as well as the protein-protein dissociating sequence. Thus, this technique is limited in a reaction scheme in which protein molecules are chemically altered. Unfortunately, steady state kinetics yields no information regarding unimolecular processes, i.e. the

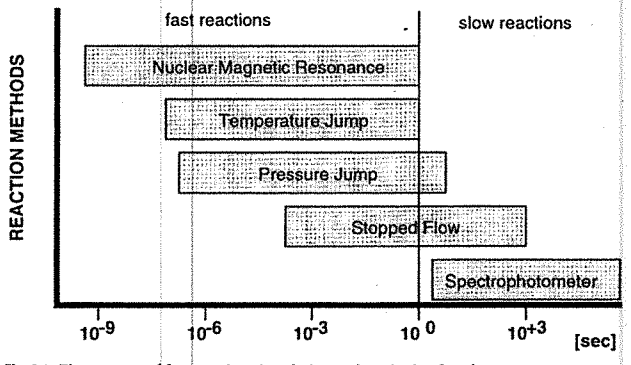

Fig. 8.1. Time ranges of fast reactions in solution and methods of study

"isomerization of complexes". The limitation of steady state kinetics resides in being unable to clarify the unique reaction mechanism amongst a plethora of possibilities which lead to the same overall rate equation. This may be removed by studying partial reactions or elementary steps directly instead of the overall reaction. For example, the spectral features of the protein–protein species involved in the isomerization process can be investigated and elementary steps can be determined. Transient kinetics, however, is a powerful tool as it permits observation of changes occurring in the molecule itself to clarify the elementary steps of the protein–protein reaction.

Techniques, which measure the rates of fast reactions in solution, are essential for transient kinetics since these steps usually occur within a few seconds. This can be accomplished by using the stopped flow method (Fig. 8.1). It is also important to examine the conformational change occurring in proteins during the reaction. The change in absorbance is most conveniently observed. Therefore, proteins having groups which absorb at specific wavelengths are best suited for transient kinetic studies. Over the years, devices have been developed which use fluorescence for detecting protein–protein interactions. Moreover, instrumentation and experimental techniques continue to be developed permitting studies with protein concentrations of as low as 10 nM. In this chapter, the use of the stopped flow method for determining protein reaction mechanisms is described.

8.3 A Manual to Measure Fast Reactions in Solution by the Stopped Flow Method

The stopped flow method involves three main components: (1) mixing two solutions rapidly to commence the reaction; (2) instantaneously stopping the

two solution streams; (3) recording changes associated with the reaction in the observation cell. Despite the simplicity of these components, there are a few basic considerations when measuring rapid protein interactions correctly with a high degree of sensitivity:

1. The mixing of the solutions must be completed before detection commences. The efficiency of mixing depends on the structure of the mixer, the flow rate and viscosity of the solution.
2. The dead time (t_d) of the apparatus must be known, during which time the reaction cannot be observed. It is usually between 0.5–1.5 ms depending on flow velocity and dead volume (v_d) of the cell.
3. The rate constant of the reaction depends on the slit width of the monochromator and maximal signal to noise ratio (S/N).

The stopped flow device can be divided into two main parts: the fluid handling system and the detection system. The fluid handling system for a piston-driven apparatus SF 61 (Hi-Tech-Scientific, Salisbury. UK) is shown schematically in Fig. 8.2.

The two solutions in the reservoir syringes (B_1) are introduced through a valve into the driving syringes of 1 ml capacity each (B_2). The driving syringes are filled by manually drawing back the block (A). A valve is then turned to connect the syringes with the mixer (C) and the driving block (A) is moved by air pressure (4–8 atm). The two solutions are rapidly combined in the mixer, flow into the observation/reaction cell (D) and are finally displaced into waste (E_1). The flow is rapidly stopped by arresting the movement of the block (E_2) and activating the switch (F) for data collection. The observation/reaction cell has optical paths of 1.5 mm and 10 mm which can be used for absorbance, transmission or light scattering measurements. The

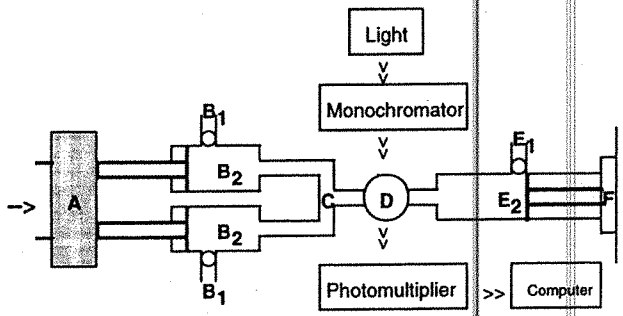

Fig. 8.2. Schematic diagram of the fluid handling system and detection system for the piston-driven SF 61 stopped flow apparatus (Hi-Tech-Scientific, Salisbury, UK): *A* pushing block; B_1 reservoir syringes; B_2 driving syringes; *C* mixer; *D* observation/reaction cell; E_1 waste; E_2 stopping block; *F* switch

dead volume (v_d) of the optical cell is 22.5 µl, and the minimum volume of the solutions needed for a single run is ≥100 µl.

The detection system consists of a light source, a monochromator, a photomultiplier, and an amplifier as well as a recording system. The light source is usually a 20 W deuterium lamp for the UV spectrum and a 75 W xenon lamp for the visible spectrum or a 100 W mercury lamp for selected peaks within the ultraviolet and visible spectrum. The reaction in the observation/reaction cell is recorded either by the change in transmittance or absorbance. The signal change detected by the photomultiplier is directly captured in digital memory after analog–digital conversion at a high sampling rate. This enables data analysis immediately after recording.

Prior to stopped flow experiments the following procedure should be adopted:

1. The magnitude of changes in e.g., absorbance, should be measured statically in a spectrophotometer. This allows the operator to set optimal detection ranges to carry out reliable kinetic measurements. A good method for testing the instrument is to mix water against water. The signal should be flat; any disturbance caused by turbulence, air bubble formation, mechanical vibration, cavitation etc. must be eliminated by optimizing the experimental protocol.
2. The reservoir syringes should be cleaned and filled with sample solutions. Three volumes of ~100 µl each should be pushed through the system to ensure equilibration of mixer and cell with the reaction solution.
3. The wavelength and slit width on the monochromator, voltage offset to compensate the voltage output and voltage of the photomultiplier should be set. The absorbance should be ideally < 1 OD; the output of the photomultiplier increases with increasing slit width or voltage of the photomultiplier; and the S/N ratio should be as high as possible.

To detect fluorescence changes the total fluorescence intensity of the reaction mixture is measured from the 'on-off' voltage difference by using a shutter which cuts off the incident light. The change in fluorescence intensity due to the reaction under the same instrumental conditions can then be read in voltage directly from the reaction signal. The ratio of the 'on/off' voltage difference indicates the percentage of the total fluorescence intensity of the reaction mixture that has actually been observed as the reaction signal. The same procedure can be carried out statically with a spectrofluorometer. In this case the fluorescence change before and after the reaction is obtained together with the total fluorescence change of the completed reaction. Fluorescence is normally detected at a 90° angle to the incident light via a filter to cut out higher order light from the excitation beam. Voltage offset is used in the same way as for absorbance measurements. The following checks are fundamental for correct measurements and interpretation of data: there should be no turbulence after the flow has stopped; unsynchronized flow stopping; temperature differences between the cell and the solution; turbidity and dust; saturation of the photomultiplier; residual effects; and the optical density of the solutions should be the same.

8.4 Analysis of Fast Protein–Protein Reactions

Changes in optical properties of proteins are categorized as changes in the absorption or fluorescence spectrum. The differences in absorption when measuring light scattering is detected at a 90° angle from the incident light. The intensity of the light scatter signal increases with a decrease in wavelength ($1/\lambda^4$). Light scattering is normally measured at 350–380 nm to avoid the specific protein absorption at ~280 nm. The changes in light scatter are of the order of 0.5–2%. Since the fluorescence emission signal of the protein is more sensitive to environmental changes than the absorption, the relative change in fluorescence is larger than the relative change in absorption for the protein undergoing reaction. Therefore, fluorescent labels bound to proteins are used in stopped flow experiments. Certain fluorophores, which are attached to a protein covalently, i.e. pyrene, NBD etc. have been used to act as probes for reporting changes (Detmers et at. 1981; Kouyama and Mihashi 1981). When a protein which contains the indicator is mixed together with another protein in a stopped flow apparatus, fluorescence quenching upon protein–protein binding will normally occur. Examples of stopped flow traces of actin binding to filamin, alpha-actinin, vinculin and talin detected by light scatter are shown in Fig. 8.3 (see pp. 166, 167).

In the study of the transient phase of protein–protein reactions, experimental data sampled by fast reaction techniques are in most cases presented by a single exponential curve for the first order reaction, or by the superposition of more than one exponential curve. Analysis of these curves permits the apparent first order rate constant (k_{app}) or the relaxation time (τ), which is the reciprocal of k_{app}, for each curve to be evaluated. Generally, the rate constant is a function of the concentration of the reacting proteins. Determining the dependence of the rate constants upon protein concentration permits both the elucidation of the reaction mechanism in terms of its elementary step and the determination of the rate constants for each of these steps. The procedure adopted is essentially similar to that employed in steady state kinetics, in which the initial rate, instead of the rate constant, is used as an index of the rate of reaction. Convenient indices for the rate of reaction are the 'initial rate' in steady state kinetics, and the 'apparent first order rate constant' or 'relaxation time' in transient kinetics. The fundamental procedures for data treatment for relaxation methods of large perturbation, i.e. stopped flow, will be considered for (1) single step reactions and (2) two-step reactions in the Appendix.

8.5 Interpretation of Kinetic Data

As mentioned in the preceding sections, the first steps in protein kinetics are: to select some plausible kinetic schemes which represents the binding mechanism; and to show that the derived rate equations account for the experimentally observed kinetic behavior, i.e. the concentration dependence of relaxation times. The next important step is the molecular interpretation of

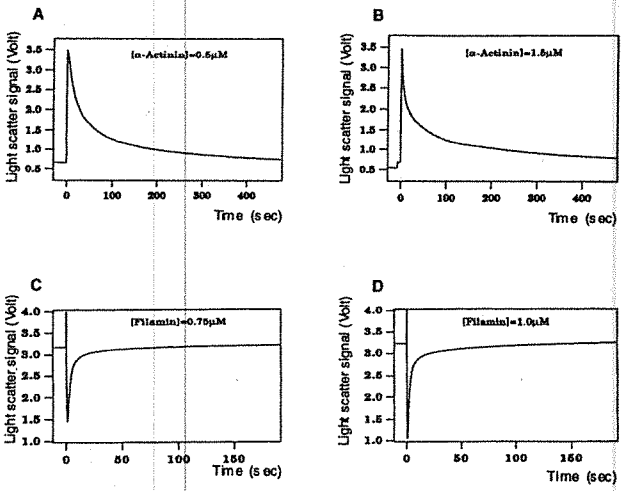

Fig. 8.3 A–H. Light scattering measurements at 90° angle to a 355 nm wavelength incident beam. All traces represent the average of five consecutive measurements in the stopped flow apparatus. Best fit to a double exponential is shown *superimposed.* A pretrigger was used to indicate the time of flow stopping and start of reaction (t = 0). The observed reaction was the binding of F-actin (3 μM) to the following proteins: **A–B** Alpha-actinin. Buffer conditions: 100 mM KCl, 2 mM $MgCl_2$ 2 mM Tris HCl, pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.005% NaN_3; T = 7 °C. Observed rates: **A** $k_1 = 0.06$ (s^{-1}); $k_3 = 0.005$ (s^{-1}); **B** $k_1 = 0.1$ (s^{-1}); $k_3 = 0.011$ (s^{-1}). **C–D** Filamin. Buffer conditions: 100 mM KCl, 2 mM $MgCl_2$, 2 mM Tris HCl, pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.005% NaN_3; T = 15 °C. Observed rates: **C** $k_1 = 0.1$ (s^{-1}); $k_3 = 0.017$ (s^{-1}); **D** $k_1 = 0.18$ (s^{-1}); $k_3 = 0.017$ (s^{-1}).

the kinetic scheme, or characterization of the mechanisms at the molecular level. A careful analysis of kinetic data from various viewpoints provides useful information or indication regarding the molecular mechanisms of reactions. To gain a deeper understanding of molecular mechanism some relevant points are described briefly below.

For a consecutive reaction, the apparent rate constant of the overall reaction (k_{app}) may be related to the rate constant of each individual step, and the slowest step in a reaction series is called the 'rate limiting step;. In general the rate constant of 'n' elementary steps in the main path of the reaction cannot be smaller than the overall reaction rate constant, if so then the relevant process is not in the main path of the overall reaction but in the side path. The rate constant, k in a bimolecular reaction $A+B \leftrightarrow AB$ is expressed as follows: $k = A \times e^{-E_a/RT}$ where, A is the frequency factor and concerns the frequency of 'effective collisions' which may lead to the reaction, and E_a, is the Arrhenius activation energy which corresponds to the height of the energy barrier to be surmounted for the reaction to occur. This equation implies that molecules which have an energy in excess of E_a, lead to the reaction. If the activation energy, E_a is zero the rate of reaction in solution will be determined simply by the frequency of effective collisions between A and B which is limited by the rate of diffusion in solution. It should be remembered that the rate constant of bimolecular association in solution cannot exceed the rate constant of a diffusion controlled reaction. Therefore, when an unreasonably high rate constant, i.e. $>10^{11}\ M^{-1} \times s^{-1}$ is obtained for a protein-protein reaction, the reaction scheme from which the rate constant was estimated should be rejected. Thus, the values of the rate constant itself could in some cases be a criterion for deciding the reaction mechanism.

Hyperbolic concentration dependence of ($1/\tau$) is often observed in protein-protein interactions. This kinetic feature is consistent with that commonly expected for a two-step reaction mechanism in which a fast bimolecular association is followed by a slow unimolecular process (see Appendix).

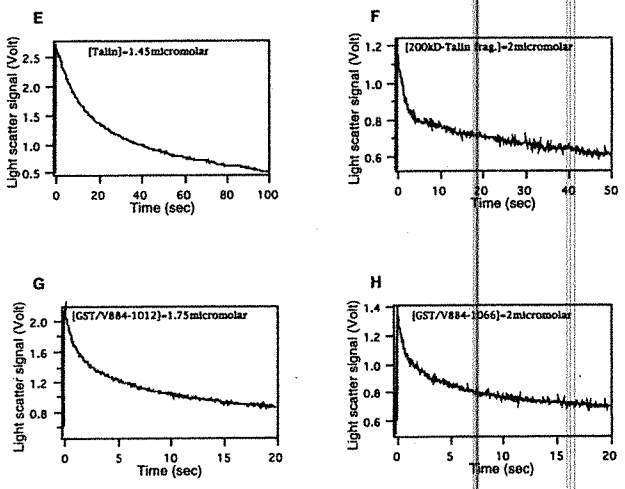

Fig. 8.3. E Talin. F-actin buffer: 2 mM $MgCl_2$, 10 mM Tris HCl, pH 7.0; 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, 0.05 mM NaN_3; T = 20 °C. Talin buffer: 20 mM Tris HCl pH 7.0, 1 mM EGTA, 0.1 mM DTT. Observed rates: $k_1 = 0.115$ (s^{-1}); $k_2 = 0.017$ (s^{-1}). **F** 200 kDa Talin fragment. Buffer conditions same as Talin; T = 20 °C; observed rates: $k_1 = 0.74$ (s^{-1}); k_2=0024 (s^{-1}). **G** Vinculin fragment (GST/V884-1012). F-actin buffer: 100 mM KCl, 2 mM $MgCl_2$, 2 mM Tris HCl, pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, 0.05 mM NaN_3. Fragment buffer: 10 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.3 mM NaN_3, 0.1 mM EDTA; T = 25 C; observed rates: k_1=1.62 (s^{-1}); k_3=0.133 (s^{-1}). **H** Vinculin fragment (GST/V884-1066). Buffer conditions same as GST/V884-1066; T = 25 °C. Observed rates: k_1=1.88 (s^{-1}); k_3=0.155 (s^{-1})

$$A + B \underset{\text{fast}}{\longleftrightarrow} A - B \underset{\text{slow}}{\longleftrightarrow} AB$$

Here, the loosely bound complex, A-B is formed very rapidly, before conversion to the more tightly and specifically bound state, AB in a unimolecular process (termed *isomerization*). This isomerization could involve: a change in the state of binding between A and B or rearrangement of A–B without any accompanying conformational changes in either A or B; a conformational change in A; a conformational change in B; or conformation changes in both A and B.

When the single reversible step, A+B↔AB is accompanied by a change in the optical property of the protein the perturbation by the stopped flow will lead to a relaxation. This relaxation is accompanied by the relaxation time (τ) and a concentration change (Δ_c). The latter is observed through the molar optical property change and this change is termed the amplitude of the relaxation. For the two-step mechanism, A+B↔A–B↔AB, two relaxation times (τ_1; τ_2) and relaxation amplitudes (Δ_{AMP1}; Δ_{AMP2}) are observed. Analyses of the relaxation amplitudes as well as the relaxation times provide useful information on the reaction mechanism. The interrelation between the observable change (relaxation time and amplitude) and elementary steps will be considered rather qualitatively for a two-step mechanism which is frequently encountered (see Appendix).

8.6 Examples of Stopped Flow Analysis of Fast Reactions

This section describes the procedures for analyzing the transient kinetics in the case of fast protein reactions. Although several excellent examples are available in the literature only four protein–protein reactions including actin are considered here. The following is a brief description of these proteins (Isenberg 1995; Maciver 1995).

1. Actin is an abundant muscle and non-muscle cytoskeletal protein of ~42 kDa molecular mass. It consists of 374/375 amino acids and exists in various isoforms. The structure has been resolved at 2.8 Å resolution. Source: rabbit skeletal muscle (Pardee and Spudich 1982).
2. Filamin is a ~280 kDa homodimeric phosphoprotein that crosslinks actin filaments and binds to lipids. It has a molecular contour length of ~160 nm, consists of 2647 amino acids, and is ubiquitous in vertebrates. *Source*: turkey gizzard smooth muscle (Hartwig and Kwiatkowski 1991).
3. Alpha-actinin is a 94–103 kDa actin cross-linking and lipid-binding protein from muscle and non-muscle cells forming antiparallel homodimers of ~30–40 nm in length. Gen-Bank: X51753 (chicken), J03486 (smooth muscle), X15804 (non-muscle), Y00689 (*Dictyostelium*). Source: chicken or turkey gizzard smooth muscle (Craig et al. 1982).
4. Vinculin is a ~117 kDa actin and lipid-binding protein. A prominent linker protein of cellular junctions and focal adhesions. *Source*: chicken gizzard smooth muscle (Geiger 1979).

5. Talin is a ~270 kDa lipid- and actin-binding phosphoprotein that nucleates actin polymerization and links microfilaments to plasma membranes. It is a dumbbell-shaped homodimer of ~51 nm contour length and consists of 2541 amino acids (mouse). Gen-Bank: X56123 (mouse fibroblast). Source: human platelets (Burridge and Connell 1983).

The dependence of the overall reaction on the protein concentration enabled the evaluation of the dissociation constant (K_d) of protein–protein complexes by static measurements. The differences in absorbance or fluorescence signal were used for measuring the binding stoichiometry and dissociation constant (K_d) of a protein–protein complex. The kinetics of actin to alpha-actinin, filamin, vinculin and talin binding were studied by the stopped flow method detecting differences in absorbance. Results obtained from actin–alpha-actinin and actin–filamin binding are given in Fig. 3A–D. In both cases, a double exponential relaxation was observed. The binding of actin to full length talin and to talin fragment (200 kDa) as well as to vinculin fragment V884-1012 and V884-1066 attached to GST (Johnson and Craig 1995) is also consistent with a two-step mechanism in which a fast bimolecular association is followed by a slower unimolecular process resulting in cross-linking and/or bundling (Fig. 3E–H). The two conformational isomers A-B and AB of the general reaction scheme (A+B↔A–B↔AB) differ in their binding mode for these species. The observed relaxation time (τ_1) and (τ_2) represent the faster and the slower binding rate, respectively.

The preceding sections have shown that transient kinetics is an indispensable method for gaining a greater understanding of the mechanisms of protein–protein reactions. Thus, the applicability of transient kinetics is limited by two factors: (1) the availability of proteins and (2) the observability of the reaction under study. Fluorescence detection has advantages in both these respects. First, lower concentrations of proteins are required for fluorescence in comparison to detection of absorption. Second, the fluorescence is far more sensitive to environmental change than absorbance. In conclusion, improvements in the performance of the apparatus used for detecting fast reactions and the development of methods for monitoring reactions based on their physical properties should make transient kinetic studies of protein–protein interactions as easy as and more efficient than steady state kinetics.

Acknowledgement. This work was supported by the Deutsche Forschungsgemeinschaft and the American Cancer Society. We thank Dr. Nathan Busch for careful reading of this manuscript.

Appendix

1. *Summary of Symbols and Terms*

(AFM)	Atomic force microscopy
(NMR)	Nuclear Magnetic Resonance
(FTIR)	Fourier Transformed infrared Spectroscopy
(FRAP)	Fluorescence Recovery After Photobleaching
(NBD)	7-chloro-4-nitrobenzeno-2-oxa-1,3-diazole
(Pyrene)	N-(1-pyrenyl)iodoacetamide

($k_{app} = 1/\tau$)	Apparent first order rate constant
(τ)	Relaxation time
($K_d = k_2/k_1$)	Dissociation constant
(OD)	Optical density
(S/N)	Signal to noise ratio
(t_d)	Dead time
(v_d)	Dead volume
(Equilibrium binding studies)	Steady state kinetics
(Rapid binding studies)	Transient kinetics
(GST/V884-1012)	Vinculin fragment (amino acids 884–1012) attached to glutathione S-transferase fusion protein (GST).
(GST/V884-1066)	Vinculin fragment (amino acids 884–1066) attached to GST.
(200 kDa Talin)	Tail fragment of the talin molecule.

2. *Michaelis–Menton Equation*

$$E + S \underset{k_2}{\overset{k_1}{\longleftrightarrow}} ES \overset{k_3}{\longleftrightarrow} E + P$$

where E = enzyme; S = substrate; P = product.

- (E) associates with (S) to form an (ES) complex at a constant rate of k_1 assuming rapid equilibrium binding. The (ES) complex can now either dissociate at a constant rate of k_2 or proceed to form a product at a constant rate of k_3. The rate of the formation of (ES) = $k_1 \times [E][S]$; the rate of the breakdown of (ES) = $(k_2+k_3) \times [ES]$ and the *Michaelis constant*, $K_M = k_2+k_3/k_1$. In a steady state the concentrations of the [ES] stay the same whilst the concentrations of [E] and [P] are changing.

3. *Single-Step Binding*

$$A + B \underset{k_2}{\overset{k_1}{\longleftrightarrow}} AB$$

- This reaction can be expressed as follows: $1/\tau = k_1 \times ([A]+[B]) + k_2$. Plotting $1/\tau$ against ([A]+[B]) the rate constants for the forward and reverse process, k_1 and k_2 can be determined from the slope and the vertical intercept, respectively. τ is equal to the reciprocal of the apparent first-order rate constant, k_{app} and is related to t such that τ at $t_{1/2}/\ln 2$. (In a special case where [A]>>[B] the concentration of A can be neglected when calculating the rate constants). Note: $K_d = k_2/k_1$.

4. *Two-Step Binding*

$$A + B \underset{k_2}{\overset{k_1}{\longleftrightarrow}} A - B \underset{k_4}{\overset{k_3}{\longleftrightarrow}} AB$$

- The relaxation times can be expressed when the bimolecular process A+B↔A–B is much faster than the unimolecular process A-B↔AB or more exactly when $k_1 \times ([A]+[B]) + k_2 >> k_3 + k_4$ as follows: $1/\tau_1 = k_1 \times ([A]+[B]) + k_2$ and $1/\tau_2 = k_3 \times ([A]+[B])/K_d + ([A]+[B]) + k_4$.

- In the case where the bimolecular process A+B↔A–B is much slower than the unimolecular process A-B↔AB or more exactly when $k_1 \times ([A]+[B]) + k_2 << k_3 + k_4$ the relaxation times can be expressed as follows: $1/\tau_1 = k_3 + k_4$ and $1/\tau_2 = k_1 \times ([A]+[B]) + k_2 \times k_4/k_3 + k_4$. For further reading see Bernasconi (1976).

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