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Distinguishing induced fit from conformational selection

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Highlights

- *Protein interactions may involve conformational changes
- *The conformational change can occur before or after the initial encounter.
- *Both mechanisms may give complex kinetics
- *Induced fit and conformational selection can be distinguished by varying the reactants' concentrations

Abstract

The interactions between proteins and ligands often involve a conformational change in the protein. This conformational change can occur before (conformational selection) or after (induced fit) the association with ligand. It is often very difficult to distinguish induced fit from conformational selection when hyperbolic binding kinetics are observed. In light of a recent paper in this journal (Vogt *et al.*, Biophys. Chem., in press) and the current interest in binding mechanisms emerging from observed sampling of distinct conformations in protein domains, as well as from the field of intrinsically disordered proteins, we here describe a kinetic method that, at least in some cases, unequivocally distinguishes induced fit from conformational selection. The method relies on measuring the observed rate constant λ for binding and varying both the protein and the ligand in separate experiments. Whereas induced fit always yields a hyperbolic dependence of increasing λ values, the conformational selection mechanism gives rise to distinct kinetics when the ligand and protein (displaying the conformational change) concentration is varied in separate experiments. We provide examples from the literature and discuss the limitations of the approach.

1. Introduction.

Mechanisms of protein-ligand interactions have been studied since the dawn of modern structure-based biochemistry. Strikingly, it is almost 60 years ago that Daniel Koshland Jr. introduced a theory to explain enzyme specificity, based on the concept that ligands may induce changes in protein structures upon binding [1]. This pioneering work, together with the subsequent studies of the group of Monod [2], lead to the establishment of what are still considered the two "standard mechanisms" in protein-ligand recognition, i.e. the concerted (nowadays more frequently called conformational selection) [2] and induced fit [3] scenarios. The field experienced a revival with the development of sophisticated NMR and molecular dynamics methods that identified high energy states in proteins that resemble ligand bound states [4–6], and allostery based on protein dynamics [7,8] rather than conformational changes, but the underlying questions remain the same [9]. Thus, it appears that proteins can sample high energy states, which are proposed to bind the ligand, while the most stable ground state does not bind the ligand. The rates of interconversion between these high and low energy states are usually fast and it is therefore hard to prove what comes first, the conformational change or the binding. This question is also of center stage in the organically growing field of intrinsically disordered proteins (IDPs) [10], where the definition of the order of events in the mechanism of binding induced folding is critical to investigate the advantages (if any) of disorder [11]. Based on different experimental and computational techniques the general consensus in the field is that the induced fit mechanism is most common [12] but conclusive evidence are scarce.

Recently, Di Cera and co-workers published three papers in which the two mechanisms, induced fit and conformational selection, were subjected to a critical appraisal [13–15]. They showed that, whilst an increase of the observed rate constant as a function of reactant concentration might be consistent with both models, a decrease in observed rate constant is a characteristic signature of conformational selection. Their analyses suggest that, in general, conformational selection is a more versatile model to describe kinetic data and the papers also offer a valuable method to distinguish between the two different scenarios.

Indeed, the first example where kinetics was used to distinguish induced fit from conformational selection clearly showed that the latter model applies to the binding of nicotinamide-adenine dinucleotide (NAD^+) to yeast glyceraldehyde-3-phosphate dehydrogenase [16]. While conformational selection is now gaining popularity, there are examples from the literature of well-established induced fit mechanisms, such as for an exonuclease-deficient mutant of T7 DNA polymerase studied by Johnson and co-workers [17–19]. This DNA polymerase represents an interesting example, in which a complex physiological mechanism is regulated kinetically by a binding step followed by a conformational change to achieve specificity towards the correct nucleotide. Binding of an incorrect nucleotide, resulting in a mismatched DNA, is characterized by a slow forward conformational change and fast reverse dissociation step, thereby allowing sufficient time for the release of the mismatched nucleotide from the polymerase active site. On the other hand, the slow release of the correct substrate and faster forward step allow the conformational change to occur and commits the enzyme towards incorporation of the nucleotide to the growing chain.

In this review we wish to complement Vogt and Di Cera's analyses [13–15] on these two mechanisms with a kinetic method that indeed can distinguish induced fit from conformational selection, even when the observed rate constant increases hyperbolically. It is our hope that the present paper will spur interest in this kinetic method which, while known since many years [20–26], has been rarely used, has never been explicitly analyzed in a dedicated paper and discussed together with its advantages and limitations.

2. How to distinguish induced fit from conformational selection

The binding kinetics of protein-ligand interactions have been reviewed previously [27,28] and Di Cera and co-workers have recently provided a comprehensive description of the kinetics for induced fit and conformational selection [13–15] (Fig. 1). In this section we complement these studies by describing a straightforward approach, which was previously used by for example Halford [20,29], Olson et al. [21], Galletto and Bujalowski [22–24] and ourselves [25,26], and that may be employed to distinguish between these two alternative scenarios.

A common method to study the time dependence of second order reactions is to carry out experiments in the presence of a very high concentration of one of the reactants. Under such conditions, commonly known as pseudo-first-order, the reaction rate (defined as the derivative of the observed signal as a function of time) will depend on the concentration of the reactant present at low concentration, with an apparent rate constant k_i' equal to the microscopic rate constant k_i multiplied by the concentration of the species at high concentration. For example, by considering a simple reaction

If $[A] \gg [B]$, the reaction rate will depend only on the concentration of B and the system will approach a first order scenario such that

We will now describe how some simple considerations regarding the pseudo-first-order assumption may be employed to distinguish between induced fit and conformational selection.

The induced fit mechanism can be described by the scheme in Fig. 1. Importantly, under pseudo-first-order conditions where $[B] \gg [A]$, this scheme will simplify to

or, if $[A] \gg [B]$

The analytical solutions of these two scenarios correspond to the following eigenvalues (denoted λ_i), which are the observed rate constants λ_1 and λ_2 .

$$\lambda_{1,2} = \frac{(k_1[A] + k_2 + k_3 + k_4) \pm \sqrt{(k_1[A] + k_2 + k_3 + k_4)^2 - 4k_1[A]k_2 - 4k_1[A]k_4 - 4k_2k_4}}{2}$$

and

$$\lambda_{1,2} = \frac{(k_1[B] + k_2 + k_3 + k_4) \pm \sqrt{(k_1[B] + k_2 + k_3 + k_4)^2 - 4k_1[B]k_2 - 4k_1[B]k_4 - 4k_2k_4}}{2}$$

It is evident from inspection of these equations that, due to their symmetry with respect to the reactants, in the case of induced fit, the solutions of the respective kinetic system are identical when pseudo-first-order experiments are performed with respect to either A or B. Thus, in both cases, the slow phase λ_2 displays a hyperbolic dependence on A or B.

On the other hand, by following the conformational selection mechanism (Fig. 1), it is postulated that the protein may explore alternative conformations in the absence of the ligand and that the different conformations are selected depending on their relative affinities for the ligand. In the simplest case with one binding-competent conformation, two alternative and different solutions may be obtained under pseudo-first order conditions for A or B, respectively. If $[B] \gg [A]$, the system will approach the following condition

with the corresponding eigenvalues of

$$\lambda_{1,2} = \frac{(k_1 + k_2 + k_3[B] + k_4) \pm \sqrt{(k_1 + k_2 + k_3[B] + k_4)^2 - 4k_1k_2 - 4k_1k_4 - 4k_2k_4}}{2}$$

On the other hand, if $[A] \gg [B]$, also the concentration of A^* will become higher than B . Thus, the reaction will resemble a simple second-order single step mechanism where B (at low concentration) and A^* (at higher concentration) will react to form AB^* . In fact, at $[A]$ tending to infinity the reaction scheme will simplify to

leading to the simple solutions

$$\lambda_2 = k_3^{app}[A] + k_4$$

where, in the case of a fast pre-equilibrium

$$k_3^{app} = k_3 \times \frac{k_1}{k_1 + k_2}$$

or expressed in terms of fraction of A^* :

$$k_3^{app} = k_3 \times \frac{[A^*]}{[A] + [A^*]}$$

Importantly, in this case, because the amplitude of the reaction is limited by the concentration of B , which is lower than A^* at high $[A]$, the linear phase (λ_2 in Fig. 2A and λ_1 in Fig. 2B) might be the only detectable phase in kinetic experiments. What

concentration of A or A* is needed to achieve pseudo first order conditions? While a 10-fold excess of one of the reactants is often used as a rule of thumb to approximate pseudo first order conditions, in practice, we usually cannot tell the concentration of A*. It is therefore crucial to go as high as possible in concentration of both $[A]_{\text{TOT}}$ and $[B]_{\text{TOT}}$ to look for differences in the kinetics (see below).

A simulation of the observed reactions with plausible experimentally accessible concentrations and rate constants are reported in Fig 2. Note that both mechanisms, induced fit and conformational selection, will both yield a hyperbolic dependence of the slow phase λ_2 , when B is varied. In other words, if a hyperbolic phase is observed, both mechanisms will equally well explain the experimental data. However, when A is varied, the values for λ will become distinct in the conformational selection scenario. As frequently noted, a hyperbolic phase going down (Fig. 2B, λ_2 , excess of B) rules out the induced fit mechanism. Finally, note that while a two-step reaction will always yield two theoretical phases (λ_1 and λ_2) only one is often observed experimentally.

3. Examples of experimental discrimination from the literature.

An early example was published by Halford already in 1971 [20]. He demonstrated that the alkaline phosphatase-catalyzed hydrolysis of 2,4-dinitrophenyl phosphate involves a conformational change in the free enzyme. Excess of enzyme over substrate is usually referred to as single turnover experiments and has been widely

used in enzymology to access intermediate steps in catalysis (see for example Fierke and Hammes [30]).

One system for which extensive kinetic experiments have been carried by Olson, Björk and co-workers is that between antithrombin and heparin. A well defined sequence of the glykosaminoglykan heparin increases the affinity of antithrombin for thrombin, leading to inhibition of the blood clotting cascade. Heparin induces a large conformational change in antithrombin resulting in an observed rate constant that increases hyperbolically [31]. Olson et al. demonstrated that the binding is via induced fit by performing stopped-flow experiments by varying both antithrombin and heparin and observing similar hyperbolic kinetics [21].

Bujalowski and coworkers performed beautiful kinetic binding experiments on the interaction between DnaC, a protein from *E. coli* involved in replication, and nucleotide cofactor analogs [22]. The binding kinetics displayed three phases with increasing concentration of the ATP analog MANT-ATP. One of the phases was linearly increasing with MANT-ATP, while the other two appeared constant. Such behavior is consistent with both induced fit and conformational selection. However, when MANT-ATP was kept constant and the concentration of DnaC was increased, one of the two constant kinetic phases disappeared, proving that this phase was related to a conformational transition in DnaC prior to binding. The same group later demonstrated that the interaction between a DNA polymerase and double stranded DNA involved induced fit rather than conformational selection [24]. Their work contains a comprehensive description of the theoretical basis for the kinetic test,

including amplitude analysis, and is highly recommended as further reading [22,23] along with Olson et al. [21].

We have previously used the kinetic test on binding of labeled peptides to PDZ domains [25,26,32], and obtained the behavior expected from both induced fit and from conformational selection. The PDZ domain typically recognizes and binds to sequences located at the C-terminus of its physiological partner [33,34]. Short peptides of 6-7 amino acids or more, mimicking the C-terminal tail of the target, are good experimental analogues for the kinetic study of such interactions [35]. It has been observed that the binding of PDZ domain to its partner, sometimes involves an allosteric behavior that is mirrored by a small conformational change [36,37]. This behavior is not a general feature of all PDZ domains and may or may not be observed in a particular case.

In one of the studies [25] we investigated the mechanism of binding in two different PDZ domains, one of which displayed a small conformational change (the second PDZ domain from PTP-BL). In line with expectations, the PTP-BL PDZ2 domain displayed a clear hyperbolic behavior of the observed rate constants as a function of either the peptide or the PDZ domain, with nearly identical observed rate constants, demonstrating that the observed kinetics resulted from a conformational change occurring after an initial recognition step, that is, induced fit.

A very different behavior was observed in another set of experiments in which the denatured state was engineered by site-directed mutagenesis to be the most stable conformation at physiological conditions. Since the ligand stabilizes the native but not

the denatured state, mixing the protein to its ligand will trigger folding. Thus, for a destabilized mutant the overall reaction may be described as:

where PDZ_D and PDZ_N represent the denatured and native PDZ domain, respectively, and P is the peptide ligand. To validate this mechanism, and to exclude that the ligand P may recognize the denatured PDZ domain, we performed the kinetic test described above, namely by performing experiments under pseudo-first-order conditions with respect to both PDZ and P (Fig 1 in ref. [32]). In line with the theory presented in the previous section, we obtained very different scenarios in the two different experimental set-ups. In particular, in the presence of high concentrations of P we observed a hyperbolic decrease of the observed rate constants while, when the experiments were performed at high concentrations of PDZ, the observed rate constants were much higher and resembled a simple one-step bi-molecular reaction (i.e., a linear increase in the observed rate constant). In the latter condition, because the total $[PDZ]$ is much higher than $[P]$ (where $[PDZ]=PDZ_N+PDZ_D$), also the concentration of PDZ_N is higher than $[P]$ and the observed kinetics follows

$$\lambda = k_3^{app}[PDZ_N] + k_4$$

The highlighted examples demonstrate that the proposed kinetic test is adequate to discriminate, at least in some cases, between induced fit and conformational selection.

4. Limitations of the method.

While the approach presented here is very simple in theory, there are several practical issues, which could make the decisive experiment difficult to perform.

Firstly, even when the hyperbolic dependence of λ_2 is clearly detected by for example varying a small molecule ligand, it might prove problematic to achieve high enough concentration of the protein, such that the two curves can be clearly assessed as identical (induced fit) or distinct (conformational selection) (Fig. 2). However, the cited examples in the previous section as well as the unpublished example below show that it is possible to achieve high enough protein concentrations to allow the test. In principle, any protein amenable to NMR, which usually requires 0.5-1 mM of protein, should be possible to use at high enough concentrations in kinetic experiments.

Secondly, how clear a certain kinetic phase will be in an experiment depends on its amplitude. To illustrate this problem we show in Fig. 3 an analysis on the interaction between the two disordered protein domains ACTR and NCBD (partially unpublished work). While we can clearly distinguish two phases when the experiment is performed under pseudo-first order conditions for ACTR, it is not possible to do so under pseudo-first order conditions for NCBD, perhaps due to high total fluorescence with excess NCBD. However, the resulting phase in the latter case (varying NCBD) is clearly an average of the two phases observed in the former case (varying ACTR). Thus, with prior knowledge about the experiments with excess ACTR, we conclude that this binding reaction displays similar kinetics under pseudo first order conditions

for both ACTR and NCBD and that the hyperbolic phase results from an induced fit conformational change. (The same conclusion was reached in the original paper [38] on other grounds). Thus, this experiment highlights the potential problem of low amplitude of kinetic phases but also show that protein-protein interactions involving disordered protein domains are amenable to the test.

Thirdly, transitions between high energy conformations and the ground state are often associated with large rate constants ($>1000\text{ s}^{-1}$), which could preclude analysis. Stopped-flow and quenched-flow methodology is usually restricted to k_{obs} values $<500\text{ s}^{-1}$. Continuous flow cover rate constants of ca. $2,000\text{--}20,000\text{ s}^{-1}$ [39] but commercial instruments are not available. Temperature jump can monitor reactions with rate constants approaching 10^5 s^{-1} (capacitor discharge) or even 10^6 s^{-1} (laser induced) but K_d for binding reactions are often insensitive to changes in temperature resulting in small kinetic amplitudes. We also wish to point out that kinetics only reflect the rate limiting step(s) for the reaction. There could be numerous induced fit and conformational selection events occurring on a fast time-scale (e.g., helix-coil transitions) that remain undetected. Thus, in terms of the current test, kinetics monitor the rate limiting step (conformational change) and the nature of the associated intermediate (free or complexed protein).

5. Conclusions

A hyperbolic increase in observed rate constant with increasing ligand concentration is consistent with both induced fit and conformational selection [13]. The method described in the current paper is a valuable test to be considered when such increase

of λ is observed (as described above for a number of cases), because it can always, in theory, distinguish induced fit and conformational selection. However, due to limitations in the experimental techniques it may be very difficult to prove a mechanism in practice. A combination of biophysical techniques and structural information may guide the experimentalist to suggest the most likely scenario, be it induced fit or conformational selection or, most likely, a multi-step binding in which both mechanisms are present. For example, upon protein-protein interactions involving IDPs it is quite possible that helices form independently of the binding partner but that the often extended conformation of the bound IDP must be guided by the surface of the partner [12]. Rapid transitions between high energy states may be almost as fast as helix-coil transitions and hard to monitor with existing techniques. Nevertheless, whenever (i) the observed rate constants are experimentally accessible, (ii) a rate limiting conformational change is reflected in kinetic data as a hyperbolic dependence, and (iii) sufficiently high concentrations of protein can be achieved, it is possible to distinguish induced fit from conformational selection [20–26].

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Figure legends

Figure 1. Reaction schemes depicting induced fit and conformational selection. The prime indicates a pseudo-first order rate constant.

Figure 2. Two examples of simulated observed rate constants for a conformational selection mechanism in which the initial step is fast (panel *A* and *D*) or slow (panel *B* and *E*) in relation to the step governed by k_4 (see Fig. 1). Panels *C* and *F* show an example of simulated observed rate constants for an induced fit mechanism. In the case of induced fit, λ_2 will always display a hyperbolic increase with either reactant A or B. Note that the kinetic amplitudes in this simulation reflects the concentration of the intermediate AB. In a real experiment involving for example fluorescence, the kinetic amplitudes depend on the concentration of each species and their respective fluorescence yield. Thus, observed kinetics may appear single exponential either because λ_1 is too high or because the total fluorescence yield of all species appear as one phase. Simulations of experimental traces were performed by defining the kinetic scheme and concentrations of the interacting species, followed by solving the differential equations numerically using Copasi (http://www.copasi.org/tiki-view_articles.php). The traces obtained were subsequently fit to either a single or double exponential equation using Kaleidagraph to obtain observed rate constants (λ) at different concentrations of A or B. Only the slow kinetic phase is shown in panel *A*, since the fast one is difficult to measure and very similar for conformational selection and induced fit. Also note that, in panel *B*, the intercept with the y-axis for λ_2 (excess B) is not k_1+k_2 , which is expected from a slow conformational change followed by fast binding [40]. The reason is that $k_4 = k_2$. When $k_4 \gg k_2$, k_{obs} at zero ligand concentration will indeed equal k_1+k_2 . On the other hand, when $k_4 < k_2$, k_{obs} at zero ligand concentration will be governed by k_1+k_4 and not by k_2 .

Figure 3. Binding experiments between the intrinsically disordered protein domain ACTR and the molten-globule like NCBD [38]. Kinetic traces were obtained by rapidly mixing (*A*) 1 μ M NCBD with 16 μ M ACTR or (*B*) 1 μ M ACTR with 16 μ M NCBD. The residuals are from fits to either a single or a double exponential equation. The large errors in the curve fitting of a double exponential in panel *B* reflect the fact that the trace is well described by a single exponential, as also shown by the residuals. (*C*) The dependence of the observed rate constants on ACTR or NCBD concentration, respectively. Parameters were obtained from fits of kinetic traces (see panels *A* and *B*) to either a single or double exponential equation. Experiments were performed in 20 mM sodium phosphate, pH 7.4, $I=1.0$ M (adjusted with NaCl). The data for excess ACTR were previously published [38]. (*D*) Structure of the complex between ACTR and NCBD (Protein Data Bank code 1KBH) [41]. The yellow ACTR domain wraps around and occupies a largely hydrophobic groove in the blue NCBD domain. The figure was made with PyMol [42].