Review

Essential role of conformational selection in ligand binding

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HIGHLIGHTS

- Conformational selection is always sufficient and often necessary to account for the relaxation kinetics of ligand binding.
- Induced fit is never necessary and only sufficient in a few cases.
- The long assumed importance and preponderance of induced fit as a mechanism of ligand binding should be reconsidered.
- Conformational selection is an essential component of any mechanism of ligand binding.

GRAPHICAL ABSTRACT

Linkage scheme containing four species is depicted in green. Conformational selection and induced fit, two special cases of the linkage scheme, are shown in blue and yellow.

ABSTRACT

Two competing and mutually exclusive mechanisms of ligand recognition – conformational selection and induced fit – have dominated our interpretation of ligand binding in biological macromolecules for almost six decades. Conformational selection posits the pre-existence of multiple conformations of the macromolecule from which the ligand selects the optimal one. Induced fit, on the other hand, postulates the existence of conformational rearrangements of the original conformation into an optimal one that are induced by binding of the ligand. In the former case, conformational transitions precede the binding event; in the latter, conformational changes follow the binding step. Kineticists have used a facile criterion to distinguish between the two mechanisms based on the dependence of the rate of relaxation to equilibrium, $K_{rel}$, on the ligand concentration, $[L]$. A value of $K_{rel}$ decreasing hyperbolically with $[L]$ has been seen as diagnostic of conformational selection, while a value of $K_{rel}$ increasing hyperbolically with $[L]$ has been considered diagnostic of induced fit. However, this simple conclusion is only valid under the rather unrealistic assumption of conformational transitions being much slower than binding and dissociation events. In general, induced fit only produces values of $K_{rel}$ that increase with $[L]$ but conformational selection is more versatile and is associated with values of $K_{rel}$ that increase with, decrease with or are independent of $[L]$. The richer repertoire of kinetic properties of conformational selection applies to kinetic mechanisms with single or multiple saturable relaxations and explains the behavior of nearly all experimental systems reported in the literature thus far. Conformational selection is always sufficient and often necessary to account for the relaxation kinetics of ligand binding to a biological macromolecule and is therefore an
The interaction between a ligand, L, and its macromolecular target, E, forms the basis of function and regulation in all biological systems. Understanding how ligands recognize their targets in terms of structure, energetics and kinetics remains a central issue of biochemistry and biophysics and a prerequisite to rationally design efficient enzymes, effective drugs, and new therapeutics [1]. The interaction involves two components: specific binding of L to E, and linked conformational changes that may precede and/or follow the binding step. The combination of binding steps and conformational transitions in any given mechanism of recognition generates the repertoire of kinetic behaviors accessible to experimental measurements. The challenge facing the experimentalist is to decipher the nature of conformational transitions involved in the recognition process from analysis of the transient behavior of the system relaxing to equilibrium [2,3]. A system of N independent species gives rise to N independent relaxations, some of which may be too fast to measure by conventional stopped-flow techniques or may be spectroscopically silent. Analysis of a ligand binding interaction must therefore rely only on the relaxations accessible experimentally and interpretation of the underlying mechanism should be based on the simplest kinetic scheme consistent with the observations.

2. Simplest binding mechanism: the lock-and-key model

Over the years, our interpretation of binding interactions has evolved alongside the emerging view of the plasticity of biological macromolecules buttressed by the successes of x-ray crystallography and NMR spectroscopy [4]. Energy landscapes portraying the conformations available to the macromolecule have replaced the static view of a single species as far as the equilibrium and kinetic properties of the system are concerned. The partition function of a system capable of binding L at a single site, as in the lock-and-key model, is linear in [L] regardless of the number of conformations accessible to the macromolecule [11,12]. Likewise, the transient properties of the system depend only on the slowest kinetic steps in the mechanism and replacing E or EL in the lock-and-key model with an ensemble of rapidly interconverting conformations does not change the observed relaxation kinetics [6]. Therefore, the lock-and-key model describes the behavior of a macromolecule that exists in a single conformation but also applies to an ensemble of rapidly interconverting conformations, in which case $k_{\text{on}}$ and $k_{\text{off}}$ represents ensemble averages of rate constants over the entire population. In general, any individual species in a kinetic scheme can be replaced by an ensemble of rapidly interconverting species without changing the kinetic properties of the system. Therefore, the dynamic nature of proteins and the existence of an ensemble of conformations will not produce a departure from the simple properties predicted by the lock-and-key model, unless the conformational transitions take place over time scales that eventually affect events of functional significance.

3. Binding coupled to conformational transitions: conformational selection and induced fit

Consider next the relevant case of the macromolecule existing in multiple conformations interconverting on the ms-μs time scale, which is directly relevant to binding/dissociation reactions [10]. Two limiting cases should be considered, depending on whether the conformational transitions precede or follow the binding step. In both cases, relaxation to equilibrium no longer follows a straight line as in the lock-and-key model because the macromolecule assumes alternative

equilibrium that is linear in [L], i.e.,

$$k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[L].$$

Measurements of $k_{\text{obs}}$ as a function of [L] yield both $k_{\text{off}}$ and $k_{\text{on}}$ as the intercept and slope of the plot, respectively. Hence, for a simple binding interaction, the value of $k_{\text{obs}}$ equals the rate of ligand dissociation for $[L] = 0$ and grows without limits as $[L]$ increases.

It has been argued for a long time that the lock-and-key model is too crude an interpretation of ligand binding because it envisions a rigid body collision between the ligand and its target, thereby neglecting the conformational flexibility of the macromolecule [2,3] recently supported by x-ray crystallography [7], NMR spectroscopy [8,9] and single-molecule fluorescence detection [10]. Although this argument has important merits, it is not completely rigorous. The existence of multiple conformations is not necessarily incompatible with the lock-and-key model. An ensemble of conformations that interconvert on a time scale faster than binding and dissociation events is indistinguishable from a single species as far as the equilibrium and kinetic properties of the system are concerned. The partition function of a system capable of binding L at a single site, as in the lock-and-key model, is linear in [L] regardless of the number of conformations accessible to the macromolecule [11,12]. Likewise, the transient properties of the system depend only on the slowest kinetic steps in the mechanism and replacing E or EL in the lock-and-key model with an ensemble of rapidly interconverting conformations does not change the observed relaxation kinetics [6]. Therefore, the lock-and-key model describes the behavior of a macromolecule that exists in a single conformation but also applies to an ensemble of rapidly interconverting conformations, in which case $k_{\text{on}}$ and $k_{\text{off}}$ represents ensemble averages of rate constants over the entire population. In general, any individual species in a kinetic scheme can be replaced by an ensemble of rapidly interconverting species without changing the kinetic properties of the system. Therefore, the dynamic nature of proteins and the existence of an ensemble of conformations will not produce a departure from the simple properties predicted by the lock-and-key model, unless the conformational transitions take place over time scales that eventually affect events of functional significance.
conformations over a time scale comparable to the binding/dissociation events of functional significance. In the first case (Fig. 1), originally discussed by Eigen as pre-equilibrium [2,3] and more recently referred to as conformational selection [13–15], the macromolecule is assumed to undergo a transition between multiple conformations from which one is selected for binding by the ligand. In this scheme, the number of conformations accessible to the macromolecule decreases upon ligation as the ligand singles out the one with optimal fit from the pre-existing ensemble. In the second case (Fig. 1), introduced by Koshland as induced fit [16], the macromolecule is assumed to first bind the ligand and then change its conformation in the bound form to optimize the complex. Here, the conformational flexibility of the macromolecule does not pre-exist. Rather, association with the ligand generates it. Two mutually exclusive philosophies emerge in the analysis of ligand binding to biological macromolecules: one where the complexity necessary to accommodate a ligand pre-exists in the ensemble of conformations available to the macromolecule, and the other where such complexity is generated after ligand binding to optimize the initial encounter. Accordingly, two distinct scenarios are expected. In the case of conformational selection, the macromolecule is expected to be trapped in multiple conformations in the absence of the ligand. The ligand selectively binds to a single conformation, stabilizing it. (E) Induced fit binding, in which the macromolecule exists as a single conformation in the absence of ligand. After binding, the ligand induces conformational rearrangements in the macromolecule to optimize the interaction. (F) Linkage scheme for binding. The macromolecule exists as multiple conformations capable of interacting with the ligand. After binding, the ligand may change the relative stabilities of the two conformations or may alter the barrier for conversion between the two bound conformations.

Fig. 1. Free energy landscape view of macromolecular structure and binding. (A) Single low energy well, indicating a single dominant conformation. (B) Multiple low energy wells separated by a free energy barrier. In this instance, two dominant conformations are present. (C) Lock-and-key mechanism of binding, where the macromolecule exists as the same species in both the free and bound states. Binding may stabilize the macromolecule, but does not alter its conformational distribution. (D) Conformational selection, where the free macromolecule exists in multiple conformations in the absence of the ligand. The ligand selectively binds to a single conformation, stabilizing it. (E) Induced fit binding, in which the macromolecule exists as a single conformation in the absence of ligand. After binding, the ligand induces conformational rearrangements in the macromolecule to optimize the interaction. (F) Linkage scheme for binding. The macromolecule exists as multiple conformations capable of interacting with the ligand. After binding, the ligand may change the relative stabilities of the two conformations or may alter the barrier for conversion between the two bound conformations.
dissociation. Ideally, information derived from structural biology and relaxation kinetics must converge to the same conclusion. Kinetic data are also necessary to derive information on the rate constants governing the recognition process.

Unlike the lock-and-key model, conformational selection and induced fit introduces a conformational transition in the kinetic scheme (Fig. 1) that increases the number of independent relaxations from one to two. The fast relaxation increases without limits with [L] because it reflects the binding step, as in the case of the lock-and-key model. The slow relaxation, on the other hand, is saturable and spans two finite asymptotes in the plot of $k_{obs}$ vs [L]. For several decades, kineticists have endorsed a facile litmus test for conformational selection and induced fit based on the dependence of this slow, saturable relaxation on the ligand concentration [L] [2,3,17–19]. An increase of $k_{obs}$ vs [L] has been considered diagnostic of induced fit, with a decrease of $k_{obs}$ vs [L] being diagnostic of conformational selection. In turn, the vast preponderance of systems relaxing to equilibrium with a $k_{obs}$ increasing with [L] to a saturable level has been used for decades as evidence of induced fit as a dominant mechanism of ligand binding [2,3,17–19]. This facile conclusion is only valid under the rather unrealistic assumption of conformational transitions being rate-limiting in the kinetic mechanism. In fact, when such assumption is dropped and the kinetic scheme of conformational transitions being rate-limiting in the kinetic mechanism in Scheme 1 (Fig. 2), i.e. [20],

$$
{k_{obs}}_{1,2} = \frac{k_{off} + k_{on} + k_{on}[L] + \sqrt{(k_{off} + k_{on}[L] - k_{on} - k_{off})^2 + 4k_{on}k_{off}[L]}}{2}
$$

We see from Eq. (2) above that the condition $k_{r} = k_{off}$ generates a second relaxation that becomes independent of [L] and equal to $k_{r}$. Elementary rearrangements show that this slower $k_{obs}$ saturates at $k_{r}$ regardless of the kinetic rate constants in the scheme and that the value at [L] = 0 is the smaller of $k_{off}$, the rate constant for ligand dissociation, or the sum $k_{r} + k_{off}$. When $k_{r} + k_{off} < k_{on}$, the value of $k_{obs}$ can only decrease with [L] from $k_{r} + k_{off}$ for [L] ≈ 0 to $k_{r}$ for large [L]. This is the scenario generated by the "rapid equilibrium approximation" where conformational transitions are slower than the rate of ligand dissociation. However, when $k_{r} + k_{off} > k_{on}$ a different scenario unfolds. When $k_{off} > k_{r}$, the value of $k_{obs}$ decreases with [L], but it otherwise increases with [L] if $k_{off} < k_{r}$. Basically, the condition $k_{off} = k_{r}$ acts as a demarcation between the decrease and increase of $k_{obs}$ with [L]. As a result of that, conformational selection covers the entire repertoire of kinetic properties of a system whose kinetics obey a single saturable relaxation. The richness of such repertoire comes at the expense of an exact resolution of the kinetic parameters involved in the mechanism. In general, when $k_{obs}$ decreases with [L], only the asymptote at saturating [L] is defined exactly as $k_{r}$. The asymptote for [L] = 0 becomes ambiguous as it may reflect either $k_{off}$ or the sum $k_{r} + k_{off}$, whichever is smaller. Hence, great care should be taken in the interpretation of such plots in practical applications. When $k_{obs}$ increases with [L], on the other hand, the lower asymptote always reflects $k_{off}$ giving a direct measurement of the dissociation rate constant of the ligand from its target. Likewise, when $k_{obs}$ is independent of [L], both $k_{off}$ and $k_{r}$ are assigned unambiguously from the plot.

The richness of kinetic behaviors associated with conformational selection is not shared by induced fit, which always produces a saturable relaxation that increases with [L]. That is readily seen from the relaxations associated with Scheme 2 (Fig. 2), i.e. [20],

$$
{k_{obs}}_{1,2} = \frac{k_{r}^2 + k_{off} + k_{on}[L] + \sqrt{(k_{off} + k_{on}[L] - k_{r} + k_{off})^2 + 4k_{off}k_{on}[L]}}{2}
$$

Although Eq. (3) is quite similar to Eq. (2) for the case of conformational selection, a difference in the last term under the square root expression produces two relaxations that always increase with [L]. There is no combination of rate constants that makes the relaxation independent of [L]. The saturable relaxation equals the sum $k_{r}^2 + k_{r}k_{off}$ for large [L] and a more convolute expression for [L] = 0 (Fig. 2) that is however always less than $k_{r}^2 + k_{r}k_{off}$.

The properties encapsulated by Eqs. (2) and (3) refer to the case where conformational transitions involve only the macromolecule E. Extension to include conformational flexibility in the ligand L becomes biologically relevant in the case where the ligand is itself a macromolecule, as observed in enzymatic cascades [21]. However, whenever the ligand is in large excess, its properties do not change significantly upon interaction with the macromolecule and the results of Eqs. (2) and (3) remain valid. The case where the macromolecule and ligand are present at comparable concentrations require modifications of the entire set of differential equations associated with the system and is beyond the scope of this review.

4. Is induced fit necessary as a mechanism of ligand binding?

The foregoing analysis has very important implications for the interpretation of relaxation kinetics. A plot of $k_{obs}$ decreasing with or independent of [L] is unambiguous proof of conformational selection and induced fit, respectively. Scheme 3 is the linkage scheme, of which schemes 1 and 2 represent special cases (see also Fig. 3).

Fig. 2. Mechanisms of ligand binding discussed in the text with corresponding asymptotic values for $k_{obs}$. Shown are the limits $[L] = 0$ and $[L] = \infty$. Schemes 1 and 2 correspond to conformational selection and induced fit, respectively. Scheme 3 is the linkage scheme, of which schemes 1 and 2 represent special cases (see also Fig. 3).
disproves induced fit automatically. On the other hand, a plot of \( k_{\text{obs}} \) increasing with [L] may be associated with induced fit or conformational selection and neither proves, nor disproves either mechanism. Therefore, conformational selection can never be disproven as a mechanism of ligand binding when a single saturable relaxation is measured experimentally, but induced fit can. Conformational selection is always sufficient and in some cases necessary to explain the relaxation kinetics for systems associated with a single saturable relaxation. On the other hand, induced fit is only sufficient in some cases and never necessary.

In addition to its limited kinetic repertoire, induced fit poses an intriguing challenge to the thermodynamicist. Validity of the induced fit hypothesis rests entirely on the assumption that the optimal bound conformation (EL in Scheme 2, Fig. 2) does not exist in the free state. However, from a thermodynamic standpoint, it is not possible to rule out that such conformation, no matter how energetically unfavorable, could pre-exist in solution prior to ligand binding. This scenario is illustrated in Fig. 3 where induced fit and conformational selection can be seen as special cases of a more general scheme (Scheme 3, Fig. 2) linking the E"-E isomerization to ligand binding. The equilibrium properties of this scheme are well known and form the basis of linkage thermodynamics [11,12]. The steady state properties have also been discussed in great detail first by Botts and Morales [22] in connection with the action of a modifier on enzyme activity and then by Hill [6] in the context of energy transduction. The linkage scheme shows that for any induced fit step in a kinetic mechanism generating a new conformation of the macromolecule in the bound form, a perfectly legitimate thermodynamic cycle can be constructed including the same conformation in the free form. Hence, the basic tenet of the induced fit hypothesis, i.e., that the ligand induces a new conformation of the macromolecule that does not pre-exist in solution, is difficult to defend on thermodynamic grounds. On the other hand, conformational selection is not invalidated by the existence of multiple bound conformations in the linkage scheme because its basic hypothesis rests on the existence of multiple conformations in the free form from which the ligand selects the optimal one for binding. Preferential rather than exclusive binding to one of the two conformations in the linkage scheme does not invalidate existence of the pre-equilibrium between E and E*, which is the pillar of the conformational selection hypothesis. Although the linkage scheme in Fig. 3 encompasses both conformational selection and induced fit as special cases, it is not the result of equal contributions from these mechanisms. Indeed, we have recently shown [23] that much of the kinetic repertoire of the linkage scheme (Scheme 3, Fig. 2) is recapitulated by conformational selection (Scheme 1, Fig. 2), but not by induced fit (Scheme 2, Fig. 2).

Analysis of the kinetic properties and thermodynamic underpinnings of induced fit raises questions about this mechanism being dominant or even necessary to explain the interaction of a ligand with its biological target. The well documented preponderance of systems associated with a single saturable relaxation that increases with [L] [18,24] can no longer be used as unequivocal evidence of induced fit and the dominance of this mechanism [20,23]. We have analyzed the relaxation kinetics of glucose binding to glucokinase, a textbook example of a protein obeying the induced fit mechanism [25], and have shown that the same experimental data fit to conformational selection [20]. The original interpretation of the binding mechanism of glucokinase has been called into question recently [26] and it is now accepted that glucokinase exists in alternative conformations in equilibrium prior to the binding of any ligands [27]. This conclusion is strongly supported by recent x-ray structures of glucokinase that reveal how ligand binding does not result in conformational changes of the enzyme but only in stabilization of the E form [28]. It is expected that several other cases previously assigned with certainty to induced fit because of a saturable relaxation increasing with [L] would allow for an alternative explanation based on conformational selection. Furthermore, the number of systems directly disproving induced fit with \( k_{\text{obs}} \) decreasing with [L] is rapidly expanding and includes alkaline phosphatase [29], glucokinase [26], several trypsin-like proteases [19,20,30–35], the immunoglobulin IgE [36], and DNA in its B to Z transition [37]. The case of trypsin-like proteases is particularly striking and biologically relevant.

5. Trypsin-like proteases obey conformational selection, not induced fit

Historically, trypsin-like proteases have been portrayed as the active embodiment of the irreversible conversion of an inactive zymogen precursor via a tightly regulated transition aimed at preventing unwanted proteolysis in the compartments where the zymogen is stored [38,39]. A key element of this conceptual framework is that the zymogen transitions to the protease through structural changes that can also be induced by binding of specific ligands or cofactors [40]. A recent extension of this view to the protease itself posits a continuum transition from zymogen-like to proteinase-like states upon ligand binding [41], based on the assumption that the protease in the free form is zymogen-like [42]. These views have fueled the expectation that the zymogen and protease would behave according to the induced fit mechanism, which is a conclusion directly refuted by a large body of structural and kinetic data [7,20,31,43,44]. Trypsin-like proteases and theirzymogens behave according to conformational selection and not induced fit, as first
reported for chymotrypsin more than forty years ago [30]. Both the zymogen and protease exist in equilibrium between inactive (E*) and active (E) forms [7] whose distribution changes upon activation or the binding of cofactors. The active form of the protease is not induced by binding of the ligand but pre-exists in solution along with the inactive form [43,44]. Likewise, the zymogen exists mainly in the inactive form E* and, to a smaller extent, in the active form E [43,44]. The case of thrombin is particularly instructive. The enzyme crystalizes in alternative conformations when free [43] and the active E form remains unchanged upon binding of ligands at the active site, exosite I, or the Na⁺ site (Fig. 4). The structure of thrombin bound to Na⁺ and hirudin to the active site and exosite I, covering 20% of the accessible surface area, shows that the enzyme retains the same E form observed when free in solution even when all three relevant binding sites are occupied (Fig. 4). Recent NMR measurements of thrombin free and bound have revealed the dynamic nature of the enzyme [45,46] and argued for the existence of conformational ensembles as a new, defining property of thrombin that should replace the static view based on a pre-equilibrium between distinct species. The dynamic nature of thrombin uncovered by NMR studies is inconsequential on the mechanism of function of the enzyme that is fully explained by two pre-existing conformations in equilibrium (see below). Each of these conformations, nicely trapped as single species by x-ray crystallography, may well be considered an ensemble of rapidly interconverting conformers, as suggested by NMR measurements. However, as already discussed in Section 2, the choice between the two interpretations is semantic because the properties of a kinetic scheme do not change if a species is replaced by an ensemble of rapidly interconverting conformers.

Kinetically, binding of different ligands to thrombin obeys the prediction of conformational selection (Fig. 5). Binding of the inhibitor p-aminobenzamide (PABA) and the substrate H-o-Val-Pro-Arg-p-nitroanilide (VPR) to the active site and Na⁺ and K⁺ to the cation-binding site produces saturable $k_{obs}$ dependencies consistent with conformational selection. All four ligands produce a saturable $k_{obs}$ that approaches the same value (60 s⁻¹) when [L] becomes large (Fig. 5A), consistent with the ligand-independent rate of isomerization from E* to E, $k_r$. When [L] = 0, PABA and K⁺, which bind at two sites separated by more than 15 Å, have nearly identical asymptotic values for $k_{obs}$ (400 s⁻¹), suggesting that this asymptote represents the ligand-independent isomerization of the free species, $k_r + k_{-r}$. The value of $k_{obs}$ for Na⁺ binding is equal to 130 s⁻¹ when [L] = 0. Since this value is lower than $k_{obs}$ at [L] = 0 for PABA and K⁺ binding, we infer that it represents $k_a$. The asymptotic value of $k_{obs}$ at [L] = 0 for VPR binding (2.5 s⁻¹) is actually lower than $k_r$, causing $k_{obs}$ to increase with [L]. Using multiple ligands to probe the conformational landscape of thrombin allows us to accurately determine both the mechanism and the individual rate constants governing binding [20]. The same strategy has been applied to two additional trypsin-like proteases present in the coagulation cascade: factor Xa (FXa) and activated coagulation protein C (aPC). FXa binding to PABA and Na⁺ produces saturable $k_{obs}$ values that decrease from approximately 350 s⁻¹ at [L] = 0 to 45 s⁻¹ as [L] approaches saturation (Fig. 5B). Again, the decreasing $k_{obs}$ provides unequivocal proof of conformational selection. Both ligands, binding to different sites, produce identical values of $k_{obs}$ when [L] = 0 and when [L] becomes large providing strong support for the identification of these asymptotes as $k_r + k_{-r}$, [L] = 0 and $k_r$ when [L] is large. The presence of conformational selection in FXa with rates of isomerization $k_r$ and $k_{-r}$ that are remarkably consistent with those of thrombin suggests that this mechanism of binding is conserved in trypsin-like proteases. Binding of Na⁺ and Ca²⁺ to aPC confirms these
suspensions. Na\(^+\) binds to aPC at a site analogous to that found in thrombin and FXa, while Ca\(^{2+}\) binds to a site defined by the 70-loop, in close proximity to exosite I in thrombin. The binding of Na\(^+\) to aPC produces a saturable \(k_{\text{obs}}\) that decreases from 50 s\(^{-1}\) to 25 s\(^{-1}\) with increasing [Na\(^+\)]. Ca\(^{2+}\) binding produces a saturable \(k_{\text{obs}}\) that increases from 5 s\(^{-1}\) to 20 s\(^{-1}\) as [Ca\(^{2+}\)] goes from zero to saturation. The convergence of \(k_{\text{obs}}\) values at high [L] suggests that both ligands are binding to the same E form of the enzyme, but the lack of agreement when [L] = 0 makes clear assignment of the sum \(k_r + k_{-r}\) impossible for aPC (Fig. 5C). The [L] = 0 asymptote of \(k_{\text{obs}}\) for Ca\(^{2+}\) binding is clearly assigned to \(k_{\text{obs}}\), but for Na\(^+\) binding that asymptote could be equal to either \(k_{\text{obs}}\) or \(k_r + k_{-r}\). The unmistakable kinetic and structural signatures of conformational selection present in this large family of proteases provides a strong case for the ubiquity of this mechanism in biological systems.

6. More complex kinetics

Conformational selection and induced fit exhaust the repertoire of two-step kinetic mechanisms linking ligand binding to conformational transitions when a single saturable \(k_{\text{obs}}\) is measured experimentally. Numerous biological systems such as the immunoglobulins IgE [47] and IgG [48], protein kinase A [49], DnA [50], CheA [51], histone deacetylase-like amidohydrolase [52], polymerase X [53], 3-hydroxybenzoate-6-hydroxylase [54], 3-chloroacrylic acid dehalogenase [55], proline utilization A protein [56], ACTR and CREB-binding protein [57], G-quadruplex folding [58] and DnA [59] exhibit multiple \(k_{\text{obs}}\) that saturate in the presence of high concentrations of their respective ligands. These data dictate expanded binding mechanisms containing a minimum of four species and requiring a more complex interpretation of the relaxation kinetics. When multiple saturable \(k_{\text{obs}}\) are present, four diagnostic scenarios are of interest: both \(k_{\text{obs}}\) increase with [L], the faster \(k_{\text{obs}}\) increases while the slower \(k_{\text{obs}}\) decreases with [L], both \(k_{\text{obs}}\) decrease with [L], and the faster \(k_{\text{obs}}\) decreases while the slower \(k_{\text{obs}}\) increases with [L]. These four scenarios are shown as the quadrants of a plot where the range of the smaller \(k_{\text{obs}}\) is plotted as a function of the range of the larger \(k_{\text{obs}}\) (Fig. 6). The range is defined as ratio of the values of \(k_{\text{obs}}\) when [L] = ∞ and [L] = 0. Adopting this approach to investigating the behavior of \(k_{\text{obs}}\) with respect to [L], the majority of the experimental systems with multiple saturable \(k_{\text{obs}}\) fall into three quadrants of the plot, leaving the quadrant where the faster \(k_{\text{obs}}\) decreases with [L] while the slower \(k_{\text{obs}}\) increases almost completely unpopulated. What mechanisms of binding are capable of explaining the experimental data? The linkage scheme, of which conformational selection and induced fit are special cases, produces three independent relaxations. The two fastest of these \(k_{\text{obs}}\) increase linearly, reflecting the two independent binding steps present in the binding mechanism. The slowest \(k_{\text{obs}}\) is saturable and may either increase or decrease with increasing [L]. Because this mechanism produces only a single saturable \(k_{\text{obs}}\), it is incapable of explaining the experimental data displayed in Fig. 6. An extension of the conformational selection scheme to include three ligand free species in equilibrium with each other with selective ligand binding occurring for a single species also produces three independent relaxations. The fastest \(k_{\text{obs}}\) once again increases linearly as [L] increases, but the remaining two slower \(k_{\text{obs}}\) are both saturable. Numerical simulations of these \(k_{\text{obs}}\) reveal that the two saturable \(k_{\text{obs}}\) can either increase or decrease with increasing [L], but never in the combination where the faster \(k_{\text{obs}}\) decreases while the slower \(k_{\text{obs}}\) increases [23]. In general, extensions of the conformational selection mechanism with any number of free species and only one single bound species produce qualitatively similar results. The largest \(k_{\text{obs}}\) will always increase linearly, reflecting the binding step while the remaining \(k_{\text{obs}}\) can either increase or decrease, but a decreasing faster \(k_{\text{obs}}\) is never coupled to a slower \(k_{\text{obs}}\) that increases with [L] (Fig. 6A). An analogous extension of the induced fit mechanism where a single free species binds to a ligand to form a complex that interconverts between three bound species fails to account for the variety of kinetic behavior seen experimentally. As with conformational selection, the first of the three relaxations increases linearly with [L]. However, the remaining two saturable \(k_{\text{obs}}\) can only ever increase, limiting the general induced fit mechanism to the upper right quadrant of the plot (Fig. 6B). The only mechanism containing four species that is capable of populating the upper left quadrant of the plot in Fig. 6 is one in which conformational selection is followed sequentially by induced fit. This mechanism, discussed previously under certain approximations [29,50], produces two saturable \(k_{\text{obs}}\) that may either increase or decrease independently of each other, thus allowing them to populate all four quadrants of the plot (Fig. 6C). Enzymes with lid-gated active sites such as ribokinase [60], adenosine kinase [61] and glucokinase [62], where a conformational change must follow the binding step [63], are particularly good candidates for application.

Fig. 5. Rapid kinetics of ligand binding to the trypsin-like proteases thrombin, FXa, and aPC. Rates of approach to equilibrium, \(k_{\text{obs}}\), determined from stopped-flow fluorescence measurements of binding of PABA (red), KCl (magenta), NaCl (blue), VPR (orange), and CaCl\(_2\) (green) to (A) thrombin, (B) FXa, and (C) aPC. Reaction conditions were as follows: (A) 5 mM Tris, 0.1% PEG8000, pH 8.0 at 15 °C, with no ChCl used due to specific inhibition of FXa by choline; and (C) 5 mM Tris, 0.1% PEG8000, pH 8.0 at 15 °C, with ionic strength kept constant at 800 mM with ChCl. Solid lines were drawn according to the eq. (2) in the text. Best-fit parameters for thrombin binding to ligand (A) are: (PABA) \(k_r = 1.7 ± 0.1 \times 10^7\) M\(^{-1}\) s\(^{-1}\), \(K_r = 9.4 ± 1.3\) × 10\(^{-2}\) M; (K\(^+\)) \(k_r = 60 ± 10\) s\(^{-1}\), \(K_r = 3.4 ± 4\) s\(^{-1}\), \(K_{\text{off}} = 7.1 ± 2\) M; (VPR) \(k_r = 340 ± 10\) s\(^{-1}\), \(K_r = 340 ± 10\) s\(^{-1}\), \(K_{\text{off}} = 7.1 ± 2\) s\(^{-1}\), \(K_{\text{on}} = 1.7 ± 0.1\) × 10\(^{-2}\) s\(^{-1}\); and (Na\(^+\)) \(k_r = 2.4 ± 0.2\) s\(^{-1}\).
of this scheme. However, an extension of the linkage scheme to include a third free species, also capable of binding, is sufficient to replicate this behavior entirely (Fig. 6D) and without the need of induced fit. The important conclusion is that all of the kinetic behaviors of a system with multiple saturable $k_{o_b}$ can be explained by a mechanism in which all possible conformations are contained within the free ensemble. Conformational selection alone is sufficient to explain the overwhelming majority of the experimental systems displaying one or more saturable $k_{o_b}$, proving essential in a wide array of them. On the other hand, induced fit is never necessary as a mechanism of ligand binding and is frequently insufficient.

7. Conclusion

Kinetic data that constitute the foundation for the induced fit hypothesis are actually consistent with conformational selection as a mechanism of binding [20,23]. The kinetic signature previously ascribed solely to induced fit can be entirely recapitulated by conformational selection, but the signature of conformational selection can never be reproduced by induced fit. A simple model of conformational selection in which one bound conformation is generated from multiple free conformations is sufficient to explain any set of experimental data where a single saturable relaxation is observed and is necessary for a large fraction of those systems. Remarkably, when multiple saturable relaxations are observed, inclusion of additional complexity in the free ensemble is sufficient to explain over 95% of existing relaxation kinetics and a scheme in which all bound conformations pre-exist in the free ensemble is entirely sufficient. The rich repertoire of kinetic behaviors associated with conformational selection makes this mechanism a far simpler unified solution to ligand binding. The complexity of the conformational ensemble pre-exists in the free form with the ligand selecting those conformations with the optimal fit. The ensemble view of the macromolecule agrees well with the fact that conformational selection can never be disproved as a mechanism of ligand binding, and with the fact that conformational selection is always sufficient and often necessary to account for the relaxation kinetics of simple and more complex systems. Pre-existence of alternative conformations is likely a fundamental property of all macromolecules.