Kinetic studies of protein–protein interactions
Gideon Schreiber

The structure of a protein–protein interaction, its affinity and thermodynamic characteristics depict a ‘frozen’ state of a complex. This picture ignores the kinetic nature of complex formation and dissociation, which are of major biological and biophysical interest. This review highlights recent advances in deciphering the kinetic pathway of protein–protein complexation, the nature of the encounter complex, transition state and intermediate along the reaction, and the effects of mutation, viscosity, pH and salt on association.

Addresses
Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, 76100 Israel; e-mail: bcgse@weizmann.ac.il

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Abbreviations
BLIP β-lactamase inhibitor protein
BSA bovine serum albumin
HEL hen-egg lysozyme
PEG polyethylene glycol
TEM1 TEM1-β-lactamase

Introduction
Specific, rapid association of protein complexes is essential for diverse processes such as signal transduction, cell regulation, the immune response, the assembly of cellular components, regulation of enzymatic activities and more. The rate of association of a protein complex is limited by diffusion and geometric constraints of the binding sites (diffusion control). The reaction process may be further slowed by subsequent chemical processes [1]. In this case, association is considered reaction, or partially reaction, explanation was given for this phenomenon, questioning the use of the viscosity dependence of $k_{on}$ as a measure for diffusion-controlled protein–protein interactions.

Contribution of cosolvents
The association of a protein complex in dilute solutions requires a long diffusion step for the two proteins to collide within the rigid geometrical constraints required for binding. Perturbing the diffusion step is possible using cosolvents. These include agents that increase solution viscosity, such as glycerol or sucrose (which slow diffusion); salts, which mask electrostatic attraction; and crowding agents (PEG, BSA, etc.), which are often used to simulate cellular conditions. The use of the last was recently reviewed [3].

Effect of viscosity on $k_{on}$
Kramers [4] has shown that the relative association rate constant, in the absence and presence of a viscous agent ($k_{on}/k_{on}^{0}$), for a bimolecular interaction is linearly dependent on the relative viscosity ($\eta/\eta_0$) of the medium, with a slope of 1 or less for a diffusion-controlled or partially diffusion-controlled reaction [1]; therefore, the dependence of $k_{on}$ on viscosity was used to determine whether a protein–protein interaction is diffusion controlled. Indeed, for some protein–protein interactions, a simple relation between $k_{on}$ and solvent viscosity with a slope of ~1 was observed [5,6]; however, slopes larger than 1 were observed for the interactions between antibodies HyHEL-5 or HyHEL-10 with hen-egg lysozyme (HEL), HyHEL-5 with bob-white quail lysozyme and barnase with barstar (with the slope of $k_{on}/k_{on}^{0}$ versus $\eta/\eta_0$ being >2) [7,8]; G Schreiber, unpublished data). Moreover, the slope of $k_{on}/k_{on}^{0}$ versus $\eta/\eta_0$ for the interaction between barnase and barstar is dependent on the magnitude of electrostatic attraction between the two proteins. No theoretical explanation was given for this phenomenon, questioning the rate of association was found to be enhanced by strong, favourable electrostatic forces.

This review highlights recent advances in the understanding and prediction of the kinetics of protein–protein interactions. It begins with a summary of the experimental methods used to perturb the rate of association and what we can learn from these studies (adding cosolvents, introducing mutations, changing the temperature or pH). It proceeds by analysing the contribution of electrostatic steering to association and includes a description of the interaction pathway. Furthermore, hot spots and the thermodynamics of the association reaction will be discussed. Experimental methods used for measuring the kinetics of protein interactions were discussed in a recent review in this journal [2]. Computational methods will be only briefly addressed, as they are the topic of a separate review in the next issue of this journal.

Ionic strength dependence of $k_{on}$
Favourable electrostatic attraction has long been recognised as a driving force for fast association [9*]. The magnitude of electrostatic attraction can be altered by mutation of the ionic strength of the solution. The relationship between ionic strength and $k_{on}$ was shown to follow the Debye–Hückel energy of interaction between a pair of proteins according to Equation 1 [10,11]:

$$\ln k_{on} = \ln k_{on}^{0} - \frac{U}{RT} \left( \frac{1}{1 + \kappa a} \right)$$

(1)

where $k_{on}$ and $k_{on}^{0}$ are the rates of association in the presence and absence of electrostatic forces, respectively, $U$ is the electrostatic energy of interaction, $\kappa$ is the inverse Debye length and $a$ is the minimal distance of approach. Hence, $k_{on}$ is the sum of two components: firstly the basal rate of association in the absence of electrostatic forces ($k_{on}^{0}$); and secondly the contribution of the electrostatic
forces between the proteins. The latter can be attended by mutation (changing $U$) or changing solution conditions. Equation 1 suggests that a plot of $k_{on}$ versus $\frac{1}{1+K_0}$ (which is proportional to the ionic strength $[I]$) is linear, with the slope being equal to $-\frac{U}{RT}$ (the electrostatic contribution of the two proteins in the absence of salt). The intercept of the line at $\frac{1}{1+K_0} = 0$ corresponds to the basal rate ($\ln k_{on}^{0}$) when electrostatic forces are shielded by salt. The intercept at $\frac{1}{1+K_0} = 1$ corresponds to $\ln k_{on}$ in the absence of salt, with the electrostatic forces being maximised. This linear relation was shown to hold for the association of TEM1-$\beta$-lactamase--$\beta$-lactamase inhibitor protein (TEM1–BLIP), interferon–receptor, hirudin–thrombin, barnase–barstar and a heterodimeric leucine zipper for all salt concentrations tested [10,12,13,14**,15]. Interestingly, the basal rate determined from extrapolating ionic strength to infinity (for barnase and barstar) has a different value from the one obtained from mutation data, when the interaction energy was extrapolated to zero [10].

**pH dependence**

Histidine is the only amino acid with a $pK_a$ of the free amino acid close to the pH at physiological conditions. Shifts of $pK_a$ of histidine residues hold valuable information about their environment. The rate of oligomisation (association) of R67 dihydrofolate reductase (DHFR) has a $pK_a$ of 6.6, which was attributed to H62. On the contrary, the dissociation reaction has a $pK_a$ under 5.5 [16*]. The shift in the $pK_a$ of H62 upon complexation is attributed to specific formation of short-range interactions, which, therefore, takes place after the rate-determining step of association. Similar results were reported for H102 on barnase, which undergoes a major $pK_a$ shift upon complexation with barstar [17].

**Probing the contribution of individual residues to $k_{on}$ by mutation**

Measuring the effect of mutation on the rate of association is a powerful tool that may be used to decipher the mechanism of association. Extensive site-directed mutagenesis of the surface residues of TEM1–BLIP, barnase–barstar, interferon–receptor, growth hormone–receptor and interleukin–4–receptor has demonstrated that only mutations involving charged residues significantly affect the rate of association (over twofold), whereas mutations of uncharged residues are neutral [13,14**,18–22]. However, the magnitude of perturbation of $k_{on}$ is not a simple measure of the change in charge, but rather it is related to the specific location of the mutation and its contribution to the electrostatic energy of interaction between the two proteins [14**,23*]. These results agree with Equation 1, which predicts that the rate of association of a specific protein–protein interaction depends on the electrostatic contribution (which can be perturbed by mutation or salt) and a basal rate, which, in most cases, is fixed for a specific protein–protein interaction; however, this rule is not absolute, as in a number of cases mutations were shown to change $k_{on}$ without affecting the net charge [24,25]. Assuming that the basal rate is related to the diffusion coefficient, the size of the mutual binding surfaces and the precise docking step (including desolvation and structural rearrangement), a change in any one of these can also affect $k_{on}$.

**Hot spots for association**

Hot spot residues in protein–protein interactions are defined as residues that, upon mutation, cause a large shift in binding affinity. Most often, these changes reflect an increasing $k_{off}$, but large shifts in $k_{on}$ (>10-fold) have been observed as well [14**,25,26**]. Theoretical calculations of the contribution of charged residues to $k_{on}$ provide an interesting insight regarding the nature of hot spot residues for association. Hot spot residues seem to be located within, or in the vicinity of, the binding site, where the charge confers a maximal efficiency towards steering the two proteins together and stabilising the activated complex. Computational studies have indicated the existence of energy funnels near binding sites. Such energy funnels can increase the number of random collisions, thus leading to faster association [27]. It was shown that hot spot residues have the largest effect on the size and depth of these energy funnels [23*]. Potential hot spot residues can now be identified computationally, by calculating the expected change in the rate of association upon introducing mutations of all of the surface-exposed residues, using the program PARE [14**]. This makes it possible to engineer pairs of proteins with much higher rates of association and affinity [14**,23*]. Other hot spots concerned with protein–protein association are residues that seemingly perturb the association pathway, for example, the T35A Ras mutant ([26**]; see below).

**Thermodynamics of protein–protein association**

The activation enthalpy ($\Delta H^\ddagger$) is determined from an Eyring plot of the temperature dependence of $k_{on}$ (or $k_{off}$), from which the activation entropy ($\Delta S^\ddagger$) at a 1 M standard state can be calculated. Small values of $\Delta S^\ddagger$ have been reported for the interactions between barnase–barstar, HyHEL-5–HEL and HyHEL-10–HEL [7,28]. Moreover, for the interaction between barnase and barstar, low activation entropies were determined for a large number of mutations, with the measured change in $\Delta H^\ddagger$ following changes in $\Delta G^\ddagger$; however, larger variations in $\Delta S^\ddagger$ were determined between mutants of D1.3/HEL and 2BS/SF8Cytc [29]. Entropic changes upon complexation can be attributed to a number of reasons: loss of rotational and translational degrees of freedom of the interacting proteins, loss of internal degrees of freedom and desolvation. The observation that $\Delta S^\ddagger$ tends to be small suggests that none of these three processes are very pronounced at the activated complex, or that their respective changes happen to cancel each other in the 1 M standard state.

**Describing the pathway for protein complex formation**

In general terms, association of a protein complex (AB) from the unbound components (A+B) can be best
mediate complexes have been experimentally observed before the main transition and an intermediate model allows the existence of a mostly solvated encounter three-state or even a two-state model. That, for some interactions, the association follows a classical example of the former was observed for the interaction of bovine \( \beta \)-trypsin with soybean trypsin inhibitor, whereby the first step of the formation of the diffusion encounter complex was found to be rate limiting [30]. More recently, Sydor et al. [31] have demonstrated the existence of a diffusion encounter complex for the interaction between Ras and the Ras-binding domain of c-Raf1. Here, a two-step association process was suggested, with an initial rapid equilibrium step followed by an isomerisation reaction occurring at several hundreds per second. As for the interaction of bovine \( \beta \)-trypsin with soybean trypsin inhibitor, the Ras–Raf1 intermediate (AB\*) was suggested to dissociate faster than complexation occurs \( k_{-1} \gg k_2 \). Interestingly, a mutation of T35A on Ras that destabilises the switch I region results in a linear dependence of \( k_{\text{obs}} \) on the protein concentration [26••], albeit with a slower \( k_{\text{on}} \). This may suggest that the switch I region funnels the association reaction through a diffusion encounter complex, which increases the overall rate of association by advancing the transition state to an earlier stage along the reaction coordinate.

For most protein–protein interactions, a diffusion encounter complex was not detected. This does not exclude its existence, as AB\* can be detected only at very high protein concentrations (tens to hundreds of \( \mu \text{M} \)), resulting in an extremely fast \( k_{\text{obs}} \). These are, or above, the detection limit of a stopped-flow machine and much higher than the detection limit of a BIAcore or similar heterogeneous phase-detection methods that measure binding in real time.

**Observing the diffusion encounter complex (AB\*)**

In a diffusion-limited association reaction in the absence of AB\*, a linear dependence of \( k_{\text{obs}} \) on the protein concentrations is expected; however, a hyperbolic dependence of \( k_{\text{obs}} \) on the protein concentration would suggest that \( k_{-1} \gg k_2 \) and the reaction is not diffusion controlled (assuming that the probe measures the second-order reaction [A+B to AB] and not the first-order reaction [AB** to AB]; see Figure 2). A classical example of the former was observed for the association of bovine \( \beta \)-trypsin with soybean trypsin inhibitor, whereby the first step of the formation of the diffusion encounter complex was found to be rate limiting [30]. More recently, Sydor et al. [31] have demonstrated the existence of a diffusion encounter complex for the interaction between Ras and the Ras-binding domain of c-Raf1. Here, a two-step association process was suggested, with an initial rapid equilibrium step followed by an isomerisation reaction occurring at several hundreds per second. As for the interaction of bovine \( \beta \)-trypsin with soybean trypsin inhibitor, the Ras–Raf1 intermediate (AB\*) was suggested to dissociate faster than complexation occurs \( k_{-1} \gg k_2 \). Interestingly, a mutation of T35A on Ras that destabilises the switch I region results in a linear dependence of \( k_{\text{obs}} \) on the protein concentration [26••], albeit with a slower \( k_{\text{on}} \). This may suggest that the switch I region funnels the association reaction through a diffusion encounter complex, which increases the overall rate of association by advancing the transition state to an earlier stage along the reaction coordinate.

Free-energy profile describing the pathway for the formation of a protein–protein complex (AB) from the free proteins A and B via the encounter complex AB\*, the transition state AB‡ and the intermediate AB** (see Scheme 1). Lines 1 and 2 describe the interaction between a pair of proteins in the absence and in the presence of favourable electrostatic forces, respectively.

**Observing the post-transition intermediate (AB**)**

The intermediate (AB**) is formed after the rate-limiting step for association \( k_{-1} \gg k_2 \); therefore, it does not affect the overall rate of association. The intermediate can be envisioned as a partially formed complex that has to reorganise to form the final complex. This reorganisation step can be fast, as for the interaction between cystatin A and papain \( (230 \text{ s}^{-1}) [32,33••] \), or slow, as for the interaction between HEL and HyHEL-10 or HyHEL-26 \( (10^{-3} \text{ s}^{-1}) [34•] \). A major problem in investigating this intermediate is to find a probe that can monitor independently the formation of the intermediate versus the formation of the final complex. For the cystatin A–papain interaction, the formation of the intermediate was monitored elegantly by the rate of inhibition of the catalytic activity of papain, whereas the evolution of the final complex was observed spectroscopically [32,33••]. Inhibition of enzymatic activity (which presumably monitors the formation of AB**) showed a linear dependence of \( k_{\text{obs}} \) on protein concentration whereas the spectroscopic probe (which monitors the evolution of AB from AB**) showed a hyperbolic dependence of \( k_{\text{obs}} \) on protein concentrations (see Figure 2).

This case emphasises the difficulty in defining whether the hyperbolic dependence on protein concentration relates to the accumulation of the encounter complex at the diffusion step or the reorganisation of the intermediate. A similar mechanism for association (initial binding of the C-terminal tail before docking of the N-terminal part) was suggested for the thrombin–hirudin interaction, albeit involving ionic tethering [35•].
A different method to investigate the post-transition intermediate was applied to the slow-forming complex between HEL and HyHEL-10 or HyHEL-26 [34•]. With this method, the rate of complex formation was monitored using a BIAcore, which probes the change of the mass bound to the surface. The intermediate formed along the association reaction was monitored by the change in amplitude of the biphasic dissociation reaction, with the amplitude of the fast dissociation step decreasing for increasing long-range electrostatic steering suggests that short-range interactions affect $k_{on}$, whereas long-range electrostatic interactions affect $k_{off}$, which was directly tested by introducing charged mutations in the vicinity of but outside the binding site of TEM1–BLIP. These mutations did increase specifically $k_{on}$ by 250-fold, but did not affect $k_{off}$ (thus, the increase in $K_D$ and $\Phi = 1$) [14••]. These data suggest that the contribution of the long-range electrostatic interactions to the rate of association lowers the free energy of the transition state by the same magnitude as the equilibrium constant. Whereas mutations of noncharged residues do not significantly affect the transition state for association, they can significantly alter $K_D$ and $k_{off}$ (see Figure 1). Moreover, the observation that $k_{off}$ was unchanged with increasing long-range electrostatic steering suggests that $k_{-1}$ (which will decrease with increasing electrostatic forces) monitors an early step along the reaction, before the main transition, and that $k_{on} > k_{off}$ [23•]. These data fit well within the general picture emerging from the salt and pH dependence of $k_{on}$, and the thermodynamics of the association reaction. These data suggest that the transition state theory was developed for unimolecular reactions; however, it is applicable to a bimolecular protein–protein interaction if we consider only the relative change in free energy between the unbound proteins and the transition state for a mutant versus the wild type, and $\Delta G(\Phi)$ is the difference in the free energy between the unbound and bound proteins for a mutant versus the wild type) [23•]. A $\Phi$ value close to one indicates that a specific interaction is formed at the transition state, whereas a $\Phi$ value close to zero indicates that the interaction is formed after the transition state.

The nature of the transition state along the association reaction

The most unstable species along a reaction pathway is the transition state, which occurs at the peak of a reaction coordinate diagram. In the transition state, chemical bonds are in the process of being made and broken. Transition state theory was developed for unimolecular reactions; however, it is applicable to a bimolecular protein–protein interaction if we consider only the relative change in the transition state as caused by perturbing the rate of association by mutation or by altering solvent conditions. Many such studies have been performed, giving valuable information on the nature of the transition state for association [7,8,10,12,14••,20,21,24,28,34•,35•,36–39].

Direct information on the structure of the transition state was obtained by measuring activation interaction energies using double mutant cycles. If the change in $k_{on}$ caused by a pair of mutations is additive, the two residues do not interact during the transition state; however, if the change is less than additive, one may assume that they interact. Probing the structure of the activated complexes of barnase–barstar and thrombin–hirudin by this method has shown that only charged residues that are in close proximity in the final complex already interact at the activated complex. No interaction was measured between uncharged residues at this stage. Masking these specific long-range electrostatic interactions by increasing the concentration of salt causes the loss of some, but not all, pairwise charge–charge interactions at the activated complex, suggesting that structural specificity of the activated complex is maintained even at high salt [13,28,35•].

A somewhat different approach to probe docking trajectories experimentally is $\Phi$ value analysis ($\Phi = \Delta G(\Phi)/\Delta G_D$, where $\Delta G(\Phi)$ is the difference in the free energy between the unbound proteins and the transition state for a mutant versus the wild type, and $\Delta G_D$ is the difference in the free energy between the unbound and bound proteins for a mutant versus the wild type) [23•]. A $\Phi$ value close to one indicates that a specific interaction is formed at the transition state, whereas a $\Phi$ value close to zero indicates that the interaction is formed after the transition state. In a study of the HyHEL-10 FAB complex, multiple replacements were made in two positions, with most of the replacements having $\Phi$ values close to zero. This was interpreted as the transition state occurring early along the reaction trajectory, before short-range interactions (which have the largest contribution to $\Delta G_D$) are formed. The notion that short-range interactions affect $k_{off}$, whereas long-range electrostatic interactions affect $k_{on}$ was directly tested by introducing charged mutations in the vicinity of but outside the binding site of TEM1–BLIP. These mutations did increase specifically $k_{on}$ by 250-fold, but did not affect $k_{off}$ (thus, the increase in $K_D$ equals the increase in $k_{on}$ and $\Phi = 1$) [14••]. These data suggest that the contribution of the long-range electrostatic interactions to the rate of association lowers the free energy of the transition state by the same magnitude as the equilibrium constant. Whereas mutations of noncharged residues do not significantly affect the transition state for association, they can significantly alter $k_{off}$ and $K_D$ (see Figure 1). Moreover, the observation that $k_{off}$ was unchanged with increasing long-range electrostatic steering suggests that $k_{-1}$ (which will decrease with increasing electrostatic forces) monitors an early step along the reaction, before the main transition, and that $k_{on} > k_{off}$ [23•]. These data fit well within the general picture emerging from the salt and pH dependence of $k_{on}$, and the thermodynamics of the association reaction. These data suggest that the transition state
state for association is stabilised by electrostatic interactions and its structure resembles that of the final complex, but is mostly solvated (Figure 3). In this case, the high-energy barrier for association is the formation of specific short-range interactions, which is accompanied by structural rearrangement and desolvation. Moreover, this would suggest that the structure of the encounter complex (AB*), which occurs before the transition state, resembles the latter and that a major contribution of electrostatic forces towards faster binding would be by stabilising the encounter complex [23*]. One has to emphasise that this picture of the encounter complex and transition state comes from the analysis of a specific set of proteins and that, for different proteins, variations of the kinetic pathway are expected.

**Calculations versus experimental data**

The rates of association of a number of protein–protein interactions have been simulated in recent years using both Brownian dynamic simulations and transition state theory (reviews in [40•] and in the next issue of this journal). The simulations were most successful in predicting the relative change in $k_{on}$ upon change in ionic strength or mutation, whereas the calculation of absolute values continues to be problematic [11,14••,41,42••,43]. Most of the Brownian dynamic simulations consider the association reaction to be diffusion controlled; however, it was noted that the difference in absolute rates for association between simulation and experiment may arise from a slow desolvation step or structural rearrangement during association [42••,43]. This would confirm experimental data that even for very fast associating protein complexes the reaction may not be solely diffusion controlled; however, simulating the kinetics of desolvation during binding, Camacho et al. [44•] suggest that the desolvation of weakly specific pathways actually increases association significantly. Whereas at first sight these two findings seem to be contradictory, they actually represent different processes that steer the reaction. Desolvation of hydrophobic patches on the surface can stimulate association by allowing for a two-dimensional local search of the mutual binding sites, whereas the desolvation of charged residues and surface rearrangement slow down association. The reason that mutation studies rarely identify noncharged residues that significantly contribute to $k_{on}$ may be attributed to the small contribution of individual sidechains to desolvation-induced association, as this has more of a global effect of the protein.

**Biological significance of binding kinetics**

Is binding affinity the major factor that dictates biological activity or are the independent contributions of the rates of association and dissociation important as well? A number of studies from recent years have actually indicated that the individual kinetic constants are important. A good example is the kinetics of the cytokine–receptor interaction, whereby binding initiates the signal transduction cascade. For the interactions of interleukin-4, human growth hormone and interferon with their respective cell-bound receptors, a linear dependence was established between the rate of dissociation and the magnitude of biological activity as long as $k_{off} > 0.02 \text{ s}^{-1}$ [21,22,45]; however, slower dissociation did not increase bioactivity [22,45]. A minimal threshold and maximal ceiling of off-rates was also observed for the dependence of the B-cell response on antigen concentration [46,47].

Most protein complexes associate at rates compatible with a diffusion-limited reaction, limited by geometrical constraints; however, some protein–protein complexes associate much faster. Fast association was found to be driven by optimisation of the electrostatic attraction between the proteins [48,49]. The most outstanding of these are the extremely fast rates measured for some enzyme–protein inhibitor interactions, where the speed of inhibition puts an evolutionary pressure on the system [13,38,50–52]. A different example is the control of signal transduction through Ras, which seems to be association controlled [31].

**Conclusions**

This review summarises the progress made in understanding the pathway of protein–protein association, emphasising the new experimental evidence of this process. The emerging association pathway includes the formation of an unstable diffusion encounter complex, which may evolve to form either the final complex directly or a second intermediate complex, but one that is already committed to evolve into the final complex. These precomplexes are
often difficult to track experimentally; thus, it is reasonable to assume that they are more abundant than has been reported so far. Much work has been done in characterising the transition state during the association reaction. These studies have clearly indicated that long-range electrostatic forces play a major role in stabilising the transition state, which can be described as a precursor state for association in which the two proteins are correctly orientated towards each other, but have a solvated, or at least partially solvated, interface. Accordingly, the transition state stems from the energetically costly process of exact structural matching and surface desolvation (especially of charged residues). This description of the transition state would actually suggest that protein–protein association is only partially diffusion controlled. Whether this description is precise, and how this would fit to the very fast rates of association measured between proteins, has to be seen.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

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