Huan-Xiang Zhou
Department of Physics, Drexel University, Philadelphia, PA 19104

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Disparate Ionic-Strength Dependencies of On and Off Rates in Protein–Protein Association

Abstract: *Electrostatic interactions have been observed to play important roles in the kinetics of protein–protein association. Ionic strength, by its ability to modulate the magnitude of electrostatic interactions, has often been conveniently used to test their presence. From experiments on a wide range of associating proteins, a common feature has emerged: the on rates show strong dependence on ionic strength whereas the off rates are relatively insensitive. Here this feature is explained by an explicit description of a transition state for the association process and the suggestion that this transition is near the final bound state of two proteins. The molecular basis of the transition state in the bimolecular process lies in the fact that the bound state is characterized by local specific (e.g., van der Waals, hydrophobic, and electrostatic) interactions, whereas the unbound state is characterized by translational and rotational freedom. In the transition state the protein–protein pair encounters a free-energy maximum since its translational-rotational entropy is reduced while the specific interactions are not yet attained. In this formalism of the protein–protein association process, the enhancement of on rates by long-range electrostatic interactions can be written (analogous to an ordinary transition-state theory) in the form* $k_{on} = k^0_{on} exp(-G^{\dagger}_{el}/k_BT)$*, where* $G^{\dagger}_{el}$ *is the electrostatic free energy of the transition state.* © 2001 John Wiley & Sons, Inc. Biopolymers 59: 427–433, 2001

Keywords: *protein–protein association; diffusion control; electrostatic interactions; electrostatic rate enhancement; transition state*

INTRODUCTION

This paper concerns a common feature that has emerged from experiments on a wide range of association proteins.[1–5] The on rates have been observed to show strong dependence on ionic strength whereas the off rates are relatively insensitive. The dependence on ionic strength signifies that electrostatic interactions are important. If electrostatic interactions are important in the association process, then there is no reason for them to be unimportant in the dissociation process. We present an explicit description of a transition state for the association process and use this concept to explain the weak ionic-strength dependence of the off rate. An essential part of the explanation is the suggestion that the transition states for these associating proteins are close to their final bound states.

Why are electrostatic interactions important in the kinetics of protein–protein association? In solution two proteins form a complex only after translational and rotational diffusion brings them together in the

Correspondence to: Huan-Xiang Zhou; email: hxzhou@einstein.drexel.edu
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appropriate relative orientation. Hence diffusion sets a limit on the on rate. The simplest model formulating this diffusion limit was proposed by Smoluchowski[6] for colloid coagulation, in which two spheres form a complex as soon as their surfaces are in contact. The rate constant for this model is

$$k_{on}^{0} = 4\pi DR \quad (1)$$

where D is the sum of the translational diffusion constants of the spheres and R is the sum of their radii. Using parameters appropriate for proteins, Eq. (1) yields an order of magnitude of $5 \times 10^9 M^{-1}\ s^{-1}$ for k_{on}. This number has often been quoted improperly as the limit of the on rate in protein–protein association. Proteins do not form a complex as soon as any parts of their surfaces come into contact. In fact, in the final complex only very specific contacts are formed between residues of the two proteins. A model more appropriate for protein–protein association is one in which only a small patch on each spherical surface is reactive (i.e., a complex is formed only when the two patches are in contact). When both reactive patches are extremely small, Berg[7] found the following simple expression:

$$k_{on}^{0} = 4\pi DR[F_1\xi_2\tan(\alpha_2/2) + F_2\xi_1\tan(\alpha_1/2)] \quad (2)$$

where α_i are the polar angles spanned by the patches, $F_i = (1 - \cos\alpha_i)/2$ and $\xi_i = [(1 + D_iR^2/D)/2]^{1/2}$ with D_i denoting the rotational diffusion constant of sphere i ($i = 1$ and 2). For a pair of spheres each with a 5° patch (covering 0.2% of the surface area), the on rate predicted by Eq. (2) is $\sim 10^6 M^{-1}\ s^{-1}$. This number, over three orders of magnitude smaller than that of the Smoluchowski model, would be a more appropriate limit for protein–protein association, if the proteins were noninteracting. Potentially such a small rate constant can be deleterious in situations where fast association (such as between barnase and barstar) is essential. The on rate can be increased by an interaction force, but the range of such a force must be long enough as to influence the translational and rotational Brownian motion of the proteins before the complex is formed. Thus electrostatic interactions provide the only mechanism for rate enhancement. Indeed Fersht and co-workers[8] have showed that barstar is even willing to pay a price of reduced stability to achieve rapid association with barnase in selecting a cluster of negatively charged residues facing barnase.

Such rate enhancement has been observed in a wide range of associating proteins.[1–5,9] In particular, the on rate of barnase–barstar association was measured to be $6 \times 10^8 M^{-1}\ s^{-1}$ at an ionic strength (I) of 25 mM.[1] Ionic strength, by its ability to modulate electrostatic interactions, provides a convenient tool for testing their presence. For barnase–barstar association, the on rate was reduced to $2 \times 10^6 M^{-1}\ s^{-1}$ at an extremely high ionic strength of 2 M,[10] where the electrostatic interactions have largely been screened out. Such a dramatic decrease is in stark contrast to the relative constancy of the off rate. Between $I = 25$–525 mM, k_{off} merely increased by a factor of 5. These disparate ionic-strength dependencies of k_{on} and k_{off} recur in a number of other protein systems (see Table I) and their explanation is the focus of the present paper.

THEORY AND RESULTS

The foundation of our explanation is the concept of transition state. For unimolecular reactions, it is easy to prescribe the state with the highest energy or potential of mean force along the reaction pathway (e.g., bond breaking formation and rotation around a bond) as the transition state. In the context of diffusion-influenced bimolecular reactions such as protein–protein association, transition state has been invoked as a general concept.[4,5,11] Is there a molecular basis for prescribing a transition state for protein–protein association? The bound state of two proteins is characterized by local specific (e.g., van der Waals, hydrophobic, and electrostatic) interactions, whereas the unbound state is characterized by translational and rotational freedom. It is thus inevitable that the protein pair will pass through a free-energy maximum where translational-rotational entropy is reduced but specific interactions have not yet attained. This state is naturally the transition state.

The presence of a transition state can be illustrated by a model consisting of two uniformly reactive spheres interacting with a potential $U(r)$ (see Figure 1). It is natural to use the center-to-center distance r as a reaction coordinate for the protein–protein association. The equilibrium probability density $p(r)$ for finding the pair at a distance r is proportional to $4\pi r^2\exp[-\beta\ U(r)]$. The density, when written as a Boltzmann distribution,

$$4\pi r^2\exp[-\beta U(r)] = C\exp[-\beta G(r)] \quad (3)$$

defines a free energy $G(r)$ for the system at a given distance r (C on the right-hand side is an arbitrary constant). This free energy consists of enthalpic and entropic terms:

Table I Ionic-Strength Dependencies of Protein–Protein Association On and Off Rates

Barnase and barstar (Schreiber and Fersht, 1993)[1]

I (mM)	25	125	225	325	525
k_{on} ($10^8 M^{-1}$ s^{-1})	6.0	1.1	0.45	0.32	0.16
k_{off} (10^{-6} s^{-1})	8.0	15		30	40

Shaker K^+ channel and peptide toxin Lq2 (Escobar et al., 1993)[2]

I (mM)	25	50	100	200
k_{on} ($10^8 M^{-1}$ s^{-1})	5.7	1.9	0.85	0.14
k_{off} (s^{-1})	0.19	0.32	0.71	1.1

E9 DNase and immunity protein Im9 (Wallis et al., 1995)[3]

I (mM)	25	75	125	175	225	275
k_{on} ($10^8 M^{-1}$ s^{-1})	57	15	3.9	1.2	0.86	0.50
k_{off} (10^{-6} s^{-1})	0.41	0.85	1.1	1.9	2.1	2.5

Heterodimeric leucine zipper (Wendt et al., 1997)[4]

I (mM)	74	103	144	175	275	525
k_{on} ($10^7 M^{-1}$ s^{-1})	7.2	3.3	2.3	1.5	0.84	0.37
k_{off} (10^{-3} s^{-1})	0.38	0.38	0.52	0.53	0.51	1.0

Acetylcholinesterase and fasciculin 2 (Radic et al., 1997)[5]

I (mM)	10	25	50	70	90	110	220	440	670
k_{on} ($10^8 M^{-1}$ s^{-1})	23	13	3.3	1.8	1.6	1.5	0.30	0.13	0.12
k_{off} (10^{-4} s^{-1})		1.0			1.3	0.2	0.7	0.6	0.5

$$G(r) = U(r) - k_B T \ln(4\pi r^2/C) \tag{4}$$

The enthalpic term $U(r)$ increases from a deep minimum at $r = R$ to zero at $r = \infty$ whereas the entropic term $-k_B T \ln(4\pi r^2/C)$ monotonically decreases to negative infinity at $r = \infty$ (representing the vast translational freedom of the two proteins at such a separation). Thus $G(r)$ usually will have a maximum somewhere between R and ∞ (see Figure 1). This point $r = R\dagger$ is the transition state.

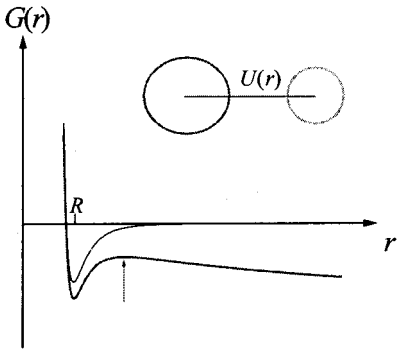

FIGURE 1 The interaction potential $U(r)$ and the resulting free-energy function $G(r)$ for the association of two proteins modeled as spheres. The upper curve shows the interaction potential $U(r)$, whereas the lower curve, with a maximum at $r = R\dagger$ as indicated by a vertical arrow, show the free-energy function $G(r)$. The position of the free-energy maximum (i.e., $r = R\dagger$) is taken to be the transition state of the association process.

The transition state separates the bound state from the unbound state. The equilibrium constant is then[12,13]

$$K = \int_0^{R^+} 4\pi r^2 \exp[-\beta U(r)]dr \tag{5}$$

The dissociation of the protein pair is actually a unimolecular reaction if one of the protein is viewed as simply providing the "potential" $G(r)$ in which the other protein moves. This is then the classical problem of escaping over a potential barrier treated by Kramers.[14] According to Kramers, the off rate is obtained by assuming a quasi-stationary distribution in which an equilibrium is established around $r = R$ while the probability at $r = \infty$ is zero. The result is

$$k_{off} = D\{\int_0^{R^+} \exp[-\beta G(r)]dr \int_R^{\infty} \exp[\beta G(r)]dr\}^{-1} \tag{6}$$

where D is the relative diffusion constant. From Eqs. (5) and (6), one can find the on rate to be

$$k_{on} = Kk_{off} = 4\pi D\{\int_R^\infty r^{-2}\exp[\beta U(r)]dr\}^{-1} \quad (7)$$

This is identical to Debye's result[15] by extending the Smoluchowski model to include an interaction potential and assuming an absorbing boundary condition at $r = R$. However, in our derivation it is not necessary to invoke the absorbing boundary at all.

The above spherical model also serves to illustrate the choice of the transition state. That is, it should be put at the outer edge of the inner potential well that defines the bound state. The dissociation–association process can be now described by the following reaction scheme:

$$b \leftrightarrow t \leftrightarrow u$$

where "b," "u," and "t" denote the bound, unbound, and transition states, respectively. Making the steady-state approximation for the transition state, one has

$$\frac{1}{k_{off}} = \frac{1}{k_{b\to t}} + \frac{K_1}{k_{t\to u}} \quad (8)$$

where $K_1 = k_{t\to b}/k_{b\to t}$ is the equilibrium constant for the first step of the reaction scheme. The overall equilibrium constant is given by the product of the equilibrium constants for the two individual steps, i.e.,

$$K = K_1K_2 = (k_{t\to b}/k_{b\to t})(k_{u\to t}/k_{t\to u}) \quad (9)$$

Dividing both sides of Eq. (8) by the equilibrium constant K, one obtains the corresponding expression for on rate:

$$\frac{1}{k_{on}} = \frac{1}{k_{u\to t}} + \frac{1}{K_2k_{t\to b}} \quad (10)$$

As a direct consequence of the transition state's location at the outer edge of the inner potential well of the bound state, the only interactions present in the transition state are electrostatic in nature. By analogy to Eq. (5), the equilibrium constant K_2 can be expressed as

$$K_2 = k_{u\to t}/k_{t\to u} = \int_{TS} \exp[-\beta U_{el}(\mathbf{x})]d\mathbf{x} \quad (11)$$

where $U_{el}(\mathbf{x})$ is the electrostatic interaction energy between the proteins at a configuration $\mathbf{x}$ (representing the relative displacement of the two proteins and their orientations) and the integration is restricted to the region of configurational space defining the transition state. The average Boltzmann factor in the transition state can be used to define the electrostatic free energy $G_{el}\dagger$:

$$\int_{TS} \exp[-\beta U_{el}(\mathbf{x})]d\mathbf{x}/V_{TS} = \exp(-\beta G_{el}^{\dagger}) \quad (12)$$

where $V_{TS} = \int_{TS} d\mathbf{x}$ is the volume of the transition-state region. Then we have

$$K_2 = k_{u\to t}/k_{t\to u} = V_{TS}\exp(-\beta G_{el}^{\dagger}) \quad (13)$$

It is important to recognize that $k_{u\uparrow t}$ [in Eq. (10)] is completely determined by the dynamics of the protein pair in the unbound state whereas $k_{t\to b}$ is completely determined by the dynamics in the bound state. In the unbound state one needs to model the overall translational and rotational Brownian motion of the two proteins, which is only influenced by long-range electrostatic interactions. The situation in the bound state is far more complicated, since internal dynamics and local specific interactions come into play. However, we can make a general statement about the effect of ionic strength on $k_{t\to b}$ when the configurational region of the bound state is small, i.e., when the transition state is close to the bound state. In this case, the screening of electrostatic interactions by ions is almost equal in all the configurations of the bound state. Hence one expects very weak ionic-strength dependence of $k_{t\to b}$. This of course will also be true for $k_{t\to b}$ and thus K_1. This weak dependence is in contrast to the strong ionic-strength dependence expected of $k_{u\to t}$. In that case one needs to compare the screening of the electrostatic interactions between two proteins in two extreme situations: in close proximity and at infinite separations.

In our earlier work[16–18] we have obtained an explicit result for the dependence of $k_{u\to t}$ on the long-range electrostatic interactions between two associating proteins. This is

$$k_{u\to t} = k_{u\to t}^0 \int_{TS} \exp[-\beta U_{el}(\mathbf{x})]d\mathbf{x}/V_{TS} = k_{u\to t}^0\exp(-\beta G_{el}^{\dagger}) \quad (14)$$

where $k^0_{u\to t}$ is the rate constant when the electrostatic interactions are completely turned off (a limit that can be reached by high ionic strengths). This result relies on the long-range nature of the electrostatic interactions and the specificity of the bound complex (i.e., the small size of the transition-state region). Hence the result for the uniformly reactive spherical model [Eq. (7)] does not conform to this relation. Comparison with Eq. (13) leads to

$$k_{t\to u} = k^0_{u\to t}/V_{\mathrm{TS}} \tag{15}$$

which means that $k_{\mathrm{t}\to\mathrm{u}}$ does not depend on electrostatic interactions at all. This result has been rationalized previously[19] by the observation that, when the size of the transition-state region is small, the reactant pair reaching the unbound state from that region will not have diffused far away. Due to the long-range nature of the electrostatic interactions, the interaction potential will be relatively smooth. Hence as far as calculating $k_{\mathrm{t}\to\mathrm{u}}$ is concerned, the reactant pair is moving in a uniform potential (or equivalently, a zero potential).

By now we have shown that all the three factors in Eq. (8) for determining the off rate are expected to have weak ionic-strength dependence, provided that the transition state is close to the bound state. This then rationalizes the experimental observations on k_{off} (see Table I). In the case of barnase–barstar association, the double mutant cycle studies of Schreiber and Fersht[11] on k_{on} have showed the presence of distinct correlations between charged residues that form stereospecific contacts in the bound state. These correlations indicate that the proteins' relative orientations in the transition state are similar to that in the bound state.

When the transition state is close to the bound state, a protein pair at the transition state will be far more likely to reach the bound state than to reach the unbound state. Then $k_{\mathrm{t}\to\mathrm{b}} \gg k_{\mathrm{t}\to\mathrm{u}}$, or equivalently, $K_2 k_{\mathrm{t}\to\mathrm{b}} \gg k_{\mathrm{u}\to\mathrm{t}}$. In this situation reaching the transition state from the unbound state becomes the rate limiting step in the association process [see Eq. (10)]. We thus conclude that a strong ionic-strength dependence of the on rate accompanied by a weak ionic strength of the off rate is a good indicator that the association is diffusion limited. This complements the classical indicator of diffusion control afforded by varying the solvent viscosity.[2,10] When an association is indeed diffusion limited, the dependence of the on rate on electrostatic interactions is fully described by

$$k_{\mathrm{on}} = k^0_{\mathrm{on}}\exp(-\beta G^{\dagger}_{\mathrm{el}}) \tag{16}$$

where k^0_{on} is the rate constant when the electrostatic interactions are turned off, which can be estimated from Eq. (2).

DISCUSSION

Experimentally the ionic-strength dependence of k_{on} has been fitted to a number of empirical functions.[5,9,10] Analytical expressions for $G_{\mathrm{el}}\dagger$ can be obtained only when the associating proteins are modeled as spheres. For example, at large separations, the electrostatic interaction energy between two spheres with charges q_{A} and q_{B} on them is[15]

$$U(r) = \frac{q_A q_B}{2\varepsilon_s}\left(\frac{e^{\kappa R_A}}{1+\kappa R_A} + \frac{e^{\kappa R_B}}{1+\kappa R_B}\right)\frac{e^{-\kappa r}}{r} \tag{17}$$

where R_{A} and R_{B} are the radii of the proteins, ϵ_{s} is the dielectric constant of the solvent, and $\kappa^2 = 8\pi N_{\mathrm{A}} e^2 I/k_{\mathrm{B}} T\epsilon_{\mathrm{s}}$. Assuming that Eq. (17) holds at the transition state (at $r = R_{\mathrm{A}} + R_{\mathrm{B}} = R$), the electrostatic free energy of the transition state is

$$G^{\dagger}_{\mathrm{el}} = -\frac{q_A q_B}{2\varepsilon_s R}\left(\frac{e^{-\kappa R_B}}{1+\kappa R_A} + \frac{e^{-\kappa R_A}}{1+\kappa R_B}\right) \tag{18}$$

Identifying k^0_{on} in Eq. (16) with $k_{\mathrm{on}}(I=\infty)$, we have

$$\ln k_{\mathrm{on}} = \ln k_{\mathrm{on}}(I=\infty) - \frac{q_A q_B}{2k_B T\varepsilon_s R}\left(\frac{e^{-\kappa R_B}}{1+\kappa R_A} + \frac{e^{-\kappa R_A}}{1+\kappa R_B}\right) \tag{19}$$

This can be rewritten as

$$\begin{aligned}\ln k_{\mathrm{on}} &= \ln k_{\mathrm{on}}(I=0) + \frac{q_A q_B}{2k_B T\varepsilon_s R}\left(2 - \frac{e^{-\kappa R_B}}{1+\kappa R_A} - \frac{e^{-\kappa R_A}}{1+\kappa R_B}\right)\\ &\approx \ln k_{\mathrm{on}}(I=0) + \frac{q_A q_B}{k_B T\varepsilon_s}\frac{\kappa}{1+\kappa R}\end{aligned} \tag{20}$$

to second order in κR. We emphasize that Eqs. (19) and (20) are presented here only for illustrative purpose and by no means represent rigorous results for the ionic-strength dependence of k_{on}. They perhaps can be used as an empirical function for fitting experimental results.[10]

In diffusion-limited protein–protein association, electrostatic interactions provide a bias toward the transition state (and thus the bound state). It is also a

bias toward the native state that makes protein folding fast.[20] There the bias is due to cooperative interactions between amino acid residues, though the exact nature of this cooperativity is still a matter of debate.[21] Roughly speaking, protein association in the absence of electrostatic interactions is like protein folding in a golf-course energy landscape, whereas protein association in the presence of electrostatic interactions is like protein folding in a funnel-shaped energy landscape.

After crossing the transition state, induced fit can occur. The internal motion that brings such induced fit is expensive to model, and, in practice, one often obtains an estimate of the on rate by calculating $k_{u\to t}$. To make this estimate as good as possible, one needs to place the transition state as close to the bound state as possible, i.e., any further "squeezing" in will introduce short-range interactions between the proteins. Specifically, we have suggested that in the transition state the two proteins are separated by one layer of solvent but have relative orientations close to that in the bound state.[22]

Our transition-state model for calculating $k_{u\to t}$ as a practical estimate of k_{on} is different from previously proposed models based on forming specific contacts.[23–25] There the bound state is reached by sequentially forming two or three "correct" contacts between the two proteins. It is difficult to rationalize how a single "correct" contact can "hold" a transient complex together. Indeed, from a study on the interfaces of a number of protein–protein complexes, McCoy et al.[26] concluded that individual charge pairs are not a good predictor for recognition. Rather, the overall electrostatic energy between the proteins is a much more reliable predictor. This supports our transition-state model in that the selected configurations are close to the bound complex in relative orientations and thus have near optimal overall electrostatic energies.

The specific-contact model appears to have two other difficulties. If forming a "correct" contact were energetically favorable, then it would be likely that "incorrect" contacts also form. Since there is no apparent mechanism for discriminating incorrect contacts, these would lead to kinetic traps. In addition, configurations satisfying a two-contact requirement can be quite distant orientationally from the bound complex. Then the transition rate from these configurations to the bound complex may not be sufficiently high. The net result is that the $k_{u\to t}$ value that one actually calculates will be a poor estimate of the on rate.

Hill[27,28] explicitly invoked the concept of transition state in his study of protein–protein association. Though he worked with spherical models of proteins, his guideline for selecting the transition state is similar to ours (i.e., transition state is at the borderline between where internal motion has to be considered and where such motion can be neglected). However, the use of the transition state in predicting the on rate is very different. Hill's expression for the diffusion-limited rate constant is modeled on Eyring's transition-state theory:

$$k_{u\to t} = (D/R_C\Lambda)(q^{\dagger}/V)(Q_A/V)(Q_B/V) \qquad (21)$$

where q_A, q_B, and $q\dagger$ are the partition functions of the two isolated proteins and the transition state, respectively, and V is volume. Eyring's frequency factor k_BT/h is replaced by $D/R_C\Lambda$, in which D is the relative diffusion constant, $\Lambda = h/(2\pi m k_BT)^{1/2}$ with m the reduced mass, and R_C is a "capture" distance. Equation (21) is only formal since Hill did not describe a procedure for calculating the capture distance. For two uniformly reactive spheres in the absence of interactions, comparing the known result $4\pi DR$ with Eq. (21) gives $R_C = R$. Hill suggested using the same value of R_C when considering the case where only a part of each surface is reactive. This leads to $k_{u\to t}{}^0 = 4\pi DRF_1F_2$, which severely underestimates the rate when the reactive patch sizes are small [the correct result is given by Eq. (2)]. When an interaction potential is present, Hill (without justification) suggested simply using the value of R_C in the absence of the potential. This, by coincidence, leads to Eq. (14). As noted earlier, Eq. (14) is a good approximation only when the size of the transition-state region is small and the interaction is long-ranged.

Janin[29] recognized the fact that during association two proteins must first sacrifice translational-rotational entropy and thus will encounter a free energy barrier. He formally wrote down the association rate constant as

$$k_{u\to t} = q_t q_r k^0_{u\to t} \qquad (22)$$

where q_tq_r is a rate enhancement factor due to electrostatic interactions. It is not clear how one can find this factor a priori. Janin did write down an explicit expression for $k_{u\to t}{}^0$. This is based on a surface fraction argument and is equivalent to the incorrect result $k_{u\to t}{}^0 = 4\pi DRF_1F_2$.

In summary, we have presented a formalism of the protein–protein association process with an explicit description of the transition state. Within this formalism we have explained the disparate ionic-strength dependencies of the on and off rates that are observed

on a wide range of associating proteins. The enhancement of the on rate by long-range electrostatic interactions can be fully accounted for by $k_{on} = k_{on}^0 \exp(-G_{el}^{\dagger}/k_B T)$, where $G_{el}^{\dagger}$ is the electrostatic free energy of the transition state.

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