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The Affinity-Enhancing Roles of Flexible Linkers in Two-Domain DNA-Binding Proteins†

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ABSTRACT: Recently many attempts have been made to design high-affinity DNA-binding proteins by linking two domains. Here a theory for guiding these designs is presented. Flexible linkers may play three types of roles: (a) linking domains which by themselves are unfolded and bind to DNA only as a folded dimer (as in a designed single-chain Arc repressor), (b) connecting domains which can separately bind to DNA (as in the Oct-1 POU domain), and (c) linking a DNA-binding domain with a dimerization domain (as in the λ repressor). In (a), the linker keeps the protein as a folded dimer so that it is always DNA-binding-competent. In (b), the linker is predicted to enhance DNA-binding affinity over those of the individual domains (with dissociation constants K_A and K_B) by $p(d_0)/K_B$ or $p(d_0)/K_A$, where $p(d_0) = (3/4\pi l_p bL)^{3/2} \exp(-3d_0^2/4l_p bL)(1 - 5l_p/4bL + ...)$ is the probability density for the end-to-end vector of the linker with L residues to have a distance d_0. In (c), the linker is predicted to enhance the binding affinity by $K_d^C/p(d_0)$, where K_d^C is the dimer dissociation constant for the dimerization domain. The predicted affinity enhancements are found to be actually reached by the Oct-1 POU domain and λ repressor. However, there is room for improvement in many of the recently designed proteins. The theoretical limits presented should provide a useful guide for current efforts of designing DNA-binding proteins.

Different domains in a protein are frequently connected by flexible linkers. In the context of DNA binding, a linker may increase the "effective concentration" of a second DNA-binding domain and enhance the overall affinity for the full binding site. This intuitive idea has in recent years prompted a flurry of design efforts in search of high-affinity DNA-binding proteins (*1*−*8*). Here we present a quantitative theory for the affinity-enhancement by flexible linkers.

Flexible linkers may play three types of roles in DNA-binding proteins (see Figure 1). (a) A domain [such as the monomers of Arc and Cro repressors and MASH-1 (*2*, *4*, *8*)] is by itself unfolded and becomes folded only as a dimer, which then binds DNA. A linker connecting two such domains ensures that the protein exists as a folded dimer and thus is always DNA-binding-competent. We have recently presented a theory for the role of the flexible linker in dimer formation (*9*). (b) Two domains can separately bind to half-sites [e.g., the POU-specific and POU homeo domains (POU_S and POU_H)[1] in the Oct-1 POU domain (*10*)]. A linker allows the two domains to bind as a single chain to the full binding site (perhaps with higher affinity). (c) A protein such as the λ repressor binds to DNA as a dimer, but dimer formation is mediated by a dimerization domain (DD), which is connected to the DNA-binding domain (DBD) by a flexible linker. Based on the earlier theory for the role of flexible linkers in dimer formation (*9*), we will present theoretical predictions for the DNA-binding affinity enhancement by flexible linkers in (b) and (c).

The introduction of a peptide linker can have complicated effects. Here we restrict to the simplest situation where the linker is truly flexible so that there is minimal interference between the linker and the tethered domains. For a dimeric protein that becomes unfolded when the subunits are dissociated, such a flexible linker was found to change the unfolding equilibrium constant K^d for the dimeric protein to that for the single-chain form, K^s, where (*9*):

$$K^d/K^s = p(d_0) \tag{1}$$

In eq 1, $p(r)$ is the probability density for the end-to-end vector of the flexible linker and d_0 is the end-to-end distance in the folded dimer. $p(d_0)$ is equivalent to the "effective concentration." We found that a flexible peptide linker consisting of L residues can be modeled as a wormlike chain, such that (*11*)

$$p(d_0) = (3/4\pi l_p l_c)^{3/2} \exp(-3d_0^2/4l_p l_c)(1 - 5l_p/4l_c + 2d_0^2/l_c^2 - 33d_0^4/80l_p l_c^3 - 79l_p^2/160l_c^2 - 329d_0^2 l_p/120l_c^3 + 6799d_0^4/1600l_c^4 - 3441d_0^6/2800l_p l_c^5 + 1089d_0^8/12800l_p^2 l_c^6) \tag{2}$$

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[1] Abbreviations: POU_S and POU_H, POU-specific and POU homeo domains in the Oct-1 POU domain; DD, dimerization domain; DBD, DNA-binding domain.

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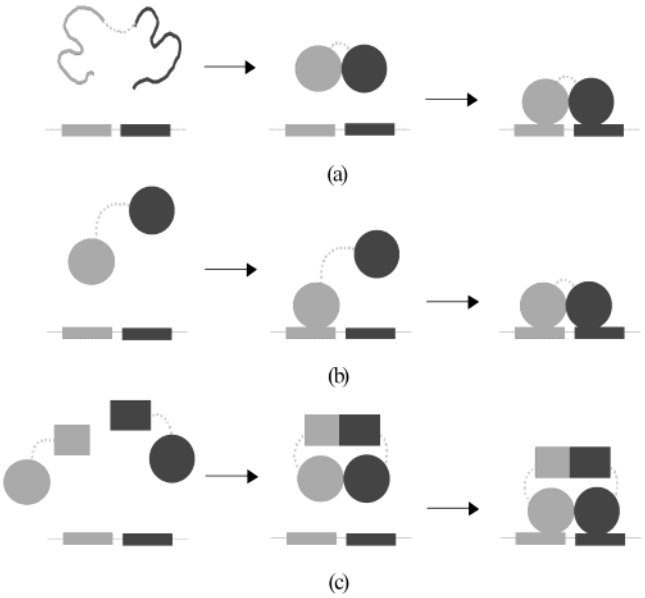

FIGURE 1: Three types of roles of flexible linkers in DNA-binding proteins. (a) A linker connects two domains which by themselves are unfolded and keeps the protein as a folded dimer and thus DNA-binding-competent. (b) A linker connects two domains which can separately bind to DNA. (c) A linker connects a DNA-binding domain to a diemrization domain. DNA-binding and dimerization domains are represented by spheres and rectangles, respectively, while linkers are represented by dashed lines. Two long boxes represent the full binding site on the DNA.

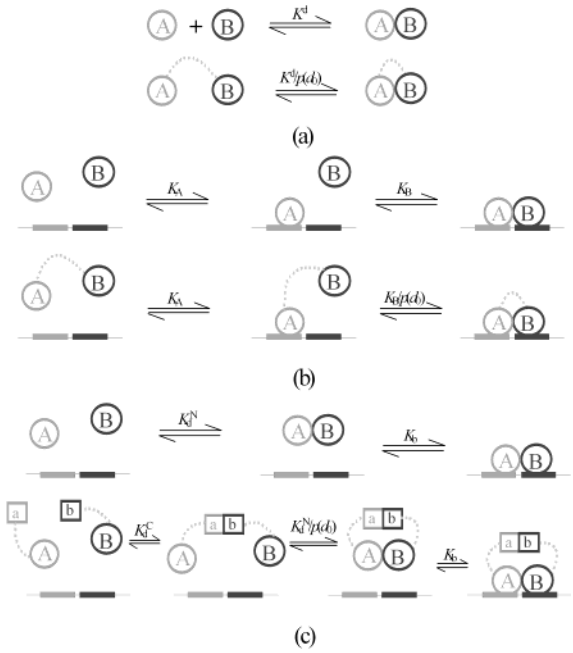

FIGURE 2: Dimerization and DNA binding with and without linkers (and dimerization domains). (a) Dimerization of two domains. (b) Sequential DNA binding of two domains. (c) Dimerization and subsequent DNA binding. In each case, the upper row refers to the linkerless form, and the lower row refers to the proteins with linkers [and dimerization domains in (c)]. Note that in (b), although we show the sequential binding of domain A followed by domain B, in principle the binding can occur in the reverse order (i.e., B followed by A). However, regardless of the order of binding, the overall dissociation constant is always $K_A K_B$ in the linkerless case and $K_{A-B} = K_A K_B / p(d_0)$ (eq 6) in the linked case.

where $b = 3.8$ Å is the nearest $C_\alpha - C_\alpha$ distance and $l_c = bL$ and $l_p = 3$ Å are the contour length and persistence length, respectively, of the peptide linker. In earlier work, we have demonstrated the utility of eq 1 on the folding stability of three dimeric proteins (*9*). In particular, for Arc repressor at 4.18 M urea and 25 °C, the dimeric protein has an unfolding equilibrium constant of 1 mM (*12*). The unfolding equilibrium constant of the single-chain form predicted by eq 1 is 0.25, agreeing well with the experimental value of 0.41 (*12*).

We will show that eq 1 also predicts well the role of the flexible linker in the unfolding of a single-chain Cro repressor. More importantly, we will develop a theory for the roles of flexible linkers in enhancing DNA-binding affinity in the two types of proteins represented by the Oct-1 POU domain and λ repressor.

THEORY

Binding between Two Spherical Domains, with and without a Flexible Linker. As the simplest model for the influence of a flexible linker, consider two spheres connected by a flexible linker (see Figure 2a). In the absence of the linker, the dimer dissociation constant is given by (*13*−*15*)

$$K^{d} = 1/\int_{\Gamma} d^3\mathbf{r} \exp[-\beta U(\mathbf{r})] \qquad (3)$$

where $\mathbf{r}$ is the vector between the two sphere centers, $U(\mathbf{r})$ is the interaction potential between the two spheres, Γ signifies the small region in $\mathbf{r}$ space defining the bound state, and β is the inverse of the product of the Boltzmann constant and the absolute temperature. Now consider the single-chain molecule formed by the presence of the flexible linker. The probability density for the end-to-end vector of the flexible linker by itself is $p(r)$. Upon tethering the two spheres and in the absence of any interactions between the linker and the two spheres, the probability density for the end-to-end vector becomes $p(r) \exp[-\beta U(\mathbf{r})]$. Then the dissociation constant for the single-chain molecule is

$$K^{s} = \int' d^3\mathbf{r} p(r) \exp[-\beta U(\mathbf{r})] / \int_{\Gamma} d^3\mathbf{r} p(r) \exp[-\beta U(\mathbf{r})] \qquad (4)$$

where the prime signifies integrating over the unbound state (occupying the entire $\mathbf{r}$ space except for the small region of the bound state). In the unbound state, $U(\mathbf{r}) \approx 0$; thus, $\int' d^3\mathbf{r} p(r) \exp[-\beta U(\mathbf{r})] \approx \int' d^3\mathbf{r} p(r) \approx 1$ [note that $p(r)$ is normalized over the $\mathbf{r}$ space]. If in the bound state $r \approx d_0$, then

$$K^{s} = 1/p(d_0) \int_{\Gamma} d^3\mathbf{r} \exp[-\beta U(\mathbf{r})] = K^{d}/p(d_0) \qquad (5)$$

which is equivalent to eq 1. Indeed, the present model for dissociation becomes one for protein unfolding when an additional step, that of the unfolding of the two spheres, is included. This step is the same in both the dimeric protein and the single-chain form and thus does not contribute to the unfolding equilibrium constants (*9*).

Equation 5 will be the critical component in the derivations below. We recognize that DNA-binding domains are by no means spherical molecules. Nonetheless, eq 5 should still reasonably model the influence of the linker as long as the distribution of its end-to-end vector is not significantly affected by the domains tethered to it.

Two DNA-Binding Domains with and without a Flexible Linker. In Figure 2b, we compare the DNA binding of two domains in the absence and presence of a flexible linker. Upon binding of the first domain (with dissociation constant K_A), the second domain binds with a dissociation constant K_B in the absence of the linker. We neglect any effect of the linker on the dissociation constant of the first domain. In the presence of the linker, eq 5 predicts that the second domain binds with a dissociation constant $K_A/p(d_0)$ (note that the first domain together with the bound DNA is equivalent to sphere A in Figure 2a). The overall dissociation constant of the single-chain protein is thus

$$K_{A-B} = K_A K_B / p(d_0) \quad (6)$$

The enhancement in binding affinity due to the flexible linker, K_A/K_{A-B} (or K_B/K_{A-B}), is then $p(d_0)/K_B$ [or $p(d_0)/K_A$].

DNA-Binding Domain Connected to a Dimerization Domain by a Flexible Linker. In Figure 2c, we compare the DNA binding of two domains without and with the mediation of two DDs. Without a DD, the DBD can conceivably bind to a half-site as a monomer. Here we restrict to the situation where only the dimerized form can bind to DNA (and occupy the full binding site). In the absence of the linked DDs, the two DBDs form a dimer with a dissociation constant K_d^N and then bind to DNA with a dissociation constant K_b. When the DDs are tethered to the DBDs, they will form a dimer with a dissociation constant K_d^C. Then the two DBDs dimerize. The dissociation constant for that is $K_d^N/p(d_0)$ according to eq 5, if the first linker, the DD dimer, and the second linker together are considered equivalent to the linker in Figure 2a. Finally, the DBD dimer binds to DNA with the dissociation constant K_b (neglecting any effect of the linkers and the DD dimer on DNA binding). The dimer dissociation constant for the intact protein (consisting of the DBD and DD connected by the flexible linker) is

$$K_d = K_d^C K_d^N / p(d_0) \quad (7)$$

In a more accurate version of eq 7, $p(d_0)$ is replaced by $p(d_1|d_0)/4\pi d_1^2$, where $p(d_1|d_0)$ is the probability density at d_1 for the distance between the C-terminals of the linkers when their N-terminals are fixed at distance d_0. A formula for $p(d_1|d_0)$ was given previously (*16*). The enhancement in DNA-binding affinity due to the DD connected to the DBD by the flexible linker is $K_d^N/K_d = p(d_0)/K_d^C$.

RESULTS

Stability and DNA Binding of Dimeric and Single-Chain Cro Repressors. Jana et al. (*4*) designed single-chain Cro repressors by connecting the C-terminal of one subunit with the N-terminal of another by 8−16-residue linkers. The melting temperature, T_m, for all the single-chain repressors measured by circular dichroism is 53 °C. In comparison, the melting temperature of the wild-type Cro repressor is concentration-dependent and is only 42 °C at 4 μM and 46 °C at 58 μM. The protein concentration required to raise T_m to 53 °C is 5.3 mM by extrapolating a linear relationship between $1/T_m$ and the logarithm of protein concentration. At T = 53 °C, we have K^s = 1 and K^d = 5.3 mM, an thus K^d/K^s = 5.3 mM.

The distance between the last residue (N61) of one subunit and the first residue (E2) of the other subunit observed in the X-ray structure of the Cro repressor dimer (*17*) is 25 Å. The last five residues of the first subunit and the first residue of the second subunit are disordered in the crystal and presumably are flexible in solution. The linker lengths in the single-chain Cro repressors designed by Jana et al. thus range from 15 to 23. With d_0 = 25 Å and L = 15−23, eqs 1 and 2 predict $K^d/K^s = p(d_0)$ = 5.9−8.0 mM. These values are in good agreement with the experimental result.

At room temperature, the single-chain Cro repressors (with T_m = 53 °C) are folded as a dimer and thus DNA-binding-competent. If the folded dimer binds DNA with dissociation constant K_b, the bound fraction θ of DNA is related to the free repressor concentration $[R_s]$ via

$$\theta = [R_s]/([R_s] + K_b) \quad (8a)$$

On the other hand, wild-type Cro repressor must first dimerize (with dissociation constant K^d) and then bind to DNA (with a dissociation constant assumed to be the same as in eq 8a). In this case

$$\theta = [R]^2/([R]^2 + K^d K_b) \quad (8b)$$

where [R] is the free repressor concentration (in monomer units). Jana et al. (*4*) performed DNA-binding titrations for wild-type and single-chain Cro repressors and found that the repressor concentrations required to achieve DNA half-saturation are $[R]_{1/2} = 8 \times 10^{-10}$ M and $[R^s]_{1/2} = 4 \times 10^{-12}$ M. According to eqs 8a and 8b, $([R]_{1/2})^2/[R^s]_{1/2} = K^d$. The experimental data on $[R]_{1/2}$ and $[R^s]_{1/2}$ thus predict $K^d = 16 \times 10^{-8}$ M at room temperature. If the concentration dependence of the melting temperature of the wild-type Cro repressor is extrapolated, we obtain $K^d = 2 \times 10^{-8}$ M at room temperature, in reasonable agreement with the dimer dissociation constant expected from the data on $[R]_{1/2}$ and $[R^s]_{1/2}$. This agreement suggests that the linkers introduced in Cro repressor to form single-chain molecules indeed mainly serve to ensure dimer formation and do not significantly affect DNA binding.

DNA Binding of Intact Oct-1 POU Domain and POU-Specific and POU Homeo Domains. Klemm and Pabo (*10*) investigated the role of the flexible linker connecting POU_S and POU_H by studying the DNA binding of the two domains without the linker. The dissociation constant (K_A) for POU_H is 1.5×10^{-7} M, and the dissociation constant (K_B) for POU_S when POU_H is already bound is 1.7×10^{-6} M. With a dissociation constant of $K_{A-B} = 7.1 \times 10^{-11}$ M for the intact Oct-1 POU domain, one has $K_A K_B/K_{A-B}$ = 3.6 mM.

The last residue (E75) of POU_S and the first residue of POU_H (R102) have a distance of 27.6 Å in the X-ray structure of the Oct-1 POU domain (*18*). With L = 26, eqs 6 and 2 predict $K_A K_B/K_{A-B} = p(d_0)$ = 5.8 mM, agreeing well with the experimental result.

Van Leeuwen et al. (*19*) have further studied the effect of linker length on the DNA-binding affinity of the Oct-1 POU domain. With L = 12, 18, 31, and 40, they found that the dissociation constants relative to that of wild type (with L = 26) are 3.4, 1.1, 1.0, and 0.8, respectively. These correlate very well with the predictions of eqs 6 and 2: 4.0, 1.4, 1.0, and 1.0.

DNA Binding of Other Two-DBD Proteins with and without Linkers. Pongor and co-workers (*20*) compared the DNA binding of the N-terminal domain R69 of the phage 434 repressor and a homodimeric single-chain protein RR69 consisting of 2 copies of R69 linked by 20 residues. The dissociation constant (K_A and K_B) for R69 is $\sim 4 \times 10^{-7}$ M whereas the dissociation constant (K_{A-B}) for RR69 is 8×10^{-10} M. Thus, $K_AK_B/K_{A-B} = 0.2$ mM. This is to be compared with the prediction of eqs 6 and 2, $K_AK_B/K_{A-B} = p(d_0) = 5.2$ mM (with $d_0 = 28.5$ Å and $L = 27$ for the present case).

A number of recent designs used zinc fingers as modules. Kim and Pabo (*3*) connected the three fingers of Zif268 with the variant NRE of Zif268 by a 11-residue linker and found $K_AK_B/K_{A-B} = 0.4$ μM. Moore at al. (*7*) connected the three fingers of Zif268 with a three-finger mutant and found $K_AK_B/K_{A-B} \sim 2$ μM. These results are much smaller than the values of $p(d_0)$ in the millimolar range.

Dimerization and DNA Binding of Intact λ Repressor and Its N-Terminal DBD. The λ repressor consists of an N-terminal DBD and a C-terminal DD connected by a flexible linker (*21*). The dimer dissociation constant (K_d) for the intact protein is 5.6 nM (*22*). In comparison, the dimer dissociation constant (K_d^N) of the N-terminal DBD is only 0.6 mM (*23*, *24*). There is experimental evidence that the dimer formed by the C-terminal DD is as stable as that formed by the intact protein (*21*); we thus assume $K_d^C \approx K_d$. Then $K_d^CK_d^N/K_d \approx K_d^N = 0.6$ mM. The last two residues (S92) of the DBD dimer (bound to DNA) have a distance of $d_0 = 13$ Å (*25*), while the first two residues (A136) of the DD dimer have a distance of $d_1 = 23$ Å (*26*). With 44 residues between the two ends of the flexible linkers, we found $p(d_1|d_0)/4\pi d_1 2 = 3.7$ mM. This predicted value for $K_d^CK_d^N/K_d$ (see eq 7 and comments that follow) is in reasonable agreement with the experimental result.

Upon forming dimers, intact λ repressor and the N-terminal DBD bind to DNA with nearly the same dissociation constant (*24*, *27*). Thus, the overall increase in DNA-binding affinity is $K_d^N/K_d = 10^5$.

DISCUSSION

We have studied the roles of flexible linkers in three types of DNA-binding proteins and presented theoretical predictions for the DNA-binding affinity enhancement in each case. The theory requires that the linkers be truly flexible so that there is minimal interference between the linkers and domains tethered to them. The interference may occur in two ways, when the tethered domains come into contact upon either dimer formation or DNA binding. The linker may adversely perturb the interactions between the domains (and their interactions with the DNA), or the domains may perturb the natural distribution of the end-to-end distance of the linker. Apparently such interference is indeed minimal for the linkers of the single-chain Arc and Cro repressors, the Oct-1 POU domain, and the λ repressor. Our theoretical predictions, without adjustable parameters, are confirmed by experimental results for the effects of linkers on dimer stability and DNA binding of these proteins.

The end-to-end distance d_0 of a linker is dictated by the complex formed between the tethered domains upon dimerization or DNA binding. For a given d_0, the linker length L can be varied to maximize $p(d_0)$. For $d_0 = 10$, 15, 20, 25, 30, 35, 40, 45, and 50 Å, the optimal linker lengths are 3, 9, 16, 26, 38, 52, 68, 87, and 107, respectively. The resulting values of $p(d_0)$ are 167, 40.5, 16.2, 8.1, 4.6, 2.9, 1.9, 1.4, and 1.0 mM. Given that $p(d_0)$ is in the millimolar range or higher, there is ample room for affinity enhancement by flexible linkers. For example, by linking a second DBD having a dissociation constant ~ 1 μM, the overall affinity for the full binding site is expected to be enhanced by 10^3 according to eq 6. Similarly, by introducing a DD having a dimer dissociation constant $\sim 10^{-8}$ M, the DNA-binding affinity is expected to be enhanced by 10^5 according to eq 7.

The theoretical predictions presented here set limits on the DNA-binding affinity enhancement that could be afforded by flexible linkers. While these limits appear to be reached by the Oct-1 POU domain and the λ repressor, they are not yet reached by recent designs based on zinc fingers (*3*, *7*). K_AK_B/K_{A-B} as predicted by eq 6 could be in the millimolar range, but is only in the micromolar range in these designs. In the poly-zinc finger design of Kim and Pabo (*3*), the individual three-finger domains have dissociation constants for their respective half-sites on the order of 10^{-11} M; eq 6 then predicts $K_{A-B} \sim 10^{-18}$ M. Such a small dissociation constant could not be measured directly; thus, K_{A-B} was deduced from the ratio of kinetic constants. It is possible that the resulting dissociation constant, 10^{-15} M, is underestimated (*3*). From a design point of view, it is important that the linker be made as flexible as possible. Any adverse effect of the linker can be checked by comparing the DNA binding of each domain to its specific half-site with and without the tether to the other domain.

The two types of DNA-binding proteins represented by Arc repressor and the λ repressor share one similarity: they both bind to DNA only after dimerization. For such proteins, one has a choice of two strategies for introducing flexible linkers: covalent linking and mediation by a dimerization domain. In the former case, the bound fraction θ of DNA is related to the repressor concentration via eq 8a. In the latter case, the bound fraction is given by eq 8b. Equation 8b gives a much sharper transition between the unbound and bound states of the DNA as the repressor concentration is increased. This sharper transition is desirable as it allows the protein concentration to be used more effectively as an off/on switch (*28*).

As noted recently by Pabo et al. (*6*), a key area of further research in DNA-binding protein design "will involve continued investigation of strategies for linker and dimer design." The theoretical considerations presented here hopefully will provide a useful guide for such efforts.

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