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Side-Chain Interactions Form Late and Cooperatively in the Binding Reaction between Disordered Peptides and PDZ Domains

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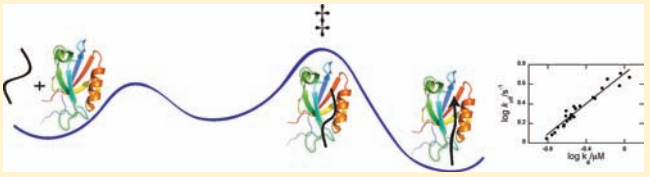
ABSTRACT: Intrinsically disordered proteins are very common and mediate numerous protein−protein and protein−DNA interactions. While it is clear that these interactions are instrumental for the life of the mammalian cell, there is a paucity of data regarding their molecular binding mechanisms. Here we have used short peptides as a model system for intrinsically disordered proteins. Linear free energy relationships based on rate and equilibrium constants for the binding of these peptides to ordered target proteins, PDZ domains, demonstrate that native side-chain interactions form mainly after the rate-limiting barrier for binding and in a cooperative fashion. This finding suggests that these disordered peptides first form a weak encounter complex with non-native interactions. The data do not support the recent notion that the affinities of intrinsically disordered proteins toward their targets are generally governed by their association rate constants. Instead, we observed the opposite for peptide−PDZ interactions, namely, that changes in K_d correlate with changes in k_{off}.



INTRODUCTION

The structure−function relationships of stably folded proteins have been studied for more than 50 years with great implications for the interpretation of biology on a molecular level as well as the understanding of drug action. It was long assumed that in order for a protein to function properly, it has to adopt a well-defined three-dimensional structure. It was not until the 1990s that an increasing amount of evidence suggested that many proteins in fact are intrinsically disordered or contain long disordered regions and at the same time are functional.[1,2] Experimental mechanistic studies on disordered proteins have appeared only recently (e.g., refs 3−10). In view of the fact that they make up a large portion of the proteins encoded by the eukaryotic genome as well as their frequent association with diseases,[11,12] understanding the role of disorder in protein−protein recognition is a key problem in modern structural biology. In particular, mechanistic data are scarce.

There are many hypotheses regarding why proteins are intrinsically disordered,[13] including the following: (*i*) it is a way of decoupling affinity and specificity; (*ii*) it allows for increased plasticity with regard to the ligand; (*iii*) a large interaction surface area is provided in a short amino acid sequence as the protein folds around its ligand. According to the so-called "fly-casting" scenario, disordered proteins may quickly form a high-energy complex with the physiological partner that would be locked in place by the subsequent folding reaction.[14] A potential advantage of the intrinsic disorder would then lie in the increased probability to capture a target ligand,[15] even with only moderate affinity. It is of critical importance to address these issues from a biophysical perspective and clarify the role of disorder in protein−ligand recognition. Such information is not only important for a general molecular understanding of cellular events but will also be crucial for future drug design directed at intrinsically disordered proteins, which have been shown to be associated frequently with different types of cancer and neurodegenerative diseases.[11,12]

Detailed experimental studies based on NMR spectroscopy,[10] fluorescence-monitored temperature jump studies,[9] and mutagenesis/stopped-flow fluorimetry[3,5] together with computational methods[14] have suggested that binding of disordered proteins takes place via a weak precomplex, possibly involving non-native interactions,[16] which then rearranges into the final complex.[2] However, evidence for conformational selection has also been provided by single-molecule spectroscopy,[4] NMR analysis,[7,8,17] and computer simulations.[18]

Linear free energy relationships (LFERs) relate the activation free energy for a reaction ($\Delta G^{\ddagger}$) with its equilibrium free

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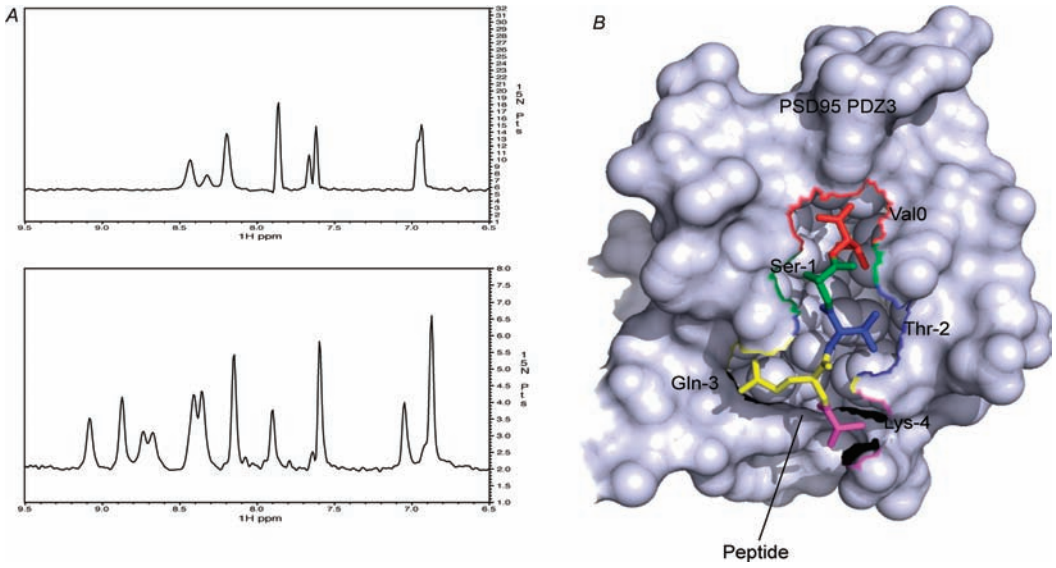

Figure 1. Structural features of free and PDZ-bound peptides. (A) 1D ^{1}H NMR titrations of peptide (GSKNYKQTSV) in the free state (upper panel) and in complex with PSD-95 PDZ3 (lower panel). The free peptide is clearly disordered since the peaks are all grouped together, a characteristic of disordered polypeptides.[55] Upon addition of saturating amounts of PSD-95 PDZ3, the ^{1}H peaks become more dispersed and are uniformly distributed, characterizing an ordered structure[55] in agreement with the crystal structure shown in panel B. (B) Crystal structure of PSD-95 PDZ3 with the peptide KQTSV.[40] PSD-95 PDZ3 is shown as a surface, and the peptide residues are shown as sticks and colored as follows: red, Val(0); green, Ser(−1); blue, Thr(−2); yellow, Gln(−3); magenta, Lys(−4). The peptide adopts an ordered β-strand conformation in the bimolecular complex. The side chain of the Lys(−4) residue is not visible in this crystal structure. The figure was drawn using Pymol.[56]

energy (ΔG^{eq}). LFERs were classically used to assess the position of the transition state during the formation of a covalent bond in physical organic chemistry.[19] By variation of the structures and thus the reactivities of the substrates, a linear relationship for $\Delta G^{\ddagger}$ versus ΔG^{eq} can be obtained, and its slope reflects the position of the transition state. However, LFERs are frequently used for noncovalent interactions in enzymology,[20] binding reactions involving allosteric regulation,[21,22] and protein folding studies.[23] Such LFERs were recently employed to suggest that affinities (K_d values) of intrinsically disordered proteins are mainly correlated with their association rate constants k_{on} while K_d values of folded proteins are mainly correlated with their dissociation rate constants k_{off}.[24]

The simplest model system for analyzing the disorder-to-order transitions in proteins involves short peptides that interact with a well-defined target. For example, PDZ domains bind to the C-termini of target proteins. This interaction leads to the formation of an intermolecular β-sheet, where the C-terminal ligand forms one β-strand. We have previously studied the interaction between peptide ligands and several PDZ domains.[25−30] The PDZ−ligand interaction is a good model system for investigating the role of disorder quantitatively and mechanistically, since the peptide ligand undergoes a structural transition from a disordered conformation in its free state to an ordered conformation in its bound state.

In this work, we used LFER analyses to study a large set of data for the PDZ−ligand interaction from the perspective of the peptide as a model of an intrinsically disordered system. Our results clearly demonstrate that affinities for PDZ−peptide interactions are governed by k_{off} rather than k_{on}, suggesting late formation of native interactions along the reaction coordinate. The implication of this result is that the proposed correlation between association rate constants and affinity constants for binding reactions involving disordered proteins[24] is not general.

MATERIALS AND METHODS

Binding Experiments. Association and dissociation kinetics for SAP97 PDZ2 and peptides were measured as previously described for PSD-95 PDZ3 and PTP-BL PDZ2.[28] Briefly, SAP97 PDZ2 with a mutation, I342W, was expressed and purified as described elsewhere.[31] Binding of four different peptides (see legend to Figure 2) to SAP97 PDZ2 was measured in an SX-20MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.) by monitoring the change in Trp fluorescence upon binding (excitation at 280 nm, emission at 330 ± 25 nm using a cutoff filter). The major binding phase corresponding to the association reaction between peptide and SAP97 PDZ2[25,31] was analyzed by a single exponential equation, and the observed rate constants were plotted versus peptide concentration to obtain the association rate constant k_{on}. Dissociation rate constants (k_{off}) were determined in displacement experiments. The PDZ−peptide complex was mixed with an excess of dansylated peptide, which traps any free PDZ domain and makes the dissociation from the unlabeled peptide irreversible. The k_{obs} value is equal to k_{off}. See refs 25, 28, and 31 for more details on the kinetic measurements. All kinetic measurements for SAP97 PDZ2 were performed in 50 mM potassium phosphate (pH 7.5) at 10 °C. Association and dissociation kinetics were also measured for 24 different site-directed mutants of SAP97 PDZ2 I342W in a similar fashion as for the pseudo-wild-type I342W and with the same four peptides.

NMR Experiments. A double-labeled ^{15}N,^{13}C peptide corresponding to the last eight residues of CRIPT[32] was expressed as a His-tagged lipoyl fusion protein in *Escherichia coli*. The fusion protein was bound onto a nickel column, washed with 50 mM Tris (pH 8.5) and 400 mM NaCl and subsequently eluted with 250 mM imidazole. The peptide was cleaved from the fusion protein with thrombin and then purified by reversed-phase HPLC. The final peptide contained an extra GS at the N-terminus resulting from the thrombin cleavage site: GSKNYKQTSV. The PSD-95 PDZ3 protein was expressed and purified as previously described.[27] ^{1}H titration experiments were performed on a Varian INOVA 600 MHz spectrometer equipped with a cryogenically cooled probe at 283 K in 50 mM potassium phosphate (pH 7.5). Peptide samples were dissolved in 10% D_2O, and 1D ^{1}H spectra for the peptide (290 μM) were recorded in the absence and

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presence of PSD-95 PDZ3 (370 μM). Data processing and analysis were done with the NMRPipe suite of software.[33]

RESULTS

We used stopped-flow fluorescence spectroscopy to obtain kinetic and equilibrium constants for interactions between peptides and PDZ domains. These constants were used to create LFERs to investigate the reaction mechanism for the binding of these disordered peptides to PDZ domains (Figure 1). Three different and well-studied PDZ domains were included in the study: SAP97 PDZ2, PTP-BL PDZ2, and PSD-95 PDZ3. For each of these PDZ domains, we selected a wild-type peptide based on previous work[32,34−38] (see legend to Figure 2 for wild-type and mutant peptides). These peptides are disordered in their unbound state, as shown by NMR analysis (Figure 1) for the peptide binding to PSD-95 PDZ3. For SAP97 PDZ2, a peptide corresponding to the disordered C-terminus of the E6 protein was used.[39] The peptide for PTP-BL PDZ2 was derived from the guanine nucleotide exchange factor RA-GEF-2.[36−38] The binding between peptides and PDZ domains involves backbone as well as side-chain interactions. Upon binding, the peptide adopts a β-strand in an extended intermolecular β-sheet[40] (Figure 1). In the present work, we used short disordered peptides, which represent the smallest binding sites of intrinsically disordered proteins.[41] However, this size of the binding region is not uncommon among disordered proteins.[15,24] In this context, we note that regions outside of the binding surface of intrinsically disordered proteins might influence their association kinetics, either through attractive or repulsive electrostatic forces. We did not address this issue here, but the association rate constant for the C-terminal domain of the E6 protein (72 residues) is almost identical to that of its C-terminus used in the present study.[25,42]

The effect of mutation in the peptide side chains was directly investigated by mutation at two or three positions (Figure 2). For PTP-BL PDZ2 and PSD-95 PDZ3, the peptides were changed in the first (0) and third (−2) positions, counting from the C-terminus (see Figure 1). These two positions (0 and −2) are known to confer both stability and specificity to PDZ−peptide interactions.[28,43,44] For SAP97 PDZ2, an additional position was mutated, namely, the fifth (−4) amino acid from the C-terminus, where the Arg(−4) residue was replaced by 2-aminopentanoic acid (Ape). This mutation removed the guanidinium moiety of the Arg side chain but left its aliphatic chain. The mutations in the peptides resulted in lower affinity (4−18-fold) toward their respective PDZ domains, except for the Ser(−2) → Thr mutation in the peptide for PTP-BL PDZ2, where the affinity did not change.

The change in affinity allowed three- or four-point LFER (Brønsted/Leffler) plots to be constructed for the wild-type and mutant disordered peptides by plotting log k_{off} or log k_{on} versus log K_d for the binding reactions between different peptides and their cognate PDZ binding domains (Figure 2). The results of these analyses were clear: the effect of the peptide mutations is mainly in the dissociation rate constant k_{off}.

Each of the three PDZ domains was subjected to site-directed mutagenesis, mainly conservative deletion mutations[45] in the protein core (e.g., Ala → Gly, Val → Ala, Ile → Val, etc.) but also a few mutations involving charged residues on the surface, (e.g., Lys or Glu → Ala). Association and dissociation rate constants were determined for the mutant proteins[28] and LFERs constructed for each PDZ with their respective wild-type and mutant peptides (Figure 3). For PSD-95 PDZ3, the slope of the log k_{off} versus log K_d plot was close to 1 for all three peptides (0.94−1.04). Both SAP97 PDZ2 and PTP-BL PDZ2 showed slight changes in k_{on} upon mutation in the PDZ domain, as reflected in their plots, but the major effect came from k_{off} (slope = 0.65−0.9). We note that when all of the data for each PDZ domain were combined into a single data set (i.e., the data in Figure 3A,C,E, in analogy with some composite data sets reported by Prakash[24]), the correlation between log K_d and log k_{off} became 0.74 (data not shown). This indicates that

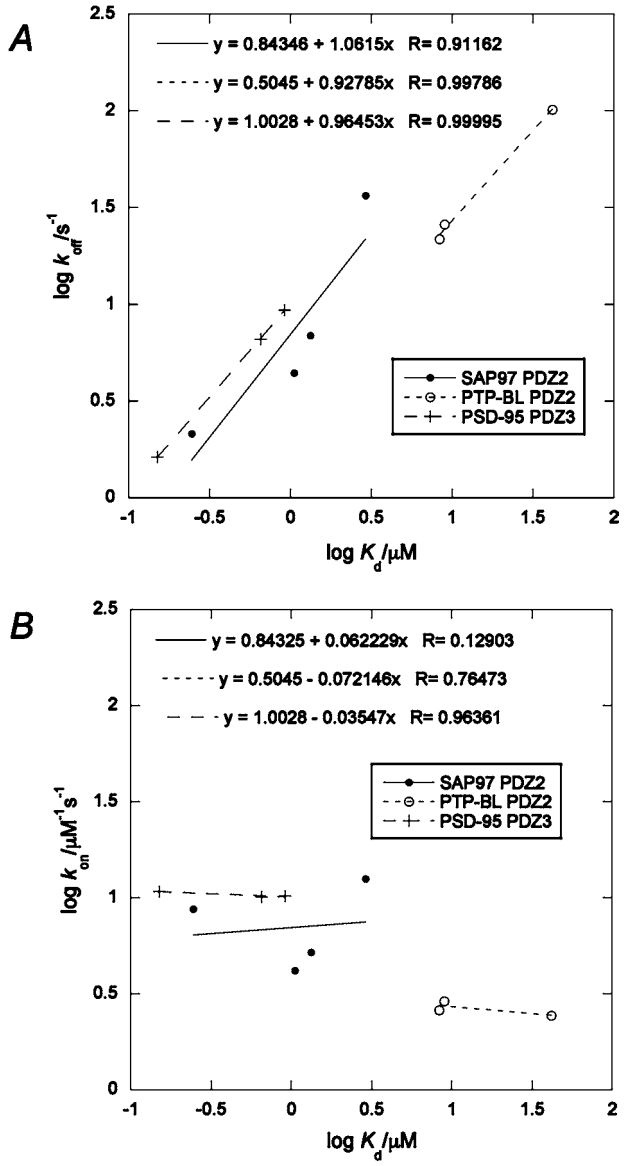

Figure 2. Linear free energy relationships for wild-type and substituted peptides for three PDZ domains. (A) Dependence of the dissociation rate constant k_{off} on the affinity constant K_d. (B) Dependence of the association rate constant k_{on} on the affinity constant K_d. The peptides were the following: LQRRRETQV, LQRRRETQ-Abu, LQRRRESQV, and LQRR-Ape-ETQV for SAP97 PDZ2; EQVSAV, EQVSA-Abu, and EQVTAV for PTP-BL PDZ2; YKQTSV, YKQTS-Abu, and YKQSSV for PSD-95 PDZ3. The peptides for PTP-BL PDZ2 and PSD-95 PDZ3 had an N-terminal dansyl group to facilitate the kinetic measurements. The kinetics for SAP97 PDZ2 were monitored through Trp fluorescence.[25] Abu is 2-aminobutyric acid, i.e., Val with one methyl group replaced by a hydrogen; Ape is 2-aminopentanoic acid, i.e., Arg with its guanidinium group replaced by a hydrogen.

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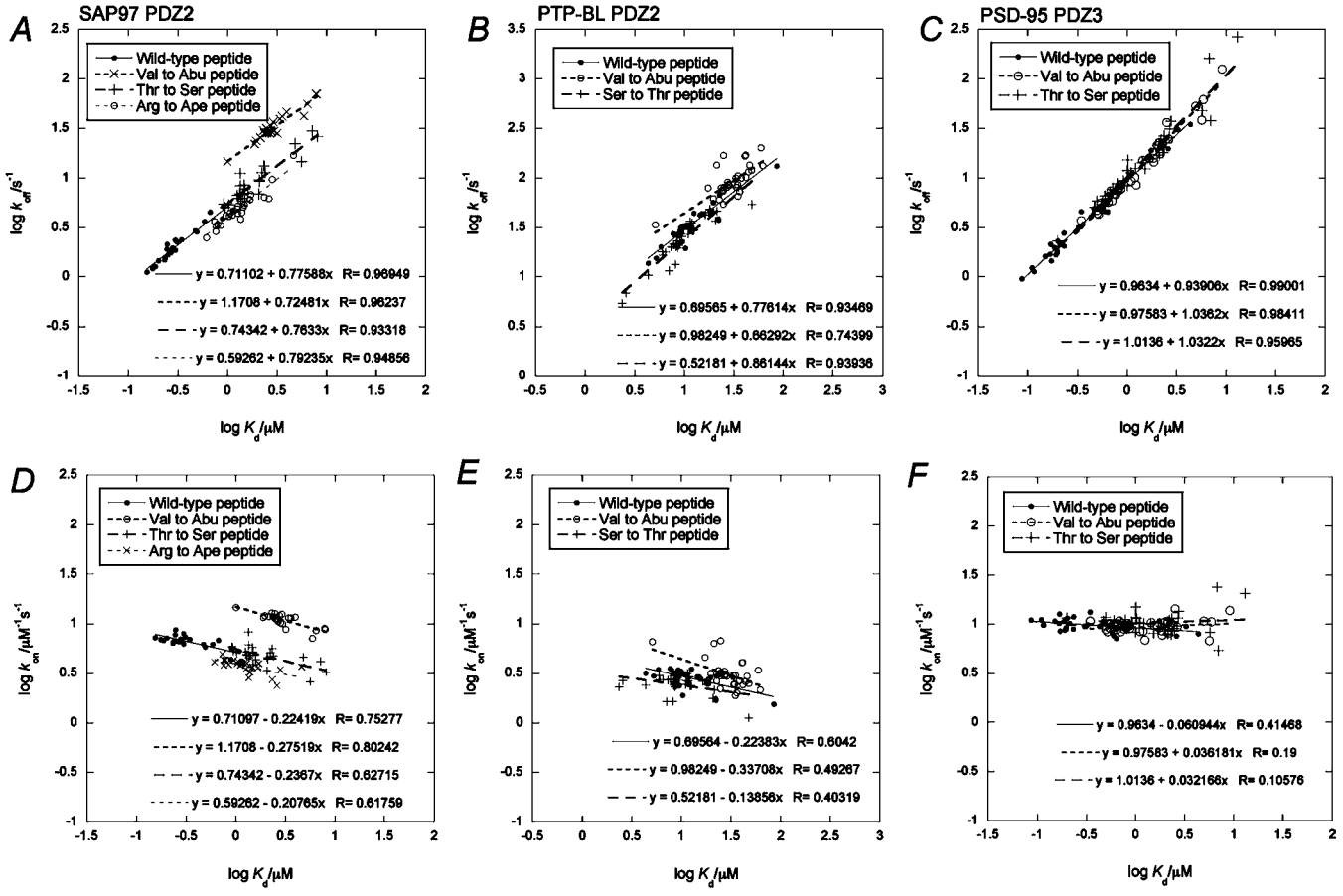

Figure 3. Linear free energy relationships for substituted peptides and mutated PDZ domains: (A, D) SAP97 PDZ2; (B, E) PTP-BL PDZ2; (C, F) PSD-95 PDZ3. The upper three panels show log k_{off} vs log K_d and the lower three panels log k_{on} vs log K_d. The data sets for PTP-BL PDZ2 and PSD-95 PDZ3 were obtained from ref 28.

details in the analysis may be lost when data from different systems are combined into one LFER.

For further comparison of our analysis with that reported by Prakash,[24] we then compiled data from 15 different peptide−PDZ interactions, including some of those in Figure 2 (Figure 4). A clear dependence of log k_{off} versus log K_d could be observed with a slope of 0.7. This value is similar to that for the combined mutant/peptide data set and similarly hides the details of the individual peptide−PDZ interactions.

Finally, binding Φ values[3] were calculated for mutations where ΔΔG for the change in K_d was >0.6 kcal/mol (Table 1). Φ values correlate the change in free energy of the transition state on binding ($\Delta\Delta G^{\ddagger}$) with that of the ground state ($\Delta\Delta G_{K_d}$). If the interaction(s) deleted by mutation is present in the transition state of the reaction as well as in the bimolecular complex, Φ =1. On the other hand, if this native interaction has not begun to form in the transition state, Φ = 0. Any intermediate values are usually interpreted as partial formation of the bond(s) broken by mutation. A sound interpretation of Φ values is to consider them as weak (0−0.3; transition state similar to reactants), intermediate (0.3−0.7), or strong (0.7−1; transition state similar to products).[46] The Φ values were generally low, but the Arg(−4) → Ape mutation in the SAP97 PDZ2 peptide resulted in an intermediate Φ value (0.5) and the value for Thr(−2) → Ser was low to intermediate (0.3) (Table 1). This suggests that the side-chain interactions of Arg(−4) and possibly those of Thr(−2) form in the rate-limiting transition state for the binding reaction. Such detail is lost even in the four-point LFER for this peptide (slope = 1.06).

■ DISCUSSION

Intrinsically disordered proteins play prominent roles in cell signaling. Such proteins may be either fully disordered or have disordered domains or even smaller unfolded regions. It has been estimated that up to 75% of mammalian signaling proteins have disordered regions longer than 30 amino acid residues and that 25% are fully disordered.[1] The binding partner of a disordered protein may be a folded, ordered protein (e.g., KIX binding to pKID[10]), but sometimes two unfolded proteins bind to each other and fold up into a well-defined structure (e.g., L27 domains[47] and ACTR/NCBD[7]). While it is well-known that these disordered regions are functional as recognition motifs,[1,2,13,48,49] little is known about the mechanism of recognition of intrinsically disordered proteins in relation to both folded and unfolded protein partners.

Small disordered peptides that become ordered upon binding have been used here as a simple model system for intrinsically disordered proteins. These peptides bind to PDZ domains[50] and adopt a β-strand structure in an extended intermolecular β-sheet in the bimolecular complex,[40] thus going from a disordered state to an ordered one (Figure 1). The advantage of this model system is that we can generate large data sets under well-defined conditions. The LFERs for the peptide−PDZ binding reactions (Figure 2) suggest that mutational destabilization has a similar effect at different positions along

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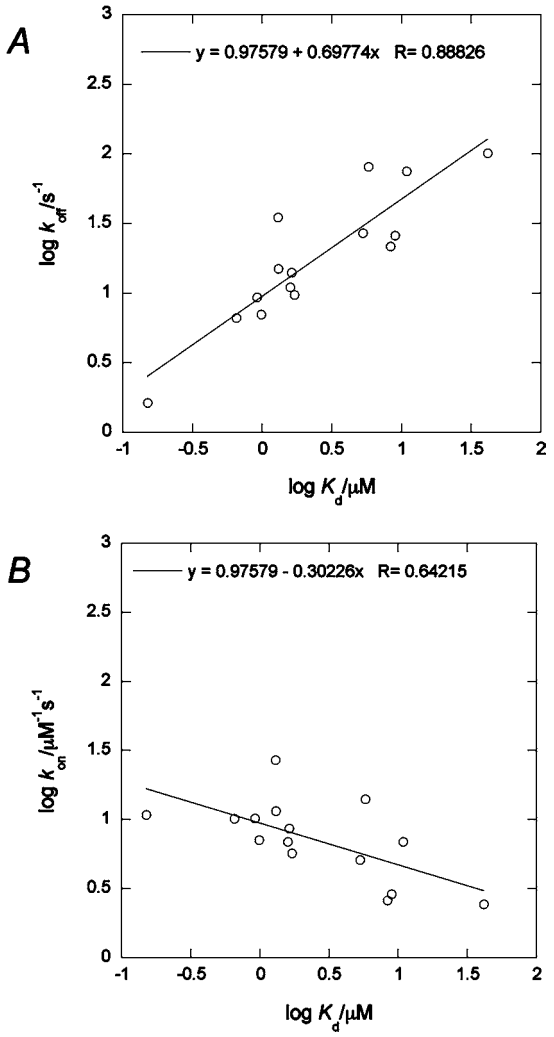

Figure 4. Linear free energy relationships for interactions between different pairs of (pseudo) wild-type PDZ domains and peptide ligands. The following were included: PSD-95 PDZ1 I100W/IESDV, PSD-95 PDZ2 I195W/IESDV,[26] PSD-95 PDZ3 F337W/**D**-YKQTSV, PSD-95 PDZ3 F337W/**D**-YQKSSV, PSD-95 PDZ3 F337W/**D**-YKQTSAbu,[28] SAP97 PDZ2 I354W/**D**-RRETQV (25 °C), SAP97 PDZ2 I354W/**D**-RRETQV (10 °C), SAP97 PDZ2 I342W/**D**-RRETQV, SAP97 PDZ2 V337W/**D**-RRETQV, SAP97 PDZ2 I354W/RRETQV, SAP97 PDZ2 I354W/RRETQL, SAP97 PDZ2 I354W/RRETQAbu,[25] PTP-BL PDZ2/**D**-EQVSAV, PTP-BL PDZ2/**D**-EQVTAV, and PTP-BL PDZ2/**D**-EQVSAAbu.[28] **D** stands for dansyl; Abu is 2-aminobutyric acid.

the peptide. In other words, the native interactions made by the peptide side chains are formed cooperatively, in analogy with the nucleation−condensation model in protein folding.[51] Furthermore, the facts that k_{off} governs the affinity (K_d) and that the slope in a log−log plot is close to 1 (Figures 2 and 3) show that the probed side-chain interactions form after the rate-limiting step of the overall binding reaction, in agreement with the proposed induced-fit binding model of peptide−PDZ interactions[25,29] and also of disordered proteins.[2]

Single-position mutations (Φ values) report on the local energetics of mutation, whereas LFERs for all mutated positions (Brønsted/Leffler plots) report on the overall mechanism, for example, nucleation−condensation or diffusion−collision in protein folding.[45,46] The binding Φ values are low for mutations at the C-terminal Val(0) and Thr(−2)/Ser(−2) residues. However, the Φ value for the Arg(−4) → Ape mutation in the SAP97 PDZ2 peptide displays an intermediate value of 0.5. This Arg(−4) residue forms hydrogen bond(s) and possibly a salt bridge, according to NMR data for the complex.[35] It is likely that Arg(−4) is involved in an attractive long-range electrostatic interaction early on the reaction coordinate and that deletion of the positive charge therefore lowers the association rate constant. The Thr(−2) → Ser mutation also had a Φ value (0.3) different from 0, and the interactions made by the γ-methyl group of Thr(−2) may thus be partially formed in the transition state. One possibility is thus that an encounter complex might involve a few nativelike contacts along with several weak non-native side-chain interactions and that their search for the most stable (native) conformations takes place as the reaction crosses the rate-limiting barrier.

It has recently been suggested that the affinity of protein−protein recognition for disordered systems is governed by the association rate constant k_{on}, a feature that does not seem to hold for ordered proteins.[24] This notion is exciting because it suggests a very basic biophysical property for intrinsically disordered proteins. But the devil is in the details: for the Cdc42/WASp data set,[5] where mutations were made only in the disordered WASp, there is indeed a strong correlation between k_{on} and K_d (Figure 5).[24] These mutations, however, involved charged groups, which are known to affect the association rate constant.[52] This electrostatic steering probably involves residues that form long-range electrostatic interactions in the transition state but not salt bridges in the product complex, according to the model of Hemsath et al.[5] In fact, the same effect was observed by rational design of the TEM1−BLIP interaction, which is an interaction between two ordered proteins. The association rate constant was changed by more than 2 orders of magnitude through mutagenesis while retaining k_{off} within a factor of 3 (Figure 5).[53] Thus, the K_d values for both the Cdc42/WASp (ordered−disordered) and TEM1−BLIP (ordered−ordered) reactions are governed by electrostatic steering in the association reaction.

■ CONCLUSIONS

We believe that LFERs using data from different studies are too crude to distinguish binding of ordered and disordered proteins from each other. One reason is that differences in experimental

Table 1. Binding Φ Values for Mutations in the Disordered Peptide

	SAP97 PDZ2		PTP-BL PDZ2		PSD-95 PDZ3	
peptide mutation	$\Delta\Delta G_{K_d}$ (kcal mol^{-1})	Φ	$\Delta\Delta G_{K_d}$ (kcal mol^{-1})	Φ	$\Delta\Delta G_{K_d}$ (kcal mol^{-1})	Φ
Val to Abu (C-terminal position)	1.4 ± 0.1	−0.15 ± 0.14	0.86 ± 0.26	0.1 ± 0.3	0.82 ± 0.11	0.04 ± 0.12
Thr to Ser/Ser to Thr	0.95 ± 0.08	0.31 ± 0.09	−0.04 ± 0.2	−[a]	1.0 ± 0.1	0.03 ± 0.10
Arg to Ape	0.82 ± 0.11	0.51 ± 0.14	–	–	–	–

[a] $\Delta\Delta G_{K_d}$ was too low to allow the calculation of an accurate Φ value.

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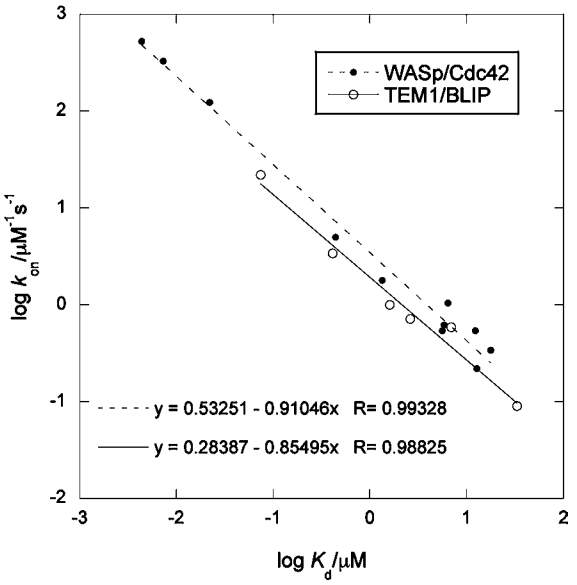

Figure 5. Linear free energy relationships for the interactions between Cdc42 and WASp[5] and TEM1 and BLIP.[53] In both cases, the changes in K_d upon mutation are due to changes in k_{on}.

conditions skew analyses in which different protein−protein interactions are plotted in the same graph, as in Figure 4. For example, differences in ionic strengths in experimental buffers may have dramatic effects on rate constants if electrostatic steering modulates the interaction.[5,52,54] We suggest that the proposal that k_{on} governs K_d for the interactions of intrinsically disordered proteins is too simplistic and that their mechanisms as well as those of ordered proteins must be assessed on a case-by-case basis.

The binding of disordered peptides in the current study follow a very clear LFER, which suggests that the native side-chain interactions in the bimolecular complex form simultaneously along the peptide following formation of a "nucleus". For the peptide−SAP97 PDZ2 interaction, this nucleus may be found around Arg(−4), that is, in the N-terminal part of the region of approximately six residues considered most important for affinity and specificity in peptide−PDZ interactions.[43,44]

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