PROTEIN DISORDER AND THE EVOLUTION OF MOLECULAR RECOGNITION: THEORY, PREDICTIONS AND OBSERVATIONS

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Observations going back more than 20 years show that regions in proteins with disordered backbones can play roles in their binding to other molecules; typically, the disordered regions become ordered upon complex formation. Thought-experiments with Schulz Diagrams, which are defined herein, suggest that disorder-to-order transitions are required for natural selection to operate separately on affinity and specificity. Separation of affinity and specificity may be essential for fine-tuning the molecular interaction networks that comprise the living state. For low affinity, high specificity interactions, our analysis suggests that natural selection would parse the amino acids conferring flexibility in the unbound state from those conferring specificity in the bound state. For high affinity, low specificity or for high affinity, multiple specificity interactions, our analysis suggests that the disorder-to-order transitions enable alternative packing interactions between side chains to accommodate the different binding targets. Disorder-to-order transitions upon binding also have significant kinetic implications as well, by having complex effects on both on- and off-rates. Current data are insufficient to decide on these proposals, but sequence and structure analysis on two examples support further investigations of the role of disorder-to-order transitions upon binding.

1 Introduction

The standard view is that protein folding into a particular 3D structure is a prerequisite for function. However, many amino acid sequences don't fit this standard view; that is, they don't fold completely on their own to form a unique 3D structure. Incompletely folded regions of proteins or incompletely folded whole proteins are herein called disordered, unstructured, flexible, unfolded, or, in reference to x-ray experiments, unobserved; otherwise, they are called ordered, structured, fixed, folded, or observed.

There are degrees of order and disorder. The ordered parts of proteins can have uneven flexibilities as reflected by variable B-factors [28]. Unstructured regions can likewise be non-uniform and vary from being highly mobile, random-coil-like structures to being slow-moving, collapsed but not-tightly-packed (e.g. molten-globular [6]) forms. Also, local regions and whole proteins can exhibit dynamic equilibria between the folded and unfolded states. Nevertheless, we propose that it

is useful to classify protein structure as either ordered or disordered for the same reasons it is useful to classify matter as solid or fluid.

Protein disorder has been revealed by x-ray crystallography [1,10,12,20,27], NMR [5,15,20], and proteolysis [16,17,25,26]. These methods and others have provided strong evidence over the years for the existence of proteins or regions of proteins that are unfolded in their native states. Paradoxically, such natively unfolded protein is often found to play a role in molecular recognition by becoming ordered upon complex formation [5,8,22,27,28,30,31].

Here we carry out thought-experiments that explore the biological implications of disorder-to-order transitions upon complex formation: these thought-experiments lead to specific proposals for sequence and structural variability in families of proteins involved in such transitions upon binding. Next, we carry out an analysis of one sequence family and one structural example to demonstrate possible starting points for testing our proposals. Finally, we discuss limitations in the current data and suggest further experiments for more completely testing whether the biological importance of disorder-to-order transitions upon binding is to uncouple specificity and affinity.

2 Methods

Multiple sequence alignment and sequence variability for a set of 8 homologous calcineurin (CaN) proteins were accomplished using the programs PILEUP and PLOTSIMILARITY, respectively, from the GCG package [23]. The analysis of the helix/helix contacts within calmodulin (CaM) was performed using the quaternion contact ribbon, which is described in more detail elsewhere [2].

3 Results

3.1 The Schulz Diagram

A simple scheme for the analysis of free energy changes in disorder-to-order transitions upon binding is presented in figure 1 [27], which we are calling a 'Schulz Diagram.' The intermediate step labeled "both solid" is a virtual state and is not on the reaction pathway. The individual solid molecules in this step are viewed to have identical structures and flexibilities as when complexed, but to be still separated when in this virtual state.

In the Schulz Diagram the left-hand limb (step 1) is the free energy change as the disorder is lost upon complex formation; this step contains enthalpic as well as entropic contributions. Consider a protein that binds to nucleic acid: folding into the virtual state without its partner involves positive enthalpy (as the positive charges are brought closer together) as well as negative entropy.

The right hand limb (step 2) is the free energy due to all the contacts between the two partners. Consider two proteins that bind to the same target, but that differ in the sum of the free energies of all of the atomic contacts with the target: for two

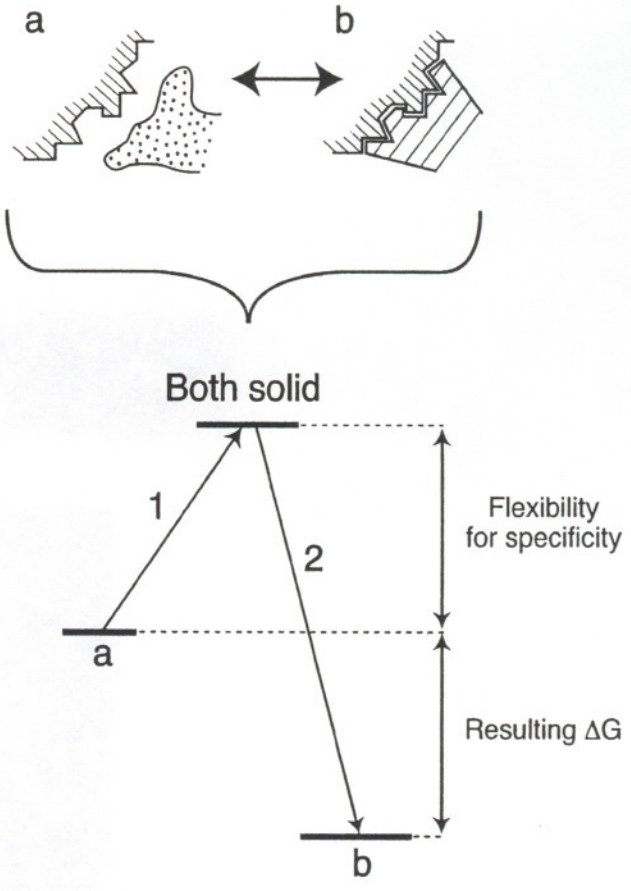

Figure 1: "Specificity Without Excessive Binding Strength. a) one of binding partners shows flexibility in solution; b) on binding, the flexible partner solidifies. Below is a free energy scheme describing state a and b as well as a virtual intermediate state where both partners are sold but not bound to each other." Both the figure legend and the diagram (with slight modifications) were copied from the paper by Schulz [27] with permission.

such proteins, the free energies of step 2 would differ. Binding to the molecule with higher contact free energy *would be more specific* (e.g. the fit would be better). Thus, comparisons of the magnitudes of the right hand limbs provide measures of the specificities for groups of closely related interactions.

As shown, the combination of steps 1 and 2 determine the overall free energy or binding affinity for the molecular interaction.

3.2 Thought-Experiments on the Evolution of Molecular Complexes

Consider the evolution of a molecular interface for which both of the interacting molecules are rigid both before and after complex formation (figure 2). Two limiting pathways would be to enlarge the region of interaction over evolutionary time (left) or to improve the fit (right). In either case, the Schulz Diagrams show that affinity and specificity increase concomitantly. This coupling of affinity and specificity for rigid partners is not generally appreciated.

Consider the kinetic implications of figure 2. The binding between two rigid interfaces is necessarily diffusion-limited. Assuming that the structural changes depicted in figure 2 are not accompanied by significant changes in diffusion constants, any increase in affinity is coupled to a decrease in the off-rate.

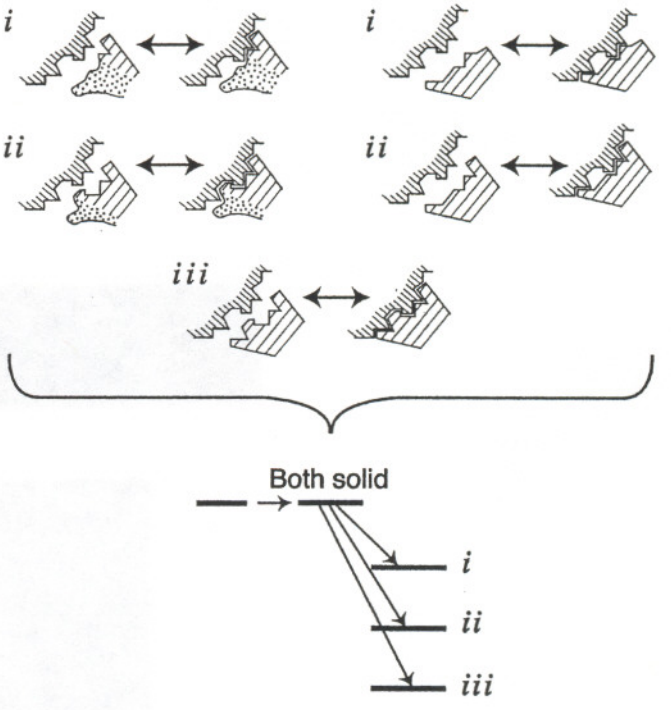

Figure 2: Schulz Diagrams for Rigid Partners. The contact free energy between a rigid molecule and a family of rigid partners can be altered by changing the extent of the contact (left) or by the goodness of fit (right) or some combination of the two. In either case, affinity and specificity would appear to be coupled.

Suppose that the same final interface evolves in steps involving changes in the amount of disorder in the unbound state (figure 3). The Schulz Diagrams indicate that, for changes in the degree or amount of disorder in the unbound state, the affinity would change, but the specificity would remain constant. A high flexibility in the unbound state leads to the combination of low affinity and high specificity.

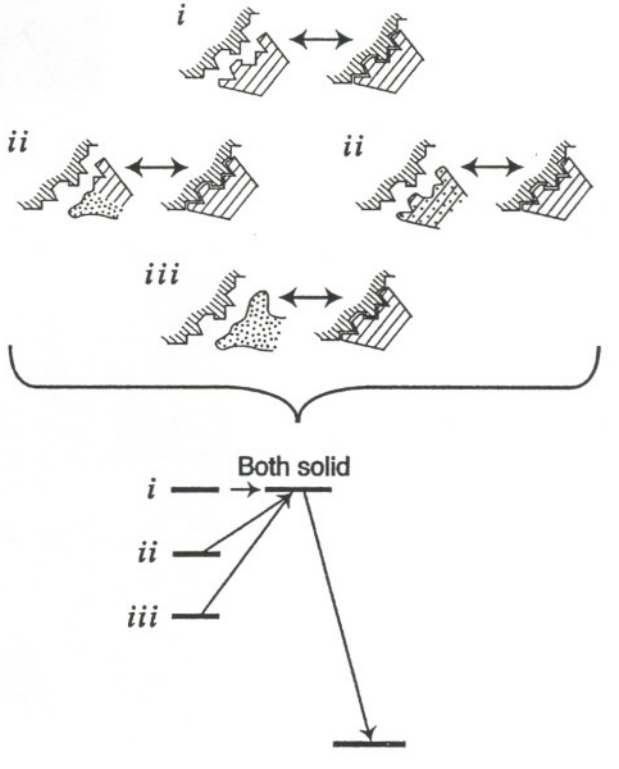

Figure 3: Schulz Diagrams for Variable Flexibility. The flexibility of a molecule in the unbound state can be altered by changing the fraction of the molecule that is disordered (left), by changing the degree of flexibility (right, the combination of dashes and dots indicate an intermediate flexibility between the two extremes), or by some combination of the two. If the same final contact results, then the affinity would change without altering the specificity.

The relationships between changes in on- and off-rates with changes in affinity and flexibility are neither simple nor straightforward. For example, if local folding were the rate-limiting step for complex formation, then increasing the flexibility would decrease the on-rate. On the other hand, if local folding were not rate-limiting, then increasing the flexibility could actually increase the on-rate. For example, increased flexibility could speed up dynamic searches of the appropriate orientation for binding or could more rapidly unmask occluded binding sites, either of which would speed up the on-rate as the flexibility increased. Similar complexities enter into considerations of off-rates. So, overall, disorder-to-order transitions upon binding provide the potential for complex control over on- and off-rates.

Suppose that changes in the binding surface of the rigid partner can be fitted with the flexible partner through alternative packing arrangements (figure 4). If recognition of multiple, similar partners were of biological advantage, co-evolution of both partners could lead to a flexible molecule that binds to a variety of partners, all with high affinity, thus yielding high affinity and multiple specificities.

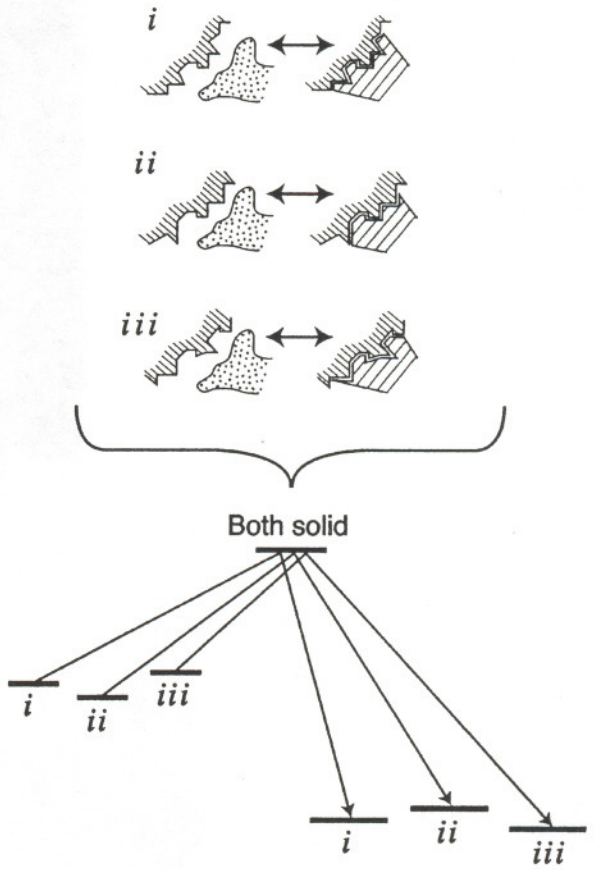

Figure 4: Schulz Diagrams for Variable Contact Surfaces. The contact surface of the rigid partner can change and the flexible partner can evolve to conform to the altered surface, thus leading to multiple specificities. The slight variations in the free energy values of the initial states are intended to signify differences that could arise from variability in the flexibilities of the surface-exposed side chains of the rigid partners. The slight differences in the free energies of the final states indicate differences in contact free energies for the different complexes.

3.3 Predictions and Observations

A complex-forming, disordered region would have two types of amino acids: those in the contacts and those not in the contacts. A prediction of the evolutionary model in figure 3 is that, to alter affinity without affecting specificity, amino acid changes would need to occur in the disordered regions, thus affecting the flexibility

of the unbound state, but not at loci involved in the contacts, thereby preserving the specificity.

To test the prediction derived from figure 3, a well-characterized disordered region involved in the formation of a molecular complex is needed. The calmodulin (CaM) target region of calcineurin (CaN) fits the criteria [12,13]. To test the prediction, eight homologous CaN sequences were aligned and their sequence variability was determined as described in the Methods.

Comparison of amino acid variability of CaN over the alignment (figure 5) shows that amino acid variability is concentrated in the disordered region at loci separate from those in contact with CaM. This observation fits the prediction.

Besides the CaM binding region, CaN has two other unobserved regions: 15 amino acids at the amino terminus and 35 amino acids at the carboxy terminus [12]. These two additional disordered regions in CaN (shaded in figure 5) are also associated with sharp drops in the Similarity Score values.

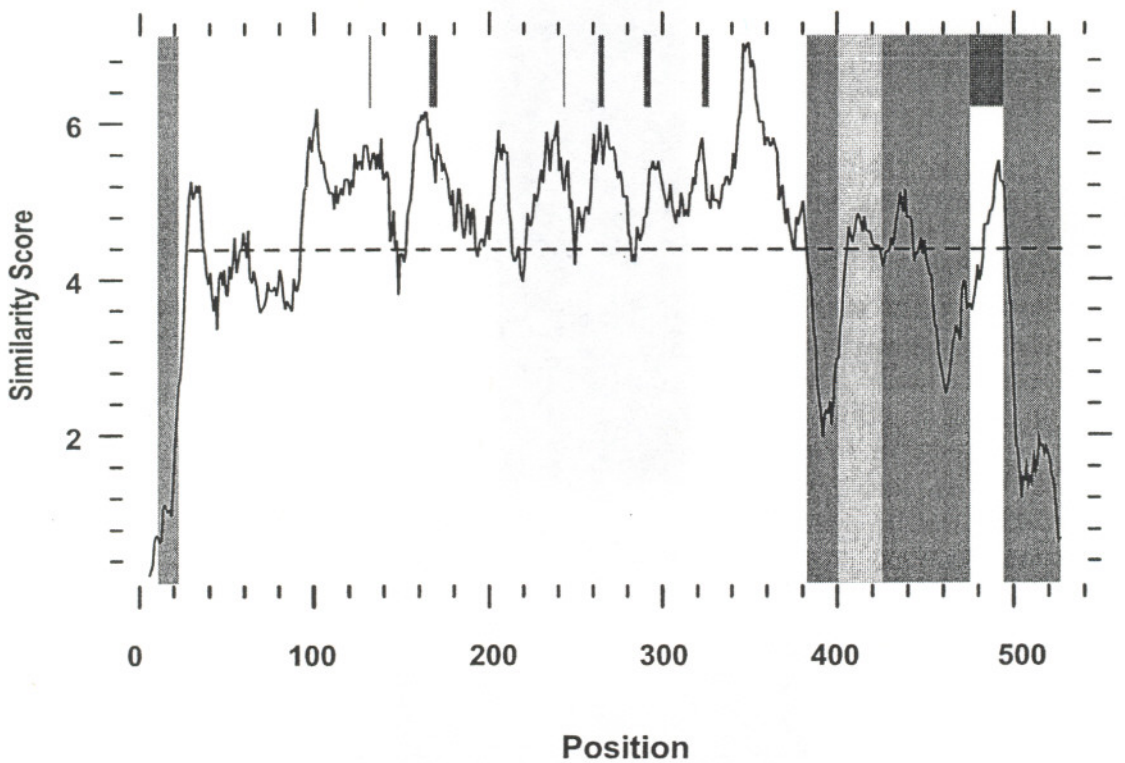

Figure 5: Sequence Variability for the CaN A Subunit. The PLOTSIMILARITY algorithm [23] was used to determine the sequence similarity scores versus the amino acid sequence positions for a multiple alignment of CaN. The CaN crystal structure has three disordered regions [12], indicated by shading. The CaM binding region (lighter shading starting near position 400) and the auto-inhibitory peptide (dark bar starting near position 475) are indicated. Also indicated are the locations of the various amino acids to which the auto-inhibitory peptide binds (series of dark bars of various widths from about position 132 to 322).

For disorder-to-order transitions to bring about multiple specificities with similar affinities, alternative packing between the protein partners must occur

(figure 4). In addition, for a protein that folds only upon binding with its partner, the internal side chain packing must lack sufficient inherent specificity for independent folding. If such inherent foldability is lacking, the flexible protein would show variability in its internal side chain packing as it binds with different protein partners.

To test the prediction derived from figure 4, data are needed on a disordered protein that binds to multiple partners; especially needed are crystal structures with different partners. CaM fulfills these requirements. CaM binds to many different protein partners [5,9,18,19,21,24]. Three crystal structures are known: CaM alone [3] and CaM bound to two different target helices [18,19].

Calmodulin qualifies as a disordered protein. It is very disordered in the absence of calcium, it becomes somewhat ordered upon calcium binding (this order is enhanced further by crystallization), and it becomes even more ordered upon calcium-induced complex formation (M. Zhang, personal communication, [31]).

To test whether binding to different targets also leads to changes in the internal packing of CaM, we used a previously developed tool [2] for the visualization of helix packing interfaces. First, the method is applied to the leucine zipper (figure 6) to acquaint the reader with this tool. The clean partition of side chains onto opposite sides of the ribbon when viewed from the edge is clearly indicated by the light side chains on one side and dark ones on the other (cell E). Furthermore, the well-known knobs-into-holes packing of the zipper is evident from the alternating light and dark side chains in the face view of the ribbon (cell F).

In contrast to the leucine zipper, the A/B interface in CaM is not knobs-into-holes packing (figure 7). Furthermore, as can be seen by comparing the E and F views for the different forms of CaM, significant changes in packing occur in the A/B interface. Side chain orientations are seen to change (compare the F-CaM cell with the F-1 cell) and even the identities of the side chains involved in the contacts vary (for example, compare the F-CaM, the F-1, and the F-2 cells with each other). These results are in agreement with the prediction derived from figure 4.

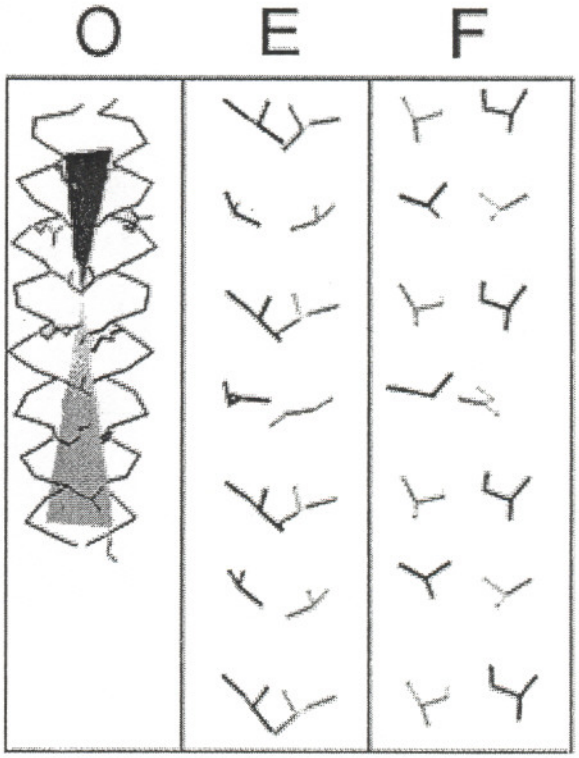

Figure 6: Application of the Contact Ribbon to the Leucine Zipper. The application of the quaternion contact ribbon [2] to the leucine zipper is shown here. The cell labeled O contains an overview showing the ribbon in place; this ribbon is used to capture the side chains in the contact. The ribbon is then flattened using quaternions. This leads to a complex spatial transformation of the side chains that simplifies the viewing of the contact. Two views of the zipper are shown. In cell E, the contact is viewed so that the edge of the ribbon faces the reader (the edge is vertical); in cell F the face view of the contact ribbon is shown, corresponding to the view from one helix axis towards the other.

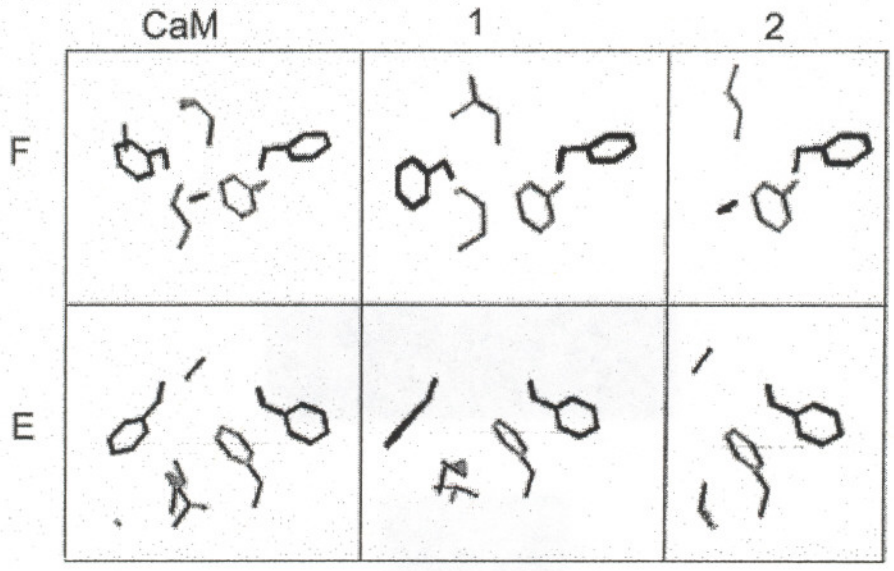

Figure 7: Packing of the A/B Helices in Calmodulin in Three Different States. Shown here is the application of the quaternion contact ribbon to the A helix/B helix interface for CaM alone or with different partners, where the side chains from A are dark and those from B are light. The column of cells labeled CaM contains two views of the A/B interface of calmodulin alone. The column labeled 1 contains the A/B interface of CaM complexed with the myosin light chain kinase target, and the column labeled 2 contains the A/B interface of CaM complexed with the protein kinase II target. The row of cells labeled F contains face views, e.g. the contact as viewed from one helix axis to the other, with the helix axes horizontal. The row of cells labeled E contains the edge-views; in these cells the edges of the ribbons are horizontal and indicated by faint lines that might not be visible.

4 Discussion

Fischer [7] suggested the lock-and-key mechanism for enzyme function, for which rigid molecules fit together by surface complementarity. Emphasizing the importance of flexibility, Koshland [14] proposed induced-fit. A glove changing shape to fit a hand is the analogy often used for induced fit.

Without reference to induced fit, Schulz [27] and Alber and coworkers [1] studied complex formation in which one of the partners underwent disorder-to-order transitions upon binding. These workers independently recognized that disorder-to-order transitions upon binding reduce the affinity of a given interaction compared to an otherwise identical interaction between two rigid structures (figure 1). This allows the advantageous combination of high specificity and low affinity [27].

A characteristic thermodynamic signature was identified for several disorder-to-order transitions, which were called both 'induced fit' and 'induced folding' [28]. The latter term is more descriptive. Indeed, induced folding doesn't conform to the hand-in-glove analogy of induced fit (e.g., there is no "glove" in the

disordered state), but a broader view of induced fit could encompass induced folding as well.

Comparing sequences and structures related by evolution provides a powerful approach for understanding protein structure and function. Since disordered regions have not been studied previously from an evolutionary point of view, it was unclear how to proceed. In an attempt to gain insight, we used the analysis of disorder-to-order transitions originally proposed by Schulz [27] (figure 1) and applied it to reasonable types of evolutionary change depicted in highly simplified diagrams (figures 2-4).

To clarify possible advantages for disordered regions, we considered both rigid partners (figure 2) and induced folding (figures 3 & 4). The rigid partner scenario is intentionally simplified; even proteins with structured backbones undergo changes in flexibility upon binding with partners as evidenced by substantially reduced sensitivity to protease digestion [17], but we suppose these contributions to the free energy would be second-order compared to the contributions from induced folding. Overall, these studies suggested that induced folding provides a means for uncoupling affinity and specificity, with concomitant effects on the kinetic properties of the interaction, thus providing a means for evolution to fine-tune complex networks of interactions. These studies also led to 2 specific predictions, which were tested (figures 5 & 7).

The sequence variability profile (figure 5) agrees with the prediction from figure 3 – namely that disordered regions have high variability in regions not involved in binding. Of course, this is just one example. Also, regions involved in binding are well-known to be more conserved than regions not involved in binding. As an example, note that auto-inhibitory peptide and the amino acids to which it binds are all localized in regions of high sequence conservation, corresponding to peaks in figure 5. Furthermore, note that all three disordered regions of CaN exhibit similarly low values for the Similarity Scores, yet there is no known binding function for the disordered ends of CaN. Thus, the sequence variability in the non-binding regions could be due to genetic drift in the absence of selection pressures instead of selection for alternative affinities and kinetic properties.

Much more data are needed to test the prediction that variability in the non-binding region arises to fine-tune networks of interactions. For example, it would be useful to determine the off- and on-rates as well as the binding constants for the interactions between CaM and the various CaN molecules and to correlate these with NMR studies and computer simulations of the disordered regions. In addition, it would be important to model networks of interactions and compare predicted and observed responses of such networks to specific, experimentally induced changes in flexibility and affinity - changes designed to substantially alter the behavior of the network. Finally, the evolution of the altered network over time would need to be followed to demonstrate re-adjustment of the network by mutation and selection in the manner proscribed by the theory presented herein.

The prediction derived from figure 4 - that the internal packing of a disordered protein would change when the disordered protein becomes ordered onto different targets – is verified (figure 7). Again, this is one example. We are searching for other examples for which the 3D structures are known for proteins complexed with different partners, but so far without success.

Of course we are not the first to recognize the importance of plasticity or flexibility for enabling one protein to bind to many partners [4,9,15,19,21,24,31].

Indeed, in recent studies, the ability of aspartate amino transferase to bind to many substrates was recognized to be a function of the flexibility of the enzyme and was called 'multi-induced-fit' [11]. Elsewhere we refer to the ability of one protein to recognize several partners as one-to-many signaling [26]. All of these terms refer to similar ideas.

It is interesting that methionine apparently plays a key role in the binding of one protein to many partners for both CaN [9,31] and also for aspartate amino transferase [11]. Evidently, the flexibility of the straight chain of methionine facilitates the accommodation of differently shaped binding surfaces.

What is novel in the studies presented here is the suggestion that high affinity in concert with low specificity and low affinity with high specificity are flip sides of the same coin, with both dependent upon induced folding. That is, induced folding is proposed herein to *uncouple* specificity and affinity.

We further suggest that induced folding leads to complex relationships between kinetics and sequence, enabling either increases or decreases in on- and off-rates, depending on the mechanistic details of the binding process. The uncoupling of specificity and affinity and potential for complex variations in the kinetic parameters may be required to enable mutation and natural selection to fine-tune networks of interactions. If so, induced folding would be expected to be extremely common. Two further observations support this expectation. First, there are many well-characterized examples of induced folding [1,5,9,12,27,28,30]. Second, our recent studies on relationships between sequence and disorder lead to the prediction of large numbers of proteins likely to have natively unstructured regions [26].

Current views of protein structure and function may need to be significantly revised as the importance of being disordered or unfolded comes to be realized [5,22,25,26]. It will be interesting to watch how views of protein structure and function unfold over the coming years.

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