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Protein dynamics and conformational disorder in molecular recognition

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Recognition requires protein flexibility because it facilitates conformational rearrangements and induced-fit mechanisms upon target binding. Intrinsic disorder is an extreme on the continuous spectrum of possible protein dynamics and its role in recognition may seem counterintuitive. However, conformational disorder is widely found in many eukaryotic regulatory proteins involved in processes such as signal transduction and transcription. Disordered protein regions may in fact confer advantages over folded proteins in binding. Rapidly interconverting and diverse conformers may create mean electrostatic fields instead of presenting discrete charges. The resultant "polyelectrostatic" interactions allow for the utilization of post-translational modifications as a means to change the net charge and thereby modify the electrostatic interaction of a disordered region. Plasticity of disordered protein states enables steric advantages over folded proteins and allows for unique binding configurations. Disorder may also have evolutionary advantages, as it facilitates alternative splicing, domain shuffling and protein modularity. As proteins exist in a continuous spectrum of disorder, so do their complexes. Indeed, disordered regions in complexes may control the degree of motion between domains, mask binding sites, be targets of post-translational modifications, permit overlapping binding motifs, and enable transient binding of different binding partners, making them excellent candidates for signal integrators and explaining their prevalence in eukaryotic signaling pathways. "Dynamic" complexes arise if more than two transient protein interfaces are involved in complex formation of two binding partners in a dynamic equilibrium. "Disordered" complexes, in contrast, do not involve significant ordering of interacting protein segments but rely exclusively on transient contacts. The nature of these interactions is not well understood yet but advancements in the structural characterization of disordered states will help us gain insights into their function and their implications for health and disease.

Keywords: conformational disorder; protein dynamics; dynamic complex; disordered complex; polyelectrostatic effect; NMR; disorder-to-order transition; Sic1

INTRODUCTION

Proteins, the dominant molecular machines of biology, require significant motion to carry out their different functions. Some of the most striking examples of dynamics in function include protein folding and unfolding, catalysis, mediation of cell motility, transport through membranes, cell replication, transcription and translation, and assembly and disassembly of protein complexes in general. Protein motions on a broad range of time-scales, with varying amplitudes and affecting a different fraction of the protein, have been characterized and linked to these and other specific tasks. Intrinsic conformational disorder is an extreme on this dynamic spectrum with a large degree of motion leading to rapid interconversion between highly heterogeneous conformers. Intrinsically disordered proteins are now widely accepted as ubiquitously occuring in all kingdoms of life (Dunker *et al*., 2000; Ward *et al*., 2004) and involved in a wide range of cellular functions including chaperoning, transport, and regulation of transcription and cell signaling (Tompa, 2002; Dyson and Wright, 2005). Specific recognition of binding partners, whether small molecules, other proteins or nucleic acids, is essential for the majority of these functions. The so-called "hub" proteins, which bind to many partners and are thus central to protein interaction networks, use conformational disorder to provide the required plasticity to interact with a large number of different proteins (Dunker *et al*., 2005; Dosztanyi *et al*., 2006; Ekman *et al*., 2006; Patil and Nakamura, 2006; Kim *et al*., 2008). Alternatively, structured hubs bind to disordered regions in their many interaction partners (Oldfield *et al*., 2008). Disorder in one of the binding partners rules out a lock-and-key mechanism of binding since it prevents the presentation of a fixed surface which could be bound by a similarly ordered complementary surface. Induced-fit mechanisms of recognition are well known but usually describe small rearrangements of few interacting groups or larger domain-domain rearrangements (Koshland, 1995). Specific binding of an intrinsically disordered protein that samples an

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ensemble of rapidly interconverting conformations mostly devoid of structure is more difficult to imagine and may even seem counterintuitive. The question emerges of whether specific recognition can occur despite conformational disorder or whether disordered protein states may even provide advantages in recognition over well-folded proteins (Meszaros *et al*., 2007). In this review, we will focus on the apparent antagonism between disorder and recognition and highlight some of the thermodynamic, steric, and evolutionary benefits afforded by disordered protein states.

To permit specific recognition, disordered targets usually undergo coupled or concomitant folding/ordering and binding (Dyson and Wright, 2002; Dyson and Wright, 2005). Ordering can occur for the whole disordered protein or large portions or only for short segments. Such short segments involved in binding can assume α-helical structure, form intermolecular β-sheets, be extended (the so-called Linear Motifs, LiMs) or be of irregular secondary structure. If they already have a propensity to sample these structural elements in the free state, the segments have been termed "preformed elements" or "molecular recognition features" (MoRFs) (Fuxreiter *et al*., 2004; Neduva and Russell, 2005; Oldfield *et al*., 2005; Sivakolundu *et al*., 2005; Vacic *et al*., 2007). MoRFs bind based on a conformational selection mechanism, in which the binding partner selects the member of the ensemble that provides the best complimentarity to its own structure. This mechanism was first proposed by Pauling to explain promiscous binding of antibodies to a variety of different antigens (Pauling, 1940) and has proven to hold for a variety of systems (Sivakolundu *et al*., 2005; Lange *et al*., 2008).

THERMODYNAMIC ADVANTAGES

While kinetic benefits of disorder have been proposed based on collision theory and with the "flycasting mechanism" suggesting that disordered states have a larger capture radius leading to faster on-rates for binding (Pontius, 1993; Shoemaker *et al*., 2000), experimental evidence for the theory is currently limited. The proposed thermodynamic effects for binding of disordered proteins are also under discussion. The affinity of an interaction is determined by entropic and enthalpic contributions, the latter of which are determined by the sum of complimentary interactions and the quality of their fit. Disorder-to-order transitions result in an entropic penalty on the free energy of binding because the previously disordered region is locked in the binding conformation instead of sampling a variety of different conformations free in solution (Dyson and Wright, 2005). The energetic cost of this loss of entropy may lead to weaker binding than expected for an interaction burying the same exposed surface area but involving already ordered proteins. The specificity, in contrast, is strongly determined by the size and complimentarity of the interface. Hence, varying the length of the interacting motif and the degree to which it is already preformed affords a means to fine-tune the affinity of the interaction while simultaneously permitting high specificity. This apparent uncoupling of affinity and specificity has been proposed to be a benefit of binding of disordered proteins since highly specific interactions of moderate affinity are often desirable in regulation and signaling where interactions need to be turned off rapidly and efficiently (Spolar and Record, 1994; Dyson and Wright, 2005). However, some intrinsically disordered proteins that fold upon binding have been reported to have high affinities, with p27 binding to a Cdk2-cyclin A complex with a K_D of 3.5 nM (Lacy *et al*., 2004). Thus, as for folded protein interaction, the relationship between affinity and specificity is affected by multiple components including the complimentarity of the interface, the associated number of favorable contacts, solvation/desolvation effects and the dynamic properties of both binding partners in free and complexed states.

A recently described thermodynamic property of charged intrinsically disordered proteins is the "polyelectrostatic" effect that enables multiple charges on a disordered protein (such as multiple phosphorylations) to influence binding affinity through long-range electrostatic effects leading to a "net charge" or "mean field" (Borg *et al*., 2007; Serber and Ferrell, 2007). Due to the rapid interconversion of a multitude of different conformations, the disordered protein does not present discrete charges to its binding partner but rather a "mean electrostatic field" that reflects the net charge of the whole disordered segment rather than presenting discrete charges in space. Disordered regions are the predominant site of phosphorylation and this may be due both to the steric access of kinases and counteracting phosphatases (Iakoucheva *et al*., 2004) and the potential to regulate interactions through the "polyelectrostatic" effect. Other post-translational modifications that modify the net charge of a protein and may be candidates for creating "polyelectrostatic" effects are acetylation of lysine residues and modification with ubiquitin or the ubiquitin-like protein SUMO.

The development of the equilibrium, mean-field theory of "polyelectrostatic" interactions emerged in order to describe the interaction of a charged, disordered protein that contains several binding motifs with a folded receptor containing one binding site (Figure 1) (Borg *et al*., 2007). In this description, in addition to a favorable contact energy from the direct association of the receptor with one binding motif on the ligand, long-range electrostatic interactions contribute to the free energy of binding. Since the charges on the disordered ligand are assumed to be dynamically distributed in a diffuse manner, they can be described by a Coulomb interaction between two charges (q_l, the net charge of the ligand, and q_r, the charge of the receptor binding site) at an effective distance $<r>$. This "polyelectrostatic" model assumes a cumulative electrostatic interaction of all charges in the ligand, whether they are bound in the binding site of the binding partner and contribute to direct, short-range electrostatic interactions or not. The assumption of the same shape and charge distribution in the free and in the bound states is clearly not warranted for long disordered ligands. Indeed, their flexibility should permit favorable conformational rearrangements due to polarizability, e.g., bringing negative charges that may be more symmetrically distributed in the free state closer to a positively charged binding surface. The polarizability should increase with the ligand length and provide even tighter binding than the "polyelectrostatic" model predicts (Borg *et al*., 2007). To avoid screening of these long-range effects by the solvent the disordered protein ensemble must be compact. The averaging of rapidly interconverting conformers may thus facilitate this "polyelectrostatic" effect, providing a thermodynamic advantage over folded protein domains and even enabling "counting" of the number of charges.

STERIC ADVANTAGES

Conformational disorder allows binding of intrinsically disordered proteins in ways sterically difficult for folded proteins. Intrinsically

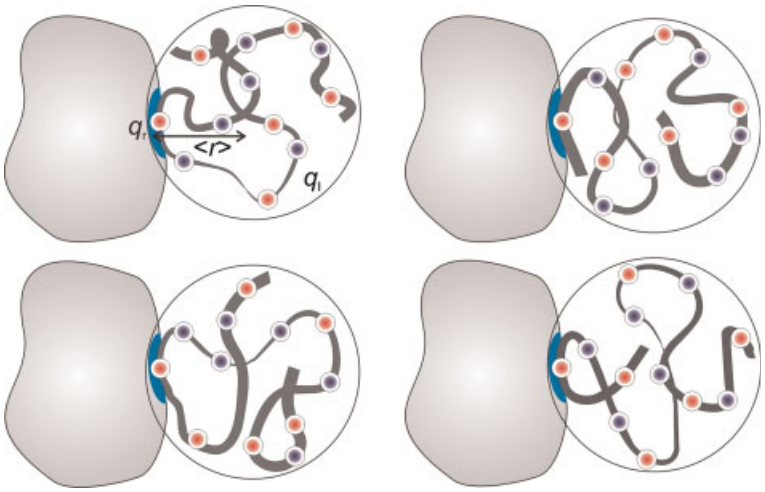

Figure 1. "Polyelectrostatic" model of interaction of intrinsically disordered proteins. Schematic of an intrinsically disordered protein (ribbon) interacting with a folded receptor (gray shape) through several distinct binding motifs and an ensemble of conformations (indicated by four representations of the interaction). The intrinsically disordered protein possesses positive and negative charges (depicted as blue and red circles, respectively) giving rise to a net charge q_l, while the binding site in the receptor (light blue) has a charge q_r. The effective distance $<r>$ is between the binding site and the centre of mass of the intrinsically disordered protein.

disordered proteins can bind on several surfaces of a binding partner, wrapping around it and thereby increasing the buried surface area (Russo *et al*., 1996; Breidenbach and Brunger, 2004; Chong *et al*., 2004; Kiss *et al*., 2008). These large buried surface areas can enable significant specificity in binding (see above). The ordered regions may still be connected by disordered linkers, facilitating numerous possibilities for regulation, e.g., by positioning two domains or subunits relative to each other, restricting inter-domain motion or inserting into catalytic or substrate binding pockets (Russo *et al*., 1996; Bhattacharyya *et al*., 2006; Kiss *et al*., 2008). Disorder also increases the plasticity and malleability of proteins and facilitates the interaction of the same protein sequence with several binding partners (Fuxreiter *et al*., 2008), possibly with opposing activities (moonlighting) (Tompa *et al*., 2005). Many protein hubs are intrinsically disordered or take advantage of disorder in their binding partners (Dunker *et al*., 2005; Dosztanyi *et al*., 2006; Ekman *et al*., 2006; Patil and Nakamura, 2006; Kim *et al*., 2008; Oldfield *et al*., 2008). Importantly, at least partial disorder is required to allow kinases and other modifying enzymes access to their targets. It also facilitates degradation, both ubiquitin dependent and independent proteasomal pathways.

EVOLUTIONARY ADVANTAGES

Disordered regions on average show higher rates of mutation, presumably because changes in protein sequence do not affect protein stability and function as severely, and only the accumulation of several mutations has deteriorating effects (Brown *et al*., 2002; Tokuriki *et al*., 2009). These regions may thus be driving forces for evolution, possibly explaining why eukaryotes, the kingdom of life with the highest degree of protein disorder, could evolve quickly to become complex, multi-cellular organisms. The modularity of many eukaryotic proteins with disordered regions between folded domains may facilitate recombination to shuffle domains and enable alternative splicing without the danger of perturbing structured regions (Romero *et al*., 2006). In addition to this, there are advantages for limiting molecular size as complexity increases. To achieve burial of the same area with the associated similar binding specificity, folded proteins would have to be considerably larger than intrinsically disordered proteins. Disorder may thus help to limit protein size, reduce molecular crowding inside cells, and also limit cell size (Gunasekaran *et al*., 2003).

"FUZZY" COMPLEXES

The concept of "fuzzy" complexes has been coined recently (Tompa and Fuxreiter, 2008) to describe the continuous spectrum of disorder possible in protein complexes from static to dynamic disorder and from segmental to full disorder. This range thus includes structurally well-defined complexes with flexible side chains, complexes containing disordered loops or longer disordered stretches, complexes in which one partner retains its disorder fully, and possibly even completely disordered dimers (Schaefer *et al*., 2000; Bhattacharyya *et al*., 2006; Frederick *et al*., 2007; Sigalov *et al*., 2007; Kiss *et al*., 2008; Mittag *et al*., 2008). "Static" disorder implies the adoption of several structurally well-defined sub-states whereas "dynamic" disorder means the rapid interconversion of a multitude of flexible conformers, with the two cases differing in the flexibility of the distinct conformations and in the time scale of their interconversion.

Another way to view this spectrum of dynamics in complexes is to compare it to the range of motional properties observed for individual proteins or protein regions (Figure 2). Protein states range from well-defined, folded proteins to molten globules to intrinsically disordered proteins containing transient secondary and tertiary structure to complete random coils, populated only under highly denaturing conditions (Figure 2). All of these states in turn can display various degrees of dynamics and conformational disorder, creating a continuous spectrum of disorder and flexibility. A folded protein can have flexible loops, display fast (ns-ps), small-scale dynamics and slower (μs-ms), larger amplitude conformational rearrangements. If, in a thought experiment, we cut the peptide chain in two, the result would be a protein complex, the interface of which is constituted by the

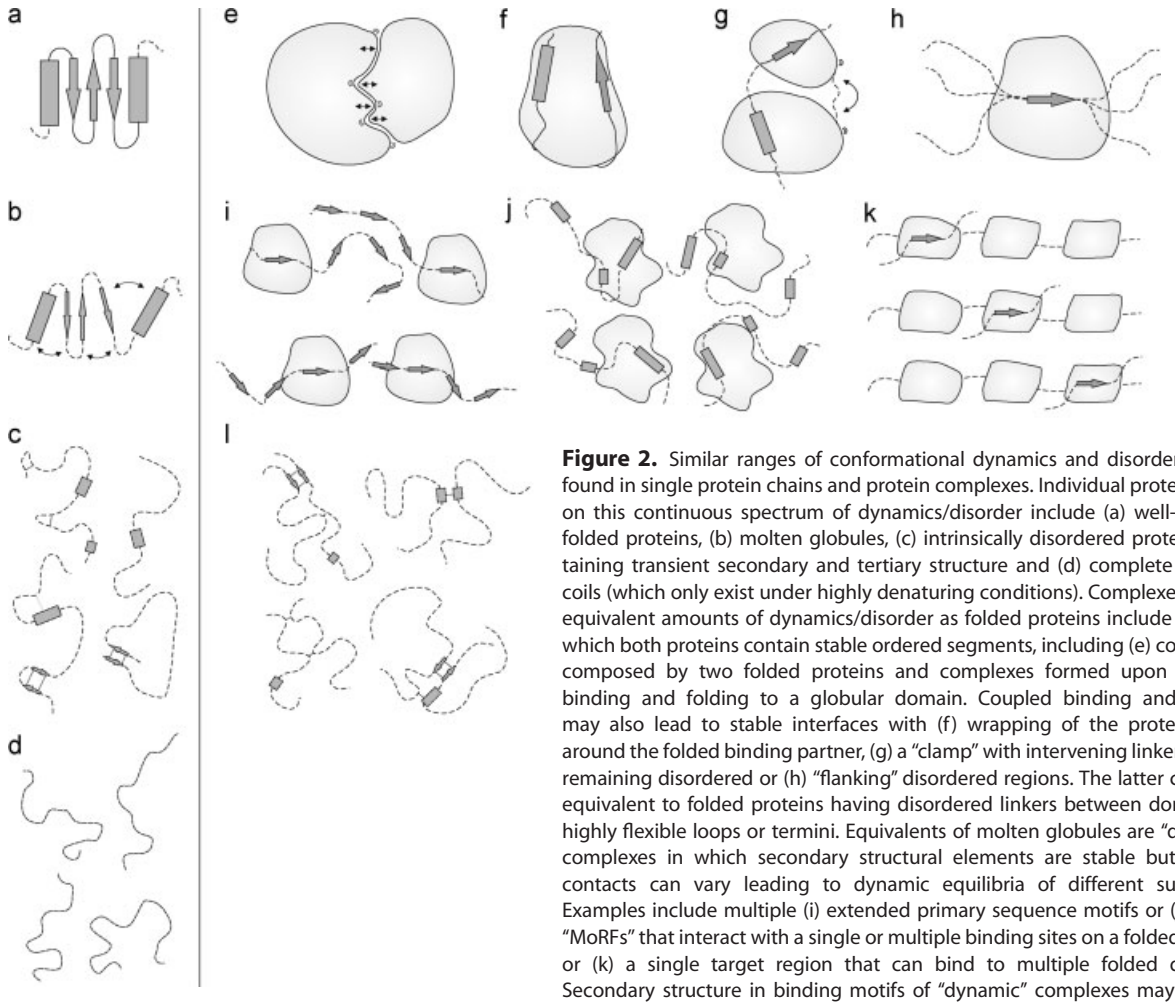

Figure 2. Similar ranges of conformational dynamics and disorder can be found in single protein chains and protein complexes. Individual protein states on this continuous spectrum of dynamics/disorder include (a) well-ordered, folded proteins, (b) molten globules, (c) intrinsically disordered proteins containing transient secondary and tertiary structure and (d) complete random coils (which only exist under highly denaturing conditions). Complexes having equivalent amounts of dynamics/disorder as folded proteins include those in which both proteins contain stable ordered segments, including (e) complexes composed by two folded proteins and complexes formed upon coupled binding and folding to a globular domain. Coupled binding and folding may also lead to stable interfaces with (f) wrapping of the protein chain around the folded binding partner, (g) a "clamp" with intervening linker regions remaining disordered or (h) "flanking" disordered regions. The latter cases are equivalent to folded proteins having disordered linkers between domains or highly flexible loops or termini. Equivalents of molten globules are "dynamic" complexes in which secondary structural elements are stable but tertiary contacts can vary leading to dynamic equilibria of different sub-states. Examples include multiple (i) extended primary sequence motifs or (j) helical "MoRFs" that interact with a single or multiple binding sites on a folded partner or (k) a single target region that can bind to multiple folded domains. Secondary structure in binding motifs of "dynamic" complexes may only be stabilized upon binding. (l) Disordered complexes that are strictly mediated by transient contacts are counterparts of intrinsically disordered proteins. There is no equivalent to random coils due to their complete lack of interactions. Disordered regions are represented by dashed lines, regions with propensities to form secondary structure are drawn with dashed edges. Ensembles are represented by three or four sub-states for simplicity.

same surface that before contributed to the intramolecular interactions defining the protein. The same physicochemical principles apply to the interactions in the single protein chain as in the protein complex (although thermodynamic and kinetic parameters differ in unimolecular and bimolecular interactions). The equivalent of a folded protein is thus a complex in which both binding partners have stable structure in at least some segments. The category contains not only complexes formed by two folded proteins, but also those made by coupled folding and binding of intrinsically disordered proteins to their folded binding partners. A molten globule is defined as a state containing stable secondary structural elements but fluctuating tertiary contacts. The equivalent is "dynamic complexes," in which either multiple binding motifs (e.g., linear motifs or α-MoRFs) interact transiently with the same binding site or one binding motif interacts with a number of binding sites in a dynamic equilibrium. Many studies of intrinsically disordered proteins have highlighted transient secondary structure and transient tertiary contacts. The equivalent is "disordered complexes" which do not contain significant stable structure but which are rather mediated by a multitude of transient interactions. A random coil protein has no equivalent among complexes as it can only be found under highly denaturing conditions.

In order to further illustrate the presence of various degrees of dynamics within protein complexes, we will describe a number of well-studied examples. While many complexes representing two folded proteins or formed by coupled folding and binding have been described, studies of "dynamic" and "disordered" complexes are just beginning to be presented. Thus, we will focus on these more dynamic complexes and will address the implications for specificity of recognition and highlight the functional roles of disorder in protein interactions.

LOW AMPLITUDE MOTION IN COMPLEXES

Complexes composed of predominantly ordered proteins nonetheless have ns-ps timescale motion, as do all folded proteins. Changes in these dynamics upon binding can have significant

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thermodynamic consequences. The free energy of binding is the sum of the changes in enthalpy and entropy of the interacting molecules and their solvent.

$$\Delta G = \Delta H - T\Delta S_{\text{bind}}$$
$$= \Delta H - T(\Delta S_{\text{protein}} + \Delta S_{\text{ligand}} + \Delta S_{\text{solvent}})$$

The contribution from the change in conformational entropy of the protein has been difficult to determine in the past although methods to address this question have been developed (Akke *et al*., 1993; Muhandiram *et al*., 1995; Wand *et al*., 1995; Yang and Kay, 1996; Millet *et al*., 2002; Skrynnikov *et al*., 2002). It was reported recently that the change in conformational entropy of the protein upon binding can be a major contributor to ΔS_{bind} (Frederick *et al*., 2007) and that NMR spectroscopy, which provides insight into protein dynamics on a large number of time scales, can be used to determine the conformational entropy through a "counting of states" implicit in the dynamics of interconversion between the different structural states (Igumenova *et al*., 2006). Fast side chain dynamics on a ps-ns time scale represent the largest contribution to the conformational entropy (Karplus *et al*., 1987) and can be probed using ^{2}H methyl relaxation experiments (Muhandiram *et al*., 1995) and interpreted in terms of degree of spatial restriction, or generalized order parameters. If the model describing the dynamics in the states being compared is the same, the results are fairly insensitive to the motional model used (Igumenova *et al*., 2006). The method was used to study binding of calmodulin to six different peptides. The binding entropies of these interactions vary widely, but show a surprisingly linear correlation with the change in conformational entropy of calmodulin upon binding, with a slope of ~0.5. (Remarkably, even different classes of sub-nanosecond motions show linear correlations or anti-correlations.) The dynamic response of calmodulin to binding six different targets, interpreted as a change in conformational entropy, therefore significantly impacts ΔS_{bind} and the free energy of binding. Folded proteins may take advantage of the modulation of their dynamics to fine-tune affinities to different targets and possibly to enable allosteric regulation (Popovych *et al*., 2006).

DISORDER IS COMPATIBLE WITH CATALYTIC ACTIVITY

Although enzymes and their complexes are usually assumed to be at the more rigid end of the spectrum, protein dynamics are utilized by many enzymes to facilitate catalytic turnover. A large number of enzymes such as protein kinases, phosphatases, and ubiquitin ligases act on proteins, but dynamic studies have only been performed on enzyme complexes with small molecules. A well-documented mechanism to achieve turnover rate accelerations is sampling of enzyme conformations required to stabilize the transition state by dynamic modes in the protein along the reaction coordinate (Eisenmesser *et al*., 2002; Butterwick *et al*., 2004; Wolf-Watz *et al*., 2004; Eisenmesser *et al*., 2005; Boehr *et al*., 2006; Henzler-Wildman *et al*., 2007b; Watt *et al*., 2007). Importantly, it was recently reported that fast time-scale (ps-ns) atomic fluctuations can facilitate slower time-scale motions (μs-ms) that have a higher amplitude and are catalytically productive (Henzler-Wildman *et al*., 2007a). The frequency of these concerted motions thus prove to be rate-limiting for the reaction.

While such larger amplitude motions are frequently found in enzyme complexes, a rigid scaffold formed by a well-defined protein fold was assumed to be essential to accommodate substrate binding sites, to allow for concerted motions in hinged loops, to change domain orientations, to vary the depth of enzymatic clefts and to allow for breaking and formation of hydrogen bonds, salt bridges, and hydrophobic contacts. This assumption has recently been challenged by the observation of catalytic activity in a disordered enzyme. A chorismate mutase mutant from *Methanococcus jannaschii*, engineered to be monomeric instead of the native dimeric form, is catalytically active although it exhibits the dynamic properties of a molten globule. Although this is an engineered enzyme and thus not naturally occurring, its investigation provides insights into the ability of disordered protein ensembles to provide specificity in recognition. The chorismate mutase mutant exists as an ensemble of loosely packed helix bundle conformers that interconvert on a μs-ms time-scale (Vamvaca *et al*., 2004). Binding of a transition-state analog (TSA) induces global ordering of the protein resulting in a similar conformation as in the native dimer, but the protein nevertheless retains a high magnitude of μs-ms dynamics throughout the sequence (Pervushin *et al*., 2007). Kinetic measurements demonstrated that binding occurs via an induced-fit mechanism, in which the molten globule binds the TSA loosely to form a low affinity complex that subsequently undergoes a slow conformational change to result in a tight complex. The disorder in the free state does not appear to significantly interfere with catalysis and the monomer achieves nearly similar rate acceleration as the wild type enzyme, but the slow conformational rearrangement of the complex that results in a catalytically competent conformation is rate limiting. It has been proposed that many enzymes may have evolved from molten globules because their associated structural plasticity would allow for relaxed substrate specificity and enzymatic promiscuity, permitting enzymes to carry out several functions in simple cells. The disorder may compromise catalysis and specificity only moderately but could facilitate evolutionary change (Vamvaca *et al*., 2004).

DISORDERED LINKERS IN COMPLEXES CAN SERVE FUNCTIONAL ROLES

Many intrinsically disordered proteins interact with their binding partners through relatively short elements that have fluctuating complementary structure and that become ordered upon binding as discussed in the introduction. Examples are the interactions of calpastatin with its cognate enzyme, calpain (Kiss *et al*., 2008) and p27 with the CDK2/cyclin A complex (Galea *et al*., 2008). If the ordered region is flanked by a segment that retains its disorder, this interaction mode has been described as a "flanking" model (Tompa and Fuxreiter, 2008). Alternatively, in the "clamp" model, two regions become ordered upon binding leaving a disordered connecting segment (Tompa and Fuxreiter, 2008). The interaction of the scaffold protein Ste5 of the mitogen-activated protein kinase (MAPK) cascade in *Saccharomyces cerevisiae* with the MAPK Fus3 is an example of a "clamp" model with two separate binding motifs that undergo ordering while their linker remains disordered (Bhattacharyya *et al*., 2006) (Figure 3). Intriguingly, this disorder is functionally important in determining the activation state of Fus3. LiMs in Ste5 and Ste7 (the MAPK kinase)

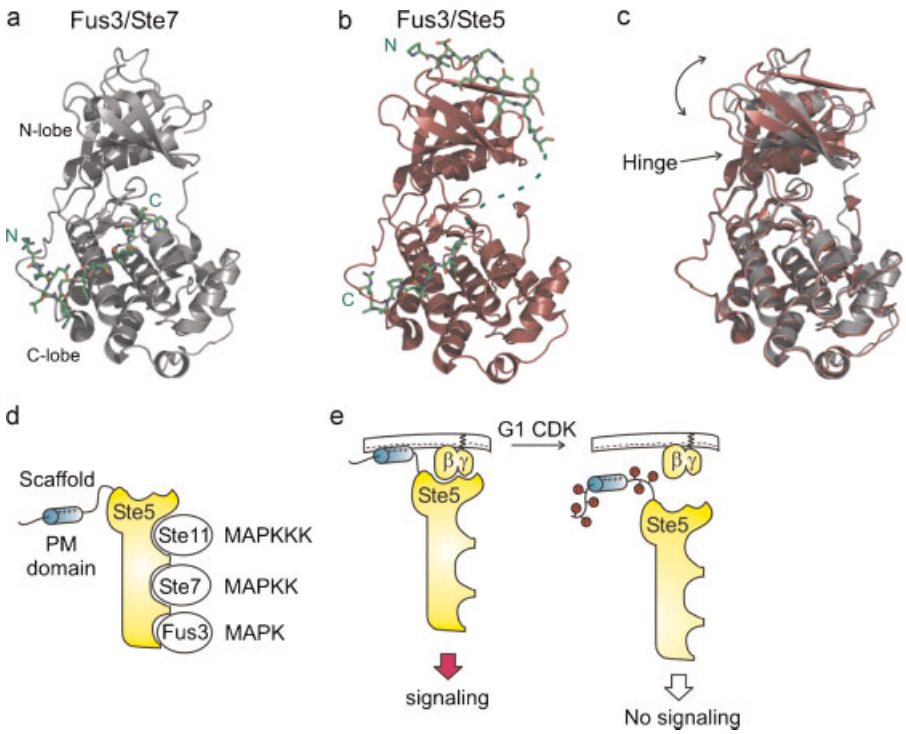

Figure 3. Functional disorder in Ste5 scaffold determines pheromone response pathway activation state. Structures of the MAPK Fus3 bound to peptides derived from (a) the MAPKK Ste7 or (b) the pathway scaffold Ste5. Ste5 binds to Fus3 in a bipartite manner with the N-terminal motif bound to the N-lobe and the C-terminal motif interacting with the C-lobe of the kinase. The intervening linker is disordered. The Ste7 peptide binds only to the C-lobe of Fus3 and in the opposite orientation to that of Ste5. (c) A comparison of the crystal structures of Fus3 with the Ste5 and Ste7 peptides reveals different orientations of the N- and C-lobe relative to each other brought about by a domain motion around a hinge. The orientation in the Fus3/Ste5 complex resembles the one in activated kinases. The disordered linker in Ste5 probably restricts the inter-lobal motions, leading to enhanced activity. (d) Domain structure of Ste5 and pathway members organized by this pheromone response MAPK scaffold. (e) Membrane recruitment and hence pathway signaling requires two weak interactions: Binding of $G\beta\gamma_2$ which is released from a G-protein coupled receptor after pheromone activation, and membrane association of an amphipathic helix in the PM domain. Cell-cycle stage dependent phosphorylation of disordered regions adjacent to the helix results in "polyelectrostatic" repulsion and dissociation of the helix from the plasma membrane. (Figure adapted from (Bhattacharyya *et al*., 2006) and (Strickfaden *et al*., 2007)).

compete for binding to Fus3. The Ste7 linear motif binds to a surface on the C-lobe of the Fus3 kinase, while Ste5 binds to Fus3 in a bipartite manner; two motifs bind to two distinct surfaces of Fus3, one in the same binding groove as Ste7, the other on the N-lobe, while the two motifs are connected by a disordered linker (Figure 3 A,B). Although the linker is flexible, it exerts an important functional role by orienting the two lobes of the kinase with respect to each other to lead to partial activation. Shorter or longer linkers fail to provide the same rate of activation (Bhattacharyya *et al*., 2006).

SIC1 FORMS A DYNAMIC COMPLEX WITH CDC4

Sic1 is an intrinsically disordered inhibitor of a cyclin dependent kinase (CDK) in *Saccharomyces cerevisiae*. Its interaction with Cdc4, the substrate recognition subunit of a ubiquitin ligase, was suggested to be a dynamic complex, based on evidence from biochemical and *in vivo* data (Nash *et al*., 2001). Sic1 degradation is required for yeast cells to enter into the S phase of the cell cycle and commit to a new round of replication. Phosphorylation of up to nine CDK phosphorylation sites in Sic1 leads to the creation of binding motifs for Cdc4, the so called Cdc4 phospho-degrons (CPDs), most of which are clustered in the N-terminal 90 residues of Sic1 (Willems *et al*., 2004). An average of any six phosphorylations is required to mediate binding to Cdc4 at affinities leading to subsequent ubiquitination and degradation ($\sim$1 μM) (Nash *et al*., 2001) while individual CPDs only impart weak affinities in the high micromolar range (Borg *et al*., 2007; Hao *et al*., 2007). Notably, Cdc4 contains only one functional binding site (Orlicky *et al*., 2003).

In the free state, the N-terminal 90 residues of both non-phosphorylated and phosphorylated Sic1 1-90 (here called Sic1 and pSic1, respectively) are intrinsically disordered. However, they sample significant yet transient secondary and tertiary structure and compact conformers (Mittag *et al*., 2008). The flexibility and disorder of pSic1 render all binding motifs accessible to Cdc4 and they indeed each interact with Cdc4, one at a time, in a dynamic equilibrium, with weak individual affinities permitting their exchange. Interestingly, the parts of the protein not interacting with Cdc4 remain intrinsically disordered. Thus, pSic1 undergoes only transient local ordering of its extended binding motifs while the rest of the protein retains its disorder.

The complex of fully phosphorylated Sic1 1-90 with Cdc4 *in vitro* is thus an example of a "dynamic complex" (Figure 4) as described above (Mittag *et al*., 2008). Remaining questions include how Sic1 binding to Cdc4 depends on the number of phosphorylated sites and whether the binding mode is similar *in*

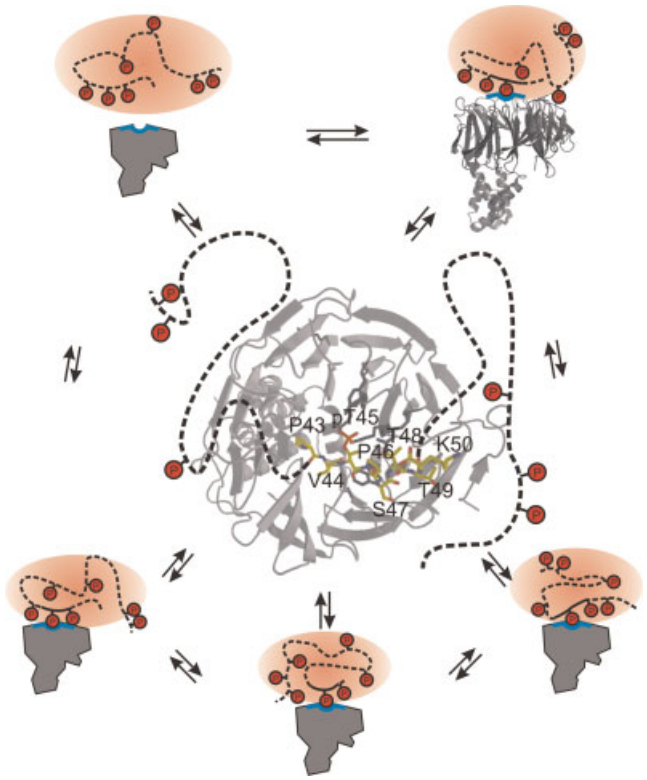

Figure 4. Dynamic complex of pSic1 and Cdc4. Multiple suboptimal binding motifs in pSic1 interact with the binding pocket of Cdc4 in a dynamic equilibrium. CPDs not directly bound at any given instance can contribute to the binding energy through "polyelectrostatic" effects giving rise to a mean electrostatic field (pale red ovals). The interaction of the pThr45 CPD is depicted in a detailed structural model, based on the crystal structure of the Cdc4-Cyclin E complex. The linear binding motif ordered upon interaction is depicted in stick representation, whereas parts of pSic1 remaining disordered in the complex are represented by dashed lines.

vivo. Biochemical and *in vivo* assays have demonstrated an intriguing non-linear dependence of the affinity on the number of phosphorylated binding motifs (Nash *et al*., 2001). Overexpression of Sic1 mutants with different phosphorylation site combinations in yeast enables a measure of their relative instability compared to wildtype Sic1 (attributed to Cdc4-mediated ubiquitination) and their ability to transition out of G1 phase. Sic1 mutants with less than about six phosphorylation sites are unable to sufficiently destabilize Sic1 to allow entry into S phase, leading to cell cycle arrests. Apparently, high-affinity Cdc4 binding requires multiple phosphorylations, which in turn allows for the observed dynamic binding mode. Why is a dynamic complex more beneficial than the association of a single linear binding motif whose sequence matches the CPD consensus and that achieves a similar affinity? The requirement for multiple phosphorylations sets a threshold for G1 phase CDK activity and converts the increase in this activity into a switch-like response for Sic1 degradation and S phase CDK activity. Assuming a distributive phosphorylation mechanism, binding of Sic1 to Cdc4 depends on the sixth power of the concentration of active G1 CDK, creating an ultrasensitive response. If recognition by Cdc4 depended on only one high-affinity CPD, the response would be graded instead. Yeast cells expressing Sic1 with only one such optimal CPD indeed show premature entry into S phase and genomic instability (Nash *et al*., 2001), probably because replication initiation is not a concerted event. The requirement for multiple phosphorylations thus allows for intricate regulation of the cell cycle, and the binding mechanism supposedly helps Cdc4 "count" the number of phosphorylations.

Phosphorylation has a dramatic effect on the net charge of Sic1, making electrostatic effects an obvious candidate for the physical basis of the increased affinity in multiply phosphorylated Sic1. Although the underlying assumptions of the "polyelectrostatic" model are clearly oversimplifying, it captures many of the properties of the interaction of Cdc4 with pSic1 or with a series of phosphopetides with different charges (Borg *et al*., 2007). This model requires compact ensembles to be populated by the intrinsically disordered protein to prevent solvent screening effects of the long-range interactions in extended conformations. Sic1 indeed contains transient structure that leads to the population of compact conformations.

Thus, there are many contributions of intrinsic disorder to the dynamic binding mode of Sic1 and Cdc4. Flexibility enables binding and release of multiple binding motifs, the dynamic interconversion of a multitude of conformers facilitates the cumulative contribution of charges from phosphorylations to the electrostatic interaction, and the flexibility of the long protein chain may allow for energetically favorable polarization effects which increase the affinity even more. The intrinsic disorder of Sic1 thus plays a crucial role in its recognition by Cdc4 and is exploited for creating a binding mechanism in which the affinity is highly sensitive to a threshold level of phosphorylation.

PHOSPHORYLATION OF STE5 MODIFIES ITS CELLULAR LOCALIZATION

Another example of the exploitation of post-translational modification as a means to modify electrostatic interactions is the scaffold protein Ste5 of the MAPK cascade in *Saccharomyces cerevisiae*, which signals as a response to mating pheromone exposure during G1 phase. Mating pathway activation requires Ste5 localization to the plasma membrane, a process which is achieved by two cooperative mechanisms, (1) binding to the $\beta\gamma$-subunit of a G-protein coupled receptor upon pheromone stimulation and (2) weak association of a plasma membrane-binding (PM) domain in its N-terminus (Figure 3 E) (Winters *et al*., 2005). The PM domain contains a short basic-rich, amphipathic α-helix that weakly interacts with acidic phospholipid membranes. Eight CDK phosphorylation sites flanking the PM domain in intrinsically disordered regions act as a sensor of the cell-cycle state; their phosphorylation in late G1 phase, when the cell has committed to another round of replication, disrupts the mating pheromone response. Apparently, the negative charges from phosphates interfere with binding to the negatively charged phospholipids, and the extent of the electrostatic repulsion is directly influenced by the net charge of the protein region rather than by its actual sequence (Strickfaden *et al*., 2007). The disordered nature of these PM-flanking regions may directly aid in this mechanism because the rapid interconversion of a multitude of flexible conformers may generate a mean electrostatic field directly reflecting the net charge of the region instead of presenting discrete charges. Since multiple charged

sites co-operate in this mechanism even when not directly bound, this is another example of the "polyelectrostatic" effect. In Ste5, "polyelectrostatic" interactions are used to abolish the rather non-specific association of the PM domain with the cell membrane and to coordinate the pheromone response with the cell cycle stage. Although phosphorylation of the disordered regions of Ste5 leads to release of weak associations rather than binding, these disordered segments are crucial for the regulation of the assembly of a signaling pathway by changing the cellular localization of Ste5. Importantly, the disorder allows access of kinases and phosphatases, the latter ones presumably playing a crucial role in restoring pheromone sensitivity after cell cycle completion.

Intrinsic protein disorder is used in the regulation of other protein interactions with Ste5, e.g., in determining the activation state of the MAPK Fus3 as discussed above. Ste5 is thus not only a passive scaffold that assembles the proper signaling proteins into a pathway but it also exerts several layers of important regulatory functions itself that are based on protein disorder.

THE REGULATORY REGION OF CFTR REGULATES CHANNEL OPENING VIA TRANSIENT CONTACTS

The cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel which is mutated in cystic fibrosis, belongs to the protein family of ABC transporters, but contains two disordered regions not commonly observed in other members of the family. Both the regulatory insertion (a short ~30 residue insertion into the nucleotide binding domain 1 (NBD1)) and the regulatory (R) region (a ~200 residue stretch C-terminal to NBD1) can be modified by PKA phosphorylation, a process required for regulating the opening of the channel. The R region samples α-helical conformations that appear to be stabilized upon binding to NBD1 and act as α-MoRFs (Baker *et al*., 2007) (Figure 5). Phosphorylation of up to nine sites, none of which are required, decreases the propensity for α-helical conformations and thus also the strength of the NBD1 binding to the R region, presumably in a rheostat manner that enables gradual regulation. Evidence from mutational and NMR studies suggests that the R region acts as a protein hub by interacting transiently with different parts of CFTR, particularly with NBD1, the N-terminal tail and possibly with NBD2, and also with other proteins such as the co-regulated chloride-bicarbonate exchanger SLC26 (Ko *et al*., 2004). According to the current model, the interactions between the R region and NBD1 inhibit the dimerization of the NBDs and therefore ATP hydrolysis and channel opening. R region segments come on and off of their targets and exchange binding partners including PKA and protein phosphatase 2A (PP2A), with the additional effect that the latter two lead to changes in phosphorylation, hence shifting the entire interaction equilibrium. The competition between several binding partners exerts a complex level of regulation on channel opening by controlling the NBD dimer equilibrium (Baker *et al*., 2007). In the context of full-length CFTR and its physiological binding partners, the large,

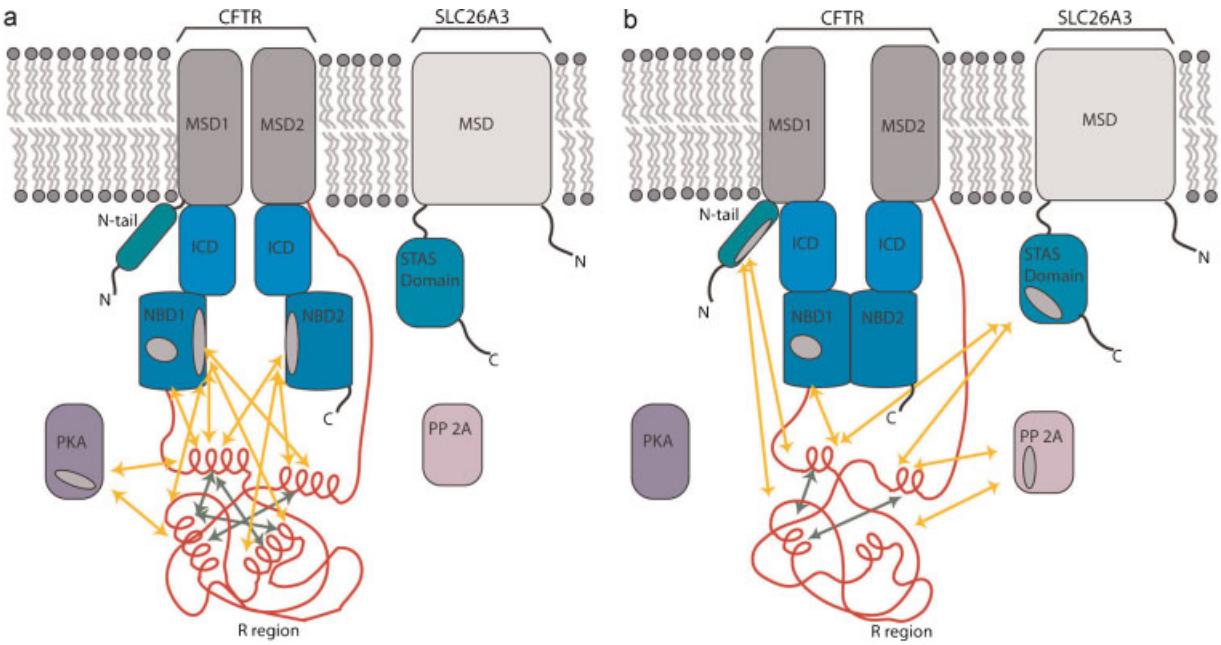

Figure 5. The regulatory (R) region of CFTR functions as an integrator by dynamically interacting with various intra- and intermolecular binding partners. The R region of CFTR is an intracellular, intrinsically disordered, ~200 residue long insertion into the usual domain structure of ABC transporters, between NBD1 and MSD2 and is shown as a red curve. (a) In the non-phosphorylated state, the R region samples stretches of α-helical conformations that are stabilized upon interaction with NBD1, blocking its dimerization with NBD2 required for channel opening. (b) Interaction with PKA leads to R region phosphorylation, reducing the propensity to sample helical conformations and the interaction with NBD1, allowing dimerization with NBD2 and increased channel opening. The interaction with NBD2 is postulated but not experimentally demonstrated. Other interaction partners include the N-tail, the STAS domain of the chloride/bicarbonate transporter SLC26A3 and PP2A. The R region is thought to function as a rheostat and an integrator of various signals. Gray ellipses, putative binding surfaces of interaction partners; green arrows, potential tertiary interactions within the R region; gold arrows, interactions with binding partners (multiple arrows represent dynamic exchange of both multiple R region binding sites and multiple interaction interfaces, without implying involvement of specific R region segments); MSDs, membrane-spanning domains; ICDs, intracellular domains; PP 2A, protein phosphatase 2A; PKA, protein kinase A.

approximately 200-residue R region and its binding sites may be interacting with more than one binding partner at any one time. The R region interactions are thus an example of a "dynamic" complex, facilitating its function as an integrator of input from multiple interactions.

INTRAMOLECULAR ETS–1 COMPLEX RETAINS DISORDER AND REGULATES DNA RECOGNITION

In the dynamic complex of Sic1 with Cdc4, linear binding motifs in Sic1 are transiently ordered. In other complexes, even transient ordering has not been observed as in the following example of an intramolecular interaction of a folded domain with an intrinsically disordered region that retains its disorder. The affinity of the transcription factor Ets–1 to DNA is allosterically regulated by the predominantly disordered serine-rich region (SRR) which is adjacent to the structured DNA-binding ETS domain and its flanking inhibitory elements, the latter two of which together are called the regulatable unit (Figure 6). The inhibitory elements undergo partial unfolding, thereby populating a DNA-binding competent conformation of the ETS domain at a low fraction (Pufall *et al*., 2005). The presence of the SRR stabilizes the regulatable unit against thermal and chemical denaturation, under native conditions shifting the dynamic equilibrium from a flexible-active to a rigid-inactive form and repressing binding to DNA (Lee *et al*., 2008) (Figure 6). Interestingly, phosphorylation of up to five serine residues in the SRR as a consequence of Ca^{2+} induced signaling increases the stability as well as autoinhibition of Ets−1 substantially in a gradual manner (Pufall *et al*., 2005). This "rheostat" behavior represents an intriguing example of precise regulation by a disordered protein region but it raises questions

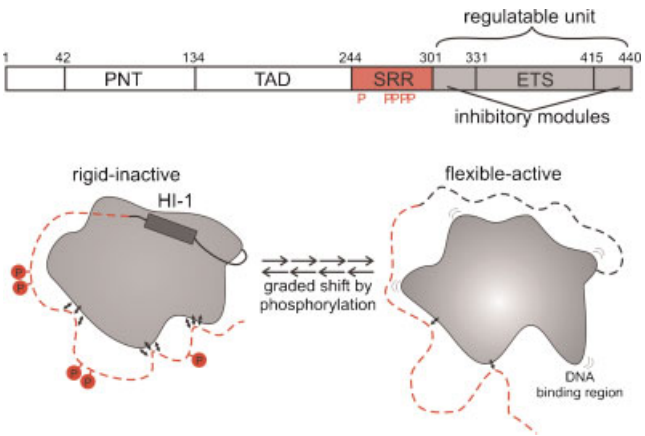

Figure 6. The transient interaction of the predominantly disordered serine-rich region (SRR) of the transcription factor Ets–1 with the regulatable unit represents a dynamic complex. Ets–1 is composed of the protein interaction (PNT) domain, the transactivation domain (TAD), the disordered serine-rich region (SRR, red) and the regulatable unit (gray), which consists of the DNA-binding (ETS) domain along with the inhibitory modules. Sites phosphorylated by CaMKII *in vitro* are indicated with "P"s. Ets–1 is in equilibrium between rigid-inactive and flexible-active (DNA-binding competent) forms, portrayed in different shapes. Different shading points to a change in dynamic properties. Phosphorylation gradually shifts the equilibrium more to the rigid-inactive form, which is stabilized by stronger transient interactions with the SRR. The inhibitory helix I of the inhibitory module is unfolded in the flexible-active form.

as to how the disordered SRR can stabilize the structured regulatable unit. Lee *et al*. demonstrated that the disordered SRR makes transient contacts with the inhibitory module and the DNA recognition helix. Phosphorylation dampens the flexibility of the SRR, thereby permitting a stronger interaction with the inhibitory module and the DNA recognition helix (Lee *et al*., 2008). Surprisingly, these interactions are not predominantly electrostatic. If they are of a hydrophobic nature instead, they may impact a network of hydrophobic residues in the regulatable unit in distinct ways for different phosphorylation states. The inhibitory mechanism could have a steric component to it, because the SRR transiently interacts with the DNA recognition helix, thereby possibly blocking or electrostatically repelling DNA binding. This mechanism is called "masking of binding sites" and is a common regulatory mechanism for disordered protein regions.

Apparently, the (intramolecular) interaction of the regulatable unit and the intrinsically disordered SRR does not require ordering of the latter. Hydrophobic clustering is not highly specific, but the intramolecular interaction and transient structure in the SRR (that is presumably also influenced by hydrophobic clustering) may aid in bringing together the right pieces, thereby providing specificity. If the stabilization of the regulatable unit depends on a multitude of short-lived, hydrophobic interactions, it is indeed conceivable that the effect can be adjusted by the number of phosphorylations in the interacting region rather than the exact combination of phosphorylation sites, creating the observed "rheostat" behavior. It remains to be tested whether or to which extent the interaction between the disordered SRR and its folded (intramolecular) partner is sequence independent, a phenomenon proposed previously for the interaction of histone tails with DNA and regulatory proteins (reviewed in (Fuxreiter *et al*., 2008)) and for the C-terminal domain (CTD) of the RNA polymerase II. Indeed, the persistent disordered nature of the SRR may permit a more fine-tuned regulation than a folded domain could provide. It permits rapid access to both kinases and phosphatases allowing for a flexible response to and integration of multiple signaling pathways, modulating transcription on the level of DNA binding.

T-CELL RECEPTOR ζ-SUBUNIT

Interactions of the T-cell receptor (TCR) ζ chain appear to be on the far disordered side of the spectrum of fuzzy complexes. It has been reported that both dimerization of the ζ chain and ζ chain binding to the Nef protein of simian immunodeficiency virus (SIV) are not accompanied by disorder-to-order transitions (Schaefer *et al*., 2000; Sigalov *et al*., 2007). The intrinsically disordered ζ chain is involved in coupling antigen recognition on the cell surface to signal transduction involving cytoplasmic downstream effectors. ζ dimerization appears to define the functional state of the TCR, while the interaction with viral proteins such as Nef induces receptor clustering by interaction with the ζ chain, possibly modulating TCR signaling. These observations render the nature of these interactions not only intriguing from a fundamentally scientific point of view but also clinically relevant.

ζ chains form several α-helical stretches upon interaction with LMPG lipid micelles (Duchardt *et al*., 2007). In contrast, neither dimerization of ζ nor its binding to Nef is accompanied by helix formation (Schaefer *et al*., 2000; Sigalov *et al*., 2007). Indeed, not

even transient ordering was observed. Although the basis of the interactions is not understood yet, the available data point to ζ forming "disordered" complexes (Figure 2L). The two interaction sites for Nef that had been identified previously using mutational analysis and the yeast two-hybrid system (Schaefer *et al*., 2000) could not be confirmed by NMR due to a lack of sequence-specific line broadening. The contacts must be of a very transient nature and the complexes must be comprised of a multitude of sub-states with low populations that do not even effect sensitive NMR chemical shifts. PRE experiments, which are exquisitely sensitive to transient contacts (as demonstrated by the detection of only transiently populated encounter complexes (Clore *et al*., 2007)) may prove useful for the future characterization of the interaction sites. Also small angle X-ray scattering of free and bound states together with computational approaches may provide insight into the "disordered" complex ensembles.

The apparent lack of ordering upon binding raises questions as to how a disordered protein can provide specificity while remaining disordered. If the interacting motif assumes an ordered state, even if only transiently, the mechanism of recognition is based on the complementarity of the binding motif and its interacting partner. However, this should lead to NMR chemical shift changes and/or line broadening, which are not observable for the ζ chain. So how does recognition of a disordered motif work? If it is based on enough short-lived interactions to escape broadening, the disordered complex ensemble would have to consist of a large number of conformers. Specificity, clearly one of the major goals in regulation, is hard to picture in such a case, as well as a desirable affinity if only small surface areas are buried at a time. If, however, every single contact is of modest specificity and affinity, their cooperation could ensure much tighter binding at increased specificity. These interactions of low affinity could be of a hydrophobic or electrostatic nature, the latter of which has been proposed to be exploited in intrinsically disordered proteins by generating a "polyelectrostatic effect" (Borg *et al*., 2007). Even co-operation of many of these low-affinity interactions would probably result in modest affinities, in agreement with the estimated K_ds of 10 and 1 μM for the ζ dimer and the ζ/Nef complex, respectively. The enhanced conformational entropy of such disordered complexes compared to complexes involving folded proteins may contribute significantly to the free energy of binding.

CONCLUSION

Our view of protein complexes is skewed by beautiful but static crystal structures of folded proteins interacting via well-ordered, complementary surfaces. However, many important regulatory processes are mediated by intrinsically disordered proteins. Protein disorder may have steric, evolutionary, and thermodynamic advantages, e.g., "polyelectrostatic" interactions that permit the utilization of phosphorylation as a means to fine-tune the electrostatic interactions of disordered protein regions according to their net charge.

Disordered protein regions do not necessarily undergo global disorder-to-order transitions upon binding. In fact, many important regulatory interactions may involve "dynamic" or even "disordered" complexes, as intrinsically disordered protein regions do not always stably fold upon binding. Such complexes may be particularly prevalent in signaling pathways as dynamic binding modes allow for the integration of complex signal inputs. Disruption of these transient interactions and their associated pathways can lead to severe pathologies such as cancer and neurodegenerative diseases as the high fraction of intrinsically disordered proteins associated with these diseases suggests. Understanding the molecular basis of these interactions and the multitude of associated functions will require detailed, residue-specific characterization of binding events, eventually leading to insight into normal cellular and pathological mechanisms. We have only just begun to examine such highly "dynamic" and "disordered" complexes and their numerous functional implications, and high-resolution structural information is missing for most of them. A combination of NMR spectroscopy, which provides atomic-resolution information even on disordered systems, small angle scattering techniques, which are unique in providing ensemble distribution properties, and computational approaches may prove useful to tackle this challenge (Bernadó *et al*., 2007; Mittag and Forman-Kay, 2007; Eliezer, 2009). Not only will structural representations of "dynamic" and "disordered" complexes help shift the long standing structure-function paradigm to more dynamic alternatives, but also they will provide insight into the variety of creative regulatory mechanisms provided by these frequently occurring complexes, maybe even leading to new therapeutic approaches.

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