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Affinity and specificity of motif-based protein–protein interactions

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It is becoming increasingly clear that eukaryotic cell physiology is largely controlled by protein–protein interactions involving disordered protein regions, which usually interact with globular domains in a coupled binding and folding reaction. Several protein recognition domains are part of large families where members can interact with similar peptide ligands. Because of this, much research has been devoted to understanding how specificity can be achieved. A combination of interface complementarity, interactions outside of the core binding site, avidity from multidomain architecture and spatial and temporal regulation of expression resolves the conundrum. Here, we review recent advances in molecular aspects of affinity and specificity in such protein–protein interactions.

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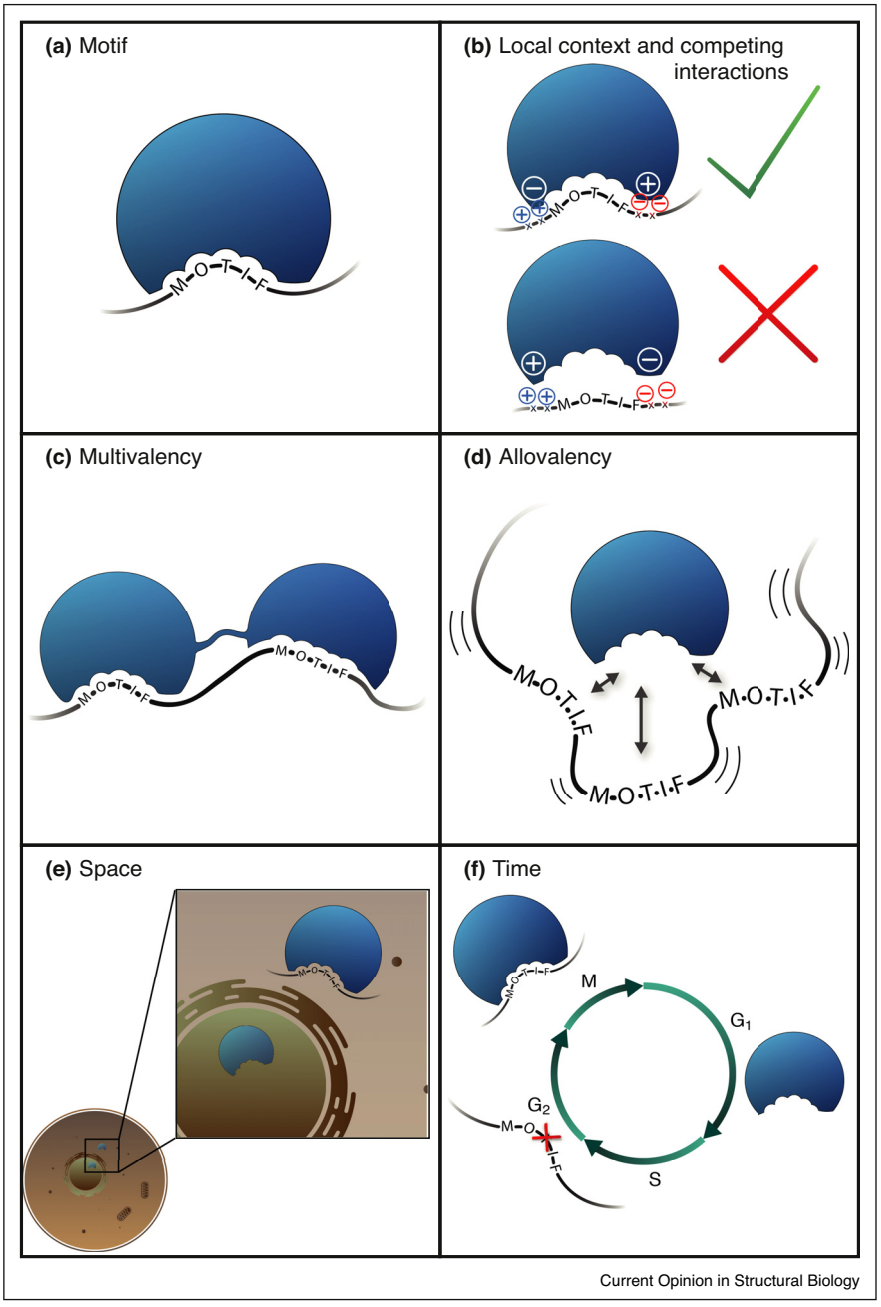
How can proteins distinguish their biologically relevant binding partner from other proteins in the cell? The answer is that the affinity of the protein–protein interaction under a given set of conditions is high enough compared to all other competing interactions. However, teasing out the details of the higher affinity for a certain protein–protein interaction is far from simple since multiple factors govern how much will be bound. We can measure the affinity using isothermal calorimetry and even dissect it into the change in strength of all individual bonds (enthalpy) and the change in entropy of the whole system, including release of water and ions upon binding, but this requires extensive experiments to give insight. Because of the complexity of even the simplest protein–protein interaction, from an experimental point of view it is often easier and more informative to assess differences in affinities, that is, specificity, for proteins with several binding partners. In other words, why is a certain interaction favored over another one? Such competition between different binding partners is also what happens in the crowded milieu of the cell. *In vivo*, spatial and temporal regulation contribute to resolving the apparent problem of overlapping specificity [1]. In this review we will focus on molecular aspects of affinity and specificity of motif-based interactions, including short linear motifs and coupled binding and folding of intrinsically disordered regions, and how they can prevail in the context of multiple competing interactions (Figure 1). The topic is of relevance for a fundamental understanding of the molecular events of endogenous processes but also from the perspective of targeting motif-based interactions for therapeutic purposes, a topic that has been receiving increasing attention in recent years [2], with the recently developed ABT-199 inibitor of BCL-2 as a prime example [3]. A classical function of interactions mediated by disordered binding motifs is to serve as binding sites for the assembly of protein complexes, often in the context of scaffolding proteins in signaling pathways [4,5]. Other functions involve guiding proteins to subcellular compartments (e.g. EB family [6]), docking of enzymes to their substrates, (e.g. MAPKSs [7,8], PP2A [9,10], Calcineurin [11], recruitment of transactivation domains to the transcriptional machinery [12–14], or the targeting of proteins for ubiquitin-dependent degradation by so-called degrons [15,16•]. Clearly, there are different requirements on the affinity and specificity of these various motif-based interactions.

Affinity and specificity of motif-based interactions

Many protein–protein interactions involve binding and folding of intrinsically disordered, that is, structurally heterogeneous regions of proteins to a folded protein domain, or less commonly to another disordered region. The shorter disordered binding regions (3–12 residues) are called short linear motifs (SLiMs), or eukaryotic linear motifs (ELMs), and are extremely common in proteomes of eukaryotic organisms. To give an idea of the numbers, more than 100 domain families have been reported to bind to SLiMs [17], and the number of SLiMs involved in protein–protein interactions in the human proteome has been estimated to be as many as 100 000 [18]. A binding motif is typically visualized as a linear string, with the main determinants of binding indicated using the one letter amino acid code, and positions of less importance indicated with 'x'. Upon binding, disordered regions

Figure 1



Schematic representations of different levels of how to achieve specificity in protein–protein interactions involving disordered binding motifs. **(a)** Side chain and backbone interactions between the core motif and the binding pocket of the folded recognition domain. **(b)** More or less-specific interactions outside of the binding pocket can increase or decrease affinity for a certain binding motif (or *vice versa*). **(c)** Multivalency as exemplified by a bivalent interaction will increase affinity and specificity further both by increasing the frequency of productive collisions and by a second intramolecular interaction, which will increase the interaction surface and slow down dissociation since both contacts must break simultaneously. **(d)** Allovalency will occur since there is a certain probability of re-binding before the protein and ligand escape their common 'sphere' and fully dissociates. This is particularly prevalent when multiple binding sites are involved, as exemplified here by three interaction motifs. **(e, f)** The place and time of expression of motifs and domains ensure that many non-specific interactions are avoided.

adopt more defined conformations, such as an α-helix, a β-sheet or a less well defined secondary structure, but sometimes remain structurally heterogeneous [19]. Thus, while graphically attractive the simplistic linear-string visualization of SLiMs in disordered regions only highlights side chains but not the tertiary structure adopted upon binding, which obviously influence affinity and specificity in the interactions. Motif-based interactions often show apparently low specificity, that is, several different SLiMs can bind to the same domain with similar affinities, and several domains can bind to the same SLiM. Clearly, it is challenging to achieve specificity in large domain families such as SH2, SH3 and PDZ, all with more than 100 members, which share structural features in their binding interface and inevitably ligand specificity. However, domain family wide studies have shown that there are gradients of specificities within the families that might contribute to the functional specificity, for example, for PDZ [20,21], SH2 [22] and SH3 [23]. Furthermore, several of the domains of a given family may bind to atypical or 'non-canonical' motifs [23]. Such interactions could be the physiological relevant ones or represent moonlighting activities, which are likely very common and a potential source for evolution of new protein–protein interactions [24,25•].

For motif-based interactions, affinity and specificity are accomplished by a combination of core motif binding determinants that provide shape and/or chemical complementarity, together with the context of the motif within the sequence, and the dynamic properties of the system including concentrations of interacting species (Figure 1, Box 1). The residues of the core motif generally interact with a set of conserved residues in the binding site of the domain. As a consequence of the limited binding interfaces (typically 500 $Å^2$), SLiM interactions typically have K_d values in the range of 1–500 μM, which is considered 'medium-to-low affinity'. Such affinities are usually associated with high dissociation rate constants and thus a rapid equilibrium [26,27•]. Affinities in this range are often, but not always, associated with relaxed specificities. This is however not a limitation set by the interaction type as the affinities and specificities of the domains and the motifs are easily evolvable [28], but rather a result of the functional demands on the interactions. Longer disordered binding motifs can have higher affinities [29] yet with high dissociation rate constants allowing equilibrium to be established rapidly enough for the cellular function (within seconds) [30,31].

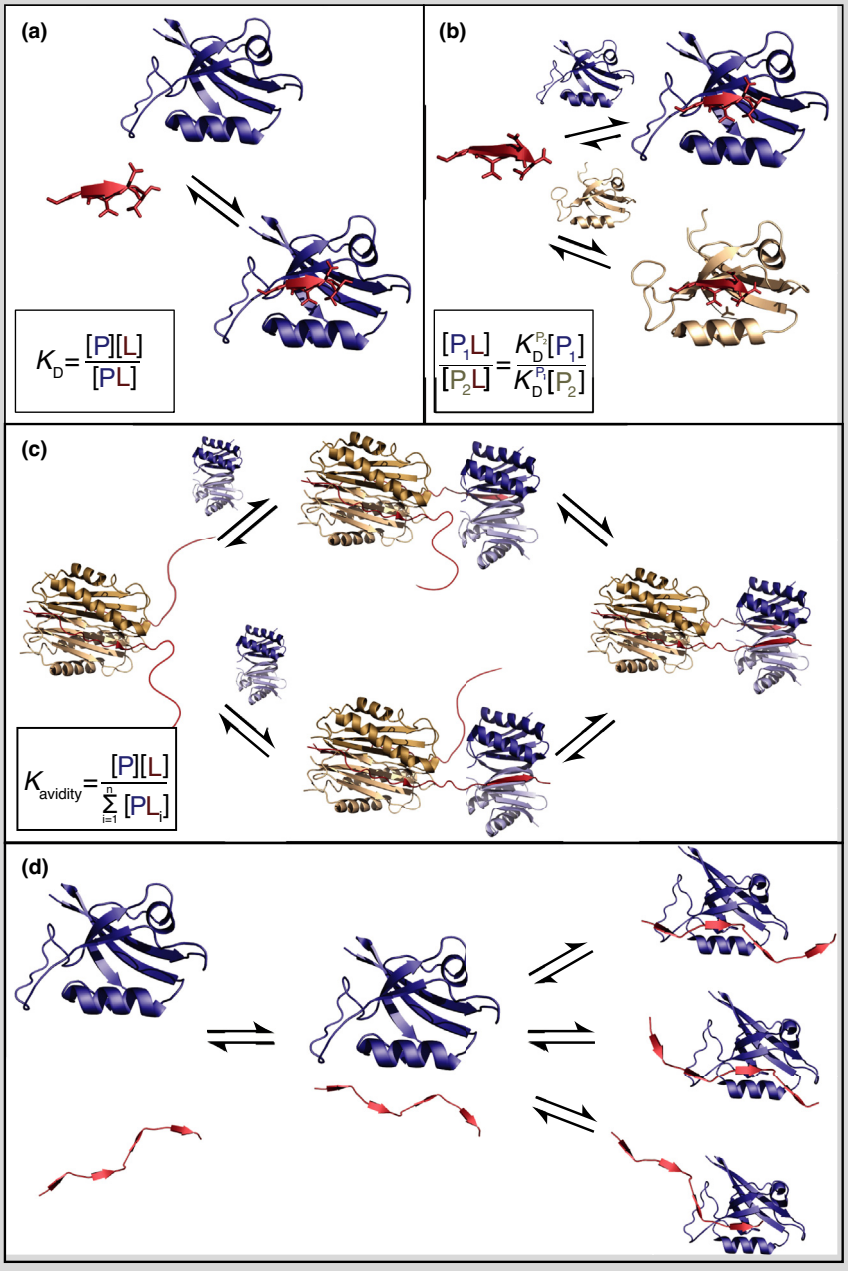
Given the variety of interactions and the conformational plasticity of disordered interaction regions, it is not surprising that there is no unique solution for how to accomplish specificity of motif-based interactions. Granting that the motif is defined by the core residues, the local context of the motif contributes to affinity. For example, for disordered motifs that adopt α-helical structures upon binding, it has been found that increasing the helical propensity of the disordered region confers an increase in affinity for the binding partners [35–38]. Motif-flanking regions can also contribute with specificity determining information in terms of permissive amino acids that increase the affinity of the interaction [39–42]. Indeed, a systematic analysis of structures of motif-based interactions revealed that the local sequence context contributes about 20% of the binding and is crucial for determining specificity [40]. As an example, the regulatory subunit B56 of protein phosphatase two recognizes an LxxIxE motif [9], which is reinforced by the presence of acidic residues located C-terminally of the motif, and/or by phosphorylation within and/or adjacent to the motif [9,10,43]. This enhancement of affinity is accomplished

Box 1 Affinity, specificity and multivalency.

(a) The affinity is usually quantified and expressed as the equilibrium dissociation constant, K_d, which has the unit of M for an interaction involving two proteins. (b) Specificity is qualitatively used to describe that a certain protein recognizes a certain molecule better than other ones. Sometimes 'selectivity' is used to indicate that a protein has several ligands, thus reserving 'specificity' for the cases where there is only one ligand. However, perfect specificity is rarely encountered in biological systems and we use the two words as synonyms. A straightforward quantitative definition of specificity is the ability of a protein P to distinguish between competing protein ligands L_1 and L_2, or *vice versa*, a ligand L to distinguish between two proteins P_1 and P_2. This is expressed as the ratio of the K_d values for the two ligands:

The advantage of looking at specificity rather than affinity is that affinity is highly dependent on the conditions: pH, ionic strength, crowding and so on. While specificity may also be modulated by these factors, it is likely that several of them affect the two interactions similarly and cancel out. Thus, *in vivo* a K_d value may differ much from that measured *in vitro*. But a 10-fold higher K_d for protein P_1 relative to protein P_2 in the test tube is more likely to remain 10-fold higher *in vivo* than the absolute K_d values. To obtain the relative abundance of the respective complex the concentrations need to be taken into account as shown in (b). Thus, a high affinity, 'specific' interaction could be outcompeted by a low affinity interaction if the concentration of the latter is high enough. Protein concentrations vary between different cell types, cell states and compartments. There are emerging techniques to quantify protein concentration with spatial resolution, for example mass spectrometry and multiplexed immunofluorescent staining [32]. (c) The affinity of multivalent interactions can be quantified by defining K_d as the product of free species divided by the sum of all bound species (two bound species in case of a 1:1 binding with two mutually exclusive binding sites or three bound species in the case of the depicted bivalent interaction).

(d) Allovalency is an extension of multivalency in which one more factor is taken into account: the escape from the common sphere where protein and ligand can re-bind with higher probability than two fully separated molecules [33,34]. PDB codes of structures used: 5VWK (a, b and d), 3QJN (yellow, b) and 3FM7 (c).



by electrostatic interactions between these negatively charged moieties and a basic patch on B56. Similarly, the affinity and specificity of the retinoblastoma protein for the LxCxE motif is enhanced by negatively charged residues, or phosphorylation of residues situated C-terminally of the core binding motif that interact with a positively charged surface surrounding the binding site [44]. Another recent study showed that phosphorylation or phosphomimetic mutations (S/T to E) within or N-terminally of the class I PDZ binding motifs (T/SxΦ-coo-) can tune the selectivity of the peptide ligand, such that it enhances the affinity of one PDZ domain while reducing the affinity of another PDZ domain [45•]. The effect can in part be explained by the presence or absence of basic residues surrounding the peptide binding pocket of the domain. The contextual specificity determinants can also function by negative selection, in other words, non-permissive residues reduce the affinity of the interaction for certain proteins. One example of achieving high specificity by such gatekeeping is the PxxP motif of the yeast protein Pbs2 that among all yeast SH3 domains only binds to the Sho1 SH3 domain, but cross reacts with SH3

domains from other species [46]. The motif has evolved to bind one-specific yeast SH3 domain not by optimizing the affinity for the target domain, but by negative selection against all other SH3 domains in yeast.

Importantly, the information content in the flanking regions is not captured in the core binding motif. Thus, although the SLiM is known, it is challenging to accurately predict ligands of motif-binding proteins, or conversely, predict motif-binding proteins for a certain ligand. Any such predictions therefore requires experimental validation [47]. Predictions of motif-based protein–protein interactions can be improved by taking evolutionary information into consideration [48•], given that functional motifs are subjected to evolutionary constraints and are more conserved than the surrounding intrinsically disordered regions in which they are typically embedded. When available, predictions can be improved when taking into consideration contextual information on non-permissive residues [42,49]. Nevertheless, experimental methods such as various display methods and peptide SPOT arrays remain in our opinion, more powerful than motif-based predictions in terms of finding interactions and exploring the specificity of motif-based interaction [50–56,57•,58]. Regardless of the approach used to identify potential endogenous motif-based interactions, it is necessary to confirm that the identified binding site is available for binding in the context of the full-length proteins and that the suggested interacting proteins can meet in the cell [47], which brings us to the next level of the contribution of the context to specificity.

The larger context

In addition to the core binding motif and the local context, the larger context contributes to affinity and specificity in terms of potential interactions between other parts of the full-length proteins. For example, intrinsically disordered regions of proteins that contain multiple recognition motifs can interact with two or more domains of a multidomain protein, or to different subunits of a multimeric protein [59,60•]. Such multivalent interactions are common for motif-based interactions [61] and give rise to avidity effects [62] due to the high local concentration of the second binding site after the initial binding, and the rebinding upon dissociation of individual binding sites (Box 1). Viral proteins often contain multiple motifs used to hijack different human protein–protein interactions [63–65]. In such cases the avidity effect could potentially be explored by designing multivalent inhibitors towards the viral protein by combining parts of the different human targets in a 'protein drug' [66,67].

Another specificity enhancing effect is allovalency, which is augmented by the presence of multiple weak affinity bindings sites on an intrinsically disordered protein that binds to one unique binding site (Figure 1, Box 1). The high local concentration of multiple binding sites results in several binding–dissociation events between the same two molecules [33,34] as illustrated using multiple phosphorylation sites binding to a receptor [68]. In this way multi-site phosphorylation was suggested to lead to ultrasensitive switches that depend on the number of binding sites available. However, a few examples are reported. In a recent study, it was shown that the Cul E3 ligase substrate adaptor KBTBD8 requires phosphorylation of multiple E3 binding motifs located in the intrinsically disordered regions to specifically interact with its target proteins [69]. While clearly multivalent, whether or not allovalent effects are present could be difficult to determine since the presence of multiple binding sites in itself will increase the apparent affinity.

Contributing to the larger context are other factors (Box 1), such as protein concentrations and cellular localization, which depend on cell type and cell state, but a detailed analysis of this is beyond the scope of this review.

Evolutionary aspects

The types of protein–protein interactions discussed here have been instrumental in the evolution of multicellular life. In particular, re-wiring of interaction networks by shuffling of interaction domains and alternative splicing can open up or close signaling pathways [70,71]. But evolution can also operate on the level of the single protein–protein interaction. It has been demonstrated that it is possible to alter the peptide binding specificity of a given domain by single amino acid substitutions [72], to generate domains with novel binding specificities [73,74] and to generate 'super binding' peptides with higher affinity than naturally occurring ligands [75] through substitution of a few amino acids. These findings highlight the plasticity of motif-based interactions and reinforce that native interactions have not evolved to be of optimal affinity. However, a recurring observation is that specificity is lost when domains are subjected to *in vitro* evolution experiment for higher affinity. For example, it was shown by high-throughput phage display that many different binding sites can be generated to bind to a given peptide, but that the artificially generated binders recognize their cognate ligands with high affinity but with low specificity [76]. In another study an engineered PDZ domain selected for binding a viral peptide [66] gained in affinity but lost specificity [77].

The motif themselves can rapidly evolve and emerge de novo from a few amino acid substitutions, as extensively reviewed recently [55]. For example, the substrates of the protein phosphatase Calcineurin in yeast and mammals differ due to recently evolved PxIxT sites, which has led to network rewiring [78]. Importantly, while evolution of the core binding motif is crucial, the context also needs to be evolutionary optimized to increase affinity and

specificity. Sometimes non-specific interactions serve as starting points for de novo motif evolution. In fact, the high concentrations of proteins in the cell leads to extensive non-specific 'quinary interactions' [79]. A few of these very low affinity interactions may provide a specific advantage for the organism and any point mutation, which increases the affinity will then be under positive selection. This can be seen for viruses, where there are numerous examples of how relatively few substitutions can turn a disordered protein region into a SLiM mimicking one of the partners in a protein–protein interaction in the host [24,80]. Because viruses evolve rapidly such adaptations can occur on very short timescales. However, similar evolutionary adaptations of new or existing protein–protein interactions involving SLiMs or longer disordered regions probably occur in all life, but on longer timescales. For example, the interaction between the two transcriptional coregulators, CREB-binding protein and NCOA, likely started as a low affinity interaction between the NCBD domain in CREB-binding protein and a disordered region in NCOA [25•].

Concluding remarks

It is a daunting task to fully understand the interaction network of our >100 000 binding motifs and their partners. Protein concentrations are regulated depending on cell type, spatially within the cell and over time to coordinate life processes. The structure and function of protein–protein interactions are addressed by several approaches and their combination in systems biology will produce ever better pictures of the entire interaction network in the coming decades. Here we have discussed recent results at the molecular end of the question, namely how the first level of specificity is achieved in the coupled binding and folding reactions of intrinsically disordered interaction regions. It is clear that the 'core binding motif' of a particular family of interactions is not sufficient to provide specificity, but it relies on further interactions close to the binding motif and between neighboring domains and other proteins [81•]. In the future, this highly active area of research should quantitatively address specificity in protein–protein interaction networks. This needs to be done at several levels: for purified proteins, in cells and ultimately *in vivo*.

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