Biophysical Perspective

The Landscape of Intertwined Associations in Homooligomeric Proteins

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ABSTRACT We present an overview of the full repertoire of intertwined associations in homooligomeric proteins. This overview summarizes recent findings on the different categories of intertwined associations in known protein structures, their assembly modes, the properties of their interfaces, and their structural plasticity. Furthermore, the current body of knowledge on the so-called three-dimensional domain-swapped systems is reexamined in the context of the wider landscape of intertwined homooligomers, with a particular focus on the mechanistic aspects that underpin intertwined self-association processes in proteins. Insights gained from this integrated overview into the physical and biological roles of intertwining are highlighted.

INTRODUCTION

Oligomers composed of identical protein subunits, also denoted as homooligomers, or homomers in short, are very common. It has been estimated that over 50% of proteins self associate to form homomers, with homodimers representing the largest fraction (1–3). A large number of studies have therefore been devoted to the analysis and classification of homomers and the evolutionary processes that may lead to their formation (4,5).

Homomers can form intertwined assemblies in which small segments or compact protein substructures (domains) are exchanged between the interacting subunits (6,7), forming various types of quaternary arrangements, ranging from dimers to higher-order oligomers and polymers. A particular subset of such assemblies, known as three-dimensional (3D) domain-swapped systems, has been thoroughly investigated (8–12). These systems undergo an interconversion between the monomeric species and an intertwined oligomer, whereby specific intramolecular interactions in the monomer are substituted for their intermolecular equivalents (13). 3D domain swapping has attracted much interest because of its potential role in protein aggregation associated with a number of neurodegenerative diseases (14–16). However, not all intertwined homomers undergo the monomer-dimer interconversion process, and it is in general unclear which ones do, as information on the corresponding monomeric structures is often unavailable. Still, overall little attention has been devoted to intertwined homomers outside the 3D domain-swapping context. The various studies that have shaped our understanding of homomer structures and their properties (17–21) have consistently ignored intertwined versions to avoid some of the confounding features of these systems. This gap was recently filled by two studies from our laboratory (22,23) that charted the full repertoire of intertwined association in homooligomers from the Protein Data Bank (PDB) (24)

In this review, we summarize the salient findings of these studies on the different categories of intertwined associations and their properties and highlight the insights gained into the physical and biological roles of these associations. In addition, our current understanding of 3D domain-swapped systems is reexamined in the context of the wider landscape of intertwined homooligomers, with a special focus on the mechanistic aspects that underlie intertwined self-association processes in proteins.

The global landscape of intertwined homomer assemblies

To chart the full landscape of intertwined protein assemblies in known protein structures (22), high-resolution x-ray structures and solutions structures of single proteins were retrieved from the 2011 release of the PDB. For each structure all subunit interfaces in the crystal, or the solution study, were examined and grouped into distinct associations. These associations were then classified into one of three possible quaternary assembly modes: dimeric, oligomeric, or polymeric (Fig. 1 *A*) as described in (22). An objective procedure (22) was applied to partition each distinct subunit association into structural domains (25,26). When these domains included residues from both subunits, they were termed noncontiguous structural domains (NCSD) and the assembly was considered as intertwined. The precise pattern of intertwining was defined by the swapping profile (Fig. 2). This profile indicates which residues are exchanged, swapped, between the subunits and defines the boundary between

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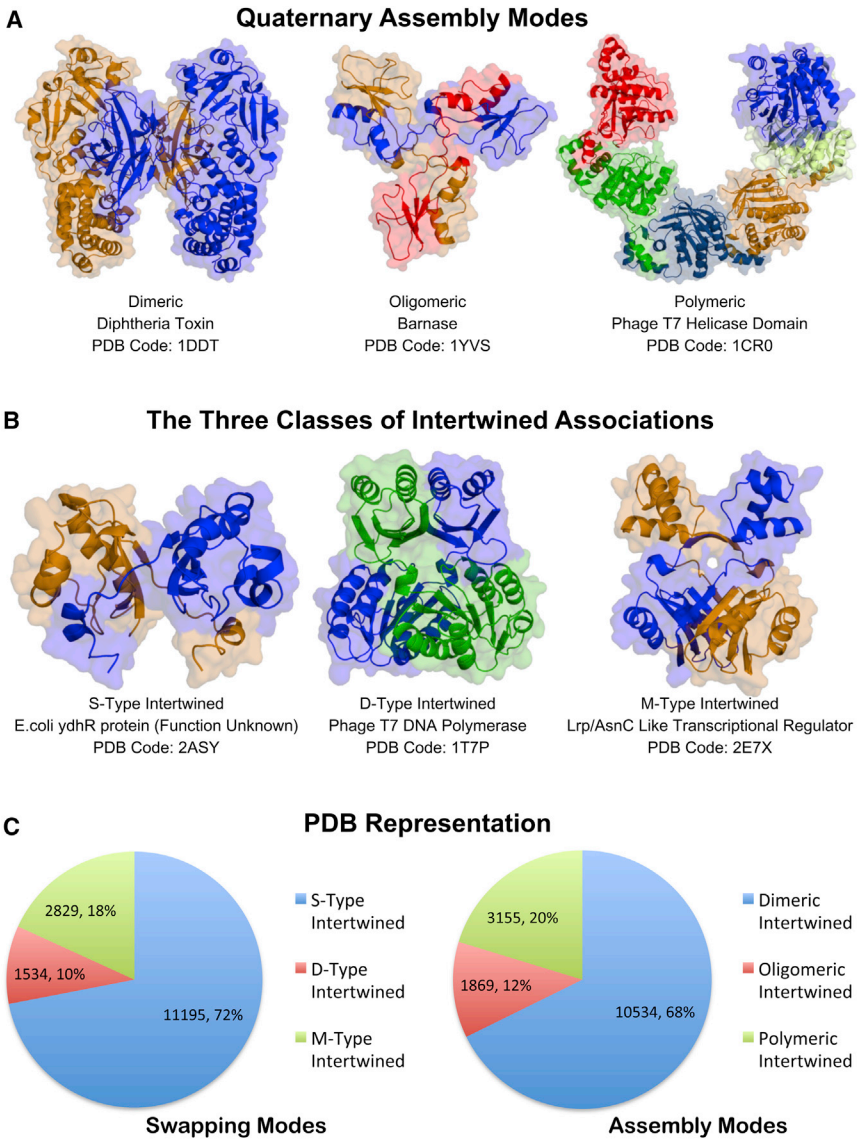

FIGURE 1 The quaternary assembly modes and classes of intertwined associations. (*A*) Examples of intertwined homomers in each of the three quaternary assembly modes: dimeric, oligomeric, and polymeric. The homomer assembly modes were derived from the crystal contacts in the PDB entries as described in (22). Each subunit is depicted by a cartoon representation of the polypeptide backbone and is shown in a different color. This enables one to see the portions of each subunit that reach toward the neighboring subunit and interact more closely with it than with its own chain. (*B*) Examples of homomers in each class of intertwined associations: S-type, comprising mostly small single-domain subunits that exchange polypeptide segments that do not correspond to structural domains; D-type, comprising multidomain subunits that exchange structural domains, and M-type comprising associations featuring a complex intertwining pattern, where subunits may exchange both segments and domains, or multiple noncontiguous segments. The cartoon representation of each subunit is depicted in a different color to highlight the portion of the chains that interact with the neighboring subunit. (*C*) Representation of the different intertwined classes and assembly modes in the PDB (22).

the swapped and nonswapped segments, referred to as the swapping hinge. Intertwined associations that exchange very short segments ($\leq$5 residues) were not considered in the downstream analyses.

Table 1 lists the total number of structures and associations identified for each of the three quaternary assembly modes, and the fraction of these that are intertwined (22) (for the full data set of intertwined associations see the Supporting Material). It reveals that intertwined associations are found in 24.3% of the analyzed structures, and are hence overall quite common. The inclusion of nonphysiological crystal contacts in the analysis contributes little to this result, as the incidence of intertwining was found to be even higher (~36%) in associations predicted to form under physiological conditions (27), whereas only 7.3% of the remaining nonphysiological crystal interfaces form intertwined assemblies (dimeric or oligomeric). Furthermore, quite strikingly over two-thirds of all dimers solved by NMR spectroscopy were intertwined. Taken together these observations underscore the common nature of intertwining and suggest that it may have a meaningful physiological role.

The different classes of intertwined associations

Intertwining covers a very diverse landscape of associations that differ in the length of the exchanged segments, in how deeply these segments integrate into the neighboring subunits, and the conformations they adopt. These segments may comprise chain ends devoid of local structure, elements of secondary or super secondary structure, protein loops, or represent complete structural domains.

To identify trends that can inform on the likely physical and functional roles of intertwining the association

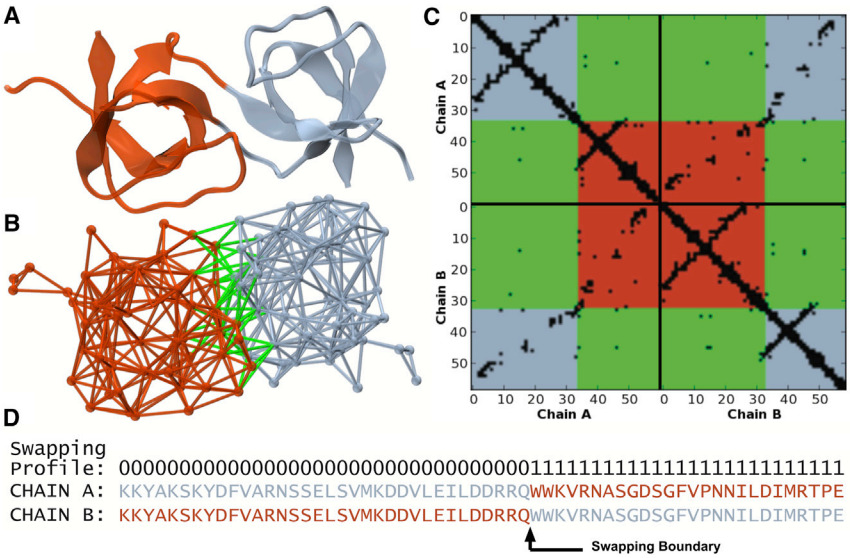

FIGURE 2 Identifying intertwined associations and defining their swapping profile (22). Ribbon diagram (*A*) and contact network representation (*B*) for an intertwined homodimer partitioned into two NCSDs. The shown example is the 3D domain-swapped SH3 domain (PDB: 1AOJ) (28). Orange and gray represent the two identified NCSDs. Each comprises chain segments from both subunits. In the contact network, vertices correspond to individual residues and edges to residue-residue contacts; contacts between the NCSDs (*green edges*); contacts within the corresponding NCSDs (*gray* and *orange edges*). (*C*) Contact map representing the same subunit association. Black dots represent Cα contacts, gray and orange colors delimit regions corresponding to contacts within each NCSD. Contacts between NCSDs are in green. (*D*) The polypeptide sequence and the computed swapping profile. Each residue subset (*orange* and *gray*) corresponds to separate polypeptide chains. A total of 26 residues, with values of 1 in the swapping profile are exchanged between subunits. The swapping boundary is defined as a discontinuity between NCSDs when following the polypeptide chain from the N- to C-terminus.

landscape was partitioned into three distinct classes denoted as D-, S-, or M- Type intertwined associations on the basis of several structural and physical properties of the structural elements exchanged between the subunits (22). Examples of homomers from each class and the relative sizes of the three classes are illustrated in Fig. 1, *B* and *C*. S-type intertwined associations represent the most common class (72%). These associations exchange chain Segments that do not correspond to structural domains and are located at the middle or the ends of a polypeptide chain, respectively. D-type associations are much less common (10%), and involve the exchange of discrete structural domains between multidomain protein subunit. The third M-type (M for mixed) class represents 18% of all intertwined association and is quite diverse. It features complex intertwining modes where structural domains as well as shorter chain segments are often both exchanged between the subunits.

The bulk of this review focuses on homomers of the S-type and D-type classes, as those were the ones most thoroughly investigated so far.

3D domain-swapped systems

3D domain-swapped systems as defined in the literature (13) represent a subset of intertwined homomers, where the portion of the polypeptide that is exchanged between subunits makes the same contacts in the oligomer as in the closed monomer, thus replacing intramolecular contacts by their intermolecular equivalents. These latter contacts form the so-called primary interface. In some of these systems part of the oligomer interface also includes new contacts not formed in the monomer, termed secondary interface (12,13).

3D domain-swapped systems can be readily identified by looking for monomeric versions of the same or a related protein whose isolated subunits are structurally similar to

TABLE 1 Intertwined Proteins in Different Assembly Modes

	All Associations		All Structures[a]		PISA-Predicted Complexes X-ray (<2.5Å)[b]		Solution Complexes NMR[c]	
Assembly Mode	Total	Intertwined (%)	Total	Intertwined (%)	Total	Intertwined (%)	Total	Intertwined (%)
Dimeric	46,146	10,534 (22.8)	29,091	9,343 (32.1)	26,695	9,194 (34.4)	377	248 (65.8)
Oligomeric	6,967	1,869 (26.8)	6,167	1,811 (29.4)	3,665	1,344 (36.7)	25	11 (44.0)
Polymeric	25,961	3,155 (12.2)	18,030	2,933 (16.3)	–	–	–	–
Total	79,074	15,558 (19.7)	56,411	13,442 (23.8)	30,360	10,538 (34.7)	402	259 (64.4)

Polymeric physiological associations were not considered, as PISA does not infer the native presence of such assemblies. The assembly mode (dimeric, oligomeric, polymeric) of a given association was determined from the crystal contacts in the PDB entry as described in reference (22).

[a]PDB structures containing the specified assembly type.

[b]Intertwined associations in assemblies predicted by PISA (27).

[c]Intertwined associations present in solution NMR structures.

the NCSDs of the intertwined homodimer. Carrying out this analysis yielded a total of only 176 3D domain-swapped systems among the larger set of 5415 S-type intertwined associations (Fig. 1 *B* and (22)). These include well-documented systems such as RNase-A, CD2, various human SH2 and SH3 domains, Cystatin, as well as many others. Applying the same analysis to D-type associations revealed only three instances of 3D domain-swapped systems: diphtheria toxin (13), βB2-crystallin (29), and calcium and integrin-binding protein (30). All three are well-documented instances of classical domain swapping. Diphtheria toxin was in fact the first such instance to be reported, which led to the proposal that 3D domain swapping may be an evolutionary mechanism for the formation of multidomain oligomers (13,31).

Four additional 3D domain-swapped systems were identified among extensively interleaved homodimers of the S-type category, which form globular assemblies that cannot be readily partitioned into identical NCSDs. They include several well-documented systems such as the B1 domain of streptococcal protein G (GB1) (32), and the fungal calcium-binding protein (33).

Thus, the so-called 3D domain-swapped systems represent a surprisingly small subset of the current repertoire of intertwined associations. Furthermore, the vast majority of these systems involve the exchange of contiguous polypeptide chain segments that do not correspond to structural domains (9,11,12). They consequently represent a subset of the class of S-type homomers defined in the objective classification of MacKinnon et al. (22). To avoid confusion it would be more appropriate to refer to these associations as 3D segment-swapped systems, and reserve the term 3D domain-swapped systems only to associations where the swapped portions actually represent structural domains.

The scarcity of 3D domain/segment-swapped systems is somewhat unexpected and is at odds with the roles that have been attributed to them early on in regulating and evolving protein association modes and function (12,13). Their low number is however likely related to the energetic properties of the molecular species and to how tightly folding and association are coupled in these systems, as will be discussed in the following sections.

Intertwining and protein stability

To investigate the possible roles played by intertwining in general, various physical and structural properties of the intertwined associations in homodimer and their interfaces were analyzed (22). The analysis was carried out independently for S-type and D-type homodimers, and the results were compared to those of control data sets of representative nonintertwined homodimers.

In general, S-type homodimers were found to have smaller subunits than those of their nonintertwined counterparts, but to feature much larger subunit interfaces, due to the nonglobular structure of the individual bound subunits. Furthermore, S-type interfaces are generally enriched in nonpolar residues relative to their nonintertwined counterparts. They have a significantly higher fraction of nonpolar interface atoms and are more densely packed than their nonintertwined counterparts. They also display a similar amino acid composition to that of strong dimeric interfaces (20), often referred to as permanent or obligate dimers (21). These trends were confirmed for sets of S-type and nonintertwined associations with similar interface size distribution, eliminating the possibility that the larger size of the S-type interfaces may have biased the observations.

These observations suggest that S-type intertwining, the most common type of intertwined associations, promotes homomer stability, and that the intertwining phenomenon itself makes a significant contribution to this effect. This contribution is not simply a consequence of the higher desolvation free energy mediated by the larger interface area of intertwined associations as compared to nonintertwined versions. Interface area calculations only consider the bound conformation of the subunits and therefore do not take into account the conformational adjustments that accompany association, which may be significant when the latter is intertwined, and can therefore lead to severely overestimating the change in accessible surface area that actually occurs upon binding. Therefore, the stability promoting character of the intertwined interfaces may also stem from their protein core-like properties and the entanglement of the polypeptide chains. The latter in particular would slow down the rate of dissociation and more generally indicate that binding and folding are coupled in these systems. Additionally, the stabilizing role of intertwining may underlie its higher incidence among smaller proteins, which may not adopt sufficiently stable structures otherwise. It also explains the prevalence of intertwining in proteins solved by NMR, which are in general much smaller than those solved by x-ray diffraction.

D-type intertwined homomers where multidomain subunits exchange structural domains, exhibit some similar trends but also important differences. Like S-type homomers, they comprise in general smaller subunits and smaller size domains than their nonintertwined counterparts, suggesting that here too intertwining contributes to protein stability. On the other hand, the amino acid composition, packing density, and the level of sequence conservation of interface residues in D-type homomers were found to be indistinguishable from those in their nonintertwined counterparts. Moreover, these properties are similar to those of weakly associated homodimers (20), indicating that folding of individual domains likely precedes association when either type of multidomain dimers (intertwined or not) are formed.

Intertwining in quaternary assemblies

Considering the particular properties of intertwined associations, an interesting question to ask is if these associations

play a role in shaping the quaternary assembly modes in which they participate. Surveying the incidence of intertwined associations in known quaternary assemblies from the PiQSi database (34), revealed that the incidence of intertwining of all categories (~70% of which are S-type) is significantly higher in interfaces with twofold symmetry (isologous), compared to other nonsymmetric (heterologous) interfaces (22). Symmetric interfaces are usually more stable, they tend to form first in the complex assembly process and are more highly conserved (4,35). These findings thus further confirm the link between intertwining and protein stability and suggest that intertwining may play a role in initiating the quaternary assembly process.

Homodimers of multidomain proteins and their intertwined version can be viewed as quaternary assemblies of four or more structural domains. These homodimers can be described as forming head-to-head (*H2H*) or head-to-tail (*H2T*) associations. These two types of association modes represent different topological relationships between the domains. Head-to-head dimers feature mainly intermolecular contacts between identical domains from the different subunits, whereas intermolecular contacts in head-to-tail dimers link dissimilar domains. On the basis of these intermolecular contacts the data set of nonredundant homodimers were classified into *H2H*, *H2T* association or a combination of both, termed Mixed (23). See Fig. 5 for a depiction of examples of the association of H2H and H2T.

H2H was found to prevail in nonintertwined multidomain homodimers, where it is twice as common as H2T associations. Interestingly, the majority of these H2H associations involve a single domain from each subunit, with the remaining domains contributing little to the overall dimer interface. In contrast, the majority of D-type intertwined homodimers were shown to form H2T associations, whereas the H2H arrangement is about an order of magnitude less frequent. Between 20–25% of both intertwined and nonintertwined homodimers adopted Mixed arrangements.

The origins of these differences in intermolecular domain-domain interaction topologies are not obvious, but may follow from the intramolecular domain-domain interactions being in general weaker in intertwined D-type dimers than in their nonintertwined counterparts, as will be further discussed in relation to the structural plasticity properties of these systems. As a result, subunits of D-type dimers, may engage both their domains in forming a twofold symmetric intermolecular interface. This interface comprises two copies of the same nonsymmetric (heterologous) intermolecular interface between a pair of dissimilar domains (A/B), implying in turn that only one new interface needs to evolve to produce the observed association. A different scenario would apply to nonintertwined homodimers, where stronger intramolecular interactions are maintained between the dissimilar domains in individual subunits. The most likely subunit association to evolve would then be by forming a new symmetric interface featuring intermolecular interactions between the same domains (A/A or B/B). Finding that only one domain from each subunit contributes to the dimer interface would follow from the smaller probability of simultaneously evolving two different stable interfaces (A/A and B/B) than a single one (A/A or B/B).

Intertwining and structural plasticity

The high degree of plasticity of proteins engaged in intertwined associations and particularly in 3D segment-swapped systems has been well documented. Studies on GB1 (the B1 domain of Staph Nucl. Protein G) revealed that upon mutation of specific residues, this protein forms respectively, a swapped dimer, a tetramer, or an amyloid fibril (32,36,37), earning this protein the reputation of protein contortionist. Three other examples are illustrated in Fig. 3. One is the classical case of the RNase-A family, whose member proteins were found to form three different intertwining modes (distinct swaps): two dimeric ones and one oligomeric (38–40). The two other examples are those of the cytochrome C and phosphotransferase HPr families.

Analyzing the full range of distinct S-type intertwined topologies or swapping modes adopted by families of related proteins, MacKinnon et al. found that the number of different swapping modes per family is limited and specific to the protein fold. This conclusion agrees with previous observations that pairs of folds interact in a few discrete ways (41) and with conclusions reached from computational analyses of the GB1 system (42). It suggests that sequence variations within a protein family only determines if one of the allowed modes may or may not form a stable intermolecular association. Yet, although limited, the set of allowed swapping modes enables the subunits to adopt a range of relative orientations and form different interfaces as highlighted for the RNase-A family (see Fig. 3 *B*). It has also been suggested that a large variety of oligomeric and polymeric arrangements may be accessible to these systems when the subunits are able to engage in two different intertwining modes simultaneously (16). For example, biochemical evidence was presented that in addition to the swapping modes identified in crystal structure (Fig. 3 *A*), RNase-A can form a polymeric arrangement in which both N- and C-terminal segments are exchanged between subunits (39).

The structural plasticity of D-type intertwined homomer, or more generally that of homooligomers of multidomain proteins, has been less well documented. Evaluating the degree of plasticity in these homomers is of particular interest if one aims at predicting the oligomeric state of multidomain proteins or the interactions between protein domains on the basis of observed association modes in known structures of related proteins (43–45). We (23) have undertaken this task by analyzing the extent to which domain-domain rearrangements, and the corresponding intermolecular interfaces in both D-type homodimers and in their nonintertwined

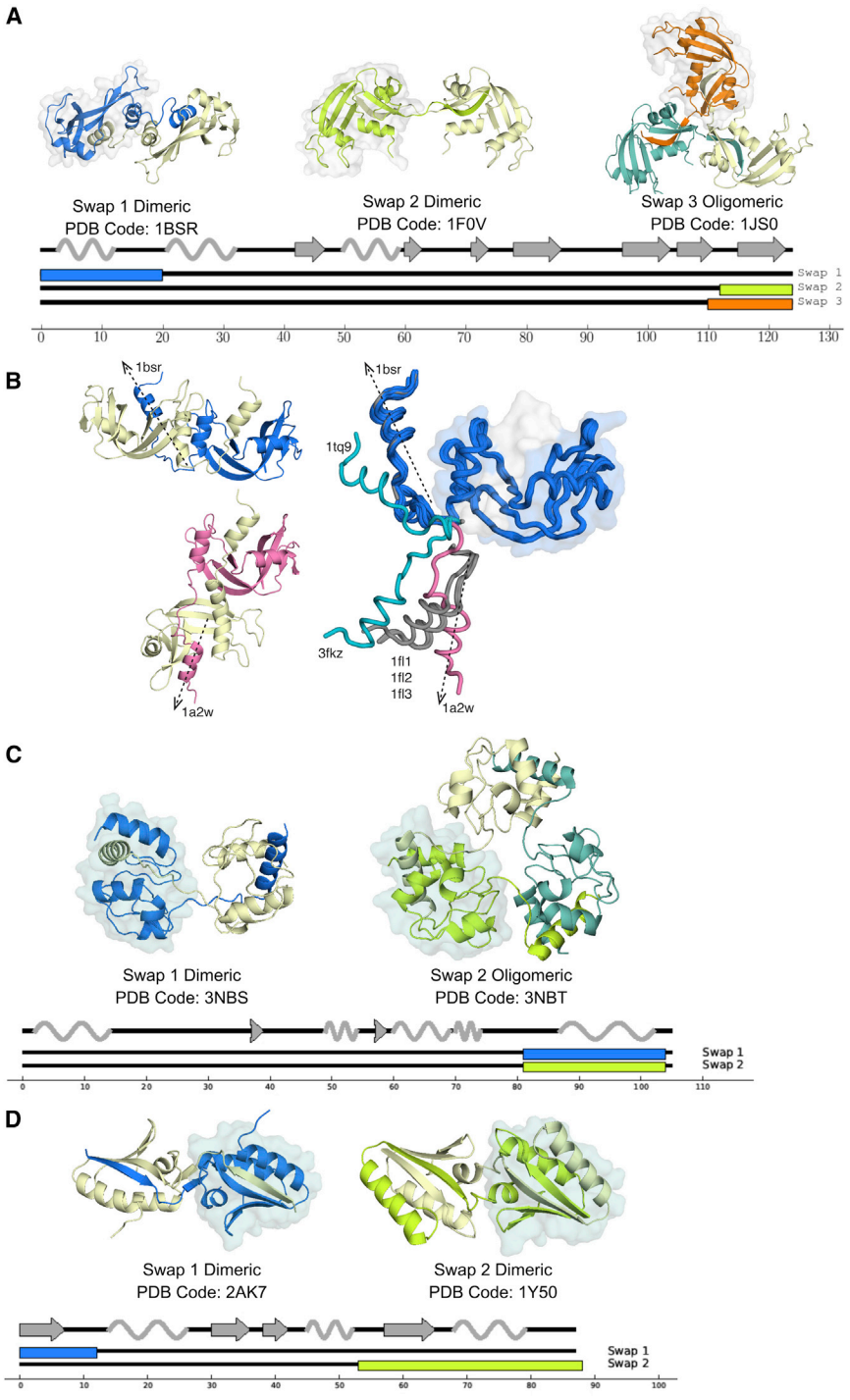

FIGURE 3 Structural plasticity in S-type intertwined associations. Distinct S-type swapping modes or swaps, in three families of related proteins. Swaps may differ in the length of the exchanged segment, or in the assembly mode of the corresponding homomer (dimeric, oligomeric, or polymeric). For each family are shown ribbon diagrams illustrating examples of the different swapping modes. Below these diagrams are depicted schematic representations of the secondary structure elements in family members and the swapping profile that corresponds to the depicted examples. The color codes of the swapping profiles match those of the ribbon diagrams. (*A*) Examples of the three swapping modes in members of the RNase-A family: two dimeric swaps and one oligomeric swap. (*B*) Illustrates the variability in the relative orientations between the swapped segment and the nonswapped portion in the different swaps of RNase-A. (*C*) Examples of the two distinct swapping modes in members in the cytochrome *C* family: a dimeric swap and an oligomeric swap. (*D*) Examples of the two distinct dimeric swaps in the phosphotransferase HPr sequence family. Each example is annotated by its RCSB PDB code.

counterparts, are conserved across structures of related proteins in the PDB.

This analysis revealed that proteins related to those forming D-type intertwined dimers are approximately four times more likely to adopt different intramolecular domain-domain arrangements (mostly different relative orientations), than those related to their nonintertwined counterparts (23). A sizable fraction of the D-type dimers exhibiting this variability (~42%), was found to have very closely related proteins (>90% sequence identity) with rather well conserved intermolecular interfaces adopting H2H or H2T intertwined topologies. This ability to conserve a particular dimer topology and intertwining mode, while undergoing changes in intramolecular domain-domain arrangements is likely favored by weaker domain-domain interactions within the subunits, although this aspect was not directly investigated.

At the other end of the spectrum, over half (~58%) of the analyzed D-type dimers had relatives with poorly conserved or nonconserved subunit interfaces. The subset with poorly conserved interfaces comprises more distant relatives that display limited variability in intramolecular domain-domain arrangements, suggesting that the poor interface conservation is probably related to protein sequence variations. The group with nonconserved interfaces comprises relatives, many of which comprised nonintertwined homomers, and monomeric proteins, whose interfaces differ significantly from that in the D-type variant. Somewhat surprisingly, these relatives display high sequence identity to the D-type variant, while sampling a wide range of intramolecular domain arrangements. In several of the proteins in this group the homodimer interface is disrupted in the relative, and reused to form a heteromeric complex with another binding partner (a protein or a small molecule) (23).

Thus, the plasticity in multidomain homodimers and the resulting variability in the corresponding interfaces and intertwining states, may be a confounding factors in predicting domain-domain interactions between or within proteins on the basis of interactions observed in related proteins, especially if the protein family comprises D-type intertwined variants. At the same time, the structural plasticity of these systems seems to be exploited by evolution to modulate function, as will be discussed below.

Mechanistic aspects of intertwining

S-type intertwined association: tight coupling of folding and binding

The more hydrophobic and densely packed interfaces of S-type homodimers assign the latter to the category of obligate protein complexes (21). Such complexes are defined as those where folding and subunit association is tightly coupled and consequently the independent subunits are not stable enough on their own. Investigating the process of association in these systems has therefore been challenging. On the other hand, the unfolding mechanism of homodimers has been extensively studied, revealing a range of behaviors, with some systems exhibiting complex unfolding kinetics, involving one or more intermediates, as reviewed by Rumfeldt et al. (46). Eleven S-type intertwined homodimers from the PDB were identified to have known unfolding pathways in the Rumfeldt review. Not unexpectedly, dimers shown to dissociate before unfolding are less extensively swapped than those with no monomeric intermediates (22).

The tight coupling of folding and association in S-type dimers also applies to the subset of these systems that are 3D domain-swapped, or rather 3D segments-swapped according to our nomenclature. This signifies that the corresponding monomeric species probably represent near native or nonnative folding intermediates with much lower stability than the intertwined dimeric form (32,47). The scarcity of 3D segment-swapped systems may therefore be attributed to the fact that the structure of such intermediates may not be readily characterized. The same reasons may contribute to the more frequent involvement of 3D segment-swapped systems (as opposed to domain-swapped systems) in misfolding and aggregation pathologies, which are usually fostered by mutations or change in conditions (pH or temperature) that significantly destabilize the monomeric state (48), as will be discussed below.

D-type intertwined association: binding is accompanied by conformational changes

With characteristics of weakly associated complexes, a D-type dimer would form by association of essentially folded subunits, although the latter may undergo significant conformational changes upon binding. Indeed, the interfaces of D-type homomer, although covering a wide range of values, are on average much larger than those of their nonintertwined counterparts and often significantly larger than those found in stable complexes (17,49). This property is usually indicative of conformational changes that take place upon association (50,51). Furthermore, the wide range of intramolecular domain-domain orientations in proteins forming D-type dimers suggests that these conformational changes would primarily involve domain rearrangements.

Assuming that subunits of D-type dimers would, to a large extent, fold on their own, it remains unclear why less than a handful of D-type homomer are found to represent 3D domain-swapped systems (even when homologs are considered). It might result from the larger evolutionary distance (e.g., a larger number of mutations, including length changes in the segments linking domains) separating the stable monomeric species from the dimeric form, making it less likely to encounter both forms among homologs in the PDB. It may also be due in part to a lower incidence of D-type intertwined associations (~10% of all intertwined associations) in comparison to that of S-type associations (~74%).

The swapping reaction: insights and hypotheses

Much of the insight accumulated over the years about the mechanism of intertwining was obtained from studies of a limited number of model systems amenable to mutagenesis experiment, folding/unfolding studies, structure determination (as reviewed in (11,12) and computer simulations (42,47)). All these systems are S-type intertwined homomers that undergo 3D segment swapping, and therefore many of the gained mechanistic insights are of general relevance to S-type intertwined associations. Two main questions were addressed by the studies on 3D segment-swapped systems. One concerned the sequence and structural features that foster swapping, and the other dealt with elucidating the detailed mechanism and energetics of the swapping process.

Sequence and structural features that impact swapping. Mutagenesis studies provided evidence that the sequence features and the length of the hinge loop, defined as the segment that links the swapped segments to the rest of the structure, can play an important role in fostering or hindering swapping. For example, shortening the hinge loop was found to favor swapping in systems such as in Staphylococcal nuclease (31), CD2 (52), or p13 suc1 (53), whereas in CI2, increasing the flexibility of such a loop through lengthening or mutagenesis was observed to favor the formation of a range of swapped variants (e.g., dimers, trimers, or high-order oligomers) (54). Proline residues in the hinge loop have been attributed the role of either favoring or disfavoring swapping (12,53). The importance of hinge prolines was also inferred from surveys of known protein crystal structures (22,55), and was suggested to result from the unique ability of prolines to adopt both the *cis* and *trans* peptide conformations, thereby allowing it to regulate monomer to intertwined dimer interconversion (56).

The more general role of the hinge loop was rationalized as resulting from the buildup or relief of strain caused in this loop by destabilizing interactions: introducing such interactions in a loop of the monomer, favors the formation of the swapped dimer, introducing them into the hinge loop of the swapped dimer shifts the equilibrium back to the monomeric state (12).

In other systems, sequence changes outside the hinge region were also found to modulate the monomer-dimer equilibrium. Mutations in the secondary interface of the CD2 swapped dimer were found to favor swapping by stabilizing the dimer state (57,58). Similarly, mutations of several residues in the core of the B1 domain of the immunoglobulin G binding protein from group G of *Streptococcus* (GB1), were shown to foster the formation of a swapped dimer or tetramer (36).

Finally, it has been argued that the protein topology, a very global feature of the protein, may be a determinant factor in enabling swapping and dictating the observed swapping modes (42), in agreement with the limited number of distinct S-type swaps found in families of related proteins in the recent surveys (22).

Proposed mechanism for the swapping reaction. Several mechanisms have been proposed for the swapping process, that of the monomer-to-dimer interconversion reaction. All were inspired by the observations that the rate of this interconversion is very slow. This was taken to imply that the two states are separated by a large energy barrier (9,53,59), often referred to as being of kinetic nature (see for example (12)).

In one of the earlier mechanisms, the transition state of the swapping reaction represents an open form of the protein structure, where the native conformation of the swapped segment is maintained but many native interactions are disrupted, significantly increasing the solvent exposure of the corresponding residues (9). Such scenario may fit the requirement for a high energy barrier in systems with some of the slowest interconversion rates, but seems to be too energetically unfavorable to be representative of most swapping reactions.

In a second category of mechanisms, swapping proceeds via a denatured state of the protein. Such mechanism has been extensively documents in p13suc1, based among other things, on the findings that mutations and experimental conditions that affect the folding rate of the proteins, also affect the monomer-to-dimer interconversion rate (60,61). A similar involvement of the denatured state in the swapping reaction was proposed for CD2 (12,58) and more recently for Cyanovirin N (62). In barnase however, swapping seems to involve the association of a folding intermediate rather than the fully denatured state of the protein (63).

A third category of mechanisms for the swapping reaction was proposed more recently on the basis massive computer simulations of detailed atomic models of the GB1 system (47,64). These simulations modeled the reaction of the monomer-to-dimer conversion of the quadruple GB1 mutant (L5V/F30V/Y33F/A34F), reported to be in equilibrium with a native-like monomer conformation (32) over timescales, which are significantly shorter than those reported for systems such as *wt* p13suc1. The monomer-dimer transition in this system involves the exchange of the C-terminal β-hairpin between the subunits (Fig. 4 *A*).

According to the proposed mechanism, swapping starts from one of the polypeptide chain ends (the C-terminus in case of GB1) and progresses by exchanging an increasing portion of the chain until a stable conformational state is reached. This exchange process does not involve unfolding, but a reptation-like mechanism (65), such that the conformational changes of individual monomers and their association are tightly coupled so as to minimize solvent exposure and maximize the total number of native contacts at all times. This orchestrated interplay acts to closely approximate the minimum energy path of the reaction (Fig. 4 *A*). The free energy profile of the reaction was computed for both the mutant GB1 that forms the swapped dimer and for the *wt* version that remains monomeric (Fig. 4 *B*). This profile indicates that the exchange reaction is a nonspecific process akin to encounter complex formation where the amino acid sequence plays a marginal role. The calculations also suggest that the mutations destabilize the GB1 monomer state, leading to the formation of an activated monomer species, which then engages in the swapping reaction (Fig. 4 *C*), in good agreement with the experimental study (32). Interestingly, the same mutations were also shown to foster nonnative intersubunit interactions, which contribute to the stabilization of the swapped dimer (47), not unlike the situation reported for CD2 (57,58).

The scenario for the swapping reaction proposed for GB1 and p13suc1 are both consistent with a tight coupling between folding and association in homomers engaged in S-type associations. They differ however in the level of

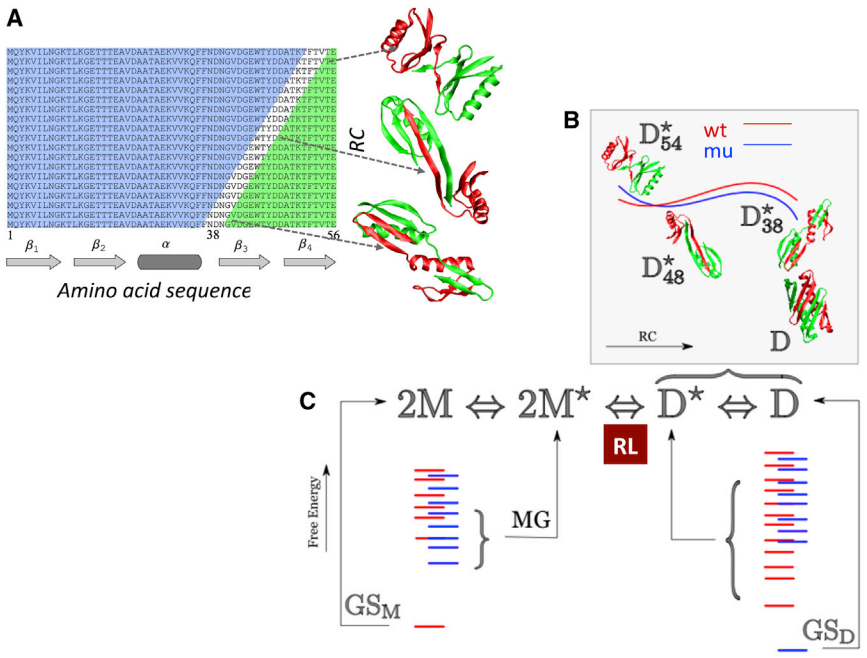

FIGURE 4 Mechanism and free energy profile of the GB1 monomer to intertwined dimer interconversion (47). (*A*) Progression of the GB1 swapping reaction along the reaction coordinate (RC). The GB1 secondary structure elements are depicted along the horizontal axis. The RC value is the residue number of the middle of the hinge regions (*white*), which moves along the chain as the reaction progresses from the C- to the N-terminus, such that the swapped chain portion (*green*) grows, whereas the unswapped portion (*blue*) decreases. Highlighted are RC = 54 (C-terminal tip of $\beta 4$ is swapped); RC = 48 (an intermediate with only $\beta 4$ is swapped); RC = 38 (entire $\beta 3-\beta 4$ hairpin is swapped). Three snapshots of swapped dimer conformations for these three RC values (indicated by the *arrows*) are displayed on the right side. (*B*) A schematic representation of the free energy profile of the swapping reaction as the RC progresses from the barely swapped activated dimer species (D^*_{54}) to the swapping intermediate (D^*_{48}) and then to the fully swapped activated dimer species (D^*_{38}) that subsequently relaxes to the ground state dimer species (D). (*C*) Schematic illustration of the energy levels corresponding to the ground state (GS) and activated species for the mutant and *wt* GB1 monomer and dimer states. The *wt* monomer (*red bars*, *left*) represents a unique stable conformation (M or GS_M), with a significant energy gap relative to other monomer conformations, whereas the GB1 mutant monomeric state (*blue bars*) features no significant gap. On the other hand, the quadruple mutant swapped dimer features an energy gap relative to all other dimer conformations, hence representing a unique stable conformation (D or GS_D), whereas the *wt* swapped dimer features no energy gap and thus adopts a less stable activated state (D*). RL denotes the rate-limiting step according to (47).

destabilization that the monomer needs to undergo to engage in the swapping reaction: is full unfolding required, or is a native-like activated state sufficient? Although the requirements could well differ between protein systems, and likely depend on the extent of intertwining and other parameters, Malevanets et al. argue that involvement of a fully unfolded species is unlikely, even for slow swapping but stable proteins such as *wt* suc1. Given the stability of *wt* suc1 (~7.2kcal/mol (66)) the population of the fully unfolded state would be too low to allow swapping at the rates observed in the experiments, whereas a more native-like activated intermediate would be sufficiently populated to allow swapping to proceed at the measured rates. Hence, the rate-limiting step of the swapping reaction in systems such as GB1 and suc1 may not be the rate for formation of the activated species, but the rate of their association (RL step in Fig. 5 *C*). Interestingly, the existence of an activated intermediate on the folding/unfolding pathway of suc1 has been reported (67), but the role attributed to it in the swapping process appears to differ.

Considering the notorious complexity of characterizing activated intermediates, it will likely be a while before the relevance of such intermediates for the swapping reaction in these and other systems can be solidly established.

Biological relevance of intertwining

Given the common incidence of intertwining, it is legitimate to ask if it plays other biological roles in addition to enhancing homomer stability. As far as S-type homomers and the corresponding 3D segments-swapped systems are concerned, the evidence for their biological role remains largely anecdotal and refers mainly to the regulation of protein function. 3D segment-swapped oligomers of RNase-A variants/mutants were observed to exhibit allosteric regulation of the enzymatic activity, not present in the monomer (68). *Pseudomonas putida* Glyoxalase I was shown to transition from an active swapped dimer to a weakly active less stable monomer in vitro upon addition of glutathione (69). Intertwining was also suggested to play a functional role in G-coupled receptors (70)

On the other hand, it is becoming increasingly plausible that S-type intertwining plays a role in protein misfolding and aggregation processes associated with amyloid fiber formation and prion diseases (14,38,59,71). Fibrils made by mixing two inactive variants of RNase-A, were shown to restore RNase-A activity through complementation between neighboring subunits (72), a demonstration that 3D segment swapping is at play. Studies on disease-associated proteins such as β2-microgolbulin (73), cystatin (74), and the prion protein (73) suggest that analogous complementation, involving segment-swapping, underpins fibril formation in these systems. The potential role of S-type associations in aggregation and fiber formation is also suggested by their relatively frequent involvement (~13%) in open-ended polymeric arrangements in structures of the PDB and by finding that some protein families are particularly prone to displaying several distinct intertwining modes, including

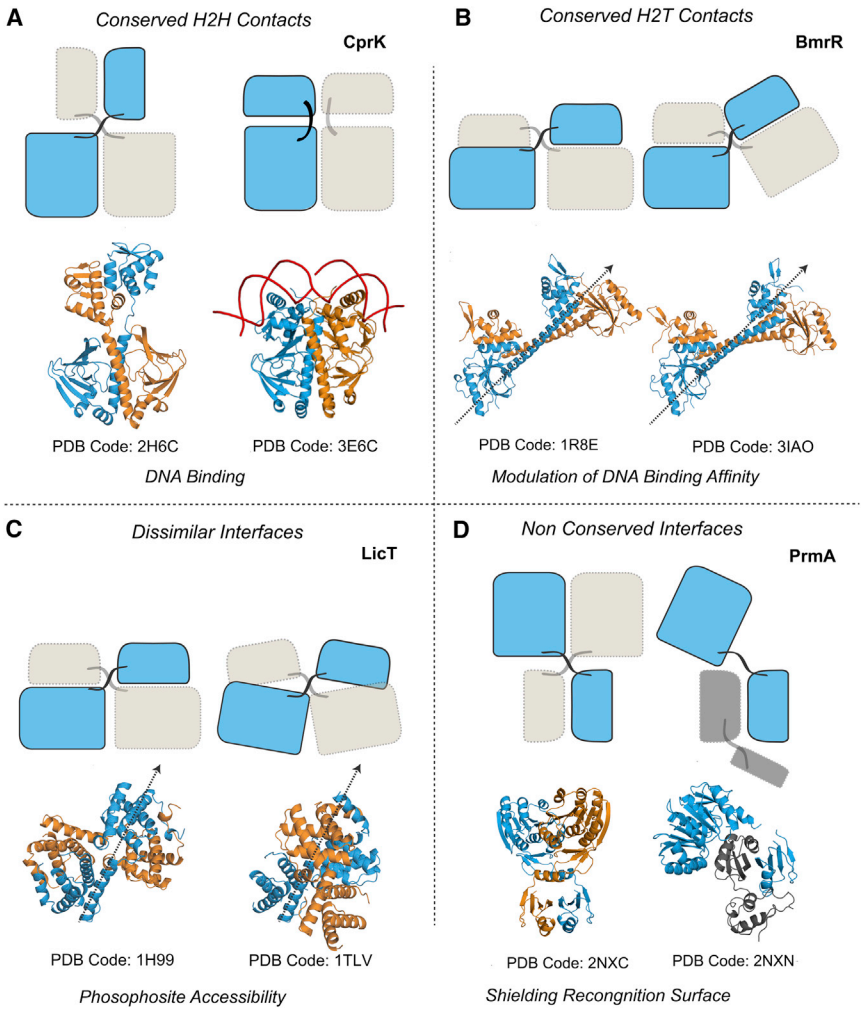

FIGURE 5 Structural plasticity of D-type intertwined dimers is exploited by evolution to regulate function. (*A*) Examples of inactive intertwined dimer (PDB: 2H6C) and active (DNA-bound) nonintertwined dimer (PDB: 3E6C) versions of the CprK transcriptional regulator protein, with conserved H2H contact topology. (*B*) Examples of the MerR transcriptional regulator proteins with conserved H2T topology, which display high (PDB: 1R8E) and low (PDB: 3IAO) DNA binding affinity. (*C*) Examples of the native inactive form (PDB: 1TLV) and the constitutively active form (1h99) of the PTS regulation domain from the LicT transcriptional antiterminator proteins, in which the H2T homodimer interface is poorly conserved. The inactive-to-active transition of the intertwined dimer involves a significant conformation change, which exposes regulatory phosphorylation sites that are buried in the native inactive dimer (77). (*D*) Examples of the *Thermus thermophilus* ribosomal protein L11 methyltransferase PrmA proteins with nonconserved dimer contacts. Shown is the apoenzyme, predicted to represent a D-type intertwined association (PDB: 2NXC), and the monomeric form bound to its substrate, the L11 protein (PDB: 3NXN). Examples in (*A*)–(*C*) represent cases where related homomers have different conformations, but a conserved quaternary state. In (*A*) and (*B*), a significant patch of the subunit interface is well conserved, whereas in (*C*) it is only partially conserved, although still maintaining an intertwined arrangement. (*D*) Shows an example where the relative is monomeric and hence the subunit interface of the dimer is completely disrupted.

open-ended assemblies. Such versatility is not very common, but was nevertheless observed in ~15% of the families that contained at least one S-type intertwined homomer (22). It cannot be ruled out that these properties, and particularly the non-negligible propensity to forming open-ended polymeric arrangements, may also play a functional role in the cell.

An altogether different picture can be drawn for D-type intertwined associations. The modular structure and the plasticity of the domain arrangements in these associations enables the corresponding homomers to adjust the spatial positions of key recognition surfaces, while maintaining or breaking their symmetric arrangement, a property that seems to be exploited by evolution to regulate function.

Our survey revealed indeed that a majority of the D-type homodimers with conserved H2H topology are DNA binding proteins involved in transcriptional regulation (22). One such system, the CprK transcriptional regulator protein, has 10 different closely related dimeric versions with well-conserved H2H contacts, but rather diverse intramolecular domain orientations corresponding to active and inactive versions of the protein (see Fig. 5 *A*). Another example from D-type dimers with conserved H2T arrangements is that of the MerR family transcriptional regulator, BmrR (Fig. 5 *B*). The D-type dimer and one of its close relatives correspond to the DNA bound (75) and unbound (76) structures of BmrR, which are believed to represent high- and low- affinity DNA-binding states, respectively.

Other examples from the category of D-type dimers with poorly or nonconserved subunit interfaces, illustrate the broader range of regulatory mechanisms afforded by the flexibility of these systems. One is the PTS regulation domain from the LicT transcriptional antiterminator (77,78). The two closely related variants of the protein adopt a similar H2T arrangement but the corresponding intermolecular interface is poorly conserved (Fig. 5 *C*). One variant corresponds to a constitutively active mutant, whereas the other is believed to represent the native, inactive state. It is the changes in the quaternary structure that seem to cause dimer activation by exposing regulatory phosphorylation sites, which are buried in the native inactive dimer.

In more extreme cases such as in *Thermus thermophilus* ribosomal protein L11 methyltransferase PrmA, the subunit interface of the D-type dimer is not conserved in another structure of the same protein or its homolog (see Fig. 5 *D*). Structures of this two-domain protein adopt five distinct intramolecular domain orientations. Three of these are observed in Apo-PrmA, which our analysis finds to be D-type intertwined dimers. All share the same H2H dimeric contacts (23) with an appreciable size interface (~1200 Å^2/subunit), but the author-assigned biological assembly mode is monomeric. The other two orientations correspond to heteromeric PrmA-L11 complexes, where the homodimer interface is lost and the catalytic domain is repositioned to act on different methylation sites of L11 (79). Remarkably, closer inspection reveals that the PrmA interface forming the heteromeric contact in the PrmA-L11 complex (PDB: 3CJT (80)), is the same as the homodimer interface detected in the apoenzyme structures (23), suggesting in turn that Apo-PrmA dimerization may act to shield the recognition surface from solvent in the absence of its substrate. This possibility was however, not considered by the authors. They may have incorrectly assumed Apo-PrmA to be a monomer, or their assumption was correct and the D-type dimer state deduced from the crystal contacts does not reflect the Apo-PrmA physiological state. These uncertainties highlight the challenge of determining the physiologically relevant assembly mode of a protein, especially from crystal structures. More generally, homodimers that undergo ligand-induced oligomeric state changes tend to be weaker, as suggested by their comparatively smaller interface area (23). This would facilitate other molecules to compete with dimer formation, and may pose a challenge for oligomeric state assignment.

CONCLUSIONS

We presented an overview of the landscape of intertwined associations in homooligomeric proteins. This overview integrated findings from recent comprehensive surveys of intertwined homomers in the PDB, with those from a significant body of experimental and computational analyses of specific systems. Until recently most of the available knowledge on intertwined homomers was limited to the so-called 3D domain-swapped systems, due in part to their likely involvement in disease-related aggregation phenomena. As highlighted here, 3D domain-swapped systems represent only a small subset of known intertwined homomers. Integrating our knowledge about these systems with insights gained on the broader landscape of intertwined homomers is therefore of great added value.

We show that this integration provides additional evidence on the role of intertwining in promoting homomer stability, and in initiating the assembly process of higher-order native oligomers. Although indirect, this evidence is in excellent agreement with previous findings on the contribution of dimer formation to protein stability (for review, see (2,81)). Indeed, proteins that undergo a single transition from native dimer to denatured monomer (two-state unfolding) were shown to include the more extensively intertwined S-type dimers (22). Such proteins feature unfolding free energy values in the range of 10–27 kcal/mol (81). These values are significantly higher than those of monomeric proteins and can in general be attributed to stabilizing contributions from intersubunit interactions, whose magnitude in turn depends on the characteristics of the subunit interface, such as its intertwined character.

Analysis of the different modes of intertwined associations (e.g., those that exchange segments (S-type) or structural domain (D-type)) and their domain-swapped equivalents, confirms certain similarities, but also uncovers significant differences. These differences, notably in the multidomain versus single-domain nature of the proteins involved, the properties of the subunit interfaces and overall structural plasticity, appear to impact their functional roles and the mechanisms underpinning the formation of the corresponding homomers.

The picture that emerges is that the vast majority of the so-called domain-swapped systems actually exchange segments between the subunits that do not correspond to structural domains. They therefore represent a subset, and a rather small one at that, of the S-type intertwined systems, with which they share most properties. An important shared property is the small size (and single domain architecture) of the proteins involved. Another is the permanent (or obligate) nature of these assemblies, entailing a tight coupling of folding/unfolding and association in these systems, as mentioned previously. However, the extent to which individual subunits need to fold or unfold to associate is unclear and probably varies across proteins. Although these features seem to privilege the involvement S-type intertwining in disease-related aggregation processes, the exact role it plays in forming or stabilizing nonnative oligomeric or polymeric aggregates is presently unclear (16). At the same time, there is currently little evidence for a native functional role of the S-type intertwined systems, save for promoting homomer stability.

In comparison, D-type intertwined associations between multidomain proteins are altogether different systems. They represent weaker associations, in which folding and binding are loosely coupled. The corresponding homomers display a surprising degree of structural plasticity, expressed mainly by the wide range of domain-domain orientations adopted by the same protein in different contexts or in its close homologs. This plasticity seems to be exploited by evolution to regulate the functional properties in these proteins. In proteins where intertwining is conserved, structural plasticity often serves to modulate DNA recognition, which requires structural adjustments while maintaining a symmetric relationship between the recognition interfaces. In other systems, like that of the ribosomal protein L11

methyltransferase PrmA, where intertwining is not conserved, structural plasticity enables the transition from an intertwined inactive homomer to an active heteromeric complex in the presence of the L11 substrate. An unexpected result has been the near absence of 3D domain-swapped systems in this category of intertwined associations, with no new systems identified in addition to the three well-known cases of diphtheria Toxin, βB2-crystallin, and calcium and integrin-binding protein. Another intriguing finding has been, that although a sizable fraction of D-type associations in the PDB gives rise to open-ended polymeric assemblies in the crystal (23), so far at least, they do not seem to be associated with detrimental aggregation phenomena. It appears in fact, that large-scale pathological aggregation of fully native proteins, as opposed to aggregation involving some level of unfolding (as in S-type intertwining), is very rare, with the sickle cell hemoglobin fibers (82) as the only prominent example.

Several hypotheses underlying these various findings were discussed, but further work is clearly required to gain a fuller understanding of these systems and the role that intertwining plays in cellular function and disease.

SUPPORTING MATERIAL

Supporting Materials and Methods and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(15)00821-8.

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