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Review

Unusual biophysics of intrinsically disordered proteins ☆

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ABSTRACT

Research of a past decade and a half leaves no doubt that complete understanding of protein functionality requires close consideration of the fact that many functional proteins do not have well-folded structures. These intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered protein regions (IDPRs) are highly abundant in nature and play a number of crucial roles in a living cell. Their functions, which are typically associated with a wide range of intermolecular interactions where IDPs possess remarkable binding promiscuity, complement functional repertoire of ordered proteins. All this requires a close attention to the peculiarities of biophysics of these proteins. In this review, some key biophysical features of IDPs are covered. In addition to the peculiar sequence characteristics of IDPs these biophysical features include sequential, structural, and spatiotemporal heterogeneity of IDPs; their rough and relatively flat energy landscapes; their ability to undergo both induced folding and induced unfolding; the ability to interact specifically with structurally unrelated partners; the ability to gain different structures at binding to different partners; and the ability to keep essential amount of disorder even in the bound form. IDPs are also characterized by the "turned-out" response to the changes in their environment, where they gain some structure under conditions resulting in denaturation or even unfolding of ordered proteins. It is proposed that the heterogeneous spatiotemporal structure of IDPs/IDPRs can be described as a set of foldons, inducible foldons, semi-foldons, non-foldons, and unfoldons. They may lose their function when folded, and activation of some IDPs is associated with the awaking of the dormant disorder. It is possible that IDPs represent the "edge of chaos" systems which operate in a region between order and complete randomness or chaos, where the complexity is maximal. This article is part of a Special Issue entitled: The emerging dynamic view of proteins: Protein plasticity in allostery, evolution and self-assembly.

1. Introduction

Recently, intrinsically disordered proteins (IDPs) and intrinsically disordered protein regions (IDPRs) gained significant attention of the researchers primarily due to the fact that the existence of such biologically active molecules without unique 3D-structure clearly contradicts to the traditional "one protein–one structure–one function" paradigm [1–7]. Before they were finally recognized as a unique and important extension of the protein kingdom, these highly dynamic proteins with important biological functions were discovered and rediscovered multiple times. The complex and lengthy pathway to recognition left a wide trail of terms used for the description of these proteins, which were depicted as floppy, pliable, rheomorphic [8], flexible [9], mobile [10], partially folded [11], natively denatured [12], natively unfolded [3,13], natively disordered [6], intrinsically unstructured [2,5], intrinsically denatured, [12] intrinsically unfolded [13], intrinsically disordered [4], vulnerable [14], chameleon [15], malleable [16], 4D [17], protein clouds [18], and dancing proteins [19], among several other terms.

This trail of terms can be considered as "prehistory" of intrinsic disorder. For early researchers, it was clear that biologically active but non-folded proteins are different from "normal" globular, transmembrane, and fibrous proteins. For a long time, each such a protein was considered as an exception from a general rule, where unique sequence defined unique 3D-structure that was crucial for unique function. The multitude of terms used to describe IDPs in past not only reflects the creativity of researchers but also indicates difficulties they faced while trying to find an appropriate way of portraying these proteins. Although none of the terms proposed for defining biologically active proteins without unique structure is perfect, the term "intrinsically disordered protein" is currently used more often than any other terms. The qualifier "disordered" is always used in the context of a comparison between a single, ideal, well-defined situation, and the actual situation which we consider to be only one of many different possibilities, none of which deserves to be singled out [20].

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Systematic bioinformatics analyses clearly indicated that IDPs are highly abundant in any given proteome [4,7,21–23]. Therefore, these proteins have moved from a category of obscure and rare exceptions to the novel class of proteins, whose functionality is determined by the lack of stable structure, and which are very common in nature. Functions of IDPs/IDPRs are complementary to functions of ordered proteins and domains [4,24,25], with disordered proteins being typically involved in regulation, signaling and control pathways [26,27]. Because of their unique functionality, dysfunctions of IDPs are known to be associated with various human diseases, such as cancer, cardiovascular disease, amyloidosis and neurodegenerative diseases [28].

One of the goals of this review is to put together a set of old and new concepts (such as the ideas that IDPs are characterized by high spatiotemporal heterogeneity; that they have rough and relatively flat energy landscapes; that IDPs might contain foldons, inducible foldons, semi-foldons and non-foldons; that some ordered proteins might have unfoldons, i.e., regions that have to undergo order-to-disorder transition in order to make protein active; that globally, there is a phenomenon of dormant disorder, where some proteins are inactive when they are ordered, and become activated when they become more dynamic; and that IDPs can be considered as the "edge of chaos" systems) that would inevitably provoke disputes and therefore would initiate new studies. I do realize that some of the concepts are not well-developed and some might be naïve. However, they are present here since they can be clearly taken as "food for thoughts".

2. Unusual biophysics of IDPs

2.1. Behold the root: Peculiarities of the amino acid sequence provide an answer to the question "To fold or not to fold?"

IDPs/IDPRs are different from ordered proteins and domains already at the level of their amino acid sequences. In fact, the sequence peculiarities define both the ability of ordered proteins to fold and the ability of IDPs to stay non-folded. Therefore, the well-known Anfinsen's dogma for foldable proteins stating that information dictating the native fold of protein domains is encoded in their amino acid sequence [29] and therefore at optimal conditions (temperature, solvent concentration and composition, etc.), the native structure represents a unique, stable and kinetically accessible minimum of the free energy, can be converted into similar statement for IDPs/IDPRs, namely, information dictating the lack of folded structure in disordered proteins is encoded in their amino acid sequence. In other words, the absence of rigid structure in IDPs may be somehow encoded in the specific features of their amino acid sequences [1,3,4,7,25,30]. In agreement with this hypothesis, the unusual amino acid sequence compositions were observed for some IDPs, which in extreme cases were unfolded at the physiological conditions due to the presence of numerous uncompensated charged groups (often negative) that defined a high net charge of these proteins at neutral pH [13,31,32], and a low content of hydrophobic amino acid residues [31,32]. In fact, based on the comparative analysis of 275 natively folded and 91 natively unfolded proteins (i.e., proteins which at physiologic conditions have been reported to have the NMR chemical shifts of a random-coil), and/or lack significant ordered secondary structure (as determined by CD or FTIR) it was revealed that the combination of low mean hydropathy and relatively high net charge represents an important prerequisite for the absence of compact structure in proteins under physiological conditions [3]. The resulting charge-hydropathy (CH) plot method can distinguish ordered and disordered proteins based only on their net charges and hydropathies [3]. From the physical viewpoint, such a combination of low hydropathy with high net charge as a prerequisite for intrinsic disorder makes perfect sense: high net charge leads to charge-charge repulsion, and low hydropathy means less driving force for protein compaction. In other words, these features are characteristic for highly disordered IDPs with the coil-like (or close to coil-like) structures, which obviously represent only a small subset of the entire IDP realm.

At the more detailed level, there are numerous differences in the amino acid compositions of ordered and disordered proteins and many IDPs clearly share at least some common sequence features [1,33]. Here, IDPs/IDPRs are significantly depleted in so-called order-promoting residues that include bulky hydrophobic (Ile, Leu, and Val) and aromatic amino acid residues (Trp, Tyr, and Phe), which would normally form the hydrophobic core of a folded globular protein, and also possess low content of Cys (which is often contribute to the protein conformational stability via the disulfide bond formation or coordination of different prosthetic groups) and Asn residues. On the other hand, IDPs/IDPRs were shown to be substantially enriched in disorder-promoting, amino acids, that were polar Arg, Gly, Gln, Ser, Glu, and Lys, and hydrophobic, but structure breaking Pro and hydrophobic Ala [4,7,24,34–36]. Based on the ability of amino acids to promote order and disorder, a special amino acid scale was introduced that was able to discriminates between ordered and intrinsically disordered proteins reasonable well [37]. Here, amino acids were ranked according to their capabilities to promote order or disorder resulting in the following scale (where amino acids are arranged from the most order-promoting to the left to the most disorder-promoting to the right): W, F, Y, I, M, L, V, N, C, T, A, G, R, D, H, Q, K, S, E, P [37].

It is clear that the amino acid sequence peculiarities of IDPs can be blamed for the unusual and unexpected behavior of IDPs. Many early IDP researchers were stunned by the peculiar features of these mysterious then members of the protein kingdom. On a personal note, my journey to the IDP field started when one sunny day, an excited colleague of mine appeared in the lab shaking a tube with a sample in his hand and shouting: "I have a funny protein here. I cannot measure its concentration. And it is extremely stable – I can boil it for a few days, but as soon as I am bringing temperature down it shows 100% activity." That funny protein was prothymosin α. Fig. 1 shows that the unusual behavior of this protein is definitely determined by its amino acid sequence. It does not have any aromatic residues and cysteins. Therefore its concentration cannot be measured spectroscopically. 64 of 111 residues in this protein have charged side groups (there are 19 D, 35 E, 2 R, and 8 K residues), whereas overall content of hydrophobic residues (L, I and V) is very low [38]. Based on this amino acid composition, it was not a big surprise to find that prothymosin α behaved as a highly disordered coil-like chain – you cannot expect that highly charged polypeptide (60% polyE/D) will have a strong tendency to fold under the physiological conditions. This luck of stable structure also explained extreme thermal and acid stability of prothymosin α — you cannot break what is already broken [38].

Differences between ordered proteins and IDPs can be further elaborated by going to the very subtle levels. However, this exercise is outside the scopes of this review. The important message is already obvious from the observations listed above, namely, sequences encoding IDPs/IDPRs are very different from sequences encoding ordered proteins and domains. In fact, these two types of sequences are so different that they can be discriminated reasonably well by numerous computational tools, where comparing and combining several predictors can provide additional insight regarding the predicted disorder [39–46]. This clearly indicates that IDPs are new and specific entities in the protein kingdom.

2.2. Sequential, structural, and spatiotemporal heterogeneity of IDPs

2.2.1. Sequence space and sequence heterogeneity of IDPs

A typical estimate of the size of the protein sequence space is 20^{100} ($\sim 10^{130}$) for a protein of 100 amino acids in which any of the normally occurring 20 amino acids can be found [47]. For a long time, discussion

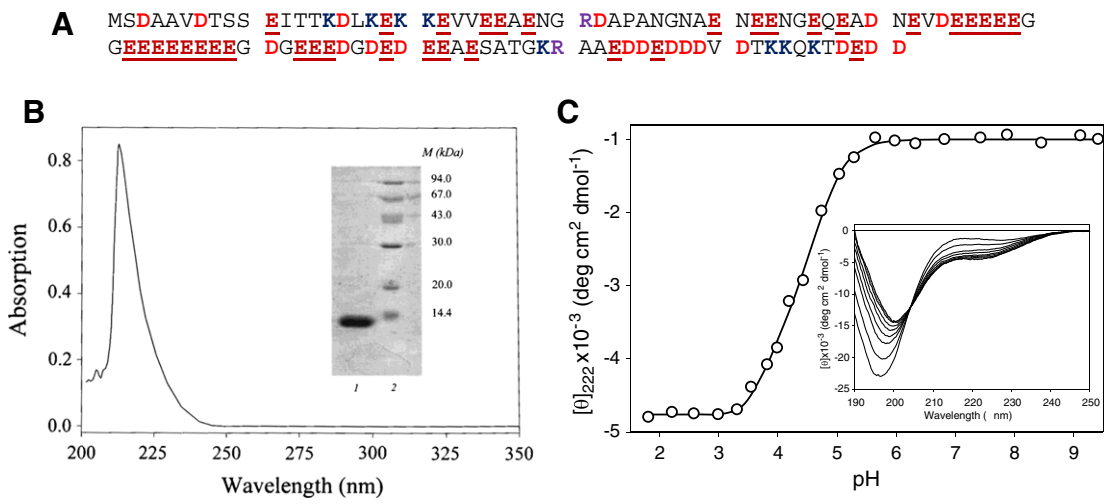

Fig. 1. "Funny" protein prothymosin α. A. Amino acid sequence of human prothymosin α. This protein contains 19 D residues (bold red characters), 35 E residues (bold dark red underlined characters), 2 R residues (bold purple characters), and 8 K residues (bold blue characters). There are no aromatic residues and cysteins. The content of aliphatic residues is very low. B. Some structural properties of the recombinant human prothymosin α. UV absorption spectrum and SDS–PAGE (inset) of the protein are shown. Due to the absence of aromatic residues in the human protein, there is no characteristic absorption peak at 260–280 nm. Electrophoresis was carried out using 12% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 (Pharmacia), washed off, and dried. Slot 1, purified human prothymosin α; slot 2, molecular mass markers (Pharmacia); M, molecular mass of proteins-markers (in kDa). Cell path length for the absorption measurements was 10 mm. C. Effect of pH on the far-UV CD spectrum of human recombinant prothymosin α monitored as the pH dependence of the $[\theta]_{222}$ value. Inset represents the far-UV CD spectra of the protein measured at various pH values.

on the available sequence space for biologically active proteins was misled by the assumption that a biologically active protein needs to have a unique folded structure in order to be functional. Obviously, "foldable" sequences represent a small portion of the entire available sequence space. There were several attempts to evaluate the size of foldable sequence space. For example, based on the simple theoretical models and evaluation of existing variation of protein sequences it was suggested that all 20 residues are not necessary for protein to fold and that the actual identity of most of the amino acids in a protein is irrelevant [48–55]. Therefore, the actual number of different amino acid residues in a given foldable sequence (i.e., the size of the amino acid "alphabet" essential for protein folding) can be dramatically reduced [47]. This simplified folding code based on the simplified amino acid "alphabet" can dramatically shrink the available sequence space. For example, the size of sequence space can be reduced to 2^{100} ($\sim 10^{30}$) and 2^{33} ($\sim 10^{10}$) based on the hypothesis that only two types of amino acid were needed to form a protein structure, hydrophilic and hydrophobic, and that the most close attention should be paid to the definition of only the surface of the protein [56]. It was also pointed out that the assumption that a protein chain needs to be at least 100 amino acids in length to be functional is not a general rule since many proteins are modular and contain domains of as few as approximately 50 amino acids thereby reducing the sequence space to 20^{50} or $\sim 10^{65}$ [57]. Combining these two hypotheses that two types of amino acids are needed for definition of the surface of the 50 residue-long foldable protein further reduces sequence space to 2^{50} ($\sim 10^{15}$) and 2^{17} ($\sim 10^{5}$), respectively. Therefore, a reduced alphabet of amino acids should be sufficient for producing all the protein folds (which count to a few thousand discrete folds, [58]) and potentially providing a scaffold capable of supporting all protein functions [47].

Of course, in this reasoning, an important simplification was made, namely, it was assumed that the space of sequences encoding for IDPs can be ignored since such proteins are assumed to usually fold upon performing their function, and therefore the distinction between spontaneously foldable proteins and IDPs is not important [47]. However, it is recognized now that many biological functions ascribed to IDPs do not require protein folding [4,5,7,25,30,59–61]. Disorder-based interactions are very different from interactions in which ordered proteins are engaged, since quite often IDPs will form fuzzy complexes, in which they will preserve significant amount of disorder [62,63]. Even for IDPs that do noticeably fold at binding, the situation is not equivalent to that of ordered proteins, since the folding code IDPs is diluted and since IDPs are depleted in stabilizing intramolecular interactions. In fact, a portion of folding code (and some time a significant part of it) that defines the ability of ordered proteins to gain spontaneously a unique biologically active structure is missing for IDPs. This missing portion of the folding code (or a part of it) can be supplemented by binding partner(s). As a result, a key difference between structured and disordered proteins is that the former fold first and then bind to their partners while that latter remain unfolded until they bind their partners. Based on these observations, it is reasonable to assume that due to the removal of restrictions posed by the need to gain ordered structure spontaneously, the sequence space of IDPs (at least those which do not completely fold at binding) is noticeably greater than that of foldable ordered proteins. Assuming that all the amino acids could be important for the IDP function, we are ending up with the original estimation, 20^{100} ($\sim 10^{130}$) for a protein of 100 amino acids. Even for proteins that are similar to "funny" prothymosin α (i.e., proteins that do not have major order-promoting residues C, W, F and Y), the potentially available sequence space is gigantic, 16^{100} ($\sim 10^{120}$).

Fig. 2 gives further support to the idea on the large sequence space of IDPs by providing a CH plot. Here, ordered proteins and extended IDPs are shown by blue squares and red circles respectively. The area accessible to sequences encoding ordered proteins are shown as a light cyan triangle, whereas the area accessible to sequences encoding IDPs is depicted as light pink pentagon. These two areas are defined by two boundaries, the known boundary separating compact proteins and extended IDPs ($\langle R\rangle = 2.785\ \langle H\rangle - 1.151$, where $\langle R\rangle$ and $\langle H\rangle$ correspond to the absolute mean charge and mean hydropathy, respectively; [3]), and the boundary showing logical limits of the CH-space ($\langle R\rangle = 1.125 - 1.125\ \langle H\rangle$). This boundary was evaluated for a series of hypothetical polypeptides containing different proportions of Ile (which is according to the Kyte and Doolittle scale is the most hydrophobic residue with the normalized hydropathy of 1 [64]) and a charge Asp (which is characterized by the normalized Kyte and Doolittle hydropathy of 0.1111 [64]). Comparison of the pink and cyan areas in Fig. 2 shows that the CH-space accessible to the compact proteins is >3-fold smaller than the CH-space accessible to the extended IDP. In reality, this difference is even bigger, since

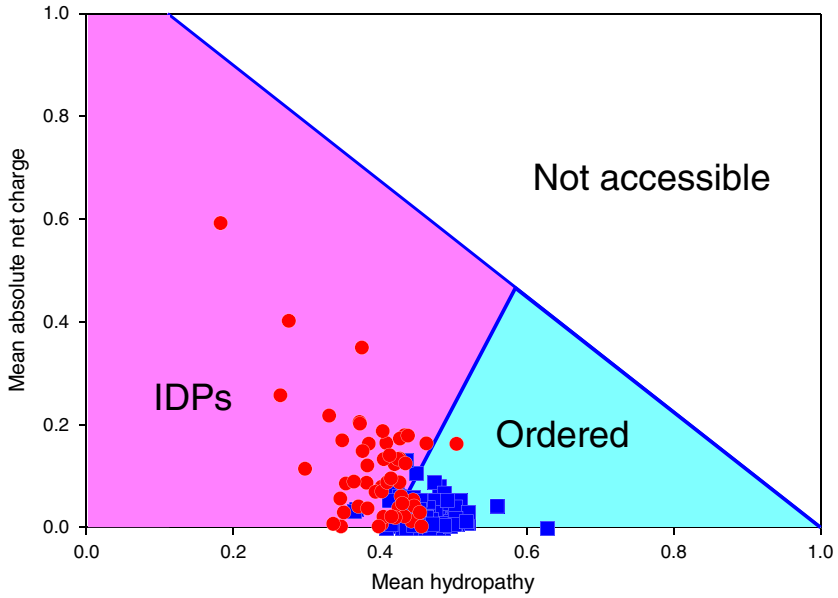

Fig. 2. Evaluation of the charge-hydropathy space available for compact proteins (blue squares) and extended IDPs (red circles). The area accessible to sequences encoding compact proteins is shown as a light cyan triangle, whereas the area accessible to sequences encoding IDPs is depicted as light pink pentagon. These two areas are defined by two boundaries, the known boundary separating compact proteins and extended IDPs ($<R> = 2.785 <H> - 1.151$, where $<R>$ and $<H>$ correspond to the absolute mean chare and mean hydropathy, respectively [3]), and the boundary showing logical limits of the CH-space ($<R> = 1.125 - 1.125 <H>$).

proteins whose hydropathy in the normalized Kyte and Doolittle scale exceeds 0.7 are unlikely to be soluble. Therefore, the sequence space of extended IDPs is at least 5-fold greater than that of sequences coding for compact soluble proteins. It is also important to remember that CH-plot differentiates extended IDPs (that cannot gain compact conformation due to the strong Coulomb repulsion and weak hydrophobic attraction) and proteins with compact conformations ("molten globule"-like IDPs and well-folded ordered proteins) [3,40].

Now, a few words about the sequence-structure heterogeneity of IDPs should be added. From the view point of distribution of the structure coding potential, amino acid sequence of an ordered single-domain protein is relatively homogeneous, since the unique 3D-structure of this protein is defined by the interplay between all its residues. Multidomain ordered proteins are a bit more heterogeneous in this respect, since in addition to the regions encoding well-folded domains they might contain regions encoding flexible linkers. IDPs are obviously on another side of the spectrum. In fact, many IDPs and functional IDPRs possess complex "anatomy" (they contain multiple, relatively short functional elements), which contributes to their unique "physiology" (an ability to be involved in interaction with, regulation of and control by multiple structurally unrelated partners) [65]. Given the existence of multiple functions in a single disordered protein, and given that each functional element is typically relatively short, alternative splicing could readily generate a set of protein isoforms with a highly diverse set of regulatory elements [65]. Overall, the complex "anatomy" of IDPs is determined by the extremely high level of their sequence heterogeneity, which is further increased due to the ability of a single IDPR to bind to multiple partners gaining very different structures in the bound state [66]. Therefore, a sequence of an IDP represents a very complex mosaic and typically contains a multitude of elements coding for potentially foldable, partially foldable, differently foldable or not foldable at all protein segments.

2.2.2. Structural heterogeneity of IDPs or spectroscopy of intrinsic disorder

Fig. 3 represents the stages in understanding of the structural heterogeneity of IDPs. First, IDPs were ignored and all (or almost all) biologically active proteins were assumed to have rigid and unique 3D structures (Fig. 3A). Obviously, the used in Fig. 3A analogy of an ordered protein with a rock is an oversimplification and over-exaggeration of the reality. In fact, although the importance of the large-scale protein flexibility has been underestimated in the past, it would be definitely wrong to assume that the dynamic nature of proteins was not known before IDPs. Then, the concept of functional disorder was introduced. For many researchers even now, the intrinsically disordered protein means completely structure-less entity, a kind of cooked noodles (Fig. 3B). However, it was recognized almost immediately that IDPs/IDPRs could be crudely grouped into two major structural classes, proteins with compact and extended disorder [3,4,7,25,30]. According to this classification, IDPs can be less or more compact and possess smaller or larger amount of flexible secondary/tertiary structure. Therefore, functional proteins can be in any of three major conformations, ordered, molten globular, and coil-like, the so-called protein trinity model [4] (Fig. 3C). Next, based on the comprehensive analysis of available structural data it was shown that the extended IDPs do not represent a uniform entity but should be grouped into two broad classes, native coils and native pre-molten globules, and the protein trinity model should be extended to protein quartet [25] (Fig. 3D). Currently available data suggest that intrinsic disorder can have multiple faces, can affect different levels of protein structural organization, and whole proteins, or various protein regions can be disordered to a different degree (Fig. 3E).

All these observations can be visualized in a form of protein intrinsic disorder emission spectra (Fig. 4), where there is a gradual transformation from the monochromic view of functional proteins as well-structured polypeptides (Fig. 4A), to bi-colored picture with ordered (folded, blue) and disordered (completely structure-less, red) proteins (Fig. 4B), to a more complex picture where differently disordered proteins can be grouped into a few discrete classes (e.g., molten globule, pre-molten globule, coil-like; an analogy to the line emission spectrum with a few spectral lines, Fig. 4C), to very complex line spectrum with a great variety of potential structural classes and subclasses (Fig. 4D), finally to a continuous spectrum of differently disordered conformations extending from fully ordered to completely structure-less proteins, with everything in between (Fig. 4E).

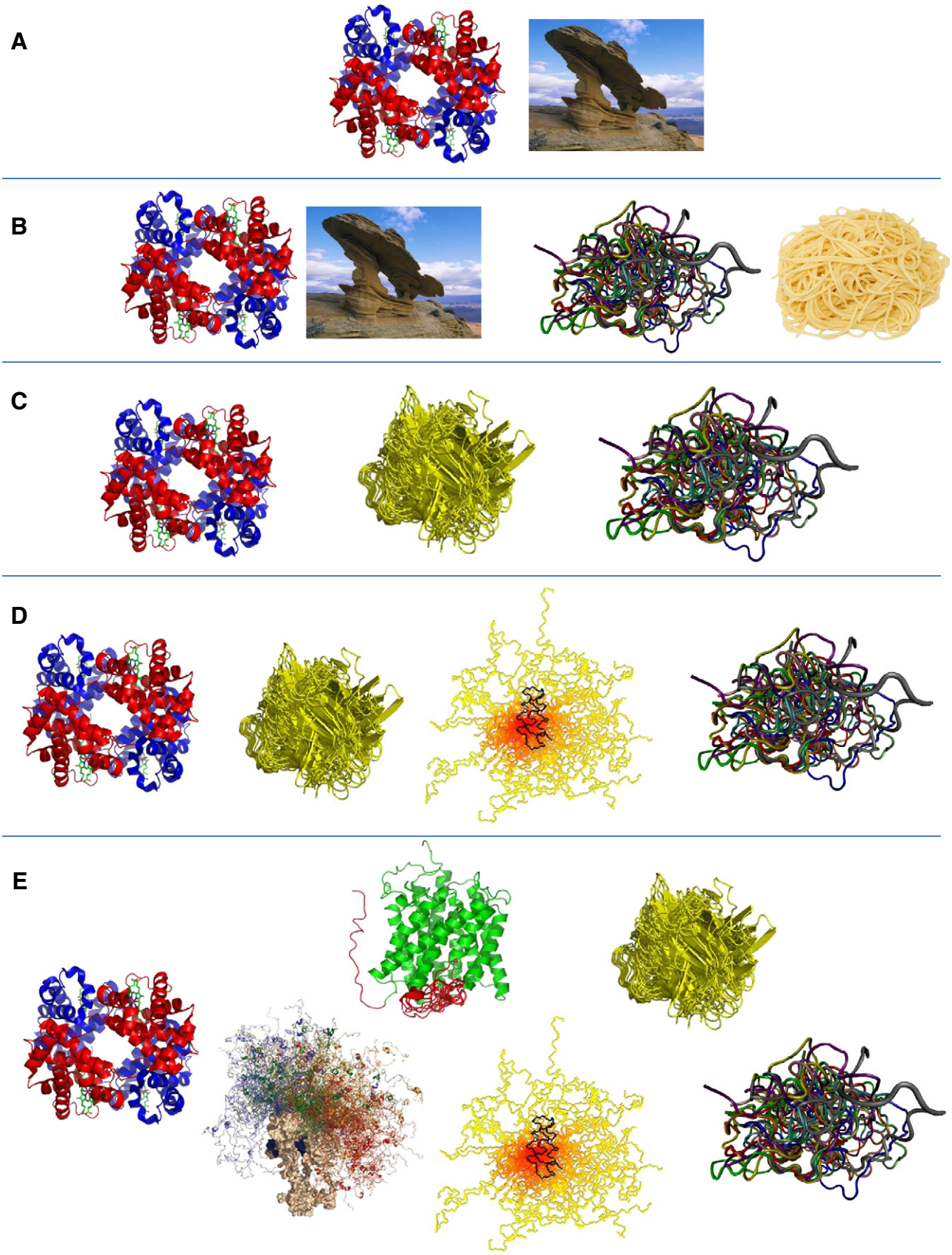

Fig. 3. Understanding the structural heterogeneity of IDPs/IDPRs. A. IDPs are ignored; all biologically active proteins have unique 3D rock-like structures. B. Concept of protein intrinsic disorder is introduced; IDPs are considered as completely structure-less entities, a kind of cooked noodles. B. Protein Trinity concept — functional proteins can be in any of three major conformations, ordered, molten globular, and coil-like. D. Protein Quartet model — extended IDPs are further subdivided to two broad classes, native coils and native pre-molten globules. E. Current view on the IDP/IDPR structure — intrinsic disorder can have multiple faces, can affect different levels of protein structural organization, and whole proteins, or various protein regions can be disordered to a different degree.

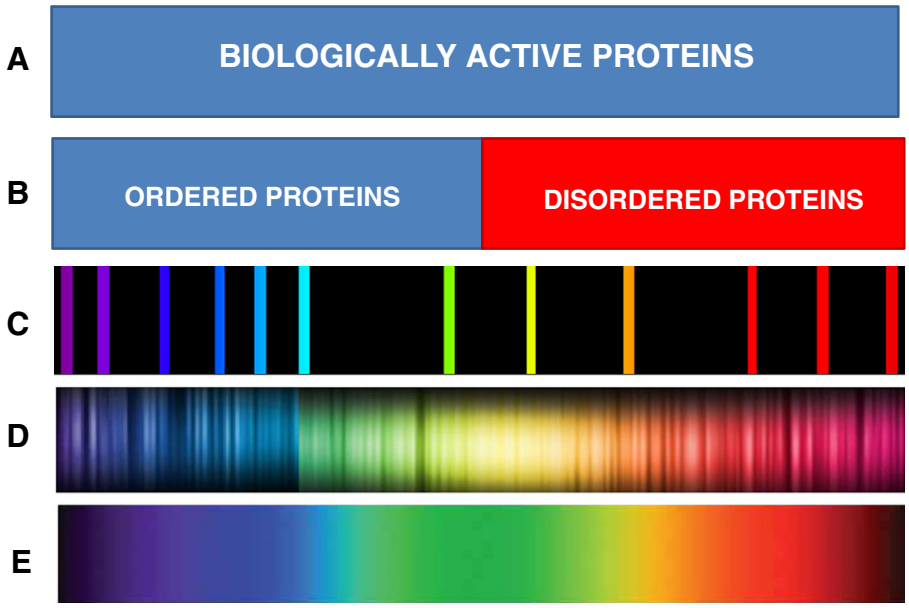

Fig. 4. Structural spectroscopy of functional proteins, where structural diversity is represented in a form of protein intrinsic disorder emission spectra. A. The monochromic view of functional proteins as well-structured polypeptides. B. Bi-colored picture with ordered (folded, blue) and disordered (completely structure-less, red) proteins. C. Simple line emission spectrum with a few spectral lines corresponding to several discrete classes of IDPs (e.g., molten globule, pre-molten globule, coil-like). D. Complex line emission spectrum reflecting a variety of potential structural classes and subclasses. E. A continuous emission spectrum representing the fact that functional disordered proteins can extend from fully ordered to completely structure-less proteins, with everything in between.

Once again, the fact that Fig. 4A represents the ordered proteins monochromatically does not mean that they were assumed to be completely non-flexible. On the contrary, the importance of conformational flexibility and the need of dynamics for the successful functionality of globular proteins (even enzymes) was emphasized in many studies over the past 55 years (e.g., Refs. [67–79]). In fact, the internal dynamics of enzymes (i.e., movement of their parts including individual amino acid residues, a group of amino acids, or even an entire domains that occurs in a wide range of time-scales, from femtoseconds to seconds) has been suggested to be linked to their mechanism of catalysis [69,75,76]. Furthermore, the existence of conformational substates (which were detected based on the atomic displacements involved in the interconversion of different local configurations of the same overall protein structure) in globular proteins potentially related to their functional conformational changes and allosteric behavior has been established [80–86].

However, ordered proteins are known to possess relatively stable 3D structure with Ramachandran angles that vary slightly around their equilibrium positions. This stable structural organization supported by the numerous crystal structures of proteins solved by X-ray diffraction resulted in a very common use of terms “unique 3D structure” and “rigid 3D structure” for the description of the structural properties of ordered proteins. Furthermore, the relative rigidity of structures of globular proteins was further supported by their high conformational stability and cooperative folding-unfolding behavior, where, for example, denaturant-induced unfolding was described as a reversible and highly cooperative “all-or-none”-type transition between native and denatured states [87], and where the temperature-induced melting was shown to be accompanied by the cooperative heat absorption related to the sharp change in the state of a protein on heating [88,89].

Notably, in representation shown in Fig. 4E, there is no boundary between ordered proteins and IDPs. Instead, structure-disorder space of a protein is considered as a continuum. This representation seems to be in contradiction with the representation of ordered proteins and IDPs based on the CH-plot, where a separation is implemented in the form of a separating line (a binary classifier, see above). However, CH-plot was developed to separate proteins located at the two extremes of the overall protein conformation space, namely compact proteins and highly extended IDPs. It is important to remember that even ordered proteins do not resemble “solid rocks,” but instead have some degree of flexibility. In fact, a protein molecule is an inherently flexible entity and the presence of this flexibility (even for ordered proteins) is crucial for its biological activity. It was also pointed out that although the entire molecule is flexible, some structural parts of ordered proteins are more rigid than others [90]. Detailed analysis of structures of ordered proteins revealed that the more rigid parts or structural units (which could be structural domains, subdomains or any structural part) are typically more compactly packed, have a stronger hydrophobic effect and have a larger stabilizing electrostatic contribution [90]. It was also indicated that the backbone movements in these more rigid structural units produce larger displacements than displacement induced by their side-chain motions. However, for some protein/protein regions thermal fluctuations of side-chains can bring about movements of the backbone [90]. Importantly, a protein with a set of stable structural units is expected to form a range of conformational isomers, peculiarities of which are expected to be dependent on the extent of its overall flexibility and the locations of the more flexible joints, whereas in a protein with unstable structural units, these thermal motions of the backbone would generate an entirely flexible molecule, which, in the extreme cases, would not retain any of the native fold [90], i.e., will behave as an IDP/IDPR.

2.2.3. Spatiotemporal heterogeneity of IDPs

Spatiotemporal heterogeneity of IDPs is an obvious consequence of their lack of fixed 3D-structure. Here, different parts of a molecule are ordered (or disordered) to a different degree and this distribution is changing in time. As a result, at any given time, an IDP molecule has a structure which is different from a structure seeing at another moment. In other words, a given segment of a protein molecule will have different structures at different time points. Therefore, IDPs act as a 4D-proteins [17], structural description of which requires time as a crucial component, since their structures are not fixed, as is generally the case for “3D proteins,” but rather defined by time and space, and since a given structure in an IDP is seeing at a given time only. Since

ordered proteins are also dynamic entities (with noticeably smaller range of fluctuations than that of IDPs and IDPRs), the concept of spatiotemporal heterogeneity is applicable for them too.

2.3. IDP energy landscapes

It was pointed out that during their biological lifetime proteins are forced to sample a variety of conformations due to the thermal fluctuations, and the probability of each conformation is determined by the topography of the underlying energy landscape [91]. This landscape describes the dependence of the free energy on all the coordinates determining the protein conformation. In this view, ordered, well-folded proteins are characterized by funnel-like energy landscapes that have a well-defined global energy minimum [92,93], since the number of conformational states accessible by a polypeptide chain is reduced while approaching the native state (Fig. 5A). However, even for ordered proteins, the bottom of the funnel-like energy landscape does not represent the only unique structure, but is rugged, and this ruggedness of the bottom of the folding funnel defines the mentioned flexibility of an ordered protein, where more rigid structural units move with respect to each other on their flexible joints [90]. It also defines the existence of a large number of the conformational substates (or nearly isoenergetic conformations) [86,94,95] and can be used to explain the protein allosterism, which is one of the major methods for regulating protein function where remote sites of a protein are energetically coupled to produce a functional response; i.e., where the ligand binding at one site is utilized to regulate the function of the protein by modulating the structure and dynamics of a distant binding site [96–104], and determines the fundamental capacity of a globular protein to undergo conformational change in response to ligand binding [105].

The free energy of an extended IDP represents a large "hilly plateau" describing the dynamic ensemble of a large number of conformations (Fig. 5B), with hills on the plateau corresponding to the forbidden conformations [28,91,106]. Therefore, the energy landscape of a well-folded ordered protein exhibits a well-defined minimum energy state corresponding to the folded conformation, whereas the energy landscape of an IDP is relatively flat and lacks such a deep energy minimum, being characterized by a very peculiar 'topology' characterized by numerous local energy minima, due to which protein tend to behave as a highly frustrated system without any stable well-folded conformation. This type of energy landscape is exceptionally sensitive to local environment (in fact, it is much more sensitive than the relatively robust funnel-like energy landscape of an ordered protein) and determines conformational plasticity of an IDP. In fact, any changes in the IDP surroundings might have very strong effect on the IDP structure. Different environmental factors might have different effects on the energy landscape making some energy minima deeper and some energy barriers higher (see Fig. 5C). This determines the ability of an IDP to fold differently depending on the environmental conditions. This also provides some clues on how an IDP can specifically interact with many ligands of different nature and to fold differently as a result of these interactions. Here, the interaction with a particular binding partner affects the IDP folding landscape in a unique way, promoting formation of a specific structure on a template-dependent manner.

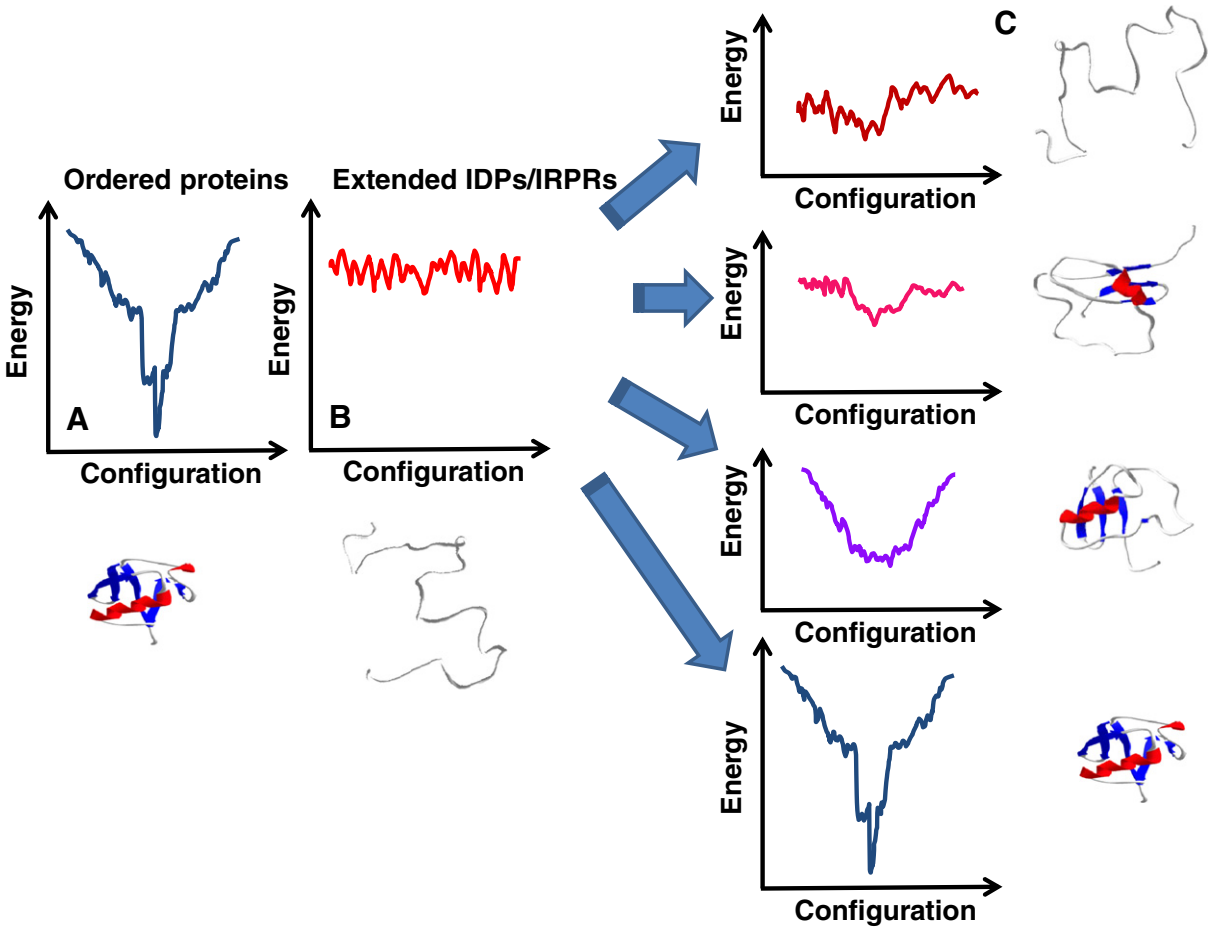

Fig. 5. Energy landscape of ordered proteins and IDPs. A diagram showing the folding energy landscapes of a typical globular protein (A) and of a typical natively unfolded protein in the absence (B) or presence of different binding partners (C). These landscapes are depicted schematically in one-dimensional cross-section. Illustrative examples of corresponding structures are also shown.

It is also necessary to keep in mind that Fig. 5C represents an oversimplified view of an IDP energy landscape, where changes are assumed to affect the entire molecule. In fact, IDPs are highly heterogeneous systems, with heterogeneity being applicable to their sequences, structural properties and spatiotemporal behavior. In terms of the energy landscape, this heterogeneity means that the different parts of an IDP might be described by individual energy landscapes, each replying to the environmental changes in its own way. Within the entire molecule, the responses of different regions can be independent, semi-dependent or dependent on each other. Furthermore, different parts of an IDP can respond differently to the different environmental stimuli. This heterogeneity of the energy landscape defines the ability of IDPs to form fuzzy complexes, were significant part of a protein preserves its intrinsically disordered state even in the bound conformation [62,63,65,107].

2.4. "Turned-out" response to the environmental changes

Unusualness of IDPs and IDPRs does not stop at their overall highly heterogeneous nature. They also have a quite unexpected response to changes in their environment. In fact, for ordered proteins, it is known for a long time that exposure to the denaturing conditions (extreme temperatures or pH) kills their biological function due to the disruption of specific structure. In other words, extreme conditions are known to induce disruption of ordered structure and globular proteins under these conditions are expected to have less structure. However, extended IDPs (so-called native coils and native pre-molten globules) are different. They possess "turned-out" response to heat and might gain some structure in a temperature-dependent manner, being typically more disordered at lower temperatures and more structured at higher temperatures [108]. Such temperature-induced folding was described for several extended IDPs, such as α-synuclein [109], caldesmon 636-771 fragment [110], phosphodiesterase γ-subunit [111], the receptor extracellular domain of nerve growth factor [112], α_s-casein [113], and several other IDPs. The structure-forming effects of elevated temperatures on extended IDPs were attributed to the increased strength of the hydrophobic interaction at higher temperatures, leading to a stronger hydrophobic attraction, which is the major driving force for folding [108].

Similarly, extended IDPs are characterized by the "turned out" response to changes in pH and gain more structure at extremely acidic and/or alkaline conditions [108]. For example, for human α-synuclein [109] and prothymosin α [38] it was shown that changes in pH induce reversible structural transformation, leading to the transition from a highly disordered coil-like conformation to a partially folded pre-molten globule-like conformation [38,109]. Similar pH-induced structural transformations have been described for such extended IDPs as pig calpastatin domain I [114], histidine rich protein II [115], naturally occurring human peptide LL-37 [116], and several other IDPs. These observations show that a decrease (or increase) in pH induces partial folding of extended IDPs due to the minimization of their large net charge present at neutral pH, thereby decreasing charge/charge intramolecular repulsion and permitting hydrophobic-driven collapse to the partially-folded conformation [108].

Therefore, the peculiarities of the amino acid sequences the extended IDPs, which are highly enriched in charged residues and noticeably depleted in hydrophobic residues represent physical basis for their "turned out" responses to changes in their environment [108].

2.5. Binding mechanisms and binding promiscuity of IDPs

IDPs and IDPRs are characterized by exceptional binding promiscuity, where one protein or regions is able to bind to multiple partners [27]. Obviously, the classical molecular recognition mechanisms cannot explain the ability of IDPs/IDPRs to bind to multiple partners [117]. In fact, the lock-and-key [118] mechanism that was developed to describe recognition behavior of ordered proteins can give a reasonable description of this multibinding capability. Furthermore, some IDPs/IDPRs were shown to adopt different structures upon binding to different partners [1,15,119–123], thereby playing a number of crucial roles in mediating protein–protein interactions (PPIs) [1,15,27,119–136]. Note, that in the induced fit model developed to describe binding behavior of some ordered proteins [137], structure of a bound protein potentially may change to fit to a different binding partner, indicating that the induced fit mechanisms is compatible with the multiple bound conformations of IDPs.

In PPI networks, there are several multitasking proteins (known as hubs) that have multiple links. With respect to temporal structure of the PPI networks, some proteins have multiple simultaneous interactions ("party hubs"), while others have multiple sequential interactions ("date hubs") [138]. From a functional perspective, date hubs may connect biological modules to each other [139], whereas party hubs may form scaffolds that enable the assembly of functional modules [138]. Involvement of intrinsic disorder is one of the reasonable mechanisms for the description of the promiscuity of hub proteins [27,124–128], where, intrinsic disorder and related disorder-to-order transitions could enable one protein to interact with multiple partners (one-to-many signaling) or to enable multiple partners to bind to one protein (many-to-one signaling) [1].

Many different IDPs can form highly stable complexes, or be involved in signaling interactions where they undergo constant "bound–unbound" transitions, thus acting as dynamic and sensitive "on–off" switches [107]. The ability of these proteins to return to their highly dynamic and pliable conformations after the completion of a particular function, and their predisposition to gain different conformations depending on the peculiarities of their environment, are unique properties of IDPs which allow them to exert different functions in different cellular contests according to a specific conformational state [7].

The recognition function of IDPs can be realized via several molecular mechanisms, being frequently associated with the disorder-to-order transition induced by binding to their partners. The binding-coupled folding of IDPs/IRDs may be either induced by the template or selected from the conformational ensemble. In other words, the IDP structure adopted in the bound form may be enforced by the partner molecule or reflect the inherent conformational preferences of IDPs. One of the models for finding intrinsic disorder-based binders, Molecular Recognition Feature (MoRF) model, involves a short binding region located within a longer disordered region [140–143]. Alternative and complementary models of MoRF-like interactions are the Short Linear Motif (SLiM) or Eukaryotic Linear Motif (ELM) based on sequence motifs that are recognized by peptide recognition domains [144]. A different approach is taken by the ANCHOR model, which identifies segments of disordered regions that are likely to fold in conjunction with a globular binding partner [145,146]. In the primary contact site (PCS) model, certain regions within the disordered ensemble are more exposed than others, and thereby may serve as the first sites of contact with the partner [147]. Some IDPs in the unbound state were proposed to have strong conformational preferences for their bound conformations; i.e., they use partially/transiently pre-formed elements for recognition [148]. In other words, although IDPs lack the hydrophobic cores typical for ordered proteins and cannot be described as single, rigid structures they still might have some local preferences for transient secondary structure elements and even for some transient tertiary contacts. Such dynamic pre-organization imposes spatial restrictions on IDPs, therefore exposing some of their potential contact sites. The existence of such pre-formed binding sites enables faster and more effective interactions of IDPs with their targets [7,59,140,148].

Another important property of IDPs/IDPRs is their ability to form fuzzy complexes, where a significant part of an IDP continues to be

disordered even in the bound state [62,63,65,107]. Many IDPs can remain predominantly disordered in bound state outside the binding interface [63,110,149,150]. Such mode of interaction is known as "the flanking fuzziness" in contrast to "the random fuzziness" when the IDP remains entirely disordered in the bound state [63,151]. An extreme case of such fuzzy complexes are "binding clouds"; i.e., specific complexes where almost no structure is formed (e.g., as in a case of the polyelectrostatic model describing the interaction between the phosphorylated and intrinsically disordered cyclin-dependent kinase inhibitor Sic1 and its ordered partner, SCF(Cdc4) ubiquitin ligase) [152]. These highly disordered complexes can be formed due to the existence of several similar binding sites combined with a highly flexible and dynamic structure of an IDP which provides any binding site of IDP with unique capability to interact with any binding site of its partner with almost equal probability, in a staccato manner [107]. However, since in such a scenario, each individual contact is characterized by a low affinity, these individual contacts are not stable and can be readily broken. This gives rise to the disordered or fuzzy complex, which is a highly dynamic ensemble in which an IDP does not present a single binding site to its partner but resemble a "binding cloud," where multiple (almost) identical binding sites are dynamically distributed in a diffuse manner. In other words, in this staccato-type interaction mode, an IDP rapidly changes multiple binding sites while probing binding site(s) of its partner [107].

2.6. Foldons, inducible foldons, semi-foldons, and non-foldons

Foldon concept was originally introduced to describe an independent foldable unit of ordered proteins, and based on the analysis of the non-homologous proteins representing different folds it was proposed that there are about 2600 foldons in the natural protein universe [153]. Since the time of first introduction, the use of the term "foldon" had several independent developments. Some researchers continued to use it to describe independently foldable domains [154], whereas others used the term exclusively to describe a small trimeric globular domain located at the C-terminal region of the bacteriophage T4 fibritin, that has a GYIPEAPRDG QAYVRKDGEW VLLSTFL sequence, forms a β-propeller-like structure with a hydrophobic interior, which is crucial for the correct coiled-coil formation [155–157]. It was recognized later that fusion of the T4 foldon domain to target coiled-coil proteins can be used to initiate the correct coiled-coil formation in various coiled-coil proteins [158–170], and also promote the formation of functional oligomers in non-coiled coil proteins [171–175].

Finally, another development in the application of the foldon concept is directly related to the topic of this review. Here, based on the analysis of the cytochrome *c* folding in a set of the equilibrium and kinetic hydrogen exchange experiments it was shown that this small, single-domain protein contains five submolecular foldon units that continually unfold and refold even under native conditions [176]. Later, other globular proteins (such as apo-cytochrome b_{562}, ribonuclease H, dimeric triosephophate isomerase, the OspA protein of Borrelia [177] and staphylococcal nuclease [178]) have been found to show similar behavior. Based on these observations it was concluded that the folding of an ordered protein can be described as the stepwise assembly of the foldon units, with previously formed foldons guiding and stabilizing subsequent foldons to progressively build the native protein [177,179–181]. Similar conclusion on the foldon existence within the structures of ordered proteins was derived from the kinetic analysis of folding of small proteins and from the analysis of their folding transition states in particular [182]. It was shown that for proteins possessing folding via a multitude of different pathways, the number of accessible pathways was linked to the number of nucleation motifs contained within the native topology. These nucleation motifs typically had size of an independent cooperative unit and were defined as "foldons" [182]. Based on these two sets of data, it is clear that ordered proteins should be considered as "modular assemblies of competing foldons" [182].

Let us apply this foldon concept to the structure of IDPs/IDPRs. The aforementioned heterogeneity of energy landscapes of IDPs and IDPRs defines their structural and spatiotemporal heterogeneities that are reflected in their 4D-protein behavior. Here, some regions of IDP are spontaneously folded, other can fold (at least in part) at interaction with binding partners, still other are always in semi-folded state, whereas some regions do not fold at all. In this respect, an IDP can be described as a modular assembly of foldons, inducible foldons, semi-foldons and non-foldons.

2.7. Unfoldons in action: Awaking of dormant disorder for function

2.7.1. Introducing unfoldon concept

As it follows from currently available data, biologically active proteins can either have 3-D structures or be devoid such specific and stable structures. Furthermore, structures of proteins can either change or remain unchanged during function. The function-related structural changes in IDPs range from the local partial folding to complete folding, and from allosteric transitions to induced fit adjustments in ordered proteins. Generally, the most common outcome of these function-related structural changes is the overall increase in the amount of ordered structure.

However, functions of some ordered proteins rely on the decrease in the amount of their ordered structure; i.e., these functions require local or even global functional unfolding of a unique protein structure. The important features of these functional alterations are their induced nature and transient character. In other words, the function-related changes in a protein are induced by transient alterations in its environment or by transient modification of its structure and are released as soon as the environment is restored or the modification is removed. These unusual features are important prerequisites of the protein functions relying on the induced unfolding or transient disorder mechanism. Here, we are talking about dormant disorder which needs to be awakened in order to make a protein functional. In line of foldons, inducible foldons, semi-foldons and non-foldons discussed above, this intricate feature can be considered as an unfoldon; i.e., a part of a protein structure that has to undergo order-to-disorder transition in order to make protein active.

2.7.2. A few illustrative examples of unfoldons

Although the topic of transient disorder is relatively new, it is already clear that nature can use different means to ensure the order-to-disorder transitions in unfoldons. In fact, any factor which can potentially unfold a structure of a folded protein is utilized here. Among these unfoldon-activating factors are changes in pH, temperature, redox potential, light, mechanical force, membrane, interaction with ligands, protein–protein interaction, various posttranslational modifications (PTMs), release of authoinhibition due to the unfolding of autoinhibitory domains or their interaction with nucleic acids, proteins, membranes, PTMs, etc. The literature on the topic of dormant disorder is vast and spread over a wide time-interval. Unfoldons and related mechanisms of protein activation are exceptionally interesting subjects and clearly deserve to be described in a focused review. Therefore, only a few illustrative examples are provided below to show how diverse the underlying unfolding-based mechanisms are.

Extended IDPs are known to gain some residual structure at acidic conditions [108]. Many ordered proteins are known to denature (i.e., lose their biological activity) at extremely low pH values. However, some ordered proteins are activated by the solution acidification. An illustrative example of this functional protein unfolding induced by changes in pH is acidification-induced activation of HdeA. HdeA is one of the smallest known chaperones, which functions as a monomer and does not require any energy factors or co-chaperones. This

chaperone is expressed by many bacteria to combat acid-induced protein unfolding and aggregation in the periplasm. At neutral pH, HdeA exists as a well-folded, but inactive dimer. However, this protein specifically senses low pH conditions (pH<3), where it partially unfolds and dissociates into chaperone-active monomers, and thus is activated by the same conditions that lead to the inactivation and aggregation of other proteins [183]. Envelope proteins of several viral families (e.g., the *Alphavirus* and *Flavivirus* genera) are another example of pH-sensing proteins. These viral envelope proteins are responsible for fusion with the membranes of endosomal compartments of the host cells in a pH-dependent fashion. Upon exposure to mildly acidic conditions (~pH 6.5), these proteins undergo extensive conformational and oligomeric state changes, which serve to tether viral and cellular membranes and pull them into the close apposition required to promote lipid mixing [184,185].

Similarly, although high temperatures are known to denature many ordered proteins, some proteins, e.g., the *Saccharomyces cerevisiae* holdase Hsp26 and the wheat holdase Hsp16.9, are activated by heat stress conditions [186]. For example, under the non-stress conditions *in vitro*, Hsp26 exists in a form of a hollow sphere of 24 subunits assembled from the 12 dimers [187]. This protein is specifically activated by the temperature increase [188], with the midpoint at ~36 °C [189]. Temperature-activated Hsp26 is characterized by the lower content of ordered secondary structure and by the noticeable rearrangements of the tertiary structure [190].

Among rather unusual factors used by nature to activate proteins via functional unfolding are light and mechanical force. For example, exposure to blue light results in the activation of the photoactive yellow protein (PYP), which is an ordered, water-soluble ~14 kDa protein that contains a thioester linked p-coumaric acid cofactor and serves as a photosensor in *Ectothiorhodospira halophila* [191,192]. Based on the high resolution NMR spectroscopic analysis it was concluded that the activated PYP possessed a large degree of disorder and existed as an ensemble of multiple conformers that exchange on a millisecond time scale [193].

Finally, some proteins serve as force sensors and undergo local unfolding induced by the mechanical forces. For example, mechanosensitive ion channels recognize and respond to the membrane tension, which is the mechanical forces applied along the plane of the cell membrane, rather than to the hydrostatic pressure perpendicular to the membrane plane [194]. Here, membrane tension induces activation via partial unfolding of some functional parts of such ion channels [195].

2.8. Functional misfolding of IDPs

IDPs/IDPRs are characterized by high conformational dynamics and flexibility, the presence of sticky preformed binding elements, and the ability to morph into differently-shaped bound configurations. One of the common interaction modes of IDPs and IDPRs is wrapping around the binding partner [107] that results in the formation of a polyvalent complex where several ordered segments of an IDP/IDPR bind to disjoint and spatially distant binding sites on the surface of the globular protein [107]. In other words, in their bound state, ordered segments of such flexible wrappers are connected by flexible linkers and almost do not have intramolecular contacts [107]. However, detailed analyses of the conformational behavior and fine structure of several IDPs in their non-bound states revealed that the preformed binding elements might be involved in a set of non-native intramolecular interactions [196]. Based on the analysis of conformational ensembles of several IDPs, a concept of functional misfolding was proposed [196]. Here, the dynamically formed elements of secondary structure (these foldons, inducible foldons, and semi-foldons mentioned above) that potentially might represent sticky and promiscuous molecular recognition sites were proposed to be protected from unwanted interactions with unwanted partners via the involvement in the intramolecular non-native interactions; i.e., via functional misfolding [196]. This possibility was described in detail for several IDPs, such as non-homologous regulators of the protein phosphatase 1 (PP1), the protein inhibitor-2 (I-2), spinophilin, the dopamine- and cyclic AMP-regulated phosphoprotein with molecular weight of 32 kDa (DARPP-32), and the N-terminal domain of the myosin phosphatase targeting subunit MYPT1. PP1-interacting domains of these proteins are highly disordered in the non-bound state, but fold at binding and wrap around PP1 interacting with it at multiple spatially distal sites [197–199]. These four IDPs possessed very different residual structures in their unbound states [200], possessing clusters of temporarily formed secondary structure elements involved in non-native interactions and therefore representing well-documented examples of functional misfolding, where the pre-populated binding sites are partially protected from the undesired contacts, being involved in extensive non-native tertiary interactions [196]. Similarly, the unbound transactivation domain of p53 was shown to lack fixed structure, possessing some residual secondary structure elements that were present in the molecule part of the time and were involved in short-lived non-native interactions [201–203]. The functional misfolding was also described for the retinal phosphodiesterase inhibitory γ-subunit [196,204], α-synuclein [196,205], cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Waf1/CiP1/Sdi1}$ [196,206], regulatory domain of the cystic fibrosis transmembrane conductance regulator [196,207], and several other IDPs. Furthermore, many extended IDPs in their unbound forms where shown to possess some partial compaction and were characterized by the presence of residual secondary structure, suggesting that they may be functionally misfolded too [196]. According to the functional misfolding concept the preformed secondary structure elements in an IDP are inevitably involved in intramolecular non-native interactions leading to the functionally misfolded state [196]. Obviously, this functional misfolding can be related to the fitness of unbound IDPs since the interaction-prone preformed secondary structure elements can be protected from unwanted interactions with the non-native partners, being sequestered inside the "non-interactive" (or at least less-interactive) cages, where they are dynamically excluded from the environment and therefore might escape unwanted interactions with the non-native binding partners [196].

The mentioned functional misfolding is related to the ensemble behavior of transiently populated elements of structure. In other words, it describes the behavior of a globally disordered polypeptide chain containing highly dynamic elements of residual structure, the interaction-prone preformed fragments, some of which could potentially be related to protein function. Both non-native intramolecular electrostatic and non-native hydrophobic interactions might contribute to the functional misfolding. Although the currently available information about fine structures of unbound IDPs in solution and about their long-range intramolecular interactions is very sparse, there is a good chance that this phenomenon is highly abundant at least among the so-called extended IDPs (native coils and native pre-molten globules) [196].

It was pointed out that functional misfolding; i.e., a process of sequestering and preserving of the interaction-prone elements, is very different from the pathological misfolding, where biologically active protein molecules adopts an aggregation-prone misfolded conformation leading to the development of various conformational diseases [196].

Recently, an idea of using functional IDP misfolding concept in the development of novel disorder-based drugs was proposed [208]. Here the ability of IDPs/IDPRs to spontaneously form a non-interactive cage sequestering interaction-prone preformed fragments was suggested to be used in the drug discovery process for finding small molecules which would potentially stabilize different members of the functionally misfolded ensemble, and therefore prevents the targeted protein from establishing biological interactions [208]. This

approach, being based on a small molecule binding to a highly dynamic surface created via the transient interaction of preformed interaction-prone fragments, can be considered as an extension of the well-established structure-based rational drug design elaborated for ordered proteins. In fact, if the structure of a member(s) of the functionally misfolded ensemble can be guessed, then this structure can be used to find small molecules that are potentially able to interact with this structure, utilizing tools originally developed for the rational structure-based drug design for ordered proteins [208].

2.9. IDPs in crowded environment

Typically, *in vitro* experiments on various IDPs are performed under the relatively ideal thermodynamic conditions of low protein and moderate salt concentrations. However, proteins have evolved to function within cells, where the concentration of macromolecules, including proteins, nucleic acids, and carbohydrates, within a cell can be as high as 400 g/L [209], creating a crowded medium, with considerably restricted amounts of free water [209–215]. Such "thick-soup-like" intracellular environment is considered to be crowded, since typically no individual macromolecular species is present at very high concentration [212,214]. In such crowded environment, the volume occupied by solutes is unavailable to other molecules, generating specific thermodynamic consequences generally known as excluded volume effects [210,216]. Volume exclusion in biological fluids may have large effects on both stability of biological macromolecules [214,217–219], and macromolecular equilibrium, including alteration of protein–protein interactions [215,220] and modulation of the rate and extent of amyloid formation [221–223].

The view that macromolecular crowding is important yet neglected variable in biochemical studies is gained attention [210,213]. Effect of excluded volume on macromolecules may be examined experimentally by using concentrated solutions of a model "crowding agent" such as polyethylene glycol, dextran, Ficoll or inert proteins [216,221]. The effect of high concentrations of different crowders on structural properties of several IDPs was analyzed. For example, molecular crowding modeled by the high concentrations (of up to 250 g/L) of the dextrans of average molecular weights 9.5, 37.5, and 77 kDa and Ficoll 70 did not induce significant folding in two IDPs, FosAD and p27ID [224]. FosAD corresponds to the C-terminal activation domain of human c-Fos (residues 216–310) that interacts with transcription factors [225]. p27ID is the cyclin-dependent kinase inhibition domain of the cell-cycle inhibitor human $p27^{Kip1}$ (residues 22–97) and is active as a cyclin A-Cdk2 inhibitor [226]. Both protein domains were shown to by intrinsically disordered as judged by several spectroscopic techniques [225,226]. In the presence of macromolecular crowding agents, none of these IDPs underwent any significant conformational change reflected in noticeable changes in either circular dichroism or fluorescence spectra. Based on these observations it has been concluded that molecular crowding effects are not necessarily sufficient to induce ordered structure in IDPs [224].

Similarly, α-synuclein was shown to preserve its mostly unfolded conformation in the presence of several crowding agents [227] and even in the periplasm of the bacterial cells [228]. The analysis of FlgM, which is a 97-residue IDP from *Salmonella typhimurium* that regulates flagellar synthesis by binding the transcription factor σ^{28}, revealed that approximately half of this IDP gained structure in the crowded environment [229]. Importantly, although free FlgM was mostly unstructured in the dilute solutions, its C-terminal half formed a transient helix in the unbound form [230], became structured on binding to σ^{28} [231] and was shown to be folded in the crowded environment [229]. Therefore, IDPs could be grouped into two classes, foldable and non-foldable, based on their response to the crowded environment. Foldable IDPs can gain structure in crowded environment (and, thus, inside the living cells) likely due to the crowding-induced formation of a hydrophobic core. Non-foldable IDPs remain mostly unstructured at the crowded conditions. Some of these non-foldable by crowding IDPs may require another protein (or DNA, or RNA, or some other natural binding partners) to provide a framework for structure formation. FlgM clearly represents a unique case of the two-faced Janus, where the first face exemplified by the C-terminal half of FlgM is structured in the crowded environment, whereas the second face exemplified by the N-terminal half of FlgM does not become structured at physiologically relevant solute concentrations [229].

Recent advantages in the in-cell NMR analysis have opened new exceptional opportunities for evaluating the structural and conformational properties of IDPs in their natural environments (i.e., within cells). Successful in-cell characterization of IDPs has been reported for both bacterial and eukaryotic cells [228,229,232,233]. For example, recent in-cell NMR analysis of α-synuclein in intact *Escherichia coli* cells clearly indicated that this protein is mostly disordered and monomeric inside *E. coli* [234].

3. IDPs as the "edge of chaos" systems

3.1. On applicability of the "edge of chaos" concept to IDPs

It is possible that IDPs/IDPRs can be considered as the "edge of chaos" systems which operate in a region between order and complete randomness or chaos; i.e., in the region where the complexity is maximal. Position at the edge of chaos (that is at the transition point between order and chaos) determines the capability of IDPs/IDPRs of being exquisitely controlled, where even small changes in their environment might generate large and diversified changes, and defines their exceptional complexity. In fact, IDPs/IDPRs can be formally defined as complex system since they seem to obey major rules proposed to describe behavior of complex systems [20]:

(i) Complex systems contain many heterogeneous components that interact nonlinearly. This means that a small perturbation may cause a large effect, a proportional effect, or even no effect at all. This also means that behavior of such a system cannot be expressed as a sum of the behaviors of its parts (or of their multiples);
(ii) The constituents of a complex system are interdependent;
(iii) A complex system possesses a structure spanning several scales and may be nested; i.e., the components of a complex system may themselves be complex systems;
(iv) A complex system is capable of emergent behavior, which is unanticipated behavior shown by the system, for example the arising of novel and coherent structures, patterns and properties during the process of self-organization;
(v) Complexity involves an interplay between chaos (disorder) and order;
(vi) Complexity involves an interplay between cooperation and competition, and complex systems contain both positive (amplifying) and negative (damping) feedbacks;
(vii) Complex systems may have a memory. In other words, the history of a complex system may be important, since due to their dynamic nature, complex systems change over time, and prior states may have an influence on present states.

Let us see now how these rules work for IDPs. Heterogenic nature of IDPs is obvious. In fact, IDPs and IDPRs are heterogeneous at multiple levels. Globally, they can be compact or extended and their major structural components are heterogeneous too, giving rise to foldons, induced foldons, semi-foldons and non-foldons. These structural components can be independent or interdependent, and they are able to interact nonlinearly. Functional misfolding represents an illustration of the interplay between cooperation and competition. The spatiotemporal complexity of IDPs/IDPRs is further increased by the

fact that they and their structural components are always moving between order and disorder. IDPs are able to sense various stimuli and response to these stimuli via corresponding structural changes, where even smallest environmental perturbations might produce large structural and functional outcomes. IDPs/IDPRs possess emergent behavior, since under some conditions they are able to undergo self-organization via stimuli-induced disorder-to-order transitions. Finally, MoRFs, SLiMs and PreSMos represent a memory of the IDP, since they are transiently populated in the non-bound state and may have a profound influence on IDP binding mechanism and on the resulting bound state. All this supports the hypothesis that IDPs/IDPs are positioned at the edge of chaos.

This brings an interesting possibility that the behavior of an IDP, being chaotic, complex, and extremely sensitive to the peculiarities of the environmental conditions, can be described in terms of the strange attractor (e.g. Lorenz attractor), where system will neither converge to a steady state nor diverge to infinity, but will stay in a bounded but chaotically defined region. Under some conditions, this system can have characteristic butterfly-like trajectories; i.e., it may behave as Lorenz attractor, where small changes in initial conditions may produce large changes in the long-term outcome (so-called butterfly effect [235]) (see Fig. 6A). Lorenz attractor, an example of a non-linear dynamic system, is a simplified mathematical model developed for the description of atmospheric convection by considering two-dimensional flow of a fluid subject to differences in temperature and gravity [236,237]. It is considered now as a foundation of chaos theory. Although it was originally developed for weather prediction, chaos theory has found its way to other scientific fields, including physics and chemistry (where the application of chaos theory has resolved some long-standing problems, such as how to calculate a turbulent event in fluid dynamics or how to quantify the pathway of a molecule during Brownian motion [238]), electronics and engineering (where chaos theory is used to describe the chaotic behavior that restricts the operating range of many electronic and mechanic devices) [239], biology (e.g., description of certain activities of the neural systems, such as odor recognition by the olfactory bulb) [240] and medicine (where chaos theory is applied to predict the occurrence of lethal arrhythmias or epileptic seizures [238]). It is tempting to hypothesize that IDPs/IDPRs might represent another example case, where chaos theory can be used to describe the complex behavior of a biological system. Here, IDPs/IDPRs can behave as Lorenz attractors; i.e., they do not converge to a steady state (do not form fully ordered state), but also do not diverge to infinity (do not behave as completely disordered polypeptide chains).

Another interesting possibility is that chaos theory potentially can be used to describe interactions between IDPs/IDPRs and their partners. Pattern formation represents one of the illustrative examples of the self-organization process; i.e., it is the emergent behavior mentioned as one of the rules defining behavior of a complex system. Pattern formation is very common in nature and occurs on many different spatial and temporal scales and several levels of complexity [241]. It was pointed out that inside the leaving cell, nonlinear reaction–diffusion dynamics allow proteins to encode for positional information [242–245], required to coordinate complex processes like cell division [246,247], cell motility [248,249], give rise to information flow within the cell [245,250], or the Min system-controlled positioning of the division septum in the cell to its center, such that it divides into two equally sized daughter cells [251].

3.2. Peculiar dynamics of the MinE–MinD system

The spatiotemporal oscillations of the Min proteins in *E. coli*, a prokaryotic protein system that is involved in the spatial regulation of the positioning of the cytokinetic Z ring [252], represent a well-studied example of the biochemical reaction–diffusion process that determines the self-organized emergent behavior [253]. In nature, the Min proteins MinD and MinE oscillate from pole to pole in the rod-shaped *E. coli* cell. The Min proteins are the members of the WAKA family (*W*alker *A* cytomotive *A*TPase; also knows as ParA), which are prokaryotic proteins that display oscillatory behavior involved in such diverse processes as spatial regulation of cell division, plasmid and chromosome segregation, and regulation of development [254]. Oscillations in the Min system emerge from the ATP-dependent interactions of three Min proteins (MinD, MinC, and MinE) with each other and with the cytoplasmic membrane [252]. MinD is a membrane-binding ATPase, MinC is an inhibitor of division, whereas MinE is the activator of ATP hydrolysis which serves as an antagonist of MinD in its membrane-bound state. It was shown that the MinD and MinE are sufficient to reproduce the oscillating behavior both *in vivo* and *in vitro* [255–257], where MinD recruits MinE to

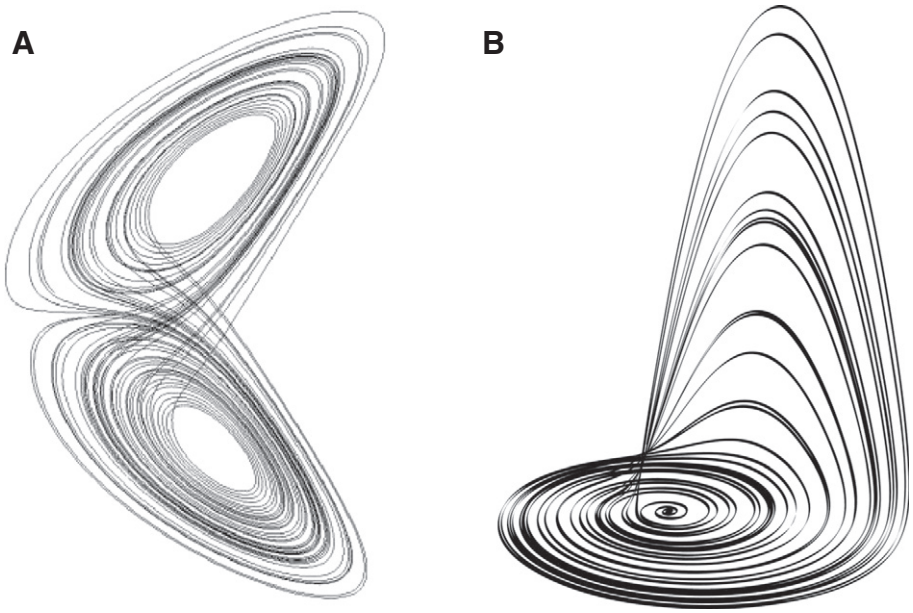

Fig. 6. IDPs/IDPRs as "edge of chaos" systems. A. Lorenz attractor potentially describing conformational behavior of IDPs. B. Rössler attractor potentially describing interactions driven by IDPs/IDPR that might define the cyclic behavior of the biochemical reaction–diffusion processes.

the membrane, leading to a coupled oscillation required for spatial regulation of the cytokinetic Z ring. *In vivo*, Min protein oscillations are characterized by the intrinsic wavelength which is similar to the size of the *E. coli* cell, about 5 μm versus 3 μm cell length and 1 μm cell diameter [258]. *In vitro*, the Min proteins are able to self-organize into mesoscale patterns in a form of traveling protein waves on a supported lipid bilayer, where protein surface waves emerge from repetitive cycles of proteins binding and detaching to and from the membrane [259,260]. It was also shown that this specific pattern of traveling protein waves is determined by the rapid rebinding and membrane interaction of MinE [260]. It was also shown that the spatial regulation of the cytokinesis by the Min oscillator is based on the MinD-dependent conformational changes in MinE [261].

Analysis of the structural properties of MinD and MinE proteins gives an intriguing hint on the potential role of intrinsic disorder in the emergent behavior of the Min system. MinD, which is an 270 residue-long ATPase required for the correct placement of the division site, binds to the membrane through the C-terminal 10 amino acids, that form an amphipathic helix that inserts into the membrane bilayer [262–264]. The biologically active unit of the *E. coli* MinD is a stable dimer where the MinE binding site is located at the dimer interface and is exposed to the cytosol [265]. This interface placement of the MinE-binding site is shown in Fig. 7A that represents a crystal structure of the *E. coli* MinE dimer bound to MinD dimer and illustrates that the α-helix of one of the MinE molecules is bound to the MinD dimer interface [261].

MinE is a small protein of 88 residues that has three functional domains and easily forms dimers [266,267]. The N-terminal region of MinE was implicated in the MinE–membrane interaction since it was shown to contain cryptic membrane-targeting sequence (MTS) that included positively charged residues at positions 10–12 [268]. The function of the MinE N-terminal domain (residues ~6–31) is to counteract the division-inhibitory activity of the MinCD complex via the formation of the α-helix that binds MinD (see Fig. 7A) [261,269]. The C-terminal domain (residues 32–88) is a topological specificity domain that is required for MinE to spatially regulate cell division [261]. Structural analysis of two intact MinE proteins and one trypsin-resistant fragment of MinE revealed that the related structures differ significantly, suggesting that they represent "snap-shots" of different conformational states accessible to this protein. A trypsin-resistant fragment of the *E. coli* MinE consists of residues 31–88 that form two long antiparallel β-strands (β_2- and β_3-strands) covered by an α-helix [270]. This fragment forms a dimer where the helices of the subunits pack together to form an antiparallel coiled-coil, and the β-strands are combined to form a four-stranded, antiparallel β-sheet (see Fig. 7B). However, the structures of the intact MinE from *Helicobacter pylori* and *Neisseria gonorrhoeae* are different. Although they also form dimers, in their dimeric form, these two MinEs contain not a four-stranded but a six-stranded antiparallel β-sheet [271,272], with additional β_1-strands (that contain part of the anti-MinCD domain) being positioned at the dimer interface where they are sandwiched between the β-strands seen in the structure of the truncated *E. coli* protein (see Fig. 7C representing structure of MinE dimer from *N. gonorrhoeae*). Therefore, in these two structures, the anti-MinCD domain is not solvent

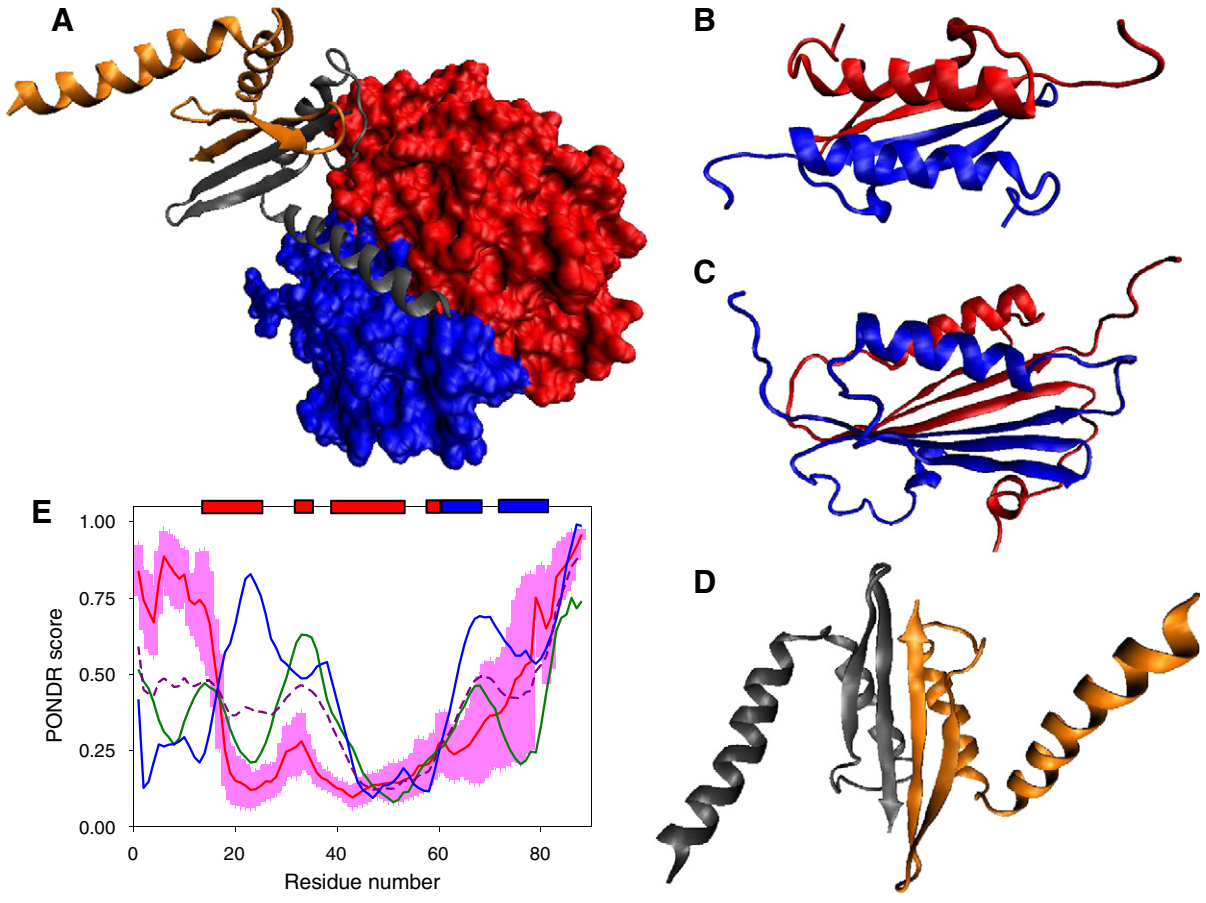

Fig. 7. Structural features of the Min system. A. Crystal structure of the MinD–MinE complex (PDB ID: 3R9J). B. NMR solution structure of the trypsin-resistant fragment of the *E. coli* MinE (PDB ID: 1EV0). C. NMR solution structure of the intact MinE from *Neisseria gonorrhoeae* (PDB ID: 2KXO). D. Crystal structure of the MinD-bound MinE dimer from *E. coli* (PDB ID: 3R9J). E. Intrinsic disorder evaluation in the *E. coli* MinE protein. Results of PONDR® VLXT, PONDR® VSL2 and PONDR-FIT are shown as blue, green and red lines respectively. The light pink shadow around PONDR-FIT prediction shows the statistical error of PONDR-FIT prediction. Elements of secondary structure detected in different crystal structures of MinE are shown as red (α-helices) and blue bars (β-strands) at the top of the plot.

accessible and therefore unavailable for binding MinD [261]. Fig. 7C shows that the *N. gonorrhoeae* MinE contains another structural element, a short N-terminal amphipathic helix (residues 3–8; residues 1–17 are not observed in the *H. pylori* structure) that is packed against the β-sheet, further masking it from the solvent.

Based on the analysis of these structures it was proposed that the interaction with MinD requires the dramatic conformational changes in MinE that would release the sequestered anti-MinCD domains [261]. This hypothesis was supported by a recent study, where the crystal structure of the *E. coli* MinD–MinE complex was solved at the 4.3 Å resolution [261]. MinE used in this study was the truncated version of the $MinE^{I24N}$ with a C-terminal his-tag, that underwent cleavage between amino acids 11 and 12 during the protein purification process following the cell lysis, whereas MinD was the $MinD\Delta10^{D40A}$ variant, which is a non-hydrolytic mutant that lacks its C-terminal amphipathic helix, that functions as an MTS. Fig. 7A represents structure of the resulting complex and shows that the MinD dimer (shown as blue and red surfaces) interacts with the MinE dimer (shown as gray and orange chains) in a rather unusual way. First of all, the MinE residues 13–26, which includes most of the residues that correspond to the β_1-strand of MinE, form an α-helix in the structure of the MinD–MinE complex. In other words, the β_1-strand (residues 21–29) undergoes transition to the α-helical structure when MinE binds to MinD. Therefore, the anti-MinCD domain of MinE contains a conformational switch that regulates and controls interaction between MinE and MinD. Fig. 7D represents the MinD-bound structure of the *E. coli* MinE to better illustrate the appearance of a new α-helix. It was also pointed out that MinE dimer bridges two MinD dimers leading to a continuous helix of alternating MinD dimers and MinE dimers [261]. Furthermore, in the MinE–MinD complex the N-terminus of the MinE contact helix is oriented toward the membrane providing possibility for the MTS to be on the same face of the complex as the MinD amphipathic helices and therefore in position to interact with the membrane [261]. Based on these observations it was suggested that that MinE senses MinD and undergoes a dramatic conformational change that releases the anti-MinCD domains and unmasks cryptic membrane-targeting sequences (MTSs) in MinE [261].

Fig. 7E represents the results of disorder prediction in the *E. coli* MinE by several predictors of the PONDR® family (PONDR-VLXT, PONDR-VSL2, and PONDR-FIT). It can be seen that this protein is predicted to possess noticeable amount of disorder, although there is a noticeable disagreement between the results of different predictors. Since the used computational tools were trained on rather different sets of attributes, they are able to "see" disorder under the different angles. Therefore, the mentioned disagreement between different predictors is a reflection of a rather complex nature of the MinE. Close consideration of the "conformational switch" region (residues 21–19) shows that it is expected to be disordered, suggesting that the intrinsically disordered nature of this region might define its capability to undergo structural transitions required for binding to MinD. Importantly, the C-terminal topological specificity domain of MinE also contains noticeable amount of disorder suggesting that intrinsic disorder might be involved in the MinE-controlled spatial regulation of cell division. This abundance of functional disorder in the MinE provides a support to the "Tarzan of the Jungle Model" proposed to describe the interaction between MinD and MinE [261]. Fig. 8 represents this model and shows that when MinE encounters MinD bound to the membrane it undergoes dramatic structural changes leading to the release of the MTSs and the β_1-strands of MinE from the six-stranded β-sheet structure and eventually resulting in formation of a four-stranded β-sheet structure. One of the released β_1-strands, together with the N-terminal flanking residues, forms a long α-helix that is involved in the MinD binding. MTS linked to this β_1-strand is involved in the membrane binding together with the two C-terminal MTSs of the MinD dimer. Another β_1-strand is likely to unfold bat can be tethered to the membrane through its linked MTS. Binding of MinE to the MinD dimer activates its ATPase activity. The ATP hydrolysis leads to the dissociation of MinD dimer and frees MinE. Now, the released MinE can be either handed off to another MinD, or dissociate from the membrane and fold back to the six β-stranded structure. Since the MinD density on

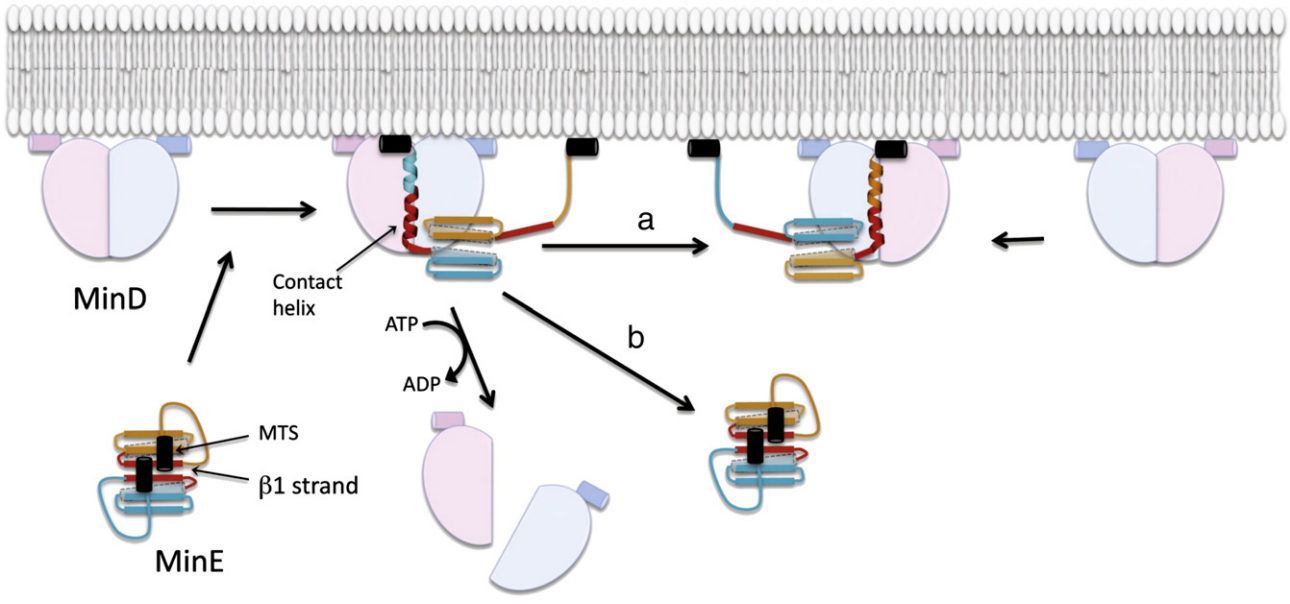

Fig. 8. "Tarzan of the Jungle Model" for the interaction between MinD and MinE. In this model, MinE encounters MinD bound to the membrane and the MTSs (black segments), and the β1 strands (red) of MinE are released from the six-stranded β sheet structure, resulting in formation of a four-stranded β sheet structure. One of the released β1 strands along with N-terminal flanking residues form an α helix that is stabilized by binding to MinD, whereas the other is tethered to the membrane through its linked MTS. The fate of MinE depends on two competing reactions (indicated by "a" and "b") following the dissociation of MinD due to ATPase stimulation. Either it is handed off to another MinD (a), or it dissociates from the membrane as it snaps back to the six β-stranded structure (b). A higher density of MinD on the membrane favors the former. Reproduced with permission from Ref. [261].

the membrane is high, the probability of MinE to be handed off to the neighboring MinD dimer is rather high [261]. Furthermore, this "Tarzan of the Jungle" mechanism explains how and MinE tracks membrane-bound MinD and why it moves toward regions of higher MinD density. It was also proposed that the described above mechanism explaining the behavior of the MinD–MinE system is likely to be applicable to other members of the WACA family [261].

It is tempting to hypothesize that the intrinsically disordered nature of MinE might be involved in the self-organized emergent behavior of the Min system and play a role in the oscillating MinD recruitment of MinE to the membrane. If this assumption is correct then varying the length of existing disordered regions in MinE should affect the intrinsic wavelength that is characteristic for the Min protein oscillations. This also opens a possibility to connect the oscillating protein intrinsic disorder-based reactions to the chaos theory. In fact, it was already shown that cyclic chemical reactions can be described in terms of the Rössler attractor (see Fig. 6B) that originally arose from studying oscillations in nonlinear, chemical reactions [273,274]. Therefore, there is a chance that the Rössler attractor (or another strange attractor) can be used to describe behavior of the biochemical reaction–diffusion processes that are based on the interactions driven by IDPs/IDPRs and that determine the self-organized emergent behavior of the related systems.

The relationship between the minE–minD system and chaotic attractors can be considered from another angle that considers both molecular and cellular aspects. At the cellular level, minE–minD oscillations constitute a reaction–diffusion system that might represent the simplest experimental realization of the Turing instability mechanism underlying morphogenesis. In fact, according to Turing, this instability driving the nonlinear dynamic system into patterns is based on diffusion, specifically, on two interacting molecular species differing significantly in their diffusion characteristics [242,253]. In subsequent development of this idea it was shown that only a restricted class of reaction–diffusion systems is capable of generating patterns, since for pattern to occur, it is necessary to have an antagonistic pair of molecular species, one of which (the activator) is self-enhancing and is characterized by the short range of action and coupled to the other one (the inhibitor) of long range [243,253]. Protein oscillations and pattern formation of MinE–MinD system in the cell are believed to be regulated by cooperative membrane binding and unbinding that serves as an energy-dependent switch [253]. However, even if the dynamics is chaotic at the cellular level and even if this chaotic dynamics defines the emerging behavior and appearance of intracellular waves that control cytokinesis in space [253], this does not necessarily imply that the dynamics must be chaotic at the molecular level. Alternatively, at this level, MinE–MinD system can be considered as an example in which the rich dynamic behavior of IDPs is used by nature for building complexity at a higher level, since the Turing mechanism is the basis for morphogenesis and cellular differentiation in multicellular organisms.

4. Concluding remarks

IDPs and IDPRs are intriguing members of the protein kingdom. Although they got into the spot-light of active research more than a decade ago and although significant progress has been achieved in this field, IDPs/IDPs continue to surprise researchers. To some extent, studies on IDPs resemble the peeling of an onion, when removing one layer uncovers another layer, which in turn hides a new level of complexity. We are slowly going through the multilayer problem, trying to reach its core; trying to understand what is so special about IDPs, their functions and regulations; trying to grasp how their amazing structural and functional complexities and the ability to be uniquely functional in the absence of unique spatial structures are encoded in the amino acid sequences of IDPs/IDPRs. The fact that IDPs and IDPRs are different from ordered proteins and domains is well-documented and rather well-accepted. Complexity and heterogeneity are the major universal characteristics of IDPs/IDPRs. These proteins are heterogeneous at multiple levels. Their sequences contain foldable, non-foldable and semi-foldable regions, which are proposed to be termed foldons, non-foldons and semi-foldons respectively. Foldons can be spontaneous (and some IDPs contain folded or partially folded regions) and can be induced (where IDPR gain structure as a result of binding to a specific partner). These sequence heterogeneity define structural heterogeneity of IDPs/IDPRs in their unbound state. IDPs/IDPRs are characterized by shallow energy landscapes with multiple local minima and without global energy minimum typical for the ordered proteins. Due to these shallow energy landscapes, IDPs/IDPRs are extremely sensitive to the changes in their environment, which further complicate these complex systems.

In addition to the active disorder, some proteins might contain dormant disorder. Here a protein or significant part of it is folded but inactive. Activization of these proteins involves functional unfolding (local or global), or, as we defined it here, awakening of dormant disorder. The corresponding "slipping beauties" are defined as unfoldons. IDPs/IDPRs can bind to multiple unrelated partners, and some of IDPs/IDPRs can gain different structures being bound to different partners.

In general, IDPs are characterized by the lack of a significant part of the folding code that defines the ability of ordered proteins to spontaneously fold into unique biologically active structure. This missing portion of the folding code (or a part of it) can be supplemented by the IDP binding partner(s). Even bound states of IDPs are structurally heterogeneous, since many of these proteins are able to preserve significant disorder after binding. This results in the formation of dynamic or fuzzy complexes.

Functions of IDPs/IDPRs can be controlled by multiple means, such as various PTMs, alternative splicing, interaction with different modulators, cleavage, etc. Therefore, functionally IDPs/IDPRs are heterogeneous too. This multilevel structural and functional complexity suggests that IDPs/IDPRs should be considered as systems at the "edge of chaos." Behavior of these systems is extremely sensitive to the environment and is characterized by the so-called butterfly effect, where small changes in initial conditions may produce large changes in the long-term outcome. Furthermore, intrinsic disorder may be related to the emergent behavior of several systems characterized by the presence of specific patterns and can be used to explain the biochemical reaction–diffusion processes.

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