

Unraveling protein's structural dynamics: from configurational dynamics to ensemble switching guides functional mesoscale assemblies

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Evidence regarding protein structure and function manifest the imperative role that dynamics play in proteins, underlining reconsideration of the unanimated sequence-to-structure-to-function paradigm. Structural dynamics portray a heterogeneous energy landscape described by conformational ensembles where each structural representation can be responsible for unique functions or enable macromolecular assemblies. Using the human p27/Cdk2/Cyclin A ternary complex as an example, we highlight the vital role of intramolecular and intermolecular dynamics for target recognition, binding, and inhibition as a critical modulator of cell division. Rapidly sampling configurations is critical for the population of different conformational ensembles encoding functional roles. To garner this knowledge, we present how the integration of (sub)ensemble and single-molecule fluorescence spectroscopy with molecular dynamic simulations can characterize structural dynamics linking the heterogeneous ensembles to function. The incorporation of dynamics into the sequence-to-structure-to-function paradigm promises to assist in tackling various challenges, including understanding the formation and regulation of mesoscale assemblies inside cells.

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Introduction

From sequence to ensemble(s) and function(s)

Proteins have a widespread and crucial role in maintaining the cell's metabolism, impacting almost every metabolic process to ensure survival and evolution. Since the first three-dimensional description of a protein at atomic

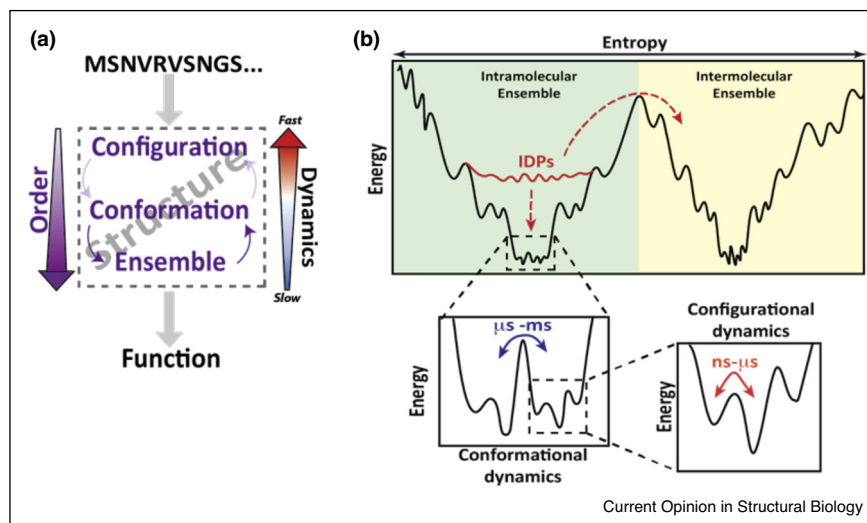
resolution obtained by Perutz in 1960 [1], the unanimated vision of a protein has led to the well-known sequence-to-structure-to-function paradigm. However, the classical textbook paradigm largely disregards the relevance of dynamical processes between these conformations; due to their inherent thermal fluctuations and chain flexibility, proteins continuously explore different configurations, reaching the accumulation of structural conformations that define the whole structural ensemble (Figure 1a). Then, dynamics not only allow reproducing what we observe as the native state, but also characterizes the proteins' function(s), properties, and regulation.

Using his seminal experimental findings, Anfinsen indirectly described the first evidence of inherent dynamics in the folding of a protein [2]. Building on that view, Levinthal [3], and later Wolynes [4], suggested that specific topological constraints from the amino acid chain must guide folding to satisfy the timescales typically observed *in vitro* and *in vivo*. The minimally-frustrated nature of proteins allows them to rapidly explore several short-lived configurations with high structural entropy and low transition energy barriers. Increasing the energetic barriers between configurations causes the adoption of a native/functional ensemble, highlighting the delicate balance between dynamics, structure, and function.

However, the discovery of proteins that show complex folding pathways leading to intricate functions has suggested a revision of this sequence-to-structure-to-function paradigm. Such is the case, for example, of proteins that dimerize via three-dimensional domain swapping (3D-DS) [5•]. These proteins contain local intrinsically disordered regions (IDRs), causing them to lack a well-defined, stable, and minimally-frustrated native ensemble. Moreover, several others are entirely disordered (IDPs) [6–10], showing highly-dynamical competing configurations (Figure 1b). While well-folded proteins show slower transitions as they jump over high energy barriers between distinct states, IDPs must be analyzed at shorter timescales to sample their different configurations due to their faster configurational dynamics.

Interestingly, for most locally or completely disordered proteins [11,12,13•], binding offers a mechanism for folding [14,15], adding a regulatory layer. For binding reactions in proteins and other macromolecules (i.e. nucleic acids) [16,17•,18,19], dynamics can exhibit dominant effects on association and/or dissociation rates by

Figure 1



Relationship between sequence, structure, dynamics, and function. **(a)** As the original paradigm stated, the linear sequence-structure-function relationship that explained the proteins' properties and functions obscure the relevance of the structural dynamics. Several meta-stable configurations are grouped into more stable conformations, defining the structural ensemble and what we observe as the native state. **(b)** Depending on the energy barriers between different structural transitions, different dynamic processes can occur between nanoseconds to milliseconds timescales depending on the energy barrier to pass. These processes conform to both intra or intermolecular ensembles. For intrinsically-disordered proteins (IDPs) (red line), the high structural heterogeneity leads to energy frustration. This heterogeneity can be decreased by employing contacts with small ligands or even other macromolecules, adopting intra- or inter-molecular structural ensembles (dashed red lines).

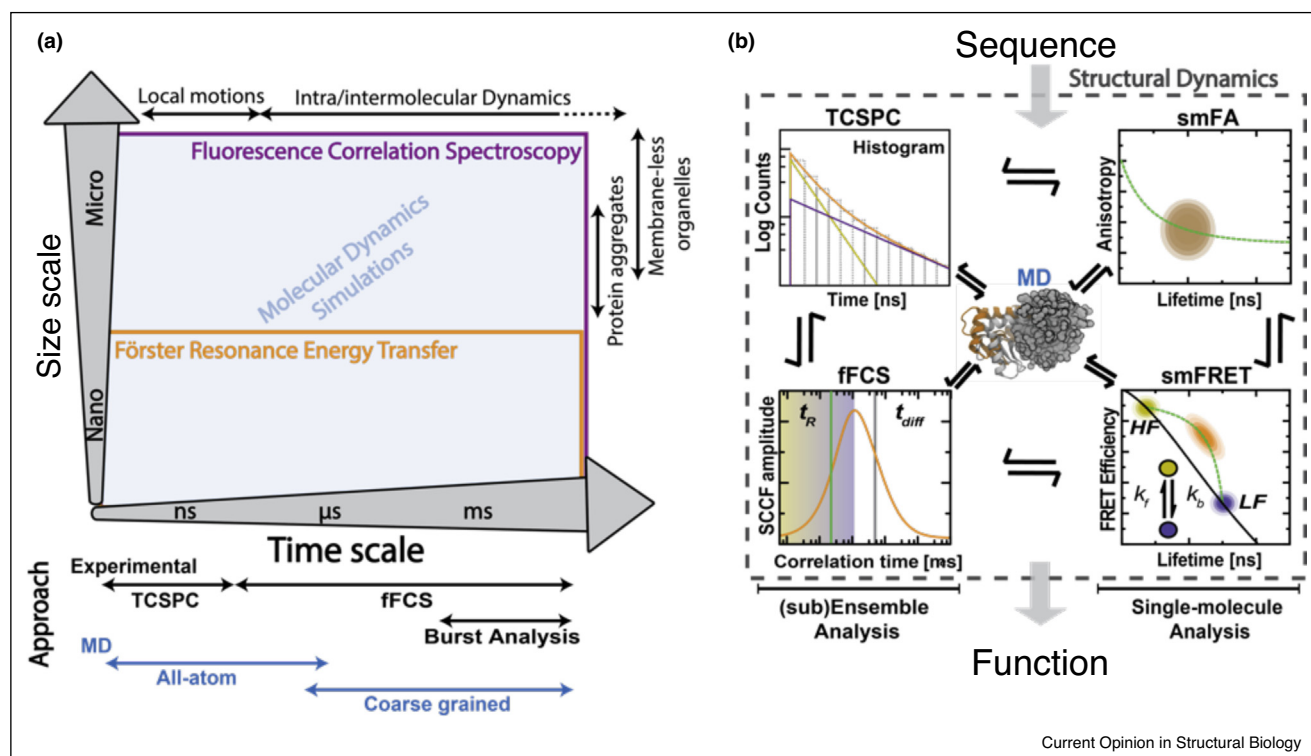
performing a pivotal role in specificity/promiscuity [20–22], thus affecting the lifetime of those complexes. In these cases, binding reactions allow switching between ensembles (Figure 1b). Additionally, binding, (un)folding, and dynamics can modulate micro and mesoscale molecular assemblies, such as membrane-less organelles [23,24[•]] and liquid phase condensates [25–27,28[•]], critical components in compartmentalization and other intricate functions within cells (Figure 1b). This new understanding of proteins fills a clear gap in the sequence-to-structure-to-function paradigm to explain numerous biological phenomena where the structure itself is insufficient.

Because solution nuclear magnetic resonance (NMR) can study molecules at the atomic level with a high temporal and spatial resolution [29], it is currently the gold standard ensemble approach to describe local and global structural changes of proteins in folding, binding, and function [30[•],31]. As such, NMR gives experimental descriptions of the intra- and intermolecular changes between pico- to milliseconds regimes [32] and dynamic behavior between micro- to milliseconds (and beyond), allowing extensive studies into their involvement in folding and binding [11,33,34] (Figure 2a). However, NMR and other classical ensemble methodologies, although possessing high temporal resolution, struggle to characterize the short-lived configurations of highly-flexible proteins due to the need

for high data throughput and ensemble averaging. For IDPs in particular, defined ensembles link to specific functions by integrating and processing signals when folded into stable structures upon binding to cellular regulatory partners, emphasizing the complexity of the (un)folding and function relationship.

The unique advantage of single-molecule methodologies is in their ability to unravel structural heterogeneity, in most cases, without ensemble averaging. Experimental results based on fluorescence are widely exploited due to their excellent structural and temporal resolution [35–37]. Taking into advantage the different approaches and experimental corrections derived from fluorescence, single-molecule multiparameter fluorescence spectroscopy (smMFS) is a robust methodology to accurately monitor and quantify local and global dynamic changes [38–40,41[•],42[•]]. When combined with (sub)ensemble approximations, such as Fluorescence Correlation Spectroscopy (FCS) and Time-Correlated Single-Photon Counting (TCSPC), smMFS allow the monitoring of structural changes in a broad time scale from nano-to-milliseconds [38–40,41[•],42[•]]. For slower processes, approaches focused on fixed molecules are ideal, monitoring real-time structural changes [41[•]]. Specifically, single-molecule fluorescence anisotropy (smFA) allows the monitoring of local changes that reflect side-chain dynamics. Also, single-

Figure 2



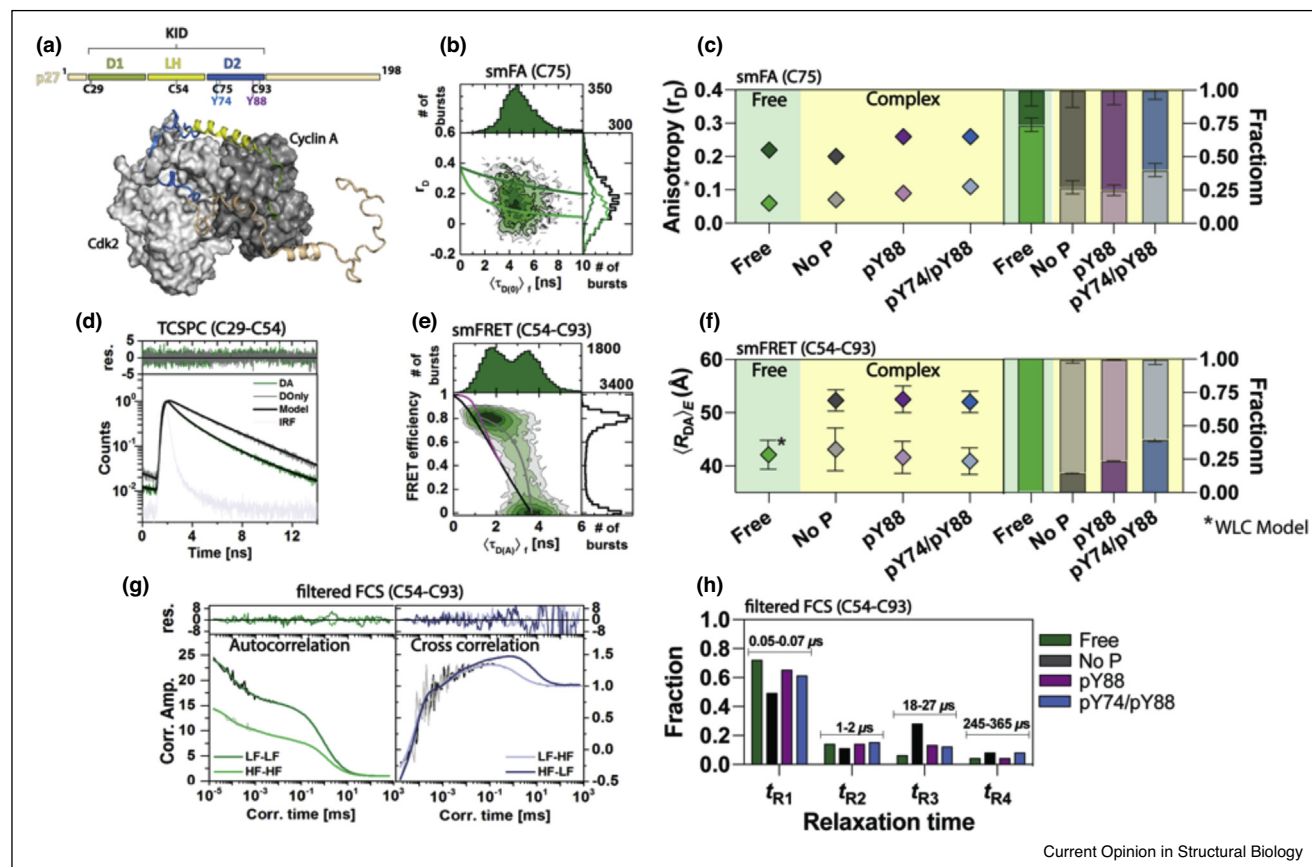
Conformational dynamics and experimental approaches to study. **(a)** Temporal and size scales covered by the combination of experimental and bioinformatic approaches. Main fluorescent methods in freely diffusing conditions (Fluorescence Correlation Spectroscopy -FCS- and Förster Resonance Energy Transfer -FRET-) are used by employing Time-Correlated Single Photon Counting (TCSPC), filtered FCS (fFCS) and burst analysis. These approaches are combined with Molecular Dynamics simulations (MD) (all-atom and coarse-grained) to cover from nano- to milliseconds in temporal resolution and from nano- to micrometers in size scale. For slower temporal scales, microscopy approaches focused on analyzing fixed molecules are ideal. **(b)** Flow chart to study structural dynamics using single-molecule multiparameter fluorescence spectroscopy (smMFS) toolbox. (Sub)ensemble (TCSPC and fFCS) and single-molecule (FRET and anisotropy) approaches, combined with MD can describe local structural changes at high temporal resolution. Each technique provides complementary information to each other, painting a complete picture across the accessible timescales: **TCSPC**: distribution of conformations present in a specified condition (monitored by FRET) that are stable on the nanoseconds timescale ($>$ fluorophore lifetime); **fFCS**: solving of different relaxation times accounting for structural changes across time; **smFA**: high sensitivity to local flexibility changes; **smFRET**: quantification of different distance changes spanning a protein or protein complex via High FRET (HF) or Low FRET (LF) states, distributions of these distances, and the kinetic forward (k_f) and backward (k_b) rates of exchange; **MD**: refinement of structural models generated by the experimental considerations.

molecule Förster Resonance Energy Transfer (smFRET), when used with time-resolved fluorescence spectroscopy (TCSPC), probes distance changes and population heterogeneity with nanoseconds resolution. When coupled to burst analysis, smFRET is sensitive to dynamics over broad time scales, from milliseconds to seconds depending on instrumentation [38–40,41,42]. Finally, filtered FCS (fFCS) becomes ideal for following exchange processes between FRET states to quantify the structural dynamics between (sub)micro- to milliseconds (Figure 2b).

Moreover, due to the comparable timescales covered by smMFS and molecular dynamics (MD) simulations (Figure 2a), the combination of experimental results with MD lead to more accurate structural dynamics

models to fully understand protein dynamics at the atomic scale [43]. In particular, coarse-grain models, by requiring less computational resources than all-atom models to manage intra- and intermolecular interactions [44–46], is preferred when modeling larger, complex, multi-protein structures, such as quinary protein structures [47,48]. As such, coarse-grain models have become instrumental in recent modeling [49–53]. By coupling the smMFS with computational approaches, the smMFS toolbox is built (Figure 2b). This toolbox allows us to monitor several aspects of protein function, including folding [36,54], super tertiary [55,56–59] and quaternary communications [5,13], and enzyme catalysis [60,61], emphasizing how those processes create more extensive, dynamic, three-dimensional systems responsible for life.

Figure 3



smMFS toolbox to study intra- and intermolecular dynamics of p27. **(a)** the topology of human p27 showing the relevant regions (D1, LH, and D2) of its Kinase Inhibitory Domain (KID). C29, C54, C75, and C93 are cysteine residues used to attach the different fluorophores, whereas Y74 and Y88 are tyrosine residues that can be phosphorylated. The ternary complex p27/Cdk2/Cyclin 2 is in cartoon. **(b)** smFA plot for free p27 monitoring C75 attached with Bodipy FI, showing the two anisotropy population of the D2 region. **(c)** Quantitative analysis of free p27 and the ternary complex with its different phosphorylation modifications (No P, pY88, and pY74/p88), showing anisotropy values and their fraction in all conditions. **(d)** TCSPC plot showing fluorescence decay of free p27 monitoring C29-C54 attached with donor and acceptor of FRET (DA), donor only labeled p27 (DOnly), and the instrument response function (IRF). Differences between DA and DOnly serves as a baseline for comparison and FRET efficiency determination. **(e)** smFRET distribution monitoring distance changes in free p27 labeled, as mentioned in (D). The black line corresponds to the static FRET line, gray line corresponds to the dynamic FRET between DOnly and high FRET, and in pink line, a worm-like chain (WLC) model considering an equilibrium between a disordered and folded protein. **(f)** Quantitative analysis of (E) shows distances and fractions in free p27 in the same conditions as mentioned in (C). For free p27, FRET distance was determined using a WLC model. **(g)** fFCS plots show both auto- and cross-correlation between low and high FRET. **(h)** Quantitative analysis from data obtained in (F) for p27 in all before mentioned conditions. Data fitting found four different exchanging times (t_R) for all conditions, showing the specific fraction for each one.

Uncovering the role of dynamics in the sequence-to-structure-to-function paradigm: conformational heterogeneity as pivotal for proteins' functions

One hallmark model highlighting the relevance of dynamics in protein function at high resolution is the human p27. This disordered protein causes cell cycle arrest when binding in a ternary complex with cyclin-dependent kinase (Cdk2) and cyclins (e.g. Cdk2/Cyclin A) [62,63] (Figure 3a). A recent integrative and collaborative work between multiple laboratories revealed how p27 morphs lead to the formation of the p27/Cdk2/Cyclin A complex. Different constructs of p27 were studied using stopped-flow kinetics and the smMFS toolbox

(Figure 2b) to identify the critical events that led to the initiation complex. An intricate combination of intra- and intermolecular dynamics seems to modulate this protein's biological function (Figure 3).

Intramolecular dynamics: structural heterogeneity of proteins as functional limiting events

Unbound or free p27 is mostly disordered while maintaining some residual alpha-helical structure in the LH subdomain consistent with prior studies [64–67]. As shown in Tsytlonok *et al.* [68[•]], free p27 adopts a compact conformation, impeding the acquisition of the ternary complex with Cdk2/Cyclin A. Hence, p27 must expand

to expose its 12 residues recognition site in the D1 subdomain [69], being crucial in the association kinetics to Cyclin A by undergoing conformational rearrangement before initial binding [68^{••}]. Similarly, the D2 region must exchange conformations for the recognition of the Cdk2 binding site. Local dynamics monitored by smFA of free p27 (Figure 3b and c) showed that free p27 shows two anisotropy values (Figure 3b and c) that reflects the flexible (low rD) and rigid (high rD) conformations. However, in the absence of target complexes, this protein is preferentially compacted (Figure 3c).

Additionally, by analyzing time-resolved fluorescence (Figure 3d) and single-molecule FRET (Figure 3e) probing various regions of p27, it was found that p27 must expand to create the ternary complex. For example, the FRET variant monitoring dynamics of regions LH and D2 (cysteines for labeling at locations C54 and C93, Figure 3a) shows a dynamic system by which p27 behaves as an intrinsically disordered protein (Figure 3e) following a worm-like-chain (WLC) model with an averaged donor-acceptor distance of 41.8 ± 2.3 Å (magenta dynamic line in Figure 3f). This result is consistent with NMR measurements, MD of the full-length p27, analytical ultracentrifugation, and small-angle X-ray scattering of the p27/Cdk2/Cyclin A complex [70,71^{••}]. The disordered nature of p27 permits jumps over low energy barriers and rapidly sample multiple configurations that can, over longer timescales, transition between distinct conformations or eventually accessing different structural ensembles, referred as ensemble switching [72,73]. ffCS can efficiently identify all these structural changes over a broad temporal domain (nano-to-milliseconds), corroborating that most of dynamical exchange occurs in the nanoseconds regime (Figure 3g).

Finally, the information derived from discrete MD (DMD) simulations, which samples the heterogeneous landscape, was used as an integrative element in the smMFS toolbox [74–80]. By using radius of gyration (R_g) and α -helical content on the same regions monitored by experimental observations, authors could compare inter-dye distances, local flexibility and polymeric behavior (like the persistence length). Thus, DMD and smMFS help each other as independent and complementary approaches without imposing physical constraints that biased either simulations or experimental observables into the attained results.

Although very useful for IDP models, this smMFS toolbox is not restricted to highly flexible proteins, but has identified transient conformations even in well-folded and minimally-frustrated models. Using the smMFS toolbox, Sanabria *et al.* [60^{••}] determined the conformations of the lysozyme of bacteriophage T4 (T4L) in the catalytic cycle progression. Three major conformations that are present in the free (E), enzyme-substrate complex

(ES), and enzyme-product (EP) bound states. These conformations exchange at few microseconds and hundreds of microseconds, extending the Michaelis-Menten mechanism and highlighting that specific conformations favor the progression of the enzymatic reaction. In contrast, for free p27, the transitions observed imply high conformational heterogeneity and flexibility according to its disordered nature (Figure 3h), which suggests that, although disordered, p27 must overcome an expansion to bind with Cdk2/Cyclin A. These examples highlight the relevance of using smMFS toolbox to temporally characterize the structural dynamics of diverse proteins.

Intermolecular dynamics: structural dynamics in multi-step binding and partial dissociation as function modulator

Once defined that p27 must extend to bind the Cdk2/Cyclin A complex, authors studied the main changes involved in forming the ternary complex. Using smFA (Figure 3c), NMR, and X-ray crystallography (X-ray), Tsytlonok *et al.* [71^{••}] discovered that p27 mostly adopts the extended conformation when it is bound to Cdk2/Cyclin A complex. Additionally, by analyzing different donor-acceptor combinations, two limiting states were obtained for p27 in complex. For example, using the FRET variant C54-C93 (covering the LH-D2 regions), authors found two distances with $\Delta R_{DA}^{E,exp}$ that go from 43.1 ± 0.1 to 52.3 ± 0.1 Å via smFRET (Figure 3f), showing a good agreement with the crystallographic structure (PDBID 1JSU). When modeling the accessible volume (AV) of the dyes in such configuration using coarse-grained simulations, results showed experimental-simulations differences within ~ 3 Å. The anterior indicates expansion from a more compact conformation to a conformation that exposes the D2 region and adds robust stabilization in the structural dynamics, as observed in ffCS (Figure 3h) by the accumulation of transitions fraction in the mid-microseconds regime. In summary, a fully formed, fuzzy ternary complex built with a simultaneous extension of p27 was identified [68^{••},71^{••}].

Furthermore, once p27 is bound to Cdk2/Cyclin A and causes cell cycle arrest, this ternary complex is finely regulated via phosphorylation of two occluded tyrosine residues by tyrosine kinases Bcr-Abl and Src for Y88 and Y74 (Figure 3a), respectively [81,82]. For these residues to be phosphorylated through dynamic anticipation, p27 exchanges between different conformations in the bound complex allow the sequential exposure of Y88, followed by Y74 anticipating phosphorylation [71^{••}]. Each of these phosphorylation conditions allow the accessibility of different conformational ensembles. The process was observed by using the smMFS toolbox (Figure 2b) and integrating other biochemical and biophysical methods, including NMR, isothermal titration calorimetry (ITC), and X-ray crystallography.

To start, smFA (Figure 3c) showed the release of Y88 followed by Y74, supported by the increase in a population with low anisotropy values, which indicates a more freely rotating fluorophore and in agreement with chemical shift assignments of the D2 domain (e.g., C75 and C93). Next, using the same FRET variant, C54-93, smFRET showed a redistribution of states occurs, shifting the population to a more extended partially released state, thus exposing the phosphorylated Y88 (Figure 3f). In this new state, Y74 is anticipated to be sequentially phosphorylated, evidenced by the release of C75 in smFA after Y88 and Y74 are phosphorylated (Figure 3c). With fFCS, a redistribution towards the accumulation of nano-seconds fraction exchange is described, suggesting that phosphorylation allows the adoption of a highly dynamic p27 is formed [71^{••}].

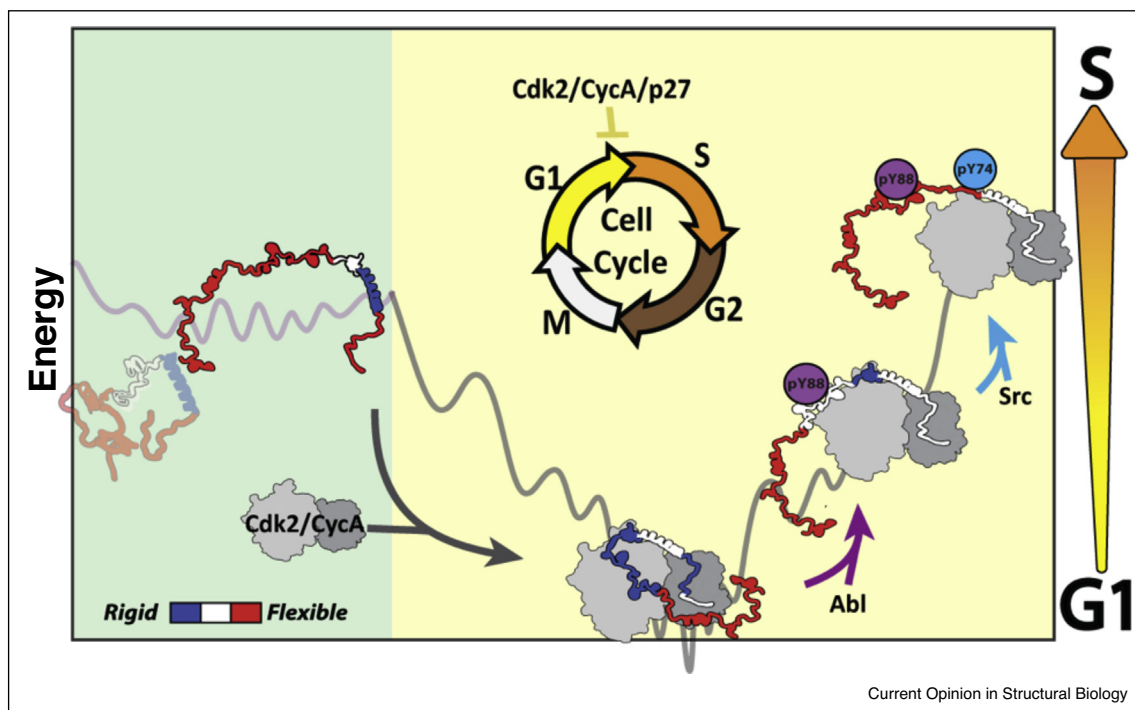
To showcase the role of partial dissociation and disorder in the structural dynamics, Medina et al. [5^{••}] studied the domain-swapped dimer of the DNA-binding domain of human FoxP1. The compact and folded dimer adopted via 3D-DS exchanges with an extended dimeric, mostly disordered, intermediate ensemble adopting heterogeneous structural changes occurring between 20 μ s to 5 ms. The extended intermediate is kinetically allowed

due to a low average energetic barrier of ~ 1 kcal mol⁻¹, resulting in the intermediate to become highly accumulated as the unfolding of the protein is promoted. This result indicates that the monomer-dimer transition overcomes the characteristic high energy barrier of three-dimensional domain swapping by containing IDRs [83,84]. Overall, the smMFS toolbox is powerful in capturing complex regulatory mechanisms from multi-step binding processes and complex folding pathways, supporting the need for updates of the current unanimated sequence-to-structure-to-function paradigm to a sequence-to-dynamics-to-function.

Perspective: from structural dynamics to function and protein assemblies

For cells to function correctly, proteins must work synergistically. Only by understanding how structural dynamics guide ensemble switching, we can understand how proteins self-assemble into multi-functional three-dimensional mesoscale architectures. Therefore, by following the relationship between dynamics and function, insights can be gained for various genetic diseases such as cancer [85–88], Huntington's [89], autism [90], spinal muscular dystrophy [91].

Figure 4



Functional connection between structural dynamics of p27 and cell cycle. The adoption of the ternary complex between p27/Cdk2/Cyclin A depends on the conformational exchange of free p27 and different conformational ensembles. The expansion of this protein enables the binding to Cdk2/Cyclin A, leading the cell cycle to arrest in phase G, impeding the development of phase S, and therefore DNA replication. Intrinsic flexibility of p27 allows phosphorylation modifications in its tyrosine residues 74 and 88, increasing the expansion and the release from the complex, and the consequent recruitment of ubiquitination proteins that finally leads to the degradation. These events allow Cdk2/Cyclin to continue their functional role in ensuring the cell cycle progression, therefore cell division.

The previous example of p27 binding with Cdk2/Cyclin A to form a ternary complex shows how intra- and intermolecular and intermolecular interactions must work together to regulate the cell cycle [62,63]. Binding only occurs due to the intramolecular behavior of p27, which allows a rapid sampling of multiple configurations to access the extended conformation (Figure 4). Intermolecular interactions with Cdk2/ Cyclin A impedes the cell division by arresting the cycle in phase G. This p27/Cdk2/ Cyclin A association has enormous metabolic significance. Further, it is tightly regulated by specific phosphorylation modifications that trigger p27 ubiquitination followed by degradation. Degradation of p27 enables cell cycle progression [70,81]. However, as discovered, all these events inherently depend on the structural dynamics that characterize p27. The p27/Cdk2/Cyclin A complex is a clear example of where dynamics lead to a change in conformations and defined ensembles that allows the complex to adapt specific functionality. This model and others [56,92,93] have recently revealed the extreme relevance of conformational dynamics as a key functional modulator.

The next logical step is understanding high-order assemblies and their role in modulating the function of the cells. So far, there are few characterized examples by which high-order complexes communicate in relevant processes [58,94]. Such is the case of the dynamics of chromatin, where nucleosome opening/closing transitions stability can severely influence the gene expression activity inside the nucleus. A combination of single-molecule approaches with molecular dynamic simulations found that binding with external proteins severely influences nucleosome dynamics [40,41*,57,95*,96], pivotal to decipher how gene expression occurs. Dynamics are also an essential part of polyfunctional molecules, where molecular adaptors must be coordinated to ensure the appropriate function depending upon the situation [97].

Future studies are required for highly dynamic and less ordered complex systems, such as biomolecular condensates, mitotic spindles, and focal adhesions [98*]. All characterized examples focus the essential role of heterogeneity in dynamics, by which molecules may explore various conformational ensembles, each with crucial consequences in those complexes and their stability. However, although much is still left to understand micro- and mesoscale assemblies within cells, current studies are focused on applying all these high-resolution approaches inside cells to increase the understanding of structural dynamics and assemblies in a real biological context [99,100]. In the near future, we anticipate that this holistic toolbox presented will continue to unravel the sequence-to-function relationship of many mesoscale assemblies in live cells.

Conflict of interest statement

Nothing declared.

Author contributions

EM and DL wrote the outline with guidance from HS. All authors contributed to writing the manuscript.

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