Modulation of Intrinsically Disordered Protein Function by Post-translational Modifications

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Post-translational modifications (PTMs) produce significant changes in the structural properties of intrinsically disordered proteins (IDPs) by affecting their energy landscapes. PTMs can induce a range of effects, from local stabilization or destabilization of transient secondary structure to global disorder-to-order transitions, potentially driving complete state changes between intrinsically disordered and folded states or dispersed monomeric and phase-separated states. Here, we discuss diverse biological processes that are dependent on PTM regulation of IDPs. We also present recent tools for generating homogenously modified IDPs for studies of PTM-mediated IDP regulatory mechanisms.

The amino acid sequences of intrinsically disordered proteins or protein regions (often simply referred to as IDPs) determine their inability to fold into stable tertiary structures under physiological conditions and instead enable them to rapidly interconvert between distinct conformations to mediate critical biological functions (1, 2). The amino acid compositions of IDPs range from very low complexity with little diversity in residue types to much higher sequence complexity that enables disorder-to-order transitions upon binding or post-translational modifications (PTMs) (3–5). This range of sequence composition leads to heterogeneous ensembles with variable hydrodynamic properties and fluctuating secondary and tertiary structure, with some IDPs able to self-associate in phase-separated protein-dense droplets (6–11). Structural heterogeneity and dynamic fluctuations endow IDPs with unique properties that do not exist in the other standard set of 20 amino acids. PTMs can modulate IDPs via chemical modification of the primary sequence (e.g. phosphorylation and acetylation) or through reversible alterations to the conformational ensemble, such as addition of the small ubiquitin-like modifier (SUMO) or addition of isopeptide bonds. The resulting chemical and structural changes resulting from PTMs can induce a range of effects, from local stabilization or destabilization of IDPs to global disorder-to-order transitions.

Due to their accessibility to modifying enzymes, IDPs are the predominant sites of PTMs, which significantly expands their functional versatility (3, 23). By changing the physicochemical properties of the primary sequence, PTMs induce a range of structural changes, from local stabilization or destabilization of transient secondary structure to more global conformational changes in disorder-to-order transitions (24, 25). As the hydrodynamic properties of IDPs are strongly affected by electrostatic effects, PTMs that change charge (e.g. phosphorylation and acetylation) can modulate compactness (9–11). PTMs can also lead to complete state changes, between disordered and folded states (26–28) or dispersed monomeric and phase-separated states (6, 8, 22). The scope of PTM-mediated structural and dynamic changes described in recent biophysical and biochemical studies of IDPs is similar to those due to binding to other proteins, nucleic acids, lipids, carbohydrates, ions, cofactors, and other small molecules (4, 29, 30).

Here we review the effects of PTMs on IDP structure and function and how such effects regulate fundamental biological processes, from PTM-mediated conformational transitions of individual IDPs to the assembly of multi-protein complexes and the formation of membraneless protein organelles. We begin by presenting the diversity of PTMs and their effects on IDP structure and function and then focus on phosphorylation, one of the most common PTMs. Because the investigation of PTM effects on IDPs is often limited by the ability to generate homogeneously modified samples, we discuss recent advances in recombinant protein expression systems and chemical synthesis strategies for producing modified IDP samples. These enable detailed biochemical and biophysical characterization using general methods for IDPs as well as those specific for IDPs with PTMs (4, 30, 31)

Diversifying the Structural Properties of IDPs via PTMs

Biological systems evolved multiple types of PTMs to expand the functional diversity of proteins, particularly of IDPs (3, 32, 33). Usually, PTM involves the addition of chemical functional groups, including phosphoryl, alkyl, glycosyl, and acyl groups, or small proteins such as ubiquitin and SUMO. In other cases, PTM involves modifying the chemical properties of amino acids through oxidation, deimidation, and deamidation. These modifications generate proteins with “new” amino acid compositions because each PTM results in novel chemical and steric properties that do not exist in the other standard set of 20 amino acids. In contrast, PTMs such as cis-trans isomerization of prolines, epimerization of serines, intein-based protein splicing, and the cleavage of the polypeptide chain at specific sites by proteases do not change the chemical nature of the amino acid side chains. IDPs can have single or multiple PTMs or a combination of different PTMs on the same or different amino acids, providing many layers of complex biological regulation.
due to the additive, cooperative, or competitive responses between these multiple modifications (34–37). Although most are catalyzed by specific enzymes, e.g. transferases and isomerases, some PTMs, including l-aspartate isomerization and l-asparagine deamidation, occur spontaneously (38).

PTMs elicit diverse effects on the biological functions of IDPs by altering the energetics of their conformational landscape and by modulating interactions with other cellular components (4, 31). PTMs can regulate IDP function by (i) altering their steric, hydrophobic, or electrostatic properties due to primary structural effects (39); (ii) stabilizing, destabilizing, or even inducing secondary structural elements (24); and (iii) inhibiting/enhancing long-range tertiary contacts between distal motifs or PTM sites within the IDPs, or with interaction partners (40). PTMs can tune all aspects of IDPs, including the nature of the disordered ensemble (i.e. extended versus collapsed), the propensity (if any) of the secondary structural elements, the types of transient intra-molecular tertiary contacts, the (in)ability to fold into stable three-dimensional structure or to interact with a binding partner or specific modular domains that interact with post-translationally modified residues during signal transduction, and the partitioning into membraneless, phase-separated, protein-dense cellular bodies (Figs. 1 and 2). Through these varied responses, biological signal inputs are written onto IDPs to change their functional output.

**Phosphorylation: The Prototypical PTM**

To provide insights into how PTMs elicit such effects on the structure and ultimately function of IDPs, we focus on one of the most common PTMs, phosphorylation. At least a third of eukaryotic proteins may be phosphorylated, and most phosphorylation sites are within intrinsically disordered regions.
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Phosphorylation and dephosphorylation of IDPs by kinases and phosphatases, respectively, provide a major regulatory mechanism in key eukaryotic processes, e.g. control of cell cycle, transcription, splicing, and translation. About 2% of the human genome is dedicated to coding for over 500 protein kinases, representing the largest class of PTM enzymes. In mammals, ~90% of phosphorylation occurs on serine with ~10% on threonine or tyrosine.

Phosphorylation replaces a neutral hydroxyl (OH) group with a tetrahedral phosphoryl group (PO₄²⁻) bearing two negative charges, drastically altering the steric, chemical, and electrostatic properties and introducing new interaction capabilities. Under physiological conditions, the double negative charge of the phosphoryl moiety together with its large hydration shell is chemically different from the acidic amino acids (Asp and Glu) often used as phospho-mimics (26, 44) that possess small hydration shells and only a single negative charge. Phosphorylation provides novel possibilities for intra- and intermolecular electrostatic interactions, including salt bridges, via charge or hydrogen-bonding networks involving up to four phosphoryl oxygens. Phosphorylated residues can form strong salt bridges with the guanido moiety of arginines, which are better able to interact with phosphoryl groups than protonated primary amines of Lys side chains due to their rigid planar structure and ability to form multiple H-bonds (45). The interaction strength depends on the proximity, orientation, and surrounding environments of the phosphoryl and guanido groups, allowing fine-tuning by the neighboring amino acid composition within the IDP. Phosphorylated residues can also interact with helix dipoles. Phosphoserine is the most stabilizing amino acid for helix dipoles. Phosphorylation leads to conformational switching.

Disorder-to-folding Transitions

Although IDPs lack the ability to fold into stable three-dimensional structures on their own under physiological conditions, many IDPs undergo some degree of folding upon ligand binding (2, 4, 50, 51). Such disorder-to-order transitions range from the stabilization or induction of secondary structural elements such as α-helices to the complete folding of an entire IDP upon binding to a partner. Binding of some IDPs to different physiological protein targets can even induce different folded states (4, 52–55). These observations demonstrate that changes in the surrounding physico-chemical environment can determine the ability of IDPs to fold and the nature of the fold itself. PTMs, by altering the physical and chemical properties of IDPs, can also provide a mechanism for partial or complete folding of IDRs or of entire IDPs, and these transitions can be exploited for regulating biology (Fig. 1). The reverse is also true in that PTM of folded proteins or stable secondary structural elements can lead to their unfolding or destabilization (27, 28). Although there are numerous examples of structural changes upon PTMs (56–60), here we describe two examples in which multi-site phosphorylation leads to conformational switching.

The E26 transformation-specific (ETS) domain-containing protein family is one of the largest groups of transcription factors with different members having distinct mechanisms of regulating DNA recognition by their ETS domains (61) (Fig. 3A). The ETS domain of Ets-1 is regulated by a flanking autoinhibitory module (IM) and an adjacent disordered serine-rich region (SRR). The IM contains four α-helices (HI-1, HI-2, H4, and H5) that pack against the ETS domain on the surface opposite to the DNA binding interface and, upon DNA binding, IM becomes more flexible with the entire HI-1 helix undergoing an order-to-disorder transition (62–65). Transient SRR interactions with both the IM and the ETS domain repress DNA binding. However, multiple phosphorylation of up to five sites in the SRR, as a result of Ca²⁺ signaling, further reduces DNA binding by dampening the flexibility of the SRR, the ETS domain, and the IM, resulting in an allosterically induced disorder-to-order transition for HI-1. Physico-chemical analysis of the SRR revealed that phosphorylation-induced autoinhibition is mediated by interactions between aromatic residues and phosphoserines, highlighting a novel intramolecular regulatory mechanism involving PTMs of IDPs (66).

Another example in which PTM-mediated conformational switching plays a critical role is that of the phosphorylation-induced folding of 4E-BP2, which controls cap-dependent mRNA translation initiation (Fig. 3B) (26). 4E-binding proteins (4E-BPs) inhibit translation by binding to eukaryotic initiation factor (eIF) 4E, which is part of the tripartite eIF4F complex (eIF4E, eIF4G, and eIF4A) that recruits the 40S ribosome to mRNA (67). 4E-BP binding to eIF4E prevents the assembly of eIF4F by competing with eIF4G because they interact at an overlapping eIF4E surface using the same YXXXXLΦ canonical binding motif (68). Previous studies support the formation of an extended, bipartite, fuzzy interaction with eIF4E, with a disorder-to-helix transition for the canonical binding motif (69–72).
Upon hierarchical multi-site phosphorylation of Thr\textsuperscript{37}/Thr\textsuperscript{46}, followed by Thr\textsuperscript{70}, Ser\textsuperscript{65}, and Ser\textsuperscript{83}, the affinity of 4E-BP2 for eIF4E is significantly weakened, enabling eIF4G interaction and translation initiation (73). Phosphorylation of Thr\textsuperscript{37}/Thr\textsuperscript{46} of 4E-BP2 reduces the affinity for eIF4E by inducing a disorder-to-order transition, resulting in the folding of residues Pro\textsuperscript{18–}Arg\textsuperscript{62}.

FIGURE 3. IDP-mediated disorder-to-order transitions to regulate protein complex formation. A, top, schematic representation of Ets-1, composed of an N-terminal protein interaction domain (PNT, gray), the transactivation domain (TAD, gray) followed by a C-terminal SRR (blue), and the IM domain (cyan) flanking the ETS DNA binding domain (red/yellow). Lower, in the apo state, helix HI-1 of the IM domain is in dynamic equilibrium between folded and disordered conformations (Protein Data Bank (PDB) codes 1R36 (65) and 1MDM (64), respectively). Upon multi-site phosphorylation on the SRR (blue to red circles), SRR dynamic fluctuations are damped, enhancing transient stabilizing interactions with the ETS and IM domains, further reducing DNA binding. B, regulation of the 4E-BP2/eIF4E complex by phosphorylation of Thr\textsuperscript{37} (pT37) and Thr\textsuperscript{46} (pT46) leads to folding of residues Pro\textsuperscript{18–}Arg\textsuperscript{62}, with further phosphorylation at Ser\textsuperscript{65}, Thr\textsuperscript{70}, and Ser\textsuperscript{83} stabilizing the folded state, enabling translation initiation. In the absence of eIF4E, non-phosphorylated 4E-BP2 is disordered albeit with significant transient secondary structural elements (69). 4E-BP2 utilizes residues from about Tyr\textsuperscript{34} to Asp\textsuperscript{90} consisting of a helical element (red) containing the canonical binding (54YXXXXL/H9021)60 motif and a flexible secondary binding site involving IPGVT82 for eIF4E (brown surface) binding. (Note: The longest observed fragment of a 4E-BP in complex with eIF4E in a crystal structure (72) is only Met\textsuperscript{49} to Ser\textsuperscript{82} (PDB code: 4UED).) This dynamic complex is represented by an ensemble of three conformers of 4E-BP2 on the surface of eIF4E.
Arg\textsuperscript{62} of 4E-BP2 into a four-stranded $\beta$-domain that is incompatible with binding, whereas phosphorylation of Ser\textsuperscript{65}, Thr\textsuperscript{70}, and Ser\textsuperscript{75} stabilizes that folded domain (26). The discovery of the phosphorylation-mediated folding of 4E-BPs exemplifies another mode of biological regulation mediated by IDP PTM.

**PTMs of Histone Tails to Generate a Histone Code for Chromatin Regulation**

Another biological process modulated by PTMs of IDRs is chromatin regulation. The fundamental building block of chromatin is the nucleosome core particle: $\sim$147 bases of DNA wrapped around an octamer of two copies each of H2A, H2B, H3, and H4 proteins. PTM of the N- and C-terminal IDRs of histones (histone tails) is a major mechanism for regulating the accessibility of the DNA within chromatin (74). PTM of histone tails is a dynamic process that transduces input signals from the cellular environment to regulate diverse genomic functions including DNA replication, transcription, repair, and recombination. Histones undergo a diverse array of PTMs including acetylation, methylation, ubiquitination, and sumoylation of lysines; methylation and citrullination of arginines; phosphorylation of serines, threonines, and tyrosines; and isomerization of prolines. For instance, acetylation of histone lysine residues neutralizes the positive charge on the lysine and produces profound changes in the interaction between histones and negatively charged DNA molecules (75). Acetylation and methylation can also allow the docking of protein machinery such as chromatin remodeling complexes that contain bromo and tudor domain interaction modules (49). Recent advances in NMR spectroscopy enabled the atomic-level structural and dynamic characterization of the nucleosome core particle and its complex formation (76), paving the way for future characterization of these diverse PTMs on the nucleosome. Future research will need to elucidate crosstalk between these different histone PTMs with respect to chromatin structure and function.

**Protein-Phospholipid Interactions**

Many non-membrane proteins are tethered to phospholipid membranes via PTM-mediated hydrophobic anchors such as myristate, palmitate, isoprenoid, or glycosylphosphatidylinositol groups (32). These PTMs enable subcellular localization required for specific spatiotemporally restricted function(s). Another mechanism of protein-phospholipid interaction involves direct lipid binding by proteins in a process that is also regulated by PTMs. A potential synergy between these two modes of protein-lipid interaction was recently demonstrated for the non-receptor tyrosine kinase c-Src (Fig. 4A) (77). Src family kinases are modular proteins consisting of a C-terminal catalytic (Src homology 1 or SH1) domain, SH2 and SH3 interaction domains, and two N-terminal IDRs, a Unique domain (UD) and an SH4 domain (78). c-Src either resides in the cytoplasm or is attached to phospholipid membranes via a myristate group attached to the SH4 Gly\textsuperscript{2}, a PTM necessary but not sufficient for membrane anchoring. The SH4 using residues \textsuperscript{14}RRR\textsuperscript{16} and the UD using the unique lipid binding region consisting of residues 60–67 can each bind phospholipids directly. However, PKA phosphorylation of the SH4 Ser\textsuperscript{17} and cyclin-de-
Ddx4, a major component of nuage (84) that plays a critical role in the Piwi-interacting RNA pathway by protecting spermatozoa and spermatids from deleterious activities of transposable elements (85). Ddx4 consists of a central DEAD box RNA helicase domain and N- and C-terminal IDRs. These Ddx4 IDRs direct its spontaneous self-association into membraneless proteinaceous organelles in HeLa cells and the isolated 250-residue N-terminal IDR phase-separates in vitro (84). Alternating charge blocks and an over-representation of FG/GF and RG/GR motifs within the positively charged blocks appear to be key to organelle formation. Phase-separated Ddx4 N terminus excluded double-stranded DNA, but enriched single-stranded DNA in the interior of the droplets, suggesting that the phase-separated state has a functional role in nucleic acid biochemi-
try (Fig. 2B). PTM by asymmetric arginine dimethylation on known conserved sites significantly inhibits phase separation by lowering the phase transition temperature by 25 °C. These in vitro data suggest that PTM is a cellular mechanism for regulating nuage assembly/disassembly in response to physiological input signals.

The phase separation that drives formation of signaling puncta based on multivalent modular binding domain-ligand interactions is also highly dependent on PTMs (Fig. 2C). As indicated above, modular binding domains recognize PTMs within IDRs, and these interactions are now implicated in phase separation. The degree of tyrosine phosphorylation (pTyr) on the disordered cytoplasmic tail of nephrin controls the phase transition of the multivalent three-component nephrin-NCK-N-WASP system. There are three potential nephrin pTyr sites that can each interact with the single SH2 domain of NCK, whereas the three SH3 domains of NCK can interact with the six proline-rich motifs of N-WASP (20). Phase separation induced by multivalent interactions of signaling proteins results in clustering of regulatory or regulated factors to facilitate a specific biological process. For instance, in the presence of the Arp2/3 complex, the phase separation of nephrin-NCK-N-WASP assembles actin filaments (21). These examples highlight the key roles PTM of IDRs play in regulating protein-based cellular organization (Fig. 2).

Generating Post-translationally Modified IDP Samples for Biochemical and Biophysical Characterization

There are many experimental tools and computational approaches to study IDPs that are applicable to those with PTMs as well, including some that are specific for these modified IDPs (4, 31). NMR spectroscopy, small angle x-ray scattering, fluorescence, circular dichroism, and other data can be acquired, and a number of computational algorithms can be used (30). However, a major impediment for rigorous quantitative biochemical and biophysical structural and dynamic characterization of PTM-mediated effects on IDP structure and function is generating the large quantities of homogeneous and site-specifically modified samples that are typically required for such studies. To overcome these challenges requires a combination of traditional biochemical, advanced molecular biology approaches, as well as improvements in chemical synthesis and semisynthetic strategies (86–89). Here we describe three strategies for generating modified IDPs.

Expressing Post-translationally Modified IDPs Using Recombinant DNA Technology

Advances in biochemical and molecular biology approaches have dramatically increased the use of recombinant DNA technology to design, express, and purify IDPs that are post-translationally modified from a range of hosts including Escherichia coli, yeast, plants, insects, or mammalian cell lines. PTMs can be introduced after mRNA translation using modifying enzymes either produced in a co-expression system (PTM within the cell) or expressed separately (in vitro PTM). Due to the possible generation of non-homogenous or nonspecific modifications from such modifying enzymes, a second approach involving amber codon suppression technologies allows direct introduction of the PTM during mRNA translation with an orthogonal tRNA/ami-noacyl-tRNA synthetase pair that genetically encodes an unnatural amino acid already containing the PTM of interest (90–92). A more complex approach is to use an unnatural amino acid that contains a selective chemical tag for later incorporation of the PTM (93). Protein expression systems provide a relatively cheap and robust mechanism for generating large quantities of high fidelity samples for a variety of peptides and proteins with homogenous PTMs, including enabling isotopic labeling (15N/13C/2H) for sophisticated NMR or mass spectrometry studies. The development of completely re-coded or re-engineered expression hosts and advances in in vitro protein expression systems will undoubtedly facilitate our ability to generate site-specific, homogenously modified IDP samples in the future (94–96).

Chemical Synthesis of Modified IDPs

One of the advantages of the chemical synthesis approach is the ability to generate IDPs with single or multiple (identical or otherwise) PTMs located anywhere along the polypeptide chain (86). This strategy involves the generation of shorter peptide fragments containing the PTM(s) of interest and their assembly into full-length IDP using the native chemical ligation (NCL) technique first introduced by Kent and co-workers (97). NCL is a chemoselective method based on the transthioesterification of two polypeptide fragments, one with an N-terminal cysteine and the other with a C-terminal thioester, and it allows two fully unprotected peptide fragments to be reacted under neutral, aqueous conditions (86, 87).

Semisynthesis: A Mélange of Chemical Synthesis and Protein Expression

Semisynthesis approaches are the most powerful methods for obtaining modified IDPs, as they combine the advantages of chemical synthesis and protein expression to generate peptide fragments for the assembly of full-length IDPs using either the NCL or the expressed protein ligation techniques (98, 99). For example, the N-terminal peptide fragment containing a C-terminal thioester can be chemically synthesized (NCL) or obtained via intein-based protein expression, whereas the C-terminal fragment with an N-terminal cysteine residue can also be chemically synthesized or expressed fused to a cleavable tag such as SUMO. As discussed above, these fragments can be modified with PTM(s) of interest using either chemical or biochemical strategies prior to NCL or expressed protein ligation (98).

Conclusion

The number and diversity of post-translationally modified IDPs are astounding; over 300 distinct PTMs occur on eukaryotic proteins, and the human proteome contains up to a million modified peptide motifs (32, 33, 100). PTMs play extensive roles in modulating the conformational properties and functions of IDPs. However, the structural, binding, and large-scale association effects of modified IDPs for most of these are currently unknown. State-of-the-art techniques in mass spectrometry and for generating homogeneously modified IDPs will increase the identification of PTMs of IDPs under various phys-
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