Illuminating the molecular operations through study of protein interactions

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ABSTRACT

Molecular mechanism research is a fascinating area involving multidisciplinary scientists. This study offers insight into the characterization of protein interactions, which is an important subject of this research. The discussion covers case studies on the principles associated with neurotransmitter release and associated factors. The concentration on methods like Nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, as well as other biophysical methods, are also included. These studies have revealed a complex cascading process leading to neurotransmitter release: (i) a tight complex of the soluble SNAP receptor (SNARE) proteins brings the plasma membranes and synaptic vesicle together, essential activity for membrane fusion; (ii) auto-inhibitory closed conformation is adopted by SNARE syntaxin-1 (iii) Munc18-1 plays a vital role by interacting with closed syntaxin-1 and with the SNARE complex; (iv) opening of syntaxin-1 is mediated by Munc13s; (v) through distinct interactions with the SNARE complex, complexions play dual roles; (vi), synaptotagmin-1 interacts simultaneously with the membranes and the SNAREs and acts as Ca21 sensor; and (vii) neurotransmitter release is modulated by a Munc13 homo-dimer to Munc13-RIM hetero-dimer switch. This review highlights the complex molecular mechanisms and explains how these mechanisms are significantly influenced by strong and weak protein interactions.

Keywords: Proteins; Interactions; Molecular mechanisms; Neurotransmission.

INTRODUCTION

As we know, complex protein machinery often controlled by immense types of biological processes are responsible for the life of living things. The main focus of the studies on molecular mechanisms is to understand how these machines work in a synchronized manner. Through this, researchers learn to gain a basic understanding of the subject, and also it can guide the design and technique of therapies for diseases that can emerge from flaws in these systems. These investigations on molecular mechanisms help to understand all the proteins that govern the biological processes, the analysis of their role and functions, and recognize correct interactions among them [1-3]. A factual mechanistic understanding can be gained through detailed characterization of these interactions. Ultimate goals and the expected outcome of such studies are often to re-constitute the relevant complexes with purified components, to find out their three-dimensional structures, and to explain how re-arrangements of these complexes are responsible for the working biology of the system. This constitutes multi-disciplinary research since knowledge of diverse techniques, methods, and expertise is required, thus involving collaborative efforts of scientists of different backgrounds. Although the outcome of the interdisciplinary research and interactions can be extremely encouraging, integration of the data generated with various approaches is impeded by the diverse views with respect to characteristic terminologies of respective disciplines[4-6]. This review serves a dual purpose that offers our outlook and perspective on (i) the study of molecular mechanisms through the lens of biophysicists and (ii) how techniques of biophysics such as nuclear magnetic resonance (NMR) spectroscopy with other tools in combination help to understand molecular mechanisms involved in protein interactions. We anticipate that this review will be of good help to biophysicists and researchers dealing with NMR spectroscopy studies, particularly those interested in the analysis of molecular mechanisms concerned with protein interactions. In addition, we also hope the examples quoted will be of

great help to researchers from other disciplines involved in biophysical studies of protein interactions and molecular mechanisms. It is beyond the scope of any review to discuss this area of research in a comprehensive manner. Instead, experiential learning from the authors is focused here through case studies on neurotransmitter release covering various related principles. We commence with a concise description of the neurotransmitter release mechanism and machinery. Next, general considerations on the analysis of protein interactions and a few basic NMR experiment concepts important to studying protein interactions are discussed followed by the case studies [7-9]. From the outset, we call the attention of the readers to the fact that all techniques have their respective advantages and limitations, which makes it imperative to combine strategies and approaches that can provide complementary information.

The neurotransmitter release machinery

A key event in inter-neuronal communication is Neurotransmitter release. The neurotransmitter release process involves sequential steps that include the attachment of synaptic vesicles to the presynaptic active region, getting the machinery ready to a release-ready state, and Ca^{2+} activated fusion of the plasma membranes and vesicle [10-12]. Of the different components belonging to the release machinery, an instrumental role is played by N-ethylmaleimide-sensitive factor (NSF), soluble NSF attachments proteins (SNAPs), the solubilized SNAP's receptor (SNARE) proteins syntaxin-1, synaptosomal, and synaptobrevin protein of 25 kDa (SNAP-25, not related to SNAP's), as well as the Sec1/Munc18 (SM) protein Munc18-1 [13]. Fusion at most intracellular membrane compartments is mediated by these proteins or their homologs, commonly accepted as a universal mechanism of fusion. Vesicle and Plasma membranes fusion is achieved through the key role of three SNAREs that form a tight 'SNARE complex' [14] while Munc18-1 is also vital for release [15], cooperating with the SNAREs in process of fusion. SNAREs are recycled after disassembling the SNARE complex by SNAPs and NSF. [16]. Fusion of synaptic vesicle is also controlled by Rab3s, small synaptic vesicle GTPases of the Rab family that are found to mediate vesicle docking, presynaptic plasticity, and targeting specificity [17]. Several specialized proteins regulate the release of neurotransmitters such as the large active zone proteins Munc13-1 and RIM, which prepare synaptic vesicle for rapid fusion in response to Ca^{2+} influx [18], the synaptic vesicle synaptotagmin-1, which plays a role of Ca2+ sensor [19], and SNARE complex tightly binding proteins complexions [20] that play key roles in priming and Ca2+ triggered release [21]. The presence of C2 domains in their sequences (referred to as C2A, C2B, etc.) is a similar characteristic of Munc13-1, synaptotagmin-1, RIM, and various proteins concerned in the release. Generally, such domains function as Ca2+ and phospholipid-binding components, although they can also act as protein-protein interaction domains when they do not bind Ca2+ [22].

Considerations on the study of protein interactions

An integral part of biological system characterization at the initial stages involves the identification of protein interactions. Protein interaction analyses using affinity chromatography on a solid, stiffer backing have been widely investigated, often for antibody-antigen binding in immunoprecipitations (IPs) or to analyze physical interactions among proteins-small molecules during pull-down tests (for instance, with the immobilized GST-fusion proteins). Fundamental and basic discoveries related to release machinery were achieved by these methods. For example, the utilization of affinity chromatography to filter syntaxin-1[23] has been permitted by the replication of synaptotagmin-1 [24]. Further, syntaxin-1 was used to isolate complexions [25] and Munc18-1 [26]. In yet another study using the same strategy, a complex of NSF and SNAPs with synaptobrevin, syntaxin-1, and SNAP-25 was identified, which was named SNAREs [27] and eventually, with the SNARE complex [28]. Another widely utilized approach is yeast-two-hybrid (Y2H), which is routinely used to examine protein binding partners. This is especially beneficial when the protein is insoluble or protein characteristics that make it challenging to manage biochemically. During the discovery of RIMs binding proteins, which form an important part of the highly insoluble cytomatrix of presynaptic active zones, Y2H assays played a definitive and key role [29]. Although techniques like pull-down assays, IPs, and Y2H assays have shown success in countless cases, their detection capabilities of established protein interactions and reproducibility with purified, well-characterized proteins often could not be established authoritatively [30]. Several reasons are listed by which these inconsistencies arise. As a simple case, protein – polypeptide fusion (for pulldowns or Y2H assays) or protein - antibody binding can result in a spatial mismatch with steric hindrance and prevent binding to the desired target. The contrasting case is also observed wherein nonspecific interaction between protein and irrelevant factor may take place due to enhanced co-operativity of weak interactions between protein and irrelevant factors such as a fused polypeptide, the antibody, or the resin itself. This issue becomes relatively important when binding involves immobilized protein in large excess and is detected by sensitive techniques such as immunoblotting with antibodies to a recognized putative target. Since these are sensitive methods that can detect a very small amount of binding, the result is likely to be artifacts (erroneous) and can be considered true only if it is proved that the interaction is saturable and stoichiometric. Experimental results can often be misleading when protein fragments characterization is insufficient and inaccurate. As a result, non-specific interactions with multiple targets are seen in the case of aggregated as well

as misfolded protein fragments because they do not correlate with a coherent folding module. Moreover, non-protein contaminants may be present in fusion proteins isolated by affinity chromatography. This is notable when further purification is not carried out and may go undetected with the classical methods of protein purity. Such impurities have the ability to mediate indirect adherence to irrelevant factors as well as mask binding to physiological targets. These problems become severe for highly positively charged proteins and thus tightly bind to DNA or RNA [e.g. the synaptotagmin-1 C2B domain [31]].

Interpretation of two-dimensional heteronuclear NMR spectra:

Discussion on the structure resolution technique is far away from the scope of this study. In this section important concepts on two-dimensional (2D) heteronuclear NMR experiments are summarized, which can enrich the information on protein interactions. These concepts will demonstrate the importance and utility of these experiments and will assist non-experts to understand some of the NMR data through illustrations and examples. Widely used 15N-labeled proteins spectra by 1 H-15N heteronuclear single quantum coherence (HSQC) technique can correlate directly bonded pairs of 1 H and 15N nuclei and hence produce one cross-peak for each amide group available in the backbone of a protein's non-proline residue, for example, this is exemplified by Rab3 effector rabphilin C2B domain's 1 H-15N HSQC spectrum [32]. This spectrum commonly shows an excellent dispersion of well-folded proteins since their amide groups possess unique and distinctive chemical environments. Thus, 1 H-15N HSQC spectra are like protein fingerprints, and any change in these fingerprints reports deviation in structural changes or binding interactions. When a small ligand binds, the selective shift in the cross-peaks is observed from residues in the vicinity of the binding site, thus revealing the site. This is illustrated in Supplementary for Ca2+ binding to the rabphilin C2B domain, which causes shifts selectively in the cross-peaks from residues near its Ca2+-binding region [33]. However, a cautionary note about the interpretation of chemical shift mapping experiments is a must since deconvolution of chemical shift changes corresponding to conformational changes that originate from the binding sites is not possible, particularly in presence of chemical shifts reflecting direct protein-protein contacts. Information in addition to 1 H-15N HSQC spectra can be obtained from titrations. It is noticed that when the exchange rate is quick compared to the changes in chemical shift generated by binding, the cross-peaks progressively transition from their free-state locations to their bound-state positions throughout the titration (that can be a range of 0-1000 Hz or more). Supplementary depicts this with a cross-peak indicated in red, that exhibits 1 H-15N HSQC spectra of synaptotagmin-1 C2A domain overlaid expansion obtained with rising Ca2+ concentrations. Thus, it can be interpreted that there is the gradual movement of red cross-peak from the Ca2+-free to the Ca2+-bound position. The studies using titration reported consecutive binding of Ca2+ at three nearby but different sites with distinct affinities revealing three components moving in different directions [34]. Elucidation of three Ca2+-binding sites was possible with the key clues provided by patterns of cross-peak movements, followed by verification with mutagenesis. For example, when a ligand (S235) was mutated to coordinate just the site having the lowest affinity, the third component of the titration was disappear.

Much to the surprise, a 1:2 stoichiometry was reported for the crystal structure of the RIM ZF domain-Munc13-1(3–150) K32E heterodimer, despite the fact that 1 H-15N HSQC spectra and ITC demonstrated a 1:1 stoichiometry in solution [35]. One of the molecules, out of the two, is one Munc13-1(3–150) K32E molecule enveloped the RIM ZF domain in the crystal structure through the C2A domain and a-helical extension at the C-terminal, matching the bona fide binding mode. In comparison to this, the other Munc13-1(3–150) K32E molecule in the crystals made relatively minimal interaction between the RIM ZF domain, and the 1:2 complex's proclivity to crystallize was the likely reason for binding. This structure is an excellent example that illustrates that, although crystal structures are normally very reliable, no method is completely perfect and free of errors. However, the structural basis for RIM-Munc13-1 binding was still revealed by the crystal structure of the heterodimer and allowed the heterodimer to disrupt the mutation's design which has strong functional effects [36-39]. Furthermore, recent research has shown that the RIM ZF domain or a Munc13 mutant with the K32E mutation may essentially rescue the vesicle priming deficiencies observed in the absence of RIMs, but not by WT Munc13 [40-42]. Hence, an unambiguous mechanism wherein homodimerization of Munc13 through the C2A domain inhibits the priming function, as well as binding of the RIM ZF domain disrupting the homodimer, is established by the blend of structural and functional studies.

CONCLUSION

The examples just discussed are glimpses from the ocean of available literature on the research of molecular mechanisms but signify the power of using the vast range of biophysical tools and techniques to obtain complementary, allied information. The continuous improvement of highly influential NMR techniques shows that NMR spectroscopy will keep playing an increasingly important role in this area. In particular, the combination of paramagnetic broadening and pseudo contact shifts measurements with highly sensitive 1 H-13C HMQC spectra of 2 H,13CH3-labeled proteins to study large protein complexes is more promising. There are obviously, ongoing technological advancements in several other fields of biophysics, such as single-molecule fluorescence spectroscopy. It will not be an exaggeration to say that the

future of the research area of molecular mechanisms is bright and promising.

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