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Collision induced unfolding of isolated proteins in the gas phase: past, present, and future

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Rapidly characterizing the three-dimensional structures of proteins and the multimeric machines they form remains one of the great challenges facing modern biological and medical sciences. Ion mobility–mass spectrometry based techniques are playing an expanding role in characterizing these functional complexes, especially in drug discovery and development workflows. Despite this expansion, ion mobility–mass spectrometry faces many challenges, especially in the context of detecting small differences in protein tertiary structure that bear functional consequences. Collision induced unfolding is an ion mobility–mass spectrometry method that enables the rapid differentiation of subtly-different protein isoforms based on their unfolding patterns and stabilities. In this review, we summarize the modern implementation of such gas-phase unfolding experiments and provide an overview of recent developments in both methods and applications.

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Introduction

The fundamental relationship between protein structure and function makes their study critical in ongoing efforts to understand both fundamental elements of biochemistry and human disease [1]. In order to understand protein structure, its role in defining function, and any changes that may occur in disease states, it is essential to explore the connective biophysical parameters that link such elements of biophysics together [2]. One such element is protein stability, often reported as a free energy of protein unfolding and represents one of the most widely utilized descriptors of protein structure [3]. Given the significance of protein stability in the framework of understanding protein structure and function, new experimental techniques that can extract such values with improved figures of merit are clearly needed.

Mass spectrometry (MS) has recently experienced a proliferation of structural biology related research, focusing primarily on heterogeneous proteins, protein complexes, and protein–ligand complexes due to its ability to access such mixtures with sensitivity, speed, and low limits of detection [4]. Ion mobility (IM), which separates ions based on their size to charge ratio and reports ion size in terms of an orientationally-averaged collision cross section (CCS), has also been widely deployed in combination with MS as a platform for structural biology [5]. Prior to this recent period of expansion, IM–MS was used to primarily assign the conformations of peptides [6] and small proteins [7] in the gas phase. However, as the size and complexity of biomolecules increases, IM-derived CCS values alone often yield insufficient information to define the structures of proteins in detail [7].

Collisional activation has long been used to probe the structure and stability of protein ions in the gas phase [8,9]. Collision induced unfolding (CIU) represents an extension of this earlier work, and is best viewed as a gas-phase analog of differential scanning calorimetry experiments often carried out in solution. In a typical CIU experiment, isolated biomolecular ions are activated through energetic collisions with a background gas (e.g. Argon) in order to increase their internal energy and cause them to change conformation (unfold) in the gas-phase, without providing sufficient energy to cause the significant dissociation of covalent bonds [10]. The progress of this CIU process is followed through IM–MS, with the former stage providing direct measurement of protein unfolding through changes in ion CCS and the latter analyzing the composition of the isolated biomolecules and enabling any collision induced dissociation (CID) products to be excluded from the analysis. Early examples of CIU include the observation of cytochrome c [7] and apomyoglobin unfolding in the gas phase [11]. Modern implementations of the technology have been extended well beyond these examples, to include detailed analyses of the CIU mechanism and applications to a range of therapeutically-relevant targets.

The potential of CIU as an analytical fingerprinting technique to study the structures and stabilities of proteins, protein complexes, and protein–ligand complexes

is now emerging. The collisional activation of protein assemblies often yields a multitude of partially folded intermediates stable on the millisecond time scale that can provide a range of diagnostic information related to the structures of the isolated protein complexes [12,13••]. In addition, CIU has been used to assay the stabilities of proteins and protein–ligand complexes in the gas phase. Although the stability measurements offered by CIU data for biomolecular ions are relative, and allow for comparisons of protein states rather than determination of absolute thermodynamic properties, they also provide valuable insight into the structure and native binding interactions of proteins and their complexes [9,14,15•,16,17]. In this review, we recount past examples of CIU as a means of illuminating both current and future applications of the technology.

Generation and analysis of CIU data

Typical CIU experiments are performed by sequentially increasing an accelerating potential difference that serves to activate ions prior to ion mobility separation. As such, IM arrival time distributions (ATDs) are acquired at each stepped potential (Figure 1a), creating a large multi-dimensional dataset. The changes in measured ATD correspond to structural transitions of the protein ion in the gas phase which, while not directly assessing solution phase structures, can be used to generate unique fingerprints (Figure 1b) that can reflect such native state structure information. Several methods to generate these fingerprints have been described [15•,18••,19,20], offering quantitative metrics for rapidly distinguishing subtle structural changes in proteins and protein complexes.

To generate a CIU fingerprint, the arrival time distribution of the *m/z* corresponding to the analyte ion must be extracted from the raw data at each collision voltage applied to create a matrix for analysis. Manual generation of this matrix can be time-consuming, and recent CIU experiments have relied more heavily upon automated extraction tools capable of creating such file structures rapidly [15•,20]. Once generated, replicates can be used to assign statistical confidence to observed deviations between fingerprints representing different protein forms, for example, between ligand-bound and unbound states. These quantitative comparisons can be leveraged to classify binding events and structural changes into biologically relevant categories, such as differentiating functional from nonspecific lipids bound to membrane proteins [15•], or determining the binding site of a ligand in systems with multiple known binding pockets [17]. As these workflows become routine and advance towards automated and high-throughput analyses, continued development of automated extraction and processing tools will be essential to realizing the full potential of CIU experiments.

Probing protein structure and stability using CIU

Collisional activation followed by IM–MS has been used to probe the conformations of proteins in the gas phase for nearly two decades [10,11]. For example, early CIU experiments probed the activation energy barriers associated the gas-phase folding and unfolding of apomyoglobin following charge manipulation, revealing clear evidence of both Coulombic and structural components for the barriers detected between the gas-phase conformers [11]. Tandem IM technology [21,22] combined with collisional activation has been used to examine similar activation energy barriers in greater detail, revealing connectivity maps between the multitude of intermediate states populated during the CIU of small proteins [23]. Overall, these early CIU experiments were aimed primarily at uncovering the biophysical rules governing gas-phase protein ions, and succeeded in significantly advancing our understanding of protein stability and structure in a solvent-free environment.

Following on from this earlier work, CIU has been implemented to study the structure and dissociation behavior of protein complexes [24]. For example, early work [25] proposed an unfolding-based mechanism for protein complex CID, in which a single subunit unfolds and is ejected bearing a large portion of the total charge of the assembly, largely through collecting indirect evidence of protein CIU. The introduction of CIU enabled the direct observation of collisionally-activated protein assemblies, confirming that they populate partially folded intermediates that are stable on the millisecond timescale [8,12,26]. Other structural rearrangements of protein complexes have been shown in the gas phase via collisional activation and IM–MS. For instance, many reports have shown evidence of compaction upon the activation of ring-like protein complexes that contain significant internal cavities [26,27]. Moreover, computational chemistry has been used to probe protein complex CIU, reproducing many of the general features of experimental data [28–30]. Recent computational approaches in this area incorporate charge hopping within coarse-grained models and mobile protons within all-atom MD simulations [31•]. Despite these recent advances, however, a complete model capable of predicting the unfolding transitions of heated protein complexes in CIU remains elusive.

Extending from these mechanistically-framed studies, CIU has been used to quantify shifts in protein complex stability upon binding large populations of both anions and cations. Early CIU work in this area indicated that buffer components of low volatility bound to intact protein complexes can act to stabilize protein complex in the gas phase [32]. These initial results were expanded upon by screening a wider range of solution additives for their ability to stabilize gas-phase protein complexes [12,33–35]. For example, CIU and CID studies incorporating a

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