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Electron transfer with no dissociation ion mobility–mass spectrometry (ETnoD IM-MS). The effect of charge reduction on protein conformation

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ABSTRACT

A novel mass spectrometry approach is reported which investigate how ion-molecule charge reduction reactions between radical anions and protein cations modulate protein conformation. An electron transfer reagent (1,3-dicyanobenzene) transfers electrons to positively charged proteins and there are no observable products of dissociation. ETnoD product ions are detected as charged-reduced species with the same molecular weight as the precursor ion, and no significant evidence for proton transfer. We present collision cross section distributions of precursor and product ions before and after exposure to radical ions. Cytochrome c and myoglobin are examined as exemplar systems under both aqueous salt and denaturing conditions before and after exposure to radical anions. We consistently observe depletion of the more compact precursor ion conformers on exposure to the ETD reagent. Remarkably, by examining the collision cross section distributions of the product ions it can be seen that the addition of a single electron can cause a dramatic rearrangement in protein conformation for charge states that are highly populated when sprayed from salty aqueous conditions. Furthermore, a given net charge on an exposed precursor and product ion favours a preferred collision cross section distribution, indicating that the distribution of charge on proteins in the gas phase dictate their conformation. An exception is reported for the low charge state of cytochrome c where compaction was seen in the radical formed post reduction compared to the electrospray generated ion under ETnoD optimised conditions. We propose a model that postulates how electron transfer to conformation stabilising salt bridges may explain our observations.

1. Introduction

Over the past 30 years a plethora of mass spectrometry based techniques have been developed to investigate protein structure following electrospray ionisation of intact proteins and protein complexes, all of which fall into the category of 'top down' analysis [1]. Electrospray ionisation of proteins produces multiply charged species where the charge state distribution is indicative of the protein's three dimensional conformation [2,3]. A compact globular conformation is relayed from a narrow distribution of low charge states while unfolded conformations exhibit a wide charge state distribution which is often multimodal [4,5]. Factors shown to influence protein conformation will also alter the charge state distribution (CSD) for example, pH [6], ionic strength [7], solvent hydrophobicity [8,9] and solution temperature [10]. Changes in these solution conditions can be made in order to denature or disrupt protein structure and this can be reflected in the CSD.

De La Mora derived an empirical relationship to determine the maximum number of charges that may be held on the surface of a quasi-spherical protein prior to their proximity causing columbic repulsion which in turn would rupture the spherical nature and cause extension away from a compact form [11]. Ion mobility mass spectrometry allows us to measure the change in conformation between a compact and an extended geometry and it has been used to prove that the De la Mora relationship holds remarkably well [12]. Whilst this provides an upper limit to the net charge that can be held on the surface of a globular protein of a given m/z, it

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does not report on the number of charged groups both protonated and deprotonated that may also exist. Globular proteins are held in such a state due to non-covalent intramolecular interactions and as a protein is desolvated during electrospray even under aqueous salt conditions, we have shown that there is invariably some contraction to the solvated form. We and others have hypothesised that this tightening is attributable to stabilising interactions between charged groups of opposite polarity on the surface of the protein, and that the lower the net charge is, the higher the probability for such stabilizing interactions [13].

Fragmentation methods employed to probe the tertiary fold of proteins include collision induced dissociation (CID) [14,15], photon induced dissociation [16,17] and electron mediated activation methods [18–20]. Of these, CID is still the most widely used and when applied to proteins produces predominantly b^+ and y^+ fragments ions due to preferential cleavage at the amide bond. The electron mediated activation methods of electron capture dissociation (ECD) and electron transfer dissociation (ETD) produce primarily c^+ and $z^{+\bullet}$ fragment ions after cleavage at the N—Cα bond. CID and ECD/ETD in combination provide complementary information however ECD/ETD have unique features which makes them attractive for the structural characterization of proteins. They allow fragmentation of peptides and proteins leaving labile post translation modifications such as phosphorylation, carboxylation, and glycosylation intact [21–24] and fragmentation of covalent bonds leaving non-covalent interactions intact [25]. Currently ETD is readily available in commercial instruments and finds its main use in the sequencing and structural characterization of peptides previously proteolytically cleaved in bottom up and middle-down approaches [26–30] although analytical use have been reported [30,31]. More recently ETD has been employed in combination with ion mobility mass spectrometry (IM-MS) [32,33].

Ion mobility is a gas phase separation technique which allows the differentiation of molecules of the same mass-to-charge but different conformations, subtle changes in structure and structural changes in chemical transformations as we will show here. This makes it a powerful tool in gas phase structural biology and as such has a wide variety of applications [34–39]. In a typical mobility experiment ions are pulsed into a mobility device filled with a neutral buffer gas and are propelled along the device with a uniform or non-uniform electric field. As the ions traverse through the gas filled device under the influence of this electric field, they experience retardation as a result of multiple collisions with the buffer gas and are separated according to charge, mass and collision cross section. The coupling of ion mobility to mass spectrometry allows the measurement of an arrival time from which the arrival time distribution (ATD) provides information on whether a molecule is more compact or extended as a more compact structure will have an earlier arrival time compared to more extended forms. In addition this arrival time can be used directly along with other known parameters to derive a rotationally-averaged collision cross section for the separated molecules [40] or a collision cross section can be determined by calibration against proteins with known cross sections in the instance when a non-linear electric field is used [41,42].

Here we used an electron transfer reagent simply to charge-reduce intact proteins which are then examined with ion mobility mass spectrometry to allow us to understand the effect of charge on the structure of isolated proteins a technique we term ETnoD. It must be noted that charge reduction is often the dominant product following exposure to electrons when performing ECD or ETD and here we make the reduced product ions the focus rather than an oft ignored bi-product of top down sequencing. Electrons are transferred from the radical anions to protein ions of both low and high charge states of cytochrome c and myoglobin generated from aqueous salt and denaturing solutions *via* nanoelectrospray ionisation. The ATDs of precursor and charge-reduced product ions are measured and converted to collision cross sections which in turn are used to deduce structural changes before and after the protein ion of interest is exposed to radical anions.

2. Experimental

2.1. Samples

Equine cytochrome c and myoglobin were purchased from Sigma Aldrich (product numbers C2506 and M1882). 1,3-Dicyanobenzene was obtained from the Waters Corporation, UK (Analytical Standards and Reagents). Ammonium acetate was purchased from Fisher Scientific, UK. Water (HPLC grade), methanol (HPLC grade, ≥99.9% purity) and formic acid were purchased from Sigma Aldrich, UK. Protein stock solutions of 1 mg/mL were prepared and stored at −20 °C. Salty solutions of both proteins were prepared in 50 mM ammonium acetate, pH 7 while denaturing solutions were prepared in 50/50 (v/v) methanol/water with 0.1% formic. Cytochrome c solutions were diluted and used for mass spectrometry analysis while myoglobin solutions were diluted and desalted using Biospin-6 columns (BioRad, USA) prior to use.

2.2. ETnoD IM-MS

All experiments were performed on a Synapt G2 Si travelling wave ion mobility mass spectrometer (Waters Corporation, Wimslow, UK). ETnoD instrument settings were optimized for each system and are given in the supporting information (SI page 1). The experimental setup for an ETnoD IM-MS experiment is illustrated in Fig. 1. Radical anions of 1,3-dicyanobenzene and analyte cations are sequentially generated and sent to the trap region of the gas filled Triwave SRIG (stacked ring ion guide) in the instrument, where the ETnoD reaction occurs. Radical anions are generated in a glow discharge source [43] with a discharge current of typically 50–75 μA in negative ion mode while analyte cations are generated via nanoelectrospray ionisation in positive mode with a capillary voltage of 1–1.6 kV. Each charge state of the analyte of interest is m/z selected in the quadrupole and then transmitted to the trap region. The Triwave SRIG is a travelling wave device which utilises a non-uniform electric field to propel ions throughout its length [44,45]. The trap SRIG wave amplitude and speed are set to 0.1–0.2 V and 300 m/s to optimise the reaction rate between radical anions and analyte cations and maximise charge reduction. All species (both unaltered and charge reduced products) are then allowed into the mobility region (Fig. 1) for separation then pulsed into a time-of-flight (TOF) mass analyser for arrival time measurement. For these experiments, ion mobility separation takes place at constant travelling wave amplitude and velocity.

2.2.1. Travelling wave ion mobility calibration

To compare the structures of unexposed precursor ions and the product ions exposed to radical anions of each data set under the same experimental conditions, collision cross sections in nitrogen buffer gas were estimated according to the protocol first published by Ruotolo et al. [41] and calibrated against helium cross sections ($^{TW}CCS_{N_2\rightarrow He}$). Calibration was done against previously measured values on drift tube instruments [46,47]. Calibration curves at wave heights of 15 V, 16 V and 17 V are shown in SI Fig. S1.