Journal of Chromatography A, 1496 (2017) 51–57

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Semi-automated screen for global protein conformational changes in solution by ion mobility spectrometry–massspectrometry combined with size-exclusion chromatography and differential hydrogen–deuterium exchange

Nicholas A. Pierson [1], Alexey A. Makarov [*,1], Christopher A. Strulson [1], Yun Mao, Bing Mao

Merck & Co., Inc., MRL, Analytical Research & Development, Rahway, NJ 07065, USA

ARTICLE INFO

Article history:
Received 12 December 2016
Received in revised form 6 March 2017
Accepted 13 March 2017
Available online 16 March 2017

Keywords:
Protein folding
SEC
HDX
IMS
Global conformational change

ABSTRACT

Development of methodologies for studying protein higher-order structure in solution helps to establish a better understanding of the intrinsic link between protein conformational structure and biological function and activity. The goal of this study was to demonstrate a simultaneous screening approach for global protein conformational changes in solution through the combination of ion mobility spectrometry–mass spectrometry (IMS–MS) with differential hydrogen–deuterium exchange (ΔHDX) on the size-exclusion chromatography (SEC) platform in a single on-line workflow. A semi-automated experimental setup based on the use of SEC on-column conditions allowed for tracking of protein conformational changes in solution as a function of acetonitrile concentration. In this setup, the SEC protein elution data was complemented by the ΔHDX profile which showed global protein conformational changes as a difference in the number of deuterons exchanged to protons. The ΔHDX data, in turn, was complemented by the changes in the drift time by IMS–MS. All three orthogonal techniques were applied for studying global higher-order structure of the proteins ubiquitin, cytochrome c and myoglobin, in solution simultaneously. The described approach allows for the use of a crude sample (or mixture of proteins) and could be suitable for rapid comparison of protein batch-to-batch higher-order structure or for optimizing conditions for enzymatic reactions.

1. Introduction

Protein function and activity are both directly related to its conformational structure in solution [1]. Therefore, the development of complementary approaches to explore and characterize conformational structure in solution is of crucial importance. There are several spectroscopic techniques that can be used to study higher-order protein structure in a solution. For example, circular dichroism (CD) and nuclear magnetic resonance spectroscopy (NMR) are often used for this purpose; however, both require relatively pure protein samples which are free from interfering proteins and impurities that can contribute to the overall spectrum [2–4]. In these cases, an approach in which higher-order structure can be simultaneously explored for a mixture of proteins is needed. This is especially important in biocatalytic [5] reaction optimization, when a low-concentration enzyme needs to be studied in the crude reaction solution.

Hydrogen deuterium exchange (HDX) mass spectrometry (MS) is a well-known technique for studying protein higher-order structure [6] based on exchange of labile protons to deuterons in amides, alcohols, carboxylic acids, or amines which are observable by MS or NMR [2]. Extensive hydrogen bonding within interior regions of folded proteins creates solvent inaccessibility, which restricts hydrogen/deuterium (H/D) exchange in those regions and allows for the evaluation of higher-order structure [7,8]. Kinetic rates of hydrogen-deuterium exchange for solvent-accessible labile protons (at certain pH and temperature) [9] occur within the liquid chromatographic timeframe (seconds to minutes), and therefore enable the use of deuterium oxide mobile phases in liquid chromatography–mass spectrometry (LC–MS) as a convenient means of performing deuterium labeling [10,11]. In contrast, H/D exchange for less solvent-accessible protons can be much slower (up to several hours) [12]. Another factor for consideration is intramolecular hydrogen bonding in a protein, which can interfere with the estimation of the number of labile solvent-accessible protons by HDX [13]; for this reason, chaotropic (liophilic) addi-

* Corresponding author at: Merck & Co., Inc., 126 East Lincoln Ave., Rahway, NJ 07065, USA.
E-mail address: alexey.makarov@merck.com (A.A. Makarov).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.chroma.2017.03.024
0021-9673/

tives can be utilized, and are well-known in their use for protein denaturation and for their disruption of hydrogen bonding [14–17].

There are two main HDX workflows: continuous and pulse HDX [7,8,18]. Continuous HDX (on the scale of hours) is primarily used to study protein structural dynamics; pulsed HDX allows for monitoring folding intermediates by rapidly transferring the protein to a particular solvent environment, thereby triggering the re-folding process on the order of milliseconds [8]. Another HDX-MS approach utilizes high pressure in an LC system as a direct probe of protein global conformational change as a function of pressure (up to 1200 bar) [19].

Size-exclusion chromatography (SEC) has been demonstrated to be a convenient platform when used in conjunction with HDX [10]. Overall SEC is one of the most frequently used techniques for protein characterization, and has a wide range of applications from the characterization of bio-similar therapeutics [20,21], to the study of protein unfolding [22,23] and metallodrug-protein interactions [24]. There have been many reports of the successful use of SEC coupled with electrospray ionization–mass spectrometry (ESI–MS) detection [10,21,25,26].

Complementary to these solution-phase characterization methods, higher-order protein structure can also be studied in the gas phase through various MS-related techniques such as gas-phase HDX-MS [27,28], and ion mobility spectrometry–mass spectrometry (IMS–MS) [29–31]. Protein conformational differences can be resolved by IMS on the basis of low-energy collisions between analyte ions and inert gas molecules during millisecond-timescale transit through an electric field region. A compact, highly folded protein undergoes fewer hindering collisions with the drift gas compared to the same protein in an extended or unfolded conformation, and hence, compact conformations will have faster ion mobility drift times than partially and fully unfolded conformations. The relevance of gas-phase biomolecule structure measured by IMS–MS has been extensively debated over the past 20 years and is the topic of numerous publications [32–36]. The aims of the present work were to monitor changes in protein conformational populations induced by varying acetonitrile content in solution, rather than to maintain "native-like" protein structure through ESI and IMS–MS; however, we do note the potential utility of this unified experiment combining SEC, on-column differential HDX, and IMS–MS in providing a direct link between solution- and gas-phase structural characterization techniques.

In this study we surveyed an approach that utilizes SEC coupled with IMS–MS and differential HDX to compare global conformational changes of proteins under different solution conditions in a semi-automated manner. The combination of structural characterization techniques in a single experiment helps to discern potential artifacts seen by each single technique. Each characterization method as a standalone technique has drawbacks; SEC can suffer from secondary analyte–stationary phase interactions.

HDX interpretation is complicated by intrinsic HDX effects. With IMS, interpretation of solution-phase protein conformations by a gas-phase measurement technique is challenging because preservation of structural equilibrium can be disrupted during both the ionization process and ion transfer through the instrument. Through the combination of differential HDX, SEC, and IMS–MS in a single experiment, these instrument-induced artifacts can be more easily recognized and help guide overall structural interpretation of the protein of interest.

2. Experimental section

2.1. Reagents and chemicals

Uracil, ammonium formate (LC–MS grade), deuterium oxide, cytochrome C (from bovine heart), myoglobin (from horse heart), ubiquitin (from bovine erythrocytes), and bradykinin acetate were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Ultrapure water was obtained from a Milli-Q Gradient A10 from Millipore (Bedford, MA, USA). Acetonitrile (MeCN), ammonium hydroxide and trifluoroacetic acid (TFA) HPLC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Sample preparation

Sample solutions of the proteins used for experiments were prepared at about 500 μM in 30 mM phosphate buffer (pH 6.8) in deuterium oxide. An Accumet AR-50 pH meter with a standard pH electrode from Fisher Scientific (Fair Lawn, New Jersey) was used for all pH measurements and adjustments. Protein samples in deuterium oxide were incubated for 48 h at 30 °C. The injection volume for different analytes was 5 μL.

2.3. Experimental conditions

Experiments were performed on a Synapt HDMS mass spectrometer with an Acquity UPLC™ (Waters Corp., Milford, MA, USA) system, with Waters MassLynx V4.1 software for instrument control and data processing, including the MaxEnt1 module for mass spectra deconvolution.

An Acquity UPLC® Protein BEH SEC-125 4.6 × 150 mm, 1.7 μm column with 125 Å pore size from Waters (Waters Corp. Milford, Massachusetts) was used in all experiments. The chromatographic conditions consisted of an isocratic run, pumped at a flow rate of 0.25 mL/min and UV detection at 230 nm. Molecular weight determination reproducibility was no more than 0.007% RSD (n = 6) based on de-convolution of multiple-charged species. The mobile phases used in the study were 50 mM ammonium formate adjusted to pH 2.0 or pH 5.5 by TFA and ammonium hydroxide in water. The organic part of the mobile phase was acetonitrile.

Each protein sample was injected at 0–50% of acetonitrile in the mobile phase (solution) with increments of 5% acetonitrile using isocratic mode, where mobile phase resulted from on-line mixing of the aqueous portion of the mobile phase with acetonitrile. Each condition was at equilibrium after passing at least 3 column volumes.

The online SEC/HDX effluent was ionized by ESI and entered the traveling-wave IMS–MS instrument, which was operated with the following source conditions: capillary 3.5 kV, sampling cone 40 V, extraction cone 5.5 V, source temperature 80 °C, desolvation temperature 400 °C, cone gas 50 L/h, desolvation gas 600 L/h. Collision energies in the trap and transfer cells were set to 6 V (non-fragmentation). The traveling-wave IMS cell was operated with a wave velocity of 300 m/s and a wave height of 9 V; the trap cell was operated with a wave velocity of 248 m/s and a wave height of 3 V. Spectra were collected at a scan rate of 1/s. 2D IMS–MS spectra were evaluated in DriftScope v 2.0. The charge state plotted in each figure was chosen based on sufficient signal intensity across the data set. IMS drift time overlays were extracted in MassLynx.

Solvent accessibility was estimated for the proteins used in this study with the computational chemistry software Discovery Studio v.3.5.0 (Accelrys Software Inc.) with input pdb (Protein Data Bank) files 2MJB, 1HRC, 5MBN for ubiquitin, cytochrome c, and myoglobin, respectively (Table 1). The solvent-accessible part of the protein sequence was defined as more than 25% of Solvent Accessible Surface (SAS) modeled by the double cubic lattice method (DCLM) [37]. Solvent inaccessibility for a protein was defined as less than 10% of SAS. The number of labile protons was calculated for the solvent-inaccessible part of the protein sequence (as determined by the software), as well as for the whole protein sequence, based on the respective pdb files (Table 1).