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Combining size-exclusion chromatography with differential hydrogen–deuterium exchange to study protein conformational changes

Alexey A. Makarov*, Roy Helmy

Department of Process & Analytical Chemistry, Merck Research Laboratories, Rahway, NJ 07065, USA

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ABSTRACT

Methods for protein characterization are being actively developed based on the growing importance of protein therapies and applications. The goal of this study was to demonstrate the use of size-exclusion chromatography (SEC) in combination with differential hydrogen–deuterium exchange (HDX) to compare protein global conformational changes at different solution conditions. Using chaotropic mobile phase additive, differential HDX was used to detect a number of solvent accessible labile protons of protein on-column at pH and temperature conditions which provided unrestricted intrinsic H/D exchange (all-or-nothing approach). Varying SEC on-column conditions allowed for protein conformational changes to be observed. Temperature and pressure were independently studied with regards to their effect on the proteins' (insulin, cytochrome C, ubiquitin, and myoglobin) conformational changes in the solution. The obtained ΔHDX profiles revealed protein conformational changes in solution under varied conditions manifested as the difference in the number of protons exchanged to deuterons, or vice-versa. The approach described in this manuscript could prove useful for protein batch-to-batch comparisons, for optimization of chemical reactions with enzyme as catalyst or for protein chemical modification reactions.

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1. Introduction

Methods for protein characterization are being developed to understand and explore protein conformational structures in regards to function and activity [1]. Several spectroscopic techniques can be used to study protein secondary structure in a solution. Circular Dichroism (CD) is one of the most commonly-used techniques for this purpose. However, CD analysis requires the protein sample to be purified and free from interfering proteins and impurities, optically active buffers, or preservatives/antioxidants, all of which may contribute to the relative spectrum [2,3]. Two spectroscopic techniques, nuclear magnetic resonance spectroscopy (NMR) and mass-spectrometry (MS), are often used to study purified protein samples in the solution. Therefore, a technique that can be applied for studying protein higher order structure in a mixture solution would be quite useful.

Hydrogen deuterium exchange (HDX) is a well-known technique for studying protein higher order structure [4]. The exchange of labile protons to deuterons in amides, alcohols, carboxylic acids, or amines is observable by MS and NMR [5]. Since there is an exchangeable NH proton in virtually every peptide bond of a protein (except proline), HDX is very useful in the studying of protein higher order structure. Hydrophobic interior regions of native proteins with significant hydrogen bonding, which restricts hydrogen/deuteron exchange in those regions, make it possible to evaluate their secondary and tertiary structure [6,7]. Kinetic rates of deuterium exchange for solvent-accessible amide protons occur readily within the chromatographic timeframe and allow the use of deuterium oxide mobile phases in LC–MS as a very convenient means of performing H/D exchange [8]. Deuterium exchange in the mobile phase for less accessible protons can be much slower (several hours) in non-denaturing conditions [9]. Continuous HDX and pulse HDX are the two main HDX workflows [10,6,7]. Both of these HDX approaches suffer from H/D back-exchange during chromatographic separation and are not efficient in the case of labile protons located on side chains or on the protein surface [6,7]. As reported by Konermann et al. (2011), continuous labeling HDX (in hour scale) primarily monitors protein structural dynamics, rather than

* Corresponding author at: Merck Research Laboratories, RY801 A103, 126 East Lincoln Av., Rahway, NJ 07065, USA.
E-mail address: alexey.makarov@merck.com (A.A. Makarov).

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actual structure; pulsed HDX is used primarily for the characterization of folding intermediates by rapidly transferring protein to a particular solvent environment, thereby triggering the re-folding process in millisecond scale [7]. Recently, another HDX approach was demonstrated using the high-pressure of an LC system in combination with differential deuterium exchange as a direct probe of protein conformational change by pressure [11]. Internal hydrogen bonding may interfere with the estimation of the number of labile solvent accessible protons by HDX [12]. Chaotropic (liophilic) additives are well known to be used for protein denaturation and for disrupting hydrogen bonding [13–16].

Size-exclusion chromatography (SEC) is one of the most frequently used techniques in protein science, from the characterization of bio-similar protein therapeutics [17,18] to the study of protein unfolding [19,20] and metallodrug–protein interactions [21]. Generally, SEC is not usually considered to be an MS-friendly technique; nevertheless, there have been several reports of the successful use of SEC coupled with ESI-MS detection [18,22] or even with ICP-MS [21]. The use of SEC-HDX approach was previously reported [23]. However, the use of SEC coupled with ESI-MS to be used for differential H/D exchange on-line requires further study.

In this study we report an evaluation of the approach that utilizes SEC coupled with MS to compare global conformational changes of proteins under different solution conditions using differential HDX. The proposed approach can be applied for protein higher order structure comparison under different conditions in a mixed sample solution during purification, batch-to-batch comparison, as well as for the optimization of the protein chemical modification reaction, and during solvent exchange.

2. Experimental

2.1. Reagents and chemicals

Ultrapure water was obtained from a Milli-Q Gradient A10 from Millipore (Bedford, MA, USA). Acetonitrile, ammonium acetate (HPLC grade), ammonium hydroxide and trifluoroacetic acid (TFA) HPLC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Uracil, guanidinium chloride (GndCl), ammonium formate (LC–MS grade), deuterium oxide, insulin (from bovine pancreas), 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).

2.2. Instrumentation and stationary phases

An Agilent 1290 quaternary pump LC system (Agilent, Santa Clara, California), able to operate up to 1200 Bar, and equipped with an 160 Hz photodiode array detector and MSD-6130 Single Quadropole Mass-Spectrometer with standard ESI source were used. The MSD-6130 mass accuracy was ±0.13 u within the calibrated mass range in scan mode, based on vendor specifications [24]. Agilent OpenLab ChemStation Edition for LC/MS C.01.05 with the de-convolution module was used for data acquisition and processing. Each spectrum was averaged based on spectral measurements across the middle of total ion chromatogram peak of the analyte. Nominal mass spectrum de-convolution parameters included molecular weight assignment based on curve fit with agreement to within 0.05%, noise cutoff 1000 counts, abundance cutoff 10%, minimum 3 peaks in set.

The estimation of proteins' solvent-accessible surface (SAS) was performed using Discovery Studio v.3.5.0 (Accelrys Software Inc., San Diego, California).

An Acquity UPLC® Protein BEH SEC-125 4.6 × 150 mm, 1.7 μm column with 125 Å pore size (Lot# 0128340381, Lot# 0134343321) from Waters (Waters Corp., Milford, Massachusetts) was used in all experiments.

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.

A pressure-restrictor apparatus (Upchurch Scientific, P-880 adjustable back pressure regulator with total volume 9 μL) was set between the column and the detector on the UHPLC system in the pressure-controlled experiments with a pressure profile.

2.3. Experimental conditions

The chromatographic conditions consisted of an isocratic run (mobile phase mixed by the Agilent 1290 pump at atmospheric pressure), pumped at a flow rate of 0.20 mL/min or 0.25 mL/min and UV detection at 230 nm. Nominal molecular weight determination reproducibility was no more than 0.0036% RSD ($n = 6$) based on de-convolution of multiple-charged species. The aqueous mobile phases used in the study were 50 mM ammonium formate or acetate adjusted to pH 2.0 or pH 5.4 by TFA and ammonium hydroxide in water or deuterium oxide. The organic part of the mobile phase was 5% acetonitrile.

In experiments with a temperature trend (profile), each protein sample was injected at 30–70 °C column temperature with increments of 5 °C after reaching equilibrium at each temperature condition (i.e., each point on the profile) for at least 30 min.

In the experiments with a pressure profile, each protein sample was injected at six different controlled column pressure settings at a constant flow rate and temperature 30 °C: 300, 400, 500, 600, 700, and 770 Bar (each about ±15 Bar), achieved with a pressure-restrictor apparatus installed after the column. Each condition (each point on the profile) was at equilibrium after passing at least 3 column volumes.

2.4. Sample preparation

Sample solutions of the proteins used for experiments were prepared at about 0.2 mg/mL in 0.1 v/v % TFA or 1.6 M guanidinium chloride (pH 7.0) in water or in deuterium oxide. All analytes were completely dissolved before injections. Protein samples in deuterium oxide were incubated for 3 h at 45 °C. The injection volumes for different analytes were 2–5 μL.

3. Results and discussion

In seeking to optimize an enzymatic reaction, a quick comparison of the effects of reaction solution environment on enzyme global conformation would be a great benefit. The goal of this study was to demonstrate in principle the ability to use size-exclusion chromatography (SEC) in combination with differential hydrogen/deuterium exchange (HDX) to compare the number of solvent accessible labile protons in a controlled solution environment in the presence of chaotropic (liophilic) additive as an indication of the relative global conformational change of protein; it was not an attempt to study protein local structural dynamics. Combining SEC with HDX may also be advantageous for evaluation of gyration radius variations of protein molecules during SEC method development. It is widely acknowledged that parameters such as temperature, pH, and organic co-solvent content may affect protein conformation in solution. It has also been demonstrated that pressure may have an observable effect on protein conformation in solution [25,26,11,27]. Different types of HDX experiments have been used to study protein conformational changes in solution [6,7,11]. However, both kinetic- and pulse- HDX approaches have caveats due to an error introduced by H/D-back-exchange during

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