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Identification of protein secondary structures by laser induced autofluorescence: A study of urea and GnHCl induced protein denaturation

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ABSTRACT

In the present study an attempt has been made to interrogate the bulk secondary structures of some selected proteins (BSA, HSA, lysozyme, trypsin and ribonuclease A) under urea and GnHCl denaturation using laser induced autofluorescence. The proteins were treated with different concentrations of urea (3 M, 6 M, 9 M) and GnHCl (2 M, 4 M, 6 M) and the corresponding steady state autofluorescence spectra were recorded at 281 nm pulsed laser excitations. The recorded fluorescence spectra of proteins were then interpreted based on the existing PDB structures of the proteins and the Trp solvent accessibility (calculated using "Scratch protein predictor" at 30% threshold). Further, the influence of rigidity and conformation of the indole ring (caused by protein secondary structures) on the intrinsic fluorescence properties of proteins were also evaluated using fluorescence of ANS-HSA complexes, CD spectroscopy as well as with trypsin digestion experiments. The outcomes obtained clearly demonstrated GnHCl preferably disrupt helix as compared to the beta β-sheets whereas, urea found was more effective in disrupting β-sheets as compared to the helices. The other way round the proteins which have shown detectable change in the intrinsic fluorescence at lower concentrations of GnHCl were rich in helices whereas, the proteins which showed detectable change in the intrinsic fluorescence at lower concentrations of urea were rich in β-sheets. Since high salt concentrations like GnHCl and urea interfere in the secondary structure analysis by circular dichroism Spectrometry, the present method of analyzing secondary structures using laser induced autofluorescence will be highly advantageous over existing tools for the same.

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1. Introduction

The efficiency of diverse functional character in proteins comes with a small window of stability which differentiates native and denatured state of the protein [1]. The intact secondary and tertiary structures which are governed by the thermodynamic, electrostatic, hydrophobic and covalent interactions decide the edge that differentiates the native and denatured state of the protein. There are several reagents used for the sequential denaturation of the protein such as SDS (Sodium dodecyl sulfate), urea, GnHCl (guanidine hydrochloride), alcohol, and acids [2–4]. These denaturants interact with different chemical groups of proteins in their unique ways to bring about distortions in electrostatic interaction, covalent modification, unfolding and sequential denaturation. Each protein has different stability and sensitivity to different denaturants based on their structural complexity and the concentration of the denaturant at which the proteins get denatured may also reflect the structural features of the proteins.

Urea and GnHCl are the well-known chaotropic agents used in molecular biology and biochemistry to denature the proteins by disrupting the hydrogen bonding network of the proteins and water [5]. To explain the urea mediated protein denaturation there are two popular mechanisms known; one is indirect mechanism wherein urea is known to disrupt the structure of water resulting in solvation of hydrophobic residues and in another the urea directly interacts with the protein backbone and side chains by forming hydrogen bonds and van der Waals interactions [6–8]. The GnHCl on the other hand is known to interact with the backbone and polar side chains of proteins for denaturation. Gn^+ ($Guanidium^+$) is a charged species [9] which contains a delocalized positive charge on its planar structure. This ionic nature of the GnHCl might be responsible for masking of electrostatic interactions and denaturation of the proteins [9,10].

Many recent studies predicted and demonstrated that urea and GnHCl have selective denaturing character towards secondary structure of proteins [9,11–14]. It was demonstrated that urea

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preferentially disrupts beta sheets first as compared to helices [9,11, 12,15] and GnHCl is more efficient in disrupting helix rich proteins [12,16–18]. Further, the autofluorescence which is highly sensitive to surrounding micro-environments of proteins has found applications in proteomics in understanding protein structure and functions [3,19–21]. The technique has the ability to capture minor structural variation in proteins reflecting it in the corresponding autofluorescence spectra. During denaturation, proteins undergo many intermediate steps depending upon their structure and stability reflecting their unique identity. Therefore, studying autofluorescence of proteins in response to varying denaturation is one of the best ways to assess protein stability and unfolding. Since urea and GnHCl have selective secondary structural denaturing capacity, proteins rich in beta sheets may be more sensitive to urea induced denaturation and at the same time proteins rich in helices are more sensitive to GnHCl. Hence, autofluorescence can act as the indicator of bulk of the secondary structure which constitutes the protein under urea and GnHCl denaturation conditions.

Autofluorescence in proteins mainly arises due to the presence of aromatic amino acid residues such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine. Among these aromatic amino acids, Trp is highly efficient in providing autofluorescence with higher molar extinction coefficients and quantum yields (5600 and 0.2) as compared to Tyr (1400 and 0.14) and phenylalanine (240 and 0.03) [22] respectively. Trp a hydrophobic amino acid, generally present in the hydrophobic core of the proteins is accordingly affecting the corresponding autofluorescence. There are several reports in the literature demonstrating protein unfolding exposing the Trp residues to the outer aqueous environment resulting in corresponding red shift of the autofluorescence due to solvent relaxation [22–26]. However, the molecular orientation of indole ring of the tryptophan can significantly contribute emission shift as well as fluorescence properties of proteins [27]. These molecular orientations of indole ring in the Trp follows the similar pattern with respect to the type of secondary structure [22,27]. Hence, the selective denaturing of proteins secondary structures by urea and GnHCl may reflect in the fluorescence properties of tryptophan residues and may be traced by close observation of the same [26,28].

The extent of shift in the autofluorescence maxima at the physiological pH may reflect the presence or absence of the secondary and tertiary characters under the influence of particular denaturant such as urea and GnHCl [26,28,29]. This technique has extensively been used to study protein folding and related phenomena. However, its application interpreting secondary structures are not much explored. Therefore, in the present study, an attempt was made to investigate the influence of secondary and tertiary structures on the autofluorescence properties of proteins under different denaturing conditions by urea and GnHCl. In order to understand more about such properties in proteins under native unfolding and digestion leading to their structural revelation, in the present study, five different globular proteins namely, BSA, HSA, Trypsin, Ribonuclease A and Lysozyme have been selected and the corresponding autofluorescence properties have been studied and validated by CD spectroscopy and ANS fluorescence assay.

2. Materials and Methods

2.1. Chemicals

Bovine serum albumin (BSA - ≥96%) (Product Number-A2153), human serum albumin (HSA - ≥97%) (PN - A9511), lysozyme (≥90%) (PN - L2876) and ANS (PN - 10417) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ribonuclease A (RNase A – 95%) (PN - MB087) Urea (PN - MB032), and GnHCl (PN - MB014) were procured from HiMedia Laboratories (India).

2.2. Sample Preparation

In the present study all the stock solutions (five globular proteins (100 μM), GnHCl (8 M), urea (10 M)) were prepared using 20 mM sodium phosphate buffer (pH 7.0). The GnHCl and urea working standards for denaturing proteins were prepared by adding various concentrations of GnHCl (2 M, 4 M, 6 M) and urea (3 M, 6 M, 9 M) stock solutions with 100 μM of the respective protein stock solutions to make 10 μM of protein concentration in the final working sample solution. The high concentrations of GnHCl and urea resulted in altered pH and therefore it was then readjusted to 7.0 by the addition of a small quantity of HCl or NaOH to the mixture. Trypsin was prepared freshly just before the experiment and Temperature was maintained at 4 °C to avoid self-digestion during denaturation experiment. Each of the sample solution was mixed by vortexing and incubated at 4–8 °C overnight. The GnHCl, urea and standard proteins are procured from Sigma Aldrich, India.

2.3. 8-Anilinonapthalene-1-Sulfonic Acids (ANS) Fluorescence

The HSA was denatured with 3, 6 and 9 M of urea and 2, 4 and 6 M of GnHCl with overnight incubation in respective denaturation buffer solutions at 4–8^{0}C. 500 μM ANS dye prepared in mili-Q water was then added to the protein denaturation mixture and incubated for 30 min at room temperature. The fluorescence spectra were then recorded using Varioscan Spectrofluorimeter (Thermo Scientific) in the spectral region 400–600 nm by exciting the samples at 380 nm maintaining the excitation slit width of the instrument at 5 nm. The pH of the sample solutions for fluorescence measurements of ANS-HSA complexes was maintained at 7.0 using 20 mM sodium phosphate buffer (pH 7.0) [30, 31].

2.4. Circular Dichroism (CD) Spectroscopic Measurements

The CD spectroscopic measurements on native and denatured HSA were performed at 25 °C using Jasco spectropolarimeter maintaining constant flow of nitrogen. The CD spectra were recorded in the far UV range between 200 and 250 nm for the 5 μM of HSA in 20 mM phosphate buffer using a cuvette of path length 2 mm. All the recorded CD spectra were then corrected by subtracting them with the CD spectra of the blank. Subsequently, the mean residue ellipticity (MRE) values for the data in deg·cm^2·dmol^{-1} were determined using the formula as shown below.

$$MRE = \theta / (10 \times C \times L \times N)$$

Where, θ, C, L and N refers to ellipticity in milli-degrees, concentration of the protein in mM, optical path length of the cuvette in mm, and the number of amino acid residues in the protein [32]. The percentage of α-helix in the protein was then calculated using the following formula [33]:

$$\%\,\alpha\text{-helix} = (MRE_{222} - 2340) \times 100 / 30300$$

The CD spectral data were further analyzed using the de-convolution program "CONTINLL" under "CDProWin7" [34–38].

2.5. Trypsin Digestion of Proteins

Each of the protein solutions (2 μM) were subjected to tryptic digestion using 50:1 protein-trypsin mixtures. Digestion was carried out by incubating the protein-trypsin mixtures at 37 °C for 3 h, followed by 30 °C for 18 h. Digestion was carried out with mass spectrometric grade trypsin (Sigma, USA).

2.6. Autofluorescence Measurements

The autofluorescence spectra of the protein samples under study were recorded at 281 nm pulsed laser excitations obtained from Nd-YAG laser (*Model LM1278 LPY 707G-10, Litron lasers, UK*) pumped frequency doubled dye laser (*Model-PULSARE Pro, FINE ADJUSTMENTS,*

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