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Second derivative tryptophan fluorescence spectroscopy as a tool to characterize partially unfolded intermediates of proteins

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Abstract

The application of second derivative tryptophan (Trp) fluorescence spectroscopy to characterize partially unfolded intermediates of proteins relevant to protein formulation was investigated. The second derivatives of the normalized emission scans of *N*-acetyl tryptophanamide (NATA), single-Trp containing proteins, somatostatin and human serum albumin (HSA), and two-Trp containing proteins previously shown to form partially unfolded intermediates, β-lactoglobulin (βLg) and interferon α-2a (IFNα2a), were studied in solution. The second derivative of NATA in water showed three bands at 340, 348 and 367 nm. The 340 nm band showed a blue shift, whereas the intensity of all three bands was affected by a decrease in solution polarity. Second derivative of single-Trp containing proteins, somatostatin and HSA, showed three negative bands, whereas, the second derivative of the two-Trp containing proteins, βLg and IFNα2a, showed four bands, two of which lie in the 320–340 nm range. These two bands were attributed to the presence of the Trps in different microenvironments. The characteristic changes in the intensities of these two bands on addition of guanidine hydrochloride (βLg) and with a decrease in solution pH (IFNα2a) were related to the presence of partially unfolded intermediates of these proteins. Thus, second derivative Trp fluorescence spectroscopy can be used as an important tool to identify partially unfolded states of proteins during formulation utilizing order of magnitude lower concentrations compared to such other technique as near UV CD.

Keywords: Second derivative; Fluorescence; Tryptophan; Proteins; Tertiary structure; Partially unfolded intermediates

1. Introduction

Protein conformation plays an important role in understanding the physical stability (unfolding, aggregation and precipitation) of proteins (Manning et al., 1989; Arakawa et al., 1993; Wang, 1999). Certain proteins can form stable partially unfolded intermediates,

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such as the "molten-globule" state, where a loss in the protein tertiary structure is observed with a minimal loss of the secondary structure (Fink, 1995; Ptitsyn, 1995; Sharma and Kalonia, 2003b). These states could have exposed hydrophobic side chains, which are otherwise buried, resulting in their enhanced propensity to undergo aggregation and thus affect the long-term stability.

Recently we reported that interferon alpha-2a has a tendency to form multiple partially unfolded states as a function of pH and temperature (Sharma and Kalonia, 2003b). The identification of these states was based on the differences observed in the circular dichroism spectroscopy data in the far UV region (representing secondary structure) and the near UV region (representing tertiary structure). It is, however, important to note that CD studies to probe structural transitions in proteins require several milligrams of protein, especially in the near UV region, to perform a complete set of experiments. This could be a limiting factor during formulation development, where the amount of protein available is often restricted and prevent a complete characterization of proteins, particularly the subtle changes in the protein tertiary structure. These subtle changes could have significant implications in the long-term stability of protein formulations.

Steady state tryptophan (Trp) fluorescence spectroscopy, a technique routinely used for protein tertiary structure characterization, requires only a few micrograms of the protein and can be used to analyze solid samples usually obtained in lyophilized formulations (Sharma and Kalonia, 2003a). However, the changes in the emission scan reflect only gross changes in the tertiary structure of the protein and are not sensitive to subtle alterations. The resolution of the Trp emission spectrum can be enhanced by its derivatization against wavelength. In fact, the properties of the second derivative of Trp fluorescence emission have been investigated in *N*-acetyl L-tryptophanamide and various proteins (Miller et al., 1982; Birmingham and Heffron, 1984; Plotkin and Sherman, 1984; Restall et al., 1986; Mozo-Villarias, 2002; Nayar et al., 2002). It was shown that the additional features observed in the second derivative could be used to characterize the native and the unfolded states of proteins.

In this study, we investigate the second derivative Trp fluorescence spectroscopy for its ability to characterize the partially unfolded states of proteins, β-lactoglobulin (βLg) and interferon alpha-2a (IFNα2a), previously shown to form these states under specific solution conditions (Ananthnarayanan and Ahmad, 1977; Sharma and Kalonia, 2003b). We show that the second derivative of Trp fluorescence emission is a sensitive and a useful technique capable of identifying subtle changes in the tertiary conformation of proteins.

2. Materials and methods

All buffer reagents were of highest purity grade available from commercial sources and were used without further purification. Ethanol and *N*-acetyl tryptophanamide (NATA) were purchased from Aldrich (Milwaukee, WI). Lyophilized β-lactoglobulin (βLg), somatostatin and human serum albumin (HSA) were purchased from Sigma (St. Louis, MO). Guanidine HCl (GuHCl) and NaCl were purchased from Fisher Scientific (Fair Lawn, NJ). Interferon alpha-2a (IFNα2a) was donated by Hoffmann-La Roche (Nutley, NJ) and was supplied as 1.6 mg/ml solution in pH 5.0, acetate buffer. Triple distilled water was used in the preparation of all solutions and each solution was filtered through 0.22 μm Millipore millex filter (Bedford, MA). Ten millimolars acetate buffer was used to prepare solutions of pHs 4.0 and 5.0, and 10 mM phosphate buffer was used to prepare solutions of pH 2.0, 3.0 and 7.4. The ionic strength of all the buffers was adjusted to 0.1 using NaCl.

Fluorescence measurements were carried out using a Perkin-Elmer LS50 Luminescence spectrometer. All studies were carried out and analyzed in triplicate. For studies involving NATA, a stock solution of NATA was prepared in ethanol. The final concentration of NATA used in all studies was 100 μM. For studies with proteins, a fixed concentration of 100 μg/ml (o.d. of less than 0.1 at 295 nm) was used to collect the emission scans. It should be noted that a lower concentration of the protein could also be used (for example, 50 μg/ml), however, such data, would require averaging of a greater number of scans to improve the signal to noise ratio.

For each scan, the excitation wavelength was fixed at 295 nm (to minimize interference from tyrosine) and fluorescence emission scans were collected from 310 to 400 nm. An excitation slit width of 10 nm and an emission slit width of 5 nm were used to collect all emission

scans. A scan speed of 20 nm/min was selected in the present studies as this effectively increased the signal to noise ratio in less time. For each solution, five spectra were accumulated using a data pitch of 0.5 nm for each spectrum, and averaged to obtain the final spectrum. All solutions were equilibrated at 25 °C, and the solutions were continuously stirred using a small magnetic bar. All emission spectra were corrected for the Raman peak by subtraction of the emission scan of the buffer from the fluorescence emission scans of the solutions containing NATA or proteins.

The fluorescence emission spectra were normalized to an intensity of 1.0 at the observed λ_{max} using FL-winlab software (Perkin-Elmer Instrument Corporation, Wellesley, MA) prior to derivatization. The normalization of the Trp emission scans was essential in order to compare the intensities and the positions of various bands that appear in the second derivatives of the fluorescence emission scans. The average of five scans was then subjected to smoothing using a 11-point smoothing average and a Savitzky-Golay algorithm using Omnic-software (Nicolet Inc., Madison, WI). Finally, the second derivative of the smoothed spectrum was obtained using the same software. A smoothing step of the normalized data was required to reduce the noise in the second derivative. The criteria for smoothing was that the overall shape and intensity of the raw emission scan is not affected following smoothing and at the same time the overall shape of the bands in the second derivative is preserved and the excess noise is removed.

3. Results and discussion

We first studied the second derivative of Trp emission scans of NATA in solutions of varying polarities to ascertain its characteristics under our instrumental and experimental parameters. NATA was used instead of L-tryptophan as it resembles the natural chemical state of Trp in proteins. The second derivative of NATA has been investigated previously; however, the results in these studies do not correlate amongst each other (Restall et al., 1986; Mozo-Villarias, 2002; Nayar et al., 2002).

The λ_{max} of Trp emission shifts to lower wavelengths as the concentration of ethanol is increased from 0% v/v ethanol (λ_{max} = 356 nm) to 100% v/v ethanol (λ_{max} = 342 nm) (Fig. 1A), as has been reported previously (Mozo-Villarias, 2002). The second derivative of the Trp emission scan of NATA in pure water features three negative bands at 340, 348 and 367 nm (Fig. 1B). The origin of the multiple bands is presumably due to the transition of the electrons back to the different vibrational levels of the ground state (Restall et al., 1986). As the concentration of ethanol is increased, the position and the intensity of the shortest wavelength band are altered. Specifically, an increase in the intensity of the shortest wavelength band is observed along with a blue shift (348 nm in 100% water and 333 nm in 100% ethanol). The position of the 352 nm and 367 nm negative bands remains largely unaltered, however, a loss in the intensity of these two bands, more pronounced for the 367 nm band, is observed with a decrease in solution polarity.

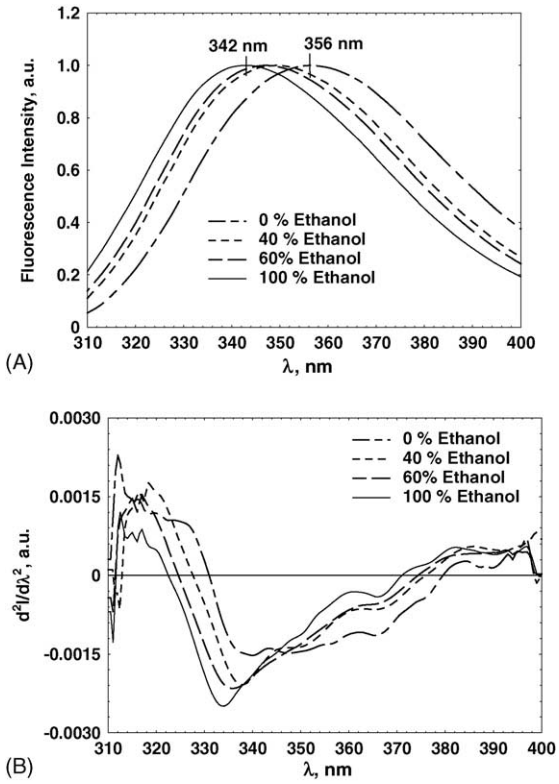

Fig. 1. Effect of solvent polarity on the fluorescence emission of NATA. (A) Trp fluorescence emission scans of NATA in ethanol/water mixtures normalized to fluorescence intensity of 1.0 at λ_{max}, λ_{exc} = 295 nm. (B) Second derivatives of the Trp emission scans of NATA in ethanol/water mixtures.

Evidently, the second derivative of the emission scans under conditions of different solvent polarity brings out several aspects otherwise unobservable in the original emission scans.

We next examined the second derivative of the Trp fluorescence emission in proteins containing single-Trp, i.e., HSA (a 66 kDa protein that has a complex secondary and tertiary structure) (Muzammil et al., 1999) and somatostatin (a small peptide with 14 amino acids with some secondary and tertiary structure) (Holladay and Puett, 1976). These studies were carried out to investigate the differences in the second derivative when the Trp is present in different microenvironments in single-Trp containing proteins as well as to compare the second derivative of Trp emission of a single-Trp containing protein with that of a two-Trp containing protein.

Fig. 2A shows the emission scans of these two proteins at pH 7.4. Fig. 2B represents the corresponding second derivative spectra. The emission λ_{max} for HSA is observed at 342 nm, indicating that the Trp is present in a relatively hydrophobic environment, whereas the emission λ_{max} for somatostatin, observed at 352 nm, indicates that its Trp is located in a relatively polar environment. The three negative bands visible in the second derivative of the Trp emission scan of HSA and somatostatin are similar to those observed in case of NATA at different concentrations of ethanol. The derivatized spectrum of HSA closely resembled that of NATA in 100% ethanol, whereas, the derivatized spectrum of somatostatin resembled that observed for NATA in 40% ethanol. Furthermore, the relative intensities of the three bands in the derivatized spectra also indicate that the Trp in HSA is present in a relatively hydrophobic environment, and the Trp in somatostatin is present in a polar environment. It is worth noting that relatively broad negative bands are observed around 352 and 367 nm when the Trp is present in a hydrophilic environment as in the case of somatostatin. A considerable loss in the intensity of these two long wavelength bands and a gain in the intensity of the short wavelength band occur when the environment of Trp becomes more hydrophobic as seen in the case of HSA.

βLg is a two-Trp containing protein, where the two Trps, Trp19 and Trp61, have been reported to lie in different microenvironments (Eftink et al., 1987). βLg has also been shown to form partially folded intermediates at low concentrations of guanidine hydrochloride

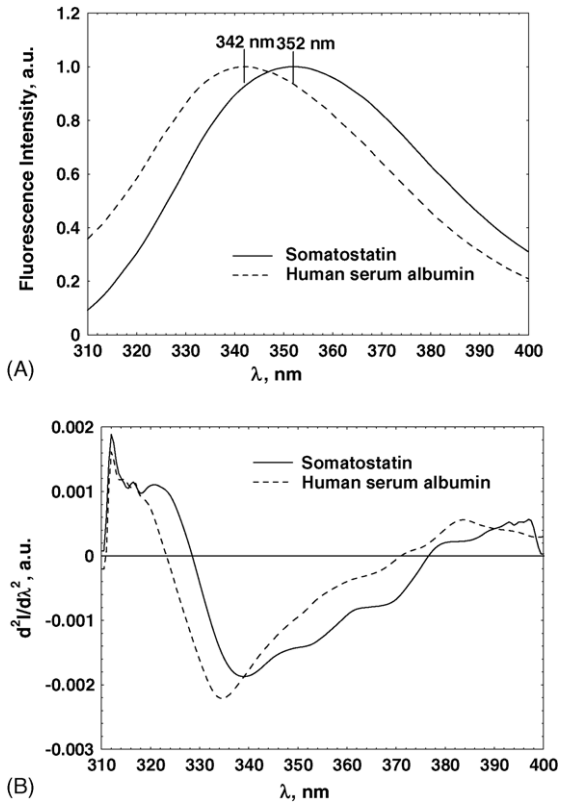

Fig. 2. (A) Trp fluorescence emission scans of somatostatin and HSA at pH 7.4 normalized to fluorescence intensity of 1.0 at λ_{max}, λ_{exc} = 295 nm. (B) Second derivatives of the normalized Trp emission scans of somatostatin and HSA.

or urea at pH 5.0 (Ananthnarayanan and Ahmad, 1977). Hence, the applicability of the second derivative of Trp emission in identifying such subtle changes in the tertiary structure of βLg was first explored.

At pH 5.0, βLg exhibits a λ_{max} of 336 nm in the absence of GuHCl (Fig. 3A) indicating that the Trps lie in a relatively hydrophobic environment. The three-dimensional structure of βLg has also indicated that the two Trps of βLg, at positions 19 and 61, are partially buried in the protein and partially exposed to the solvent (Uhrinova et al., 2000). The emission λ_{max} is shifted to higher wavelengths as the concentration of GuHCl is increased because of the unfolding of the protein (Fig. 3A). The second derivative spectra of βLg at pH 5.0 shows four negative bands appearing at 324, 336, 352 and 367 nm (Fig. 3B). The two bands appearing at 324 and 336 nm presumably arise due to the presence

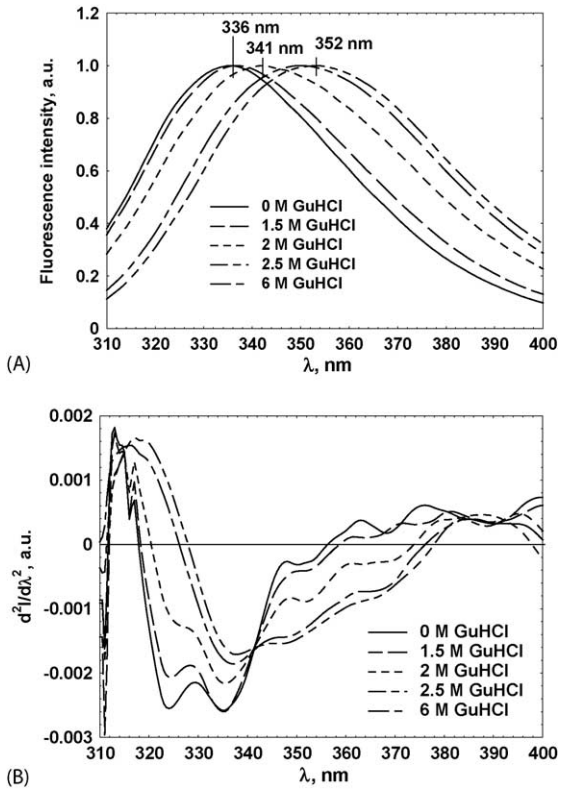

Fig. 3. Effect of different concentrations of GuHCl on the fluorescence emission of βLg in solution. (A) Trp fluorescence emission scans at pH 5.0 normalized to fluorescence intensity of 1.0 at λ_{max}, λ_{exc} = 295 nm. (B) Second derivatives of the normalized Trp emission scans.

of two Trps in somewhat different microenvironments with one of the Trps in a relatively more hydrophobic environment than the other. Note that the single-Trp containing proteins, e.g. human serum albumin and somatostatin, showed a single band in the 320–340 nm region along with the 352 and 367 nm band.

From the second derivatives (Fig. 3B), it is observed that the negative band at 324 nm is most sensitive to the changes in the tertiary structure of βLg on addition of GuHCl. Distinct difference in the second derivative at this wavelength is apparent between 0 and 1.5 M GuHCl and subsequent loss in the intensity is observed on further increasing the GuHCl concentration. The loss in the intensity at 324 nm with no change at 336 nm at 1.5 M relates to the partial unfolding of this protein. These differences (between 0 and 1.5 M GuHCl) are not evident in the original Trp emission scans (data not shown) and only slightly revealed in the normalized Trp emission scans (Fig. 2A). Previous reports have indicated similar changes in βLg tertiary structure at less than 2.0 M GuHCl concentration, i.e., formation of an intermediate, observed by optical rotatory dispersion measurements (Ananthnarayanan and Ahmad, 1977). Overall, the extent of loss in the intensity of the second derivative at 336 nm is relatively less as compared to that at 324 nm indicating that the microenvironment of one Trp is affected more than that of the other Trp. At 6 M GuHCl, the second derivative of the Trp emission in βLg appears similar to that of NATA in water (Fig. 1B). This is due to the homogeneous emission from the two Trps in this protein because of its complete unfolding.

As mentioned earlier, IFNα2a forms partially unfolded states as the pH is lowered from 5.0 to 2.0, characterized by a complete loss in the near UV signal with a minimal loss in the far UV spectrum (Fig. 4) (Sharma and Kalonia, 2003b). Hence, the generality of the second derivative approach in identifying partially unfolded states associated with subtle changes in protein tertiary structure was further explored in IFNα2a under these pH conditions.

Fig. 5A shows the fluorescence emission scans of IFNα2a at pHs 5.0, 4.0, 3.0 and 2.0. The λ_{max} of emission at pH 5.0 appears at 336 nm. The emission λ_{max} of IFNα2a is observed to be red shifted to 339 nm at pH 3.0 and appears at 340 nm at pH 2.0. The emission scan of IFNα2a at pH 4.0 almost completely overlays

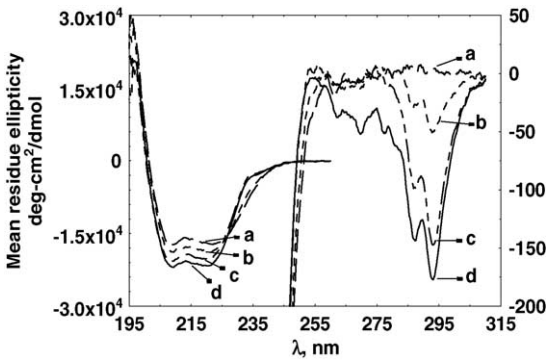

Fig. 4. pH dependence of far UV and near UV CD spectra of IFNα2a at 25 °C. The pH was varied from pH 7.4 to 2.0 and the spectra were recorded at pH (a) 2.0, (b) 3.0, (c) 3.5, and (d) 5.0. The left *y*-axis corresponds to the far UV CD spectra and the right *y*-axis corresponds to the near UV CD spectra (Sharma and Kalonia, 2003b).

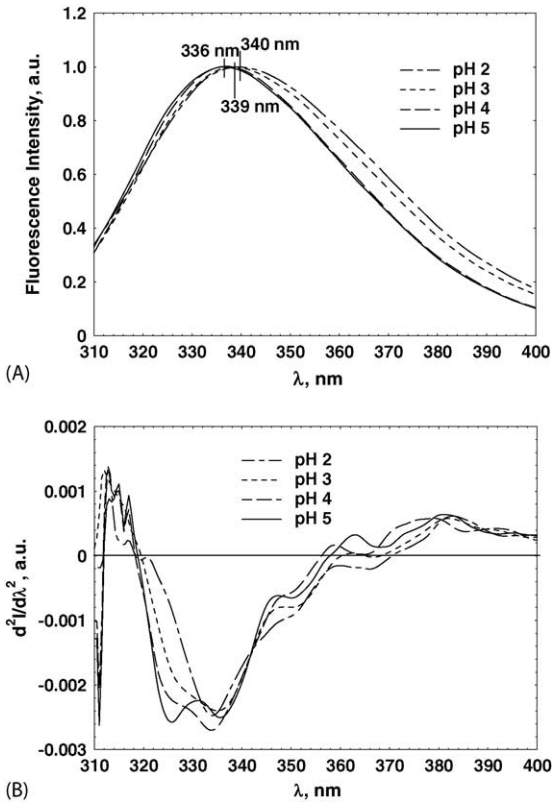

Fig. 5. Effect of pH (2.0, 3.0, 4.0 and 5.0) on the fluorescence emission of IFNα2a. (A) Trp fluorescence emission scans normalized to fluorescence intensity of 1.0 at λ_{max}, λ_{exc} = 295 nm. (B) Second derivatives of the normalized Trp emission scans.

the emission scan at pH 5.0. Significant differences are observed in the second derivative spectra of the Trp emission of IFNα2a under these different pH conditions (Fig. 5B). It is observed that the second derivative of IFNα2a at pH 5.0 features four negative bands that appear at 325, 335, 352 and 367 nm. A gradual decrease in the intensity of the band appearing at 325 nm is observed when the pH is lowered from 5.0 to 4.0 with an increase in the band intensity at 335 nm. These differences are not evident in emission scans, since the emission λ_{max} of IFNα2a at both pHs 5.0 and 4.0 appears at 335 nm and the emission spectra almost completely overlay each other. Hence, the difference in the second derivative spectra, between pH 5.0 and 4.0, clearly indicates changes in the microenvironment of the two Trps, reflective of subtle changes in the protein tertiary structure. As the pH is lowered further, the band appearing at 325 nm is completely lost, suggesting that the two Trps of IFNα2a lie in the same environment and are emitting homogeneously. However, the intensity of the band at 335 nm is only marginally altered compared to that observed at pH 5.0. A complete loss in protein structure should result in significant changes in the intensity of these two bands as was observed in βLg at 6.0 M GuHCl (Fig. 3B). Thus large changes in the 325 nm band combined with only slight alterations in the 335 nm band, as the pH is lowered, indicate formation of partially unfolded states; the formation of latter being confirmed earlier independently by using CD studies.

The studies provided herein, show that the second derivative Trp fluorescence spectroscopy can be a useful tool during protein formulation to identify partially unfolded states of proteins, associated with subtle tertiary structure changes, utilizing order of magnitude lower concentrations compared to such other techniques as near UV CD. Additionally, the fluorescence derivative spectroscopy can potentially provide significant insight into the tertiary structure changes of proteins in solid powders where other spectroscopic techniques cannot be used.

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