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Probing the conformation of protein (bFGF) precipitates by fluorescence spectroscopy

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Abstract: Aggregation and precipitation are major events in the handling and aging of most protein pharmaceuticals. We demonstrate the utility of fluorescence spectroscopy in determining protein conformation in precipitates using basic fibroblast growth factor (bFGF) as an example. Conversion of the native to the soluble denatured form by chaotropes was accompanied by an increase in tryptophan emission. The emission spectra of resuspended precipitates were as reproducible as the spectra of the soluble form. The sum of emission spectra of native soluble bFGF and denatured precipitated bFGF was superimposable on the spectrum of the unfractionated suspension, suggesting that quantitative analysis of denatured aggregates in turbid protein formulations is possible. The ratio of tryptophan to tyrosine emissions increased with increasing extent of denaturation both in solution and in suspension. For example, salting out by ammonium sulphate increased the fluorescence index (indicative of denaturation) which was reversible upon dissolution. In addition, aging (35°C) of bFGF in the presence of sulphated ligands produced precipitates with native-like fluorescence index, in contrast to denatured precipitates formed without ligands.

Keywords: *FGF; protein; fluorescence; aggregation; precipitation; unfolding; structure.*

Introduction

Aggregation and precipitation limit the development of protein drugs because of potential alteration in immunogenicity, toxicity, efficacy, and aesthetic appearance. Both irreversible and reversible aggregation/precipitation can occur as a result of physical stress (e.g. by agitation) and chemical reactions (e.g. cleavage, oxidation), as well as salting out, interaction with excipients, and exposure to a pH near the pI or to the solubility limit of the protein. Not all types of stress produce denatured precipitates so that evaluation of the structure of proteins within precipitates would be useful in understanding the pathway that led to the degradation, for example when screening formulations. Solid state NMR rotational resonance method has been used to show the existence of cis-amide bonds in the insoluble amyloid peptide deposits [1]. Laser Raman spectroscopy, which is sensitive to vibrational modes, was used to address the secondary structure of protein precipitates that were induced by inorganic salts and showed an increase in β sheet content [2, 3]. In the same study, electron paramagnetic resonance, which is sensitive to rotational mobility of the spin probe, suggested that tight folding was maintained during salting out, particularly in the active sites of the proteins that were studied. Light scattering measurements have provided information about the particle size distribution of aggregates but could not provide structural information [4]. Moreover, none of these methods could easily estimate the amount of aggregates/precipitates.

Fluorescence spectroscopy is a conventional method for quantitative analysis of changes in the protein's tertiary structure when in solution [5]. Fluorescence can also be detected in the solid state, for example when studying photo-oxidation of dyes that coat tablets [6] or measuring dye-sensitized photovoltages on solid semiconductor surfaces [7]. For quantitative analyses of turbid solutions (e.g. lipid emulsions and cell suspensions), front surface fluorometry is generally used [8]. There are no reports, however, on the application of cuvette fluorometry in assessing protein's structural stability in aging drug formulations which are susceptible to aggregation/precipitation.

In this report we describe the use of cuvette fluorometry to probe the folded state of

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aggregated/precipitated forms of bFGF and also show that the method is amenable to estimating the extent of denaturation in suspension.

Materials and Methods

Recombinant human basic FGF (154 amino acids, pI~9.2) was isolated from an *E.coli* expression system and purified to >98% purity as assessed by HPLC and other analytical methods [9]. Protein concentration was determined by UV spectroscopy using $E^{0.1\%}$ of 0.936 ml/mg.cm at 277 nm. The amount of protein in precipitates was estimated by the difference in absorbance between the total protein initially placed in the vial and that found in the filtered sample.

Fluorescence spectroscopy was performed on either a Shimadzu model RF540 or a Perkin–Elmer model LS-50B fluorometer using a quartz cuvette (2 mm excitation and 5 mm emission pathlengths). Only the latter instrument produced spectral corrections for lamp profile. Spectra were obtained using 5 nm slit widths and 240 nm/min scan speed. A 1 mg ml^{-1} protein solution was prepared in 100 mM phosphate, 1 mM EDTA buffer at pH 6.5 (Buffer A) unless otherwise indicated. Spectra of soluble protein were measured at 0.1 mg ml^{-1} (protein) in order to remain in the linear detection range. When precipitates were present, they were centrifuged (3000***g***, 22°C, 2–5 min) and washed three times with buffer. Spectra were obtained after uniform resuspension also at ~0.1 mg ml^{-1} to minimize scattering artefacts and quenching.*

Protein denaturation by chaotropes was achieved by dilution of a 10 mg ml^{-1} stock solution to the appropriate concentration of chaotrope and 1 mg ml^{-1} protein. Highest purity GnHCl (Ultrapure, ICN) was used. After incubation at 22°C for 6–18 h, samples were diluted to 0.1 mg ml^{-1} with the appropriate placebo buffer for spectroscopy.

Salting out was performed by addition of nine parts of a saturated ammonium sulphate solution (4.1 M) to one part protein solution. The precipitates were centrifuged (3000***g***, 2 min) and the supernatant was removed for UV spectroscopic determination of remaining protein concentration. The precipitate was then either resuspended in saturated ammonium sulphate or redissolved in 10% ammonium sulphate.

Heparin affinity HPLC was conducted on a TSK-gel column (7.5 × 75 mm; Toso Haas Inc.) using a 150 mM min^{-1} NaCl gradient at pH 6.5 as described previously [10, 11]. Ion exchange HPLC (HP-IEC) was performed on a polyaspartate column (PolyCat A 46 × 200 mm, Poly LC Inc.) using a 6.8 mM min^{-1} ammonium sulphate gradient at pH 6.

Results and Discussion

Fluorescence spectrum of native and denatured soluble bFGF

The fluorescence excitation spectrum of bFGF when monitored at 350 nm emission showed one asymmetric component that peaked at 277 nm (Fig. 1A). Excitation at 277 nm gave a broad emission spectrum which peaked at 304–306 nm (Fig. 1B). bFGF has seven tyrosines [12] which in the native state gave a strong emission spectrum, whereas the emission of the single tryptophan near the surface of the protein globule is quenched [10]. Tryptophan-specific excitation at 290 nm gave rise to a broad peak near the background (Fig. 1B).

Denaturation of bFGF with GnHCl (5.6 M, 5 h 22°C) resulted in a dramatic rise in emission at 350 nm (Fig. 1B) and excitation at 280 nm (Fig. 1A). In addition, a slight decrease in tyrosine emission was found as has

*For both soluble and aggregated protein, the extent of quenching of the emission intensity (EM_{corr}/EM) at a given wavelength, λ_{em}, due to inner filter effect (reabsorption of excitation and emission light by the sample) can be estimated from the Beer-Lambert Law according to:

$$EM_{corr} = EM\,[10(OD_{ex} \times l_{ex} + OD_{em} \times l_{em})]$$

where OD_{ex} and OD_{em} are total sample absorbances at the excitation wavelength, λ_{ex} and λ_{em}, respectively, l_{ex} and l_{em} are excitation and emission path lengths in the cuvette relative to those used for absorbance measurements. In the case of bFGF, OD_{em} for the range of λ_{em} = 306–350 nm was less than 1% of OD_{ex} for λ_{ex} = 277 nm. Thus, for l_{ex} = 0.2 and l_{em} = 1.0, less than 10% quenching occurred when OD_{ex} was less than 0.2. This corresponded to less than 0.2 mg ml^{-1} soluble or 0.14 mg ml^{-1} aggregated protein since 0.2 mg ml^{-1} aggregated form produced a 30% increase in absorbance at 277 nm relative to the soluble form. Precipitated bFGF concentrations <0.1 mg ml^{-1} also minimized contribution from scattering (which appeared as a decaying curve at the onset of the emission spectrum, for example in Fig. 4), though peak wavelength did not change for concentrations up to ~1 mg ml^{-1}.

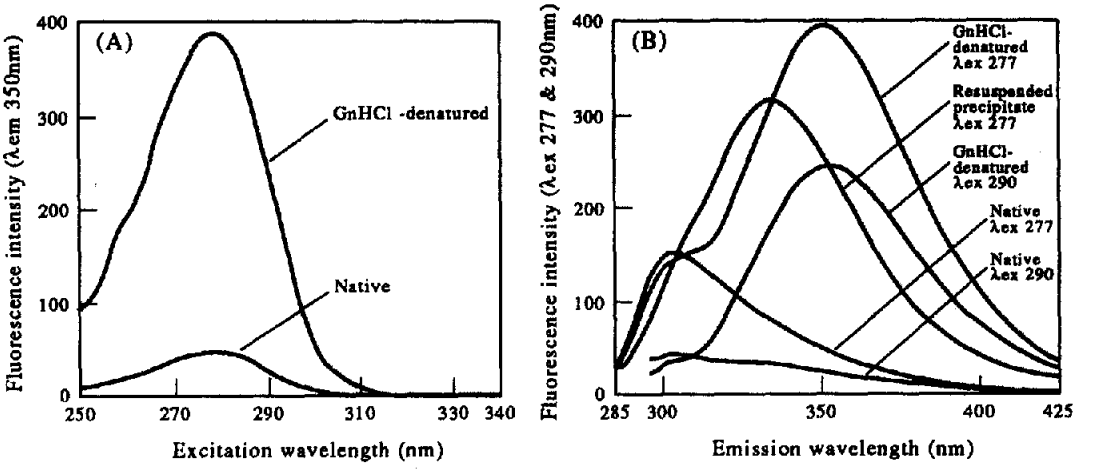

Figure 1
Fluorescence spectra of soluble bFGF. (A) The excitation spectra of native (in Buffer A) and denatured (in 5.6 M GnHCl in Buffer A) bFGF. (B) The emission spectra of native and denatured soluble bFGF, as well as 3× washed precipitates found after storage (35°C, 2.5 days). Background Raman scattering from placebo buffer has been subtracted.

been noted for other proteins [13]. A homologous member of FGF family, aFGF, has a similar behavior to bFGF [14]. The fluorescence behavior of bFGF and aFGF were unlike other proteins such as albumin in which tyrosine emission is quenched in the native state due to energy transfer to tryptophans [5].

The ratio of peak intensities of tryptophan to tyrosine emissions is a sensitive, concentration-independent index of protein denaturation. The GnHCl denaturation profile of bFGF is shown in Fig. 2. The equilibrium denaturation profile was consistent with a two-state model (χ^2 of 0.01 for a least-squares sigmoidal fit)

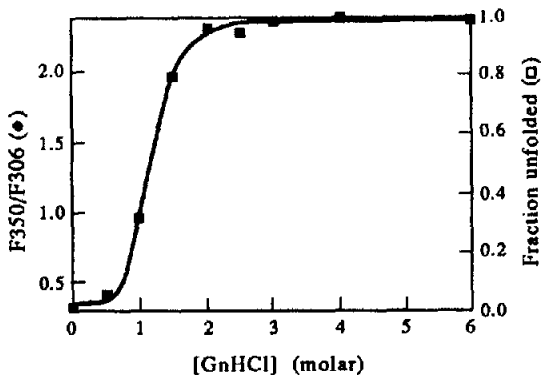

Figure 2
Equilibrium denaturation profile of bFGF. The ratio of emissions of tryptophan to tyrosine as a function of GnHCl concentration (C) is shown. The following sigmoidal least squares fit the data was obtained:

$$F350/F306 = 2.38 - \frac{2.03}{1 + (C/1.16)^{5.43}}$$

Fraction of denatured protein was calculated as described. Samples were in Buffer A.

with a calculated midpoint at 1.16 ± 0.02 M GnHCl. Though confirmation of lack of folding intermediates awaits other measurements of bFGF structure (e.g. by CD spectroscopy), with a few exceptions [13, 16], these intermediates generally have milli- to micro-second lifetimes and are not expected to be detectable by steady-state measurements. From these data, f_D (C) or the fraction of denatured by bFGF at a given GnHCl concentration, C, can be calculated according to:

$$f_D(C) = \frac{F_C - F_0}{F_\infty - F_0}, \qquad (1)$$

where F_0 and F_∞ are unitless fluorescence indices (F350/F306) at zero and 4 M GnHCl concentrations, respectively, and F_C is the fluorescence index at the GnHCl concentration, C. The reproducibility of fluorescence ratio measurements was ±3%.

Fluorescence spectrum of aggregated/precipitated bFGF

The above fluorescence behavior of a soluble protein was used to pioneer an investigation of the protein's conformation in precipitates. Incubation of bFGF at 35°C and pH 5 (well below bFGF melting temperature of 53°C [17] resulted in precipitation of 63% of the protein within 2 days. The emission spectrum of isolated resuspended precipitates showed a large tryptophan signal and no tyrosine signal, consistent with the presence of denatured protein (Fig. 1B). The peak emission of precipitated protein occurred

at 334 nm or 16 nm blue shifted compared to the maximum of 350 nm in the presence of chaotropes. This suggested a more hydrophobic environment of tryptophan in the packed aggregates compared to the dissociated, more solvent-exposed condition in the chaotrope-containing solution. Because of this shift in the emission maximum, the extent of denaturation in turbid samples would be under-estimated by ~20% when F350/F306 is used.

Fluorescence spectroscopy was also applied to examine the folded state of salt-induced precipitates as might occur during protein purification. Addition of 90% saturated ammonium sulphate to a 1 mg ml^{-1} solution of bFGF resulted in complete precipitation (no UV absorbing material remaining in the supernatant of centrifuged samples). The fluorescence index of these precipitates (1.37) indicated considerable denaturation which was completely reversible upon dissolution (index

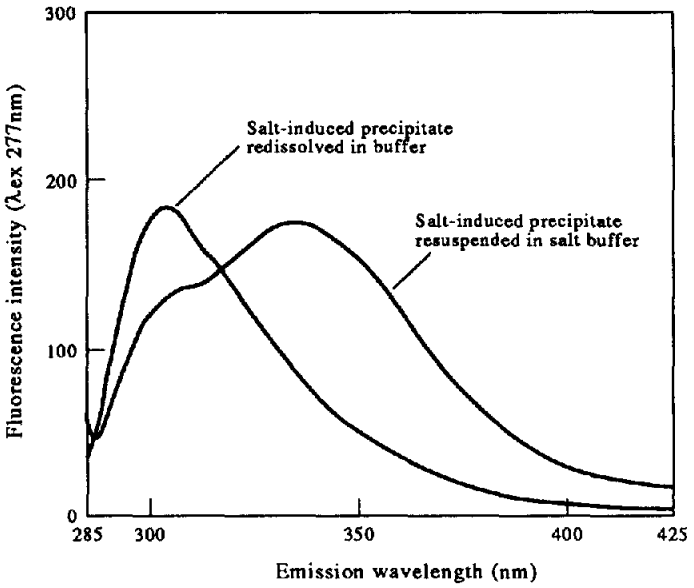

Figure 3
Emission spectra of salted out bFGF resuspended in 90% ammonium sulphate or dissolved in 10% ammonium sulphate.

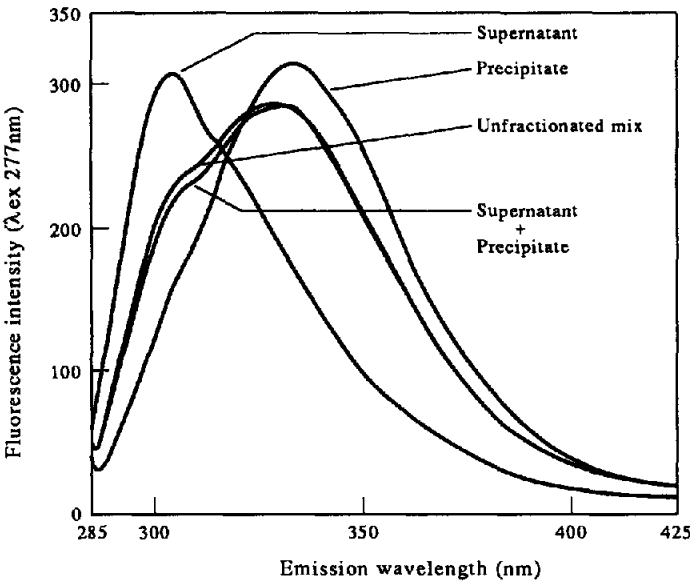

Figure 4
Emission spectra of bFGF precipitates. Spectra of supernatant and precipitates (both corrected to 0.1 mg ml^{-1}) formed after incubation (35°C, 2.5 days) of bFGF in Buffer A. The spectra were additive such that the normalized sum spectrum superimposed on the spectrum of the unfractionated turbid sample (also at 0.1 mg ml^{-1}).

of 0.32; Fig. 3). The above data collectively demonstrate the utility of fluorescence spectroscopy to differentiate the structural consequences of protein precipitation and assess their structural reversibility upon dilution.

Quantitation of denatured bFGF

To show that fluorescence spectroscopy of aggregates and precipitates is amenable to quantitative analysis, the possibility of estimating the amount of denatured bFGF in a turbid mixture was investigated. Duplicate samples of a 1 mg ml^{-1} bFGF solution in citrate, pH 5 were incubated at 35°C for 2.5 days. Control samples were kept at 4°C. The samples were centrifuged, the supernatant, two washes, and the precipitate were separated. Sixty-three per cent of total protein was in precipitate and 37% in supernatant. Figure 4 shows the emission spectra of supernatant, precipitate, and an unfractionated sample all diluted to 0.1 mg ml^{-1}. The reproducibility of peak intensity at 306–350 nm in spectra from seven separate preparations of resuspended precipitates was 2–3% RSD, comparable to that found in seven replicates of soluble native protein at 306 nm (2.6%). This data suggests that, given reproducible pipetting and uniform resuspension, the fluorescence method is not limited by the presence of low concentrations of scattering materials. The sum spectra of supernatant and precipitate (after correction to 0.1 mg ml^{-1} protein: fluorescence index of 1.09) superimposed on the spectrum of the unfractionated sample (fluorescence index of 1.06), demonstrating additivity of the two spectral components and lack of physical or chemical interference of the precipitated and soluble forms of bFGF. Thus, the amount of precipitates in bFGF formulations can be estimated provided that the fluorescence index is corrected for the 20% under-estimation due to a blue shift in emission maximum.

A slightly progressive increase in the fluorescence index of the washes was noted (0.32, 0.47, 0.78 for the first, second, and third wash, respectively) as total protein concentration decreased. The data indicated dissolution of only ~1% denatured protein from precipitates during the three washes, confirming irreversible denaturation.

To show directly that the fluorescence index is an indicator of protein denaturation both in solution and in suspension, the recovery of bFGF that was subjected to various denaturing conditions was measured by HPLC. Two methods, heparin affinity and ion exchange HPLC, were used for recovery of only the native form as described in previous studies [11]. For soluble bFGF which was denatured by chaotropes or organic solvents, a strong inverse correlation (slope = −48.98, R = 0.985) was found between the fluorescence index and the HPLC recovery (Fig. 5).* For aged bFGF suspension, an inverse correlation was also found with a slope (−45.01, R = 0.983) that was similar to that of the soluble denatured bFGF (Fig. 5). Thus, overall correlation between the fluorescence index and HPLC recoveries was independent of the source of denaturation or the aggregation state.

Detection of native bFGF precipitates

Previous formulation studies had suggested structural stabilization of bFGF by sulphated ligands such as heparin, although precipitation was not prevented [11]. For example, isolated heparin-induced precipitates (35°C, 31 days, in Buffer A) had maintained a native-like fluorescence index (0.27) compared to precipitates without excipients (2.06). Hence, precipitation

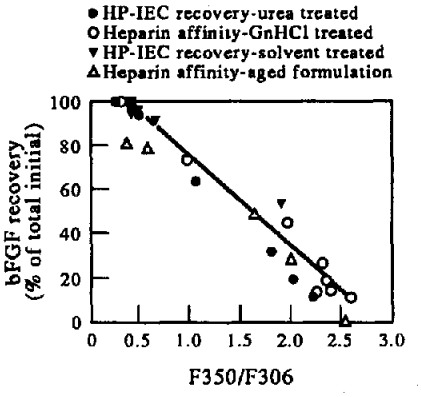

Figure 5
Correlation between HPLC recovery of native bFGF and fluorescence index. Recoveries by heparin affinity HPLC or HP-IEC of GnHCl-treated (0–6 M), urea-treated (0–6 M), organic solvent-treated (10% acetonitrile with 0–0.08% TFA), and aged bFGF (in isotonic 20–500 mM Na-acetate buffer, pH 5, 35°C over a 3 day period) are shown. The fluorescence index of aged samples was corrected for the shift in the emission maximum (increased by 20%).

* In contrast, the recovery of such denatured bFGF on a denaturing HPLC method such as RP–HPLC was not affected [11].

Table 1
Detection of native precipitates in the presence of sulphated ligands

Ligand; Condition	F350/F306	Denatured* (%)	Precipitate† (%)
None; fresh	0.34	0	0
None; aged	1.45	68	71
Inositol hexasulphate (10 mM); aged	0.30	0	19
Sucrose octasulphate (20 mM); aged	0.448	10	39
Na sulphate (45 mM); aged	0.30	0	12

*Calculated from the fluorescence ratio using equation 1 and corrected for 20% under-estimation due to blue-shifted emission maximum.

†Estimated by subtracting the amount of total protein from soluble bFGF that was recovered by heparin affinity HPLC.

One milligram per millilitre bFGF samples in isotonic acetate buffer, pH 5 containing one of the following ligands were incubated at 35°C for 21 days. Precipitation was noted in all samples. The recovery of soluble, native bFGF was measured by heparin affinity HPLC of filtered samples. The fluorescence ratio of unfiltered samples was measured. The % precipitate (HPLC) corresponded well with the % denatured bFGF (fluorescence) in the absence of ligands, but not in the presence of ligands where native precipitates appeared.

did not necessarily corroborate protein denaturation, and fluorescence spectroscopy rapidly and non-invasively distinguished the native from the denatured forms of precipitates. In a study of aging of bFGF in the presence of small sulphated ligands, little denatured protein was detected by fluorescence spectroscopy whereas marked loss of soluble protein was noted by HPLC (Table 1). When significant denaturation was noted (e.g. in the absence of excipient, Table 1), excellent correspondence with the amount of precipitation was found (Table 1). Thus, if consideration can be given to modifications in proteins that involve alteration in conformation, fluorescence index can be a stability indicator.

The advantage in using fluorescence index to assess the conformation of bFGF was that the ratio was independent of protein concentration and showed greater sensitivity to denaturation than emission from either tyrosine or tryptophan. In other proteins, the presence of tryptophans and tyrosines and the response of these chromophores to denaturation would determine whether a fluorescence index or the emission from individual chromophores should be used.

Conclusion

We have extended the utility of cuvette fluorometry to evaluate the conformation of bFGF within precipitates and to directly quantitate them without fractionation in cases where precipitates were denatured protein. Because of the sensitivity of fluorescence spectroscopy to the chromophore's local environment, the general applicability of the method needs to be evaluated on a case-by-case basis.

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