Temperature- and pH-Induced Multiple Partially Unfolded States of Recombinant Human Interferon-α2a: Possible Implications in Protein Stability

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Purpose. To study the effect of solution conditions on the structural conformation of recombinant human interferon-α2a (IFNα2a) to investigate its tendency to form partially unfolded intermediates.

Methods. The structural properties of IFNα2a were studied at various pH values (2.0–7.4) and temperatures (5°C–80°C) using Trp fluorescence emission, fluorescence quenching, near- and far-UV circular dichroism (CD) spectroscopy, and DSC.

Results. Fluorescence intensity measurements as a function of temperature indicated the onset of the thermal unfolding of IFNα2a, denoted by T_d, around 60°C above pH 4.0. T_d was not observed at pH 3.5 and below. Acrylamide and iodide quenching studies indicated partial unfolding of protein with decrease in pH and with increase in temperature up to 50°C. Near-UV CD studies indicated a significant loss in the tertiary structure of protein on increase in temperature from 15°C to 50°C at all solution pHs. DSC scans supported results obtained from fluorescence and CD studies at pH 4.0 and below. DSC, however, was insensitive to changes that occurred at moderate temperatures at pH 5.0 and 7.4.

Conclusions. IFNα2a has a tendency to acquire multiple partially unfolded states with structural conformations sensitive to solution pH and temperature. These states were formed at moderate temperatures, and it is speculated that these partially unfolded states could play an important role in the aggregation of proteins during the long-term storage of aqueous protein formulations.

KEY WORDS: interferon α2a, solution conformation, fluorescence quenching, partially unfolded states, aggregation.

INTRODUCTION

As a result of advances in biotechnology, several proteins and peptides have been identified for specific therapeutic targets; however, the development of these macromolecules as stable formulations still remains a big challenge. Formulation problems in solution arise from the complex native structure of protein molecules and often manifest in the form of physical instability such as unfolding, aggregation, and/or precipitation (1–3). Physical instability results mainly from perturbation of the native structure of the protein molecule, which can easily be caused by alteration of solution conditions such as changes in pH, temperature, and ionic strength, presence of cosolvents, and other additives. The result of this perturbation is often some kind of change in protein conformation and in most cases leads to unfolding of the protein molecule before any further protein degradation.

Protein unfolding traditionally has been believed to be a cooperative process, where only the native and the unfolded states are stable. It has been shown recently that certain proteins can form stable partially folded intermediates under acidic conditions or in the presence of mild denaturants (4–12). The description of these intermediates differs from author to author and has been a subject of debate over the years. According to one point of view, all proteins unfold through the formation of a compact partially unfolded state called the "molten-globule" state (4,6), which has also been termed the third thermodynamic state (13,14). In terms of the structural conformation, it is believed that the molten globule's secondary structure is as compact as that of the native state; however, it lacks the general tight packing of the tertiary structure of the native state. Although the concept of "molten globule" is well accepted, the similarity in the structural conformation of the molten-globule states of different proteins has not been very well established. A more general definition has been provided by some authors for the partially unfolded states of proteins (5,7). According to this view, the "compact intermediates" have a broad range of conformations, degrees of unfolding, and compactness. In fact, these intermediates can range from having a native-like compact structure to almost completely unfolded and expanded conformations.

From the pharmaceutical point of view, these partially folded intermediates have significant implications. Because partial unfolding of a given protein usually results in the exposure of hydrophobic side chains, it can enhance the propensity of the protein to undergo aggregation. Hence, it is necessary for the development of stable protein formulations that subtle changes, which can occur in protein conformation under moderate solution conditions, be investigated in detail because these changes can affect the overall long-term stability.

The goal of the present investigation was to study the structural transitions that occur in recombinant human interferon-α2a (IFNα2a) as a function of pH and temperature using fluorescence spectroscopy, circular dichroism (CD) spectroscopy, and differential scanning calorimetry. Few studies on hybrid interferons or mixture of various interferon-α subtypes have been reported in literature (15–18), and these studies did not address the effect of solution conditions on structural conformation in detail.

Interferons belong to the family of cytokines that exert antiviral, antiproliferative, and immunoregulatory activities in human body (19,20). IFNα2a, one of the many subtypes of interferons, contains 165 amino acids with four cysteines and two disulfide linkages and has a molecular weight of 19.225 kDa. The three-dimensional (3-D) solution structure of IFNα2a has been determined by NMR at pH 3.5, and it was revealed that IFNα2a is an all-helical protein containing six α-helices (21).

We report in this paper that IFNα2a has a tendency to form multiple partially unfolded states/intermediates whose conformations are sensitive to solution pH and temperature. These partially unfolded states can play an important role in the aggregation and hence long-term stability of IFNα2a in solution.

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ABBREVIATIONS: DSC, differential scanning calorimetry; CD, circular dichroism; IFNα2a, recombinant human interferon-α2a.

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MATERIALS AND METHODS

All buffer components and chemical reagents used in the present studies were of highest purity grade and obtained from commercial sources. IFNα2a was donated generously by Hoffmann La Roche (Nutley, NJ) as a 1 mg/ml solution in acetate buffer, pH 4.5–5.5. Electrophoresis grade acrylamide (99.9% assay) was purchased from Fisher Scientific, and potassium iodide was purchased from Aldrich. For pH studies, 0.01 M acetate buffer (pH 4.0, 4.5, and 5.0) and 0.01 M phosphate buffer (pH 2.5, 3.0, 3.5, and 7.4) were used. The ionic strength was adjusted to 0.14 M using NaCl.

Fluorescence Studies

Steady-State Trp Fluorescence Studies

Trp fluorescence studies were carried out on a Perkin Elmer Luminescence Spectrometer, Model LS 50. The protein concentration used for all fluorescence studies was 0.050 mg/ml. The excitation wavelength was fixed at 295 nm, and the fluorescence emission scans were collected from 300 to 450 nm using a scan speed of 50 nm/min at an excitation slit width of 5 nm and emission slit width of 2.5 nm. Thermal denaturation studies were performed by heating the protein solutions from 20°C to 80°C at a rate of 0.5°C/min and then cooling back to 20°C. Trp fluorescence intensity was recorded at 336 nm during the thermal cycle. Emission scans were collected before and after completion of each heating/cooling cycle.

Fluorescence Quenching Studies

Trp fluorescence quenching studies were performed by addition of small aliquots of stock solutions of acrylamide (final concentrations ranging from 0 to 1.0 M) and potassium iodide (final concentration ranging from 0 to 0.3 M) to the protein solution. A small amount of sodium thiosulfate (10^{-4} M) was added to potassium iodide stock solutions to prevent iodide oxidation. Fluorescence intensities were then measured at emission maximum of 336 nm and were corrected for dilution (not exceeding 25%) from addition of quencher solutions. For acrylamide quenching studies, fluorescence intensities were also corrected for the inner filter effect produced by its absorption at the excitation wavelength of 295 nm (22).

The data for fluorescence quenching were analyzed using the Stern-Volmer equation, which, for a system of two heterogeneously emitting Trps in a protein, can be written as (23,24):

$$\frac{F_0}{F} = \left(\frac{f_1}{1 + K_1 \cdot Q} + \frac{f_2}{1 + K_2 \cdot Q} \right)^{-1} \quad (1)$$

where F_0 is the fluorescence intensity in the absence of the quencher, and F is the intensity in the presence of the quencher; Q is the quencher concentration; f_1 and f_2 are the fractional contributions of each Trp to total fluorescence, and K_1 and K_2 are the dynamic (collisional) quenching constants for each Trp. Because $f_1 + f_2 = 1$, f_2 in eq. (1) can be replaced by $1 - f_1$. The parameters that are obtained by fitting of eq. (1) are f_1, K_1, and K_2. These parameters provide information about the fractional contribution of each Trp to the total fluorescence quantum yield and the extent of quenching that each Trp experiences from the external quencher. Equation (1) was used in the present studies when the Stern-Volmer plot was either downward curving or linear (see explanation below). If an upward-curving plot was observed, which indicates homogeneously emitting Trps (see explanation below), the following equation was used:

$$\frac{F_0}{F} = (1 + K \cdot Q)\exp(V \cdot Q) \quad (2)$$

where K is the collisional quenching constant and V is the static quenching constant. The two parameters K and V can be simply obtained by fitting the above equation to the experimental data.

Information about the structural conformation can also be obtained by observing the shape of the Stern-Volmer plot in a two-Trp–containing protein (24–26). The shape of the plot can vary from downward curving to linear to upward curving, and each shape represents the relative exposure of the two Trps to the quencher and hence to the solvent. A downward curve indicates heterogeneous emission and hence a significant difference in the quenching constants for two Trps as a result of different accessibility of the quencher to the Trps. A linear plot will result from either heterogeneous or homogeneous emission. A linear plot results from heterogeneous emission if the extent of difference in the quenching of two Trps by external quencher is small, as compared to the previous case. A homogeneous emission will result in a linear plot if the external quencher has equal accessibility to both the Trps and only collisional quenching takes place with no contribution from static quenching. An upward curvature in the Stern-Volmer plot results from homogeneous emission in which at higher quencher concentrations both collisional and static quenching take place. This type of plot is usually observed when both Trps are more or less completely exposed to solvent and hence are equally accessible to the external quencher. Hence, changes in the shape of the Stern-Volmer plots with changes in solution conditions provide important information about subtle perturbations in the protein tertiary structure.

Circular Dichroism Studies

CD measurements were carried out using a Jasco-710 Spectropolarimeter. The far-UV CD studies were carried out in a 0.05-cm path-length cell using a protein concentration of 0.25 mg/ml and a scan speed of 20 nm/min from 185 to 260 nm. The near-UV CD studies were carried out in a 1.0-cm path-length cell using a protein concentration of 0.5 mg/ml and a scan speed of 50 nm/min from 240 to 310 nm. Each scan was a result of five accumulations, and all scans were normalized for concentration and number of amino acid residues to convert the obtained ellipticities into mean residue ellipticities.

Differential Scanning Calorimetry Studies

DSC studies were carried out at a scan rate of 1°C/min on a differential scanning microcalorimeter (MicroCal Inc., Northampton, MA) using 0.5 ml of solution and an IFNα2a concentration of 1.0 mg/ml. All DSC scans were normalized for concentration and baseline subtracted for comparisons. The scans obtained were not acceptable for thermodynamic analysis because the protein aggregated in all cases during unfolding. The scans were analyzed mainly for the onset of unfolding and for the overall shape of the thermogram.

RESULTS AND DISCUSSION

Steady-State Trp Fluorescence Spectroscopy

Figure 1A shows the Trp fluorescence emission scans of IFNα2a at pH 5.0, before and after completion of the heating/

cooling cycle. At pH 5.0, the λ_{max} of Trp emission was observed at 336 nm, indicating that the two Trps were partially buried in the hydrophobic core of the protein (the λ_{max} of Trp emission can range from 302 nm to 350 nm depending on whether the Trp is buried entirely in hydrophobic environment or whether it is completely exposed to water) (23,27). On completion of the heating/cooling cycle, the λ_{max} was red-shifted to 340 nm, indicating that at least one of the two Trps has now moved to a relatively polar environment attributed to the unfolding of IFNα2a.

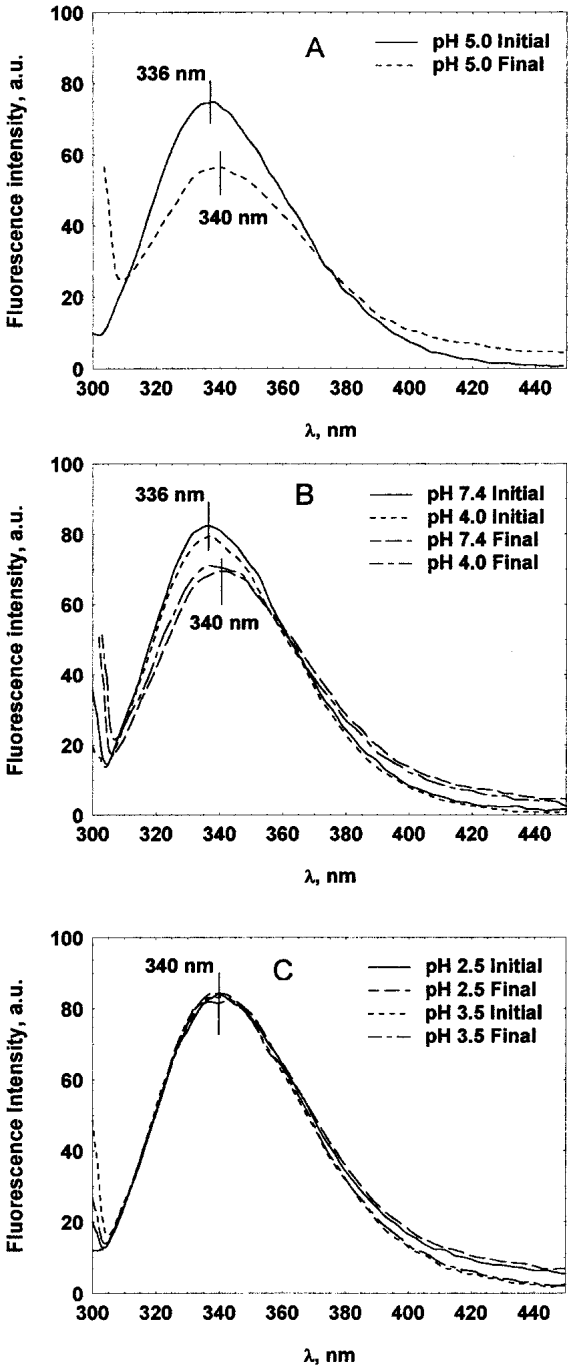

Fig. 1. Trp fluorescence emission spectra of IFNα2a at 20°C before and after completion of the thermal heating/cooling cycle. (A) pH 5.0, (B) pH 7.4 and 4.0, and (C) pH 2.5 and 3.5.

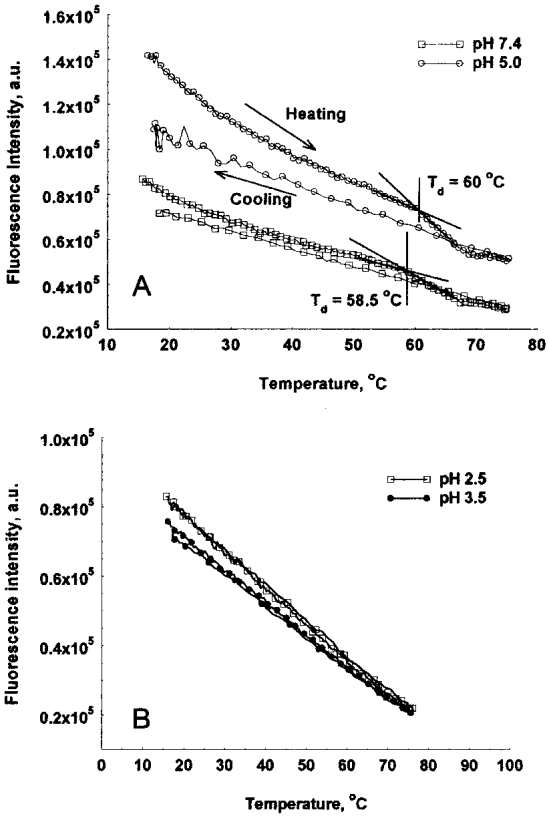

Fig. 2. Thermal scans of IFNα2a indicating temperature dependence of intrinsic fluorescence intensities of IFNα2a during the heating/cooling cycle at different solution pH values. λ_{ex} = 295 nm, and λ_{em} = 336 nm. (A) pH 5.0 and 7.4 and (B) pH 2.5 and 3.5.

Figure 1B shows the emission scans of Trp fluorescence before and after the heating/cooling cycle at pH 4.0 and 7.4, and Fig. 2C shows similar scans at pH 2.5 and 3.5. The results obtained for pH 4.0 and 7.4 were similar to those obtained for pH 5.0 because the λ_{max} was observed to be shifted from 336 nm to 340 nm on completion of heating/cooling cycle. In the cases of pH 2.5 and 3.5, however, the λ_{max} was observed at 340 nm initially, and no shift was observed after the completion of heating/cooling cycle. These results indicated that at 20°C, the two Trps reside in a relatively hydrophobic environment in solutions at pH 4.0 and above, and at the end of the thermal cycle a red shift was observed as a result of irreversible unfolding of the protein. At pH 3.5 and below (Fig. 1C), no change was observed in λ_{max}, which indicated that there was no change in the microenvironment of the Trps at the end of heating/cooling cycle. This may be because the Trps were already exposed to the solvent, and hence, the thermal cycle did not induce any change in the polarity around these Trps. Alternatively, if there were any structural changes that did take place in the protein at this pH, it apparently did not affect the microenvironment around the Trps.

Thermal denaturation studies of IFNα2a at different pHs were carried out by following changes in the Trp fluorescence intensities during the heating/cooling cycle. Figure 2A shows the dependence of fluorescence intensity of IFNα2a on temperature for pH 5.0 and 7.4. The initial decrease in the fluorescence intensity up to about 60°C was attributed to the temperature dependence of the solvent collisional quenching

of Trp fluorescence. Beyond a certain temperature, the fluorescence intensity further dropped, which resulted in a discontinuity in the curve and was attributed to the onset of the unfolding of IFNα2a. This temperature, denoted as T_d, was observed at 60°C at pH 5.0 and decreased to 58.5°C at pH 7.4. The discontinuity in the curve that is related to onset of unfolding at T_d occurred mainly as a result of the additional solvent quenching effect beyond the temperature dependence of solvent collisional quenching because unfolding of the protein exposes the Trps more to the solvent as compared to the Trps in the native state. The discontinuity can also result from a shift in the λ_{max} of Trp emission to 340 nm because the intensity measurements were still carried out at 336 nm. On cooling it was observed that the intensity–temperature curve did not superimpose the heating curve, indicating irreversible unfolding of IFNα2a. Hysteresis was observed in the heating/cooling scans and was higher for pH 5.0 than for pH 7.4, indicating a varying extent of conformational changes at these pHs.

Interestingly, at pH 2.5 and 3.5, no point of discontinuity was observed during the thermal cycle, as shown in Fig. 2B. The overall linear decrease in the fluorescence intensity with an increase in temperature was attributed to the temperature dependence of the solvent collisional quenching. On cooling, no hysteresis was observed in the intensity–temperature curves at these pHs, which was simply a result of the reversal of temperature dependence of collisional quenching. A lack of discontinuity in the thermal denaturation profiles of $IFNa_{2a}$ at pH 3.5 and below can indicate any of the following: (a) there is no unfolding of the protein with temperature; (b) the protein could already be in the unfolded state, which does not undergo any further change on increase in temperature; and (c) the unfolding may take place in such a way that it does not affect the microenvironment of the Trps. The latter two reasons seem to be more appropriate to the reality because the emission scans indicate that at pH 3.5 and below, the λ_{max} is already shifted to 340 nm as compared to 336 nm at pH 4.0 and above. Ionic strength up to 0.3 M did not affect the thermal denaturation behavior of IFNα2a at any pH (data not shown).

Several questions came up from the fluorescence studies on IFNα2a. First, the initial decrease in the fluorescence intensities during thermal studies at pH 4.0 and above showed a curvature unlike linear plots obtained at pH 3.5 and below. This curvature was not characteristic of the temperature dependence of collisional quenching of Trp fluorescence by solvent. There could be a possibility of subtle conformational changes taking place at temperatures below the observed T_d of the protein. Also, the slope of the fluorescence intensity–temperature curves below the observed T_d was different for pH 5.0 as compared to pH 7.4 (Fig. 2A) indicating that the extent of conformational changes below the observed T_d of the protein was different for different pHs. Second, even though thermal denaturation studies indicated no change in the microenvironment of Trps at lower pH, it still remained to be ascertained if there were changes taking place in other parts of the protein as well with increase in temperature. Fluorescence quenching and CD studies were conducted in an attempt to answer these questions.

Fluorescence Quenching Studies

It was speculated that the initial curvature observed during thermal fluorescence studies below T_d at pH 4.0 and above could have resulted from subtle changes in the overall conformation of IFNα2a induced by increase in temperature. These changes could have affected the relative exposure of the two Trps to the solvent molecules. As a result, the solvent quenching effect should vary differently toward each of the Trps with increase in temperature. If this were the case, one would see a similar effect with use of an external quencher whose accessibility to the two Trps in IFNα2a will change following changes in the protein conformation.

Fluorescence quenching studies were used to investigate the subtle changes in the conformation of IFNα2a that may take place below its observed T_d at different pHs. Acrylamide was used as a neutral quencher, and iodide was used as an anionic quencher. The quenching data were analyzed using Stern-Volmer equations as described earlier.

Figure 3A shows the Stern-Volmer plots for quenching of IFNα2a by acrylamide at 15°C at pH 4.0, 5.0, and 7.4, and Fig. 3B shows similar plots for pH 2.5, 3.0, and 3.5. The points represent the experimental data, and the lines represent the fitting of the Stern-Volmer equation to the data points. The fits were obtained by using Eq. (1) for all pHs because all the plots were either downward curving (pH 3.0–7.4) or linear (pH 2.5). The values of various parameters, i.e., f_1, f_2 $(1 - f_1)$, K_1, and K_2, are listed in Table I for all pHs. Although the absolute value of each parameter could not be compared at this point for different pHs because the lifetimes of the two Trps were not available for all pH values, some general conclusions could be drawn. The values of f_1 and f_2 indicated that the emission from two Trps was extremely heterogeneous in IFNα2a. One of the Trps contributed in the range of 4–17% to the total fluorescence, whereas the other Trp contributed in the range of 83–96% to the total fluorescence intensity depending on the pH of the solution. It was also clear from the K_1 and K_2 values that the less fluorescing Trp is quenched to a much higher extent than the one that contributes significantly toward the overall fluorescence.

A significant amount of information can be obtained by observing the change in the curvature of the Stern-Volmer plots and the ordinate value of F_0/F for different pH values studied. The downward curvature in the Stern-Volmer plots in Fig. 3A at higher pH indicated that the two Trps were present in different microenvironments. As the pH was decreased to 2.5 (Fig. 3B), the downward-curving Stern-Volmer plots changed to linear plots, and this change was accompanied by an increase in the overall extent of quenching as seen by an increase in the ordinate value of F_0/F. The changes in the shape of the Stern-Volmer plot as well as the increase in the ordinate value indicated changes in the conformation of protein on lowering of pH as a result of unfolding of the protein.

Figure 3C shows iodide quenching of Trp fluorescence in IFNα2a at 15°C as a function of pH. Iodide did not quench in the range of pH 4.0–7.4, which was because of either the presence of a negative microenvironment around the Trps or inaccessibility of large hydrated iodide ion to buried Trps. A slight upward curvature was observed in the Stern-Volmer plot at pH 3.5, which changed to a downward curvature at pH 3.0 and 2.5. This can be explained on the basis of partial unfolding of IFNα2a at these pHs. At pH 3.5, it is possible that only one Trp gets exposed to iodide ion and undergoes both collisional and static quenching at higher iodide concentrations, resulting in an upward-curving Stern-Volmer plot. At pH 3.0 and 2.5, further unfolding of protein led to expo-

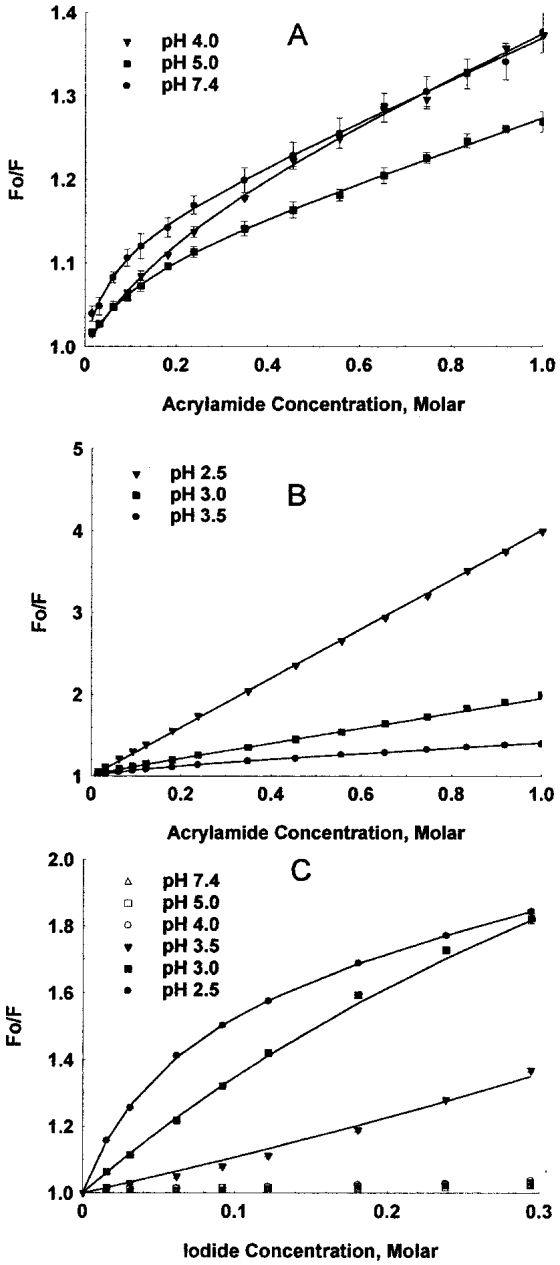

Fig. 3. Effect of pH on the quenching of intrinsic Trp fluorescence by external quenchers, acrylamide and iodide, in IFNα2a at 15°C. The markers represent the experimental data, and the lines are the fits obtained for the Stern-Volmer equation [see text, Eq. (1)]. (A) Acrylamide quenching at pH 4.0, 5.0, and 7.4; (B) acrylamide quenching at pH 2.5, 3.0, and 3.5; and (C) iodide quenching at pH 2.5, 3.0, 3.5, 4.0, 5.0, and 7.4.

sure of both Trps to iodide; however, different accessibility of iodide to the two Trps led to downward-curving Stern-Volmer plots. The fact that a higher ordinate value of F_0/F was obtained indicated that the extent of unfolding of IFNα2a was higher at pH 3.0 and 2.5 than at pH 3.5. The downward curvature that was observed at pH 3.0 and 2.5 again indicated that even at pH 2.5, IFNα2a remained only partially unfolded with limited access of the iodide ion toward the two Trps.

Figure 4 shows the quenching of Trp fluorescence by acrylamide at different temperatures (15–50°C) with pH ranging from 3.0 to 5.0. Interestingly, in all cases the shape of the Stern-Volmer plots changed either from downward-curving to linear (pH 4.0 and 5.0) or from downward-curving to linear to upward-curving (pH 3.0 and 3.5). The downward-curving and linear plots were fitted using Eq. (1), and the upward-curving plots were fitted using Eq. (2) (pH 3.0 at 30, 40, and 50°C, pH 3.5 and 4.0 at 50°C). In general, the extent of dynamic quenching is higher at elevated temperatures because of increased collisional quenching, whereas the extent of static quenching decreases with an increase in temperature (23). However, in the present studies, the static component appeared on an increase in temperature. This kind of behavior could be explained on the basis of the difference in the relative quenching constants and the change in the conformation of IFNα2a on increase in temperature. At low temperatures, there is a considerable difference in K_1 and K_2 (as shown in Table I). This difference relates to the appearance of downward curvature at low temperatures. At low quencher concentrations, the higher exposed Trp is quenched, and at higher quencher concentrations, the other Trp is quenched, although to a much smaller extent because it is not exposed to that degree. Hence, the static quenching component is almost absent. However, on increase in temperature, as the protein partially unfolds, the relative exposure of both Trps to the solvent and hence to the quencher increases, resulting in the possibility of static quenching as well. Therefore, the static component is seen only at higher temperatures.

Table I. Parameters for Acrylamide Quenching of IFNα2a for Different Solution pHs at 15°C Obtained by Fitting Equation 1 to the Experimental Data

Solution pH	f_1	K_1, mol^{-1}	f_2 ($1-f_1$)	K_2, mol^{-1}
7.4	0.12	19.95	0.88	0.215
5.0	0.096	9.942	0.904	0.165
4.0	0.166	4.323	0.834	0.197
3.5	0.102	8.538	0.898	0.286
3.0	0.041	35.8	0.959	0.882
2.5	0.063	36.1	0.937	2.68

Overall, these plots showed that at all pH values studied, the conformation of IFNα2a was affected to different extents because of partial unfolding of IFNα2a with the increase in temperature up to 50°C. From the ordinate values it was also obvious that the extent of unfolding increased as the pH was lowered at any given temperature. It was quite clear at this point that the initial curvature in the thermal denaturation studies at pH 4.0 and above resulted from the subtle changes in the protein conformation leading to partial unfolding of IFNα2a. Because of the partial opening of the structure in the microenvironment of the Trps as a result of decrease in solution pH, no further change occurred in the Trp intrinsic fluorescence intensity on increase in temperature at lower pH, and this resulted in a linear decrease in the fluorescence intensity. However, changes in protein conformation were evident at lower pH with increase in temperature, as shown by the change in the shape of the Stern-Volmer plots indicating the presence of some sort of initial structure.

Circular Dichroism Studies

CD studies were conducted to follow changes in the secondary structure and overall tertiary structure of IFNα2a with

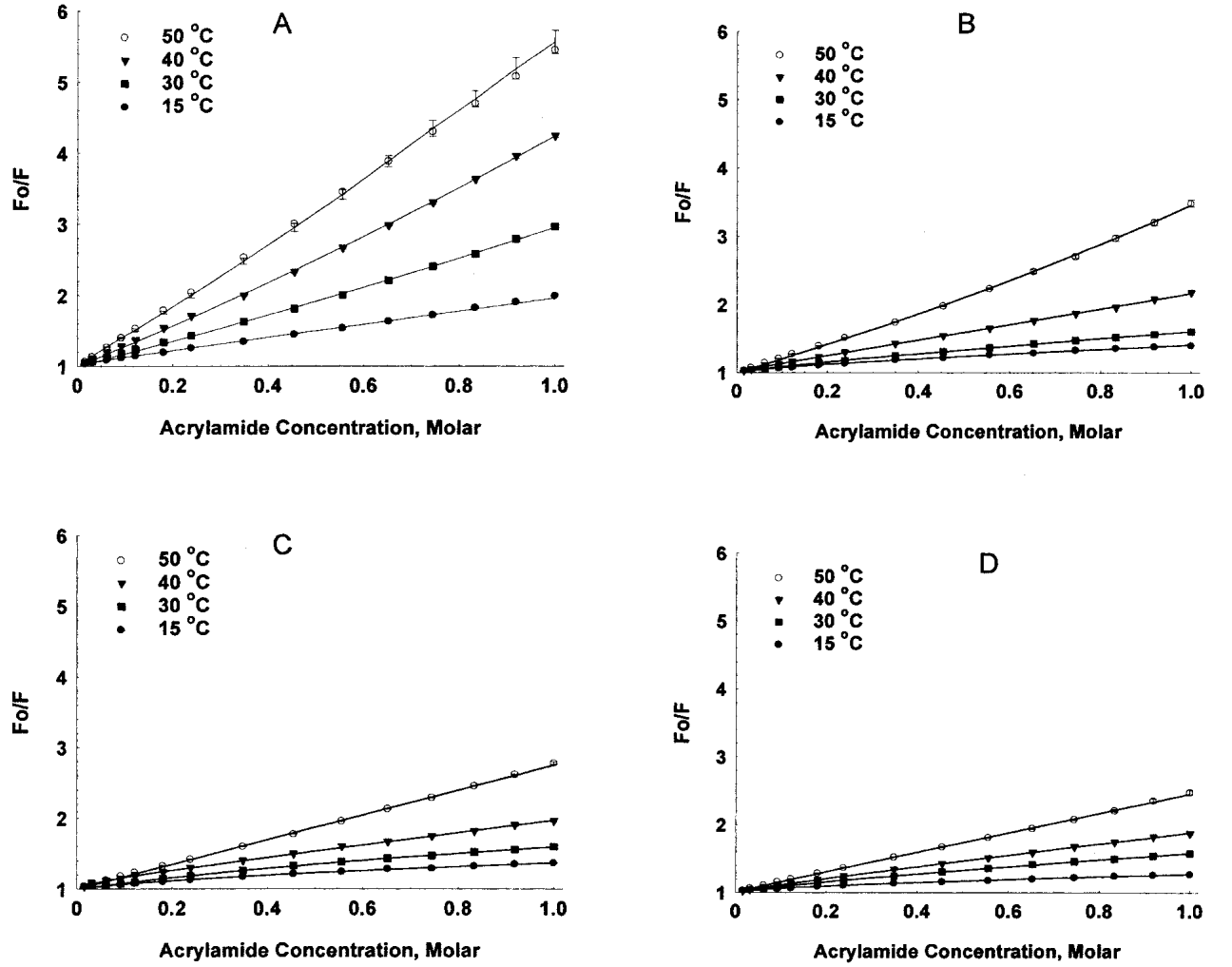

Fig. 4. Effect of temperature on the quenching of intrinsic Trp fluorescence by acrylamide in IFNα2a at different solution pHs. The markers represent the experimental data, and the lines are fits obtained for the Stern-Volmer equation [see text, Eqs. (1) and (2)]. The downward-curving and linear plots were fitted using Eq. (1), and the upward-curving plots were fitted using Eq. (2) (see text for details). (A) pH 3.0, (B) pH 3.5, (C) pH 4.0, and (D) pH 5.0.

changes in temperature and pH. CD spectroscopy is especially useful to characterize intermediates, such as the molten-globule state, because the molten-globule state exhibits a far-UV CD spectrum (representative of secondary structure elements) similar to that of the native protein and a near-UV CD spectrum (representative of the tertiary structure) similar to that of the fully unfolded state (4,5).

Figure 5A shows the far-UV and near-UV CD spectra of IFNα2a at pH 5.0, 15°C. The far-UV CD spectrum was typical of an α-helical protein with observed minima at 222 nm and 208 nm. This was consistent with the 3-D NMR solution structure of IFNα2a and corresponded to about 65% α-helical content as analyzed by SELCON (self-consistent method for the analysis of secondary structure of proteins using circular dichroism data) (28,29). The near-UV CD spectrum of IFNα2a showed two dominant negative bands at 293 nm and 287 nm. These bands were assigned to the two Trps present in IFNα2a. The two bands also confirmed that these two Trps were present in microenvironments of different asymmetries. The other minima in the spectra appeared to result from Phe, Tyr, and disulfide bonds (30). Figure 5B shows the changes in the far- and near-UV CD spectra of IFNα2a as the pH is lowered from 7.4 to 2.0. These results clearly showed that IFNα2a underwent a complete loss in tertiary structure on lowering of pH, with only about 20% loss in the secondary structure, which indicated the presence of partially unfolded states of this protein at lower solution pH. At pH 2.0, for example, no near-UV CD spectrum was observed, whereas the far-UV CD spectrum clearly indicated significant α-helical content in the protein. The changes that were observed in the tertiary and secondary conformations of IFNα2a on lowering of pH were found to be completely reversible, as shown in Fig. 5C. Both the near-UV and the far-UV CD spectra were completely superimposable at pH 7.4 at the beginning and at the end of the pH change cycle.

Figure 6 shows the changes in the near-UV and far-UV CD spectra of IFNα2a at pH 3.5, 4.0, and 5.0 with increase in temperature. At all pH values, loss in both secondary and tertiary structure was observed with an increase in temperature. The changes in the near- and far-UV CD spectra were found to be gradual with increasing temperature, and even at lower temperatures (less than 50°C) some loss in secondary and tertiary structure was evident. The overall extent of unfolding represented by a loss in tertiary and secondary struc-

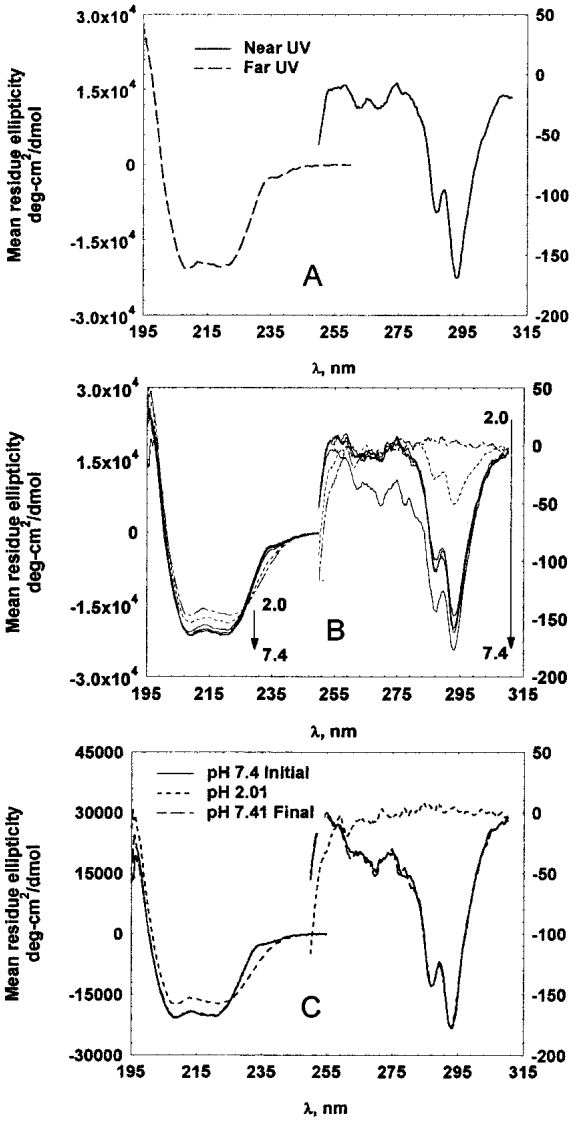

Fig. 5. (A) Far-UV and Near-UV CD spectra of IFNα2a at 15°C at pH 5.0. (B) pH dependence of far-UV and near-UV CD spectra of IFNα2a at 15°C. The pH was varied from 7.4 to 2.0, and the spectra were recorded at pH 7.4, 5.0, 4.0, 3.5, 3.0, and 2.0. (C) Reversibility of unfolding of IFNα2a with respect to change in pH from 7.4 to 2.0 and then back to 7.4 as shown in the far-UV and near-UV CD spectra. The initial and final spectra completely overlap each other. The left y-axis corresponds to the far-UV CD spectrum, and the right y-axis corresponds to the near-UV CD spectrum.

ture varied with pH. A decrease in pH caused further loss in the overall conformation of IFNα2a and, hence, indicated a higher degree of unfolding. Consistent with the quenching results, changes in the IFNα2a conformation were evident in the 5–50°C temperature range at all pHs studied. These changes corresponded to partial unfolding of protein because there was only a partial loss in secondary and tertiary structures. It was also clear from these spectra that in this temperature range, the loss in tertiary structure was observed to a much higher extent than loss in secondary structure. It can be concluded from the CD studies that the IFNα2a 3-D solution conformation strongly depended on solution conditions such as pH and temperature. In fact, at each solution pH and temperature defined, IFNα2a had the ability to attain a different conformation.

It may be argued that the partially unfolded states of IFNα2a could represent the kinetic intermediates. However, it is important to note that these studies were not stopped-flow studies, and the time difference between the preparation of the samples and the spectroscopic measurements was in the range of 15–30 min. The rate of change in conformation during unfolding of the smaller monomeric proteins is usually observed in the time scale of milliseconds to seconds and at

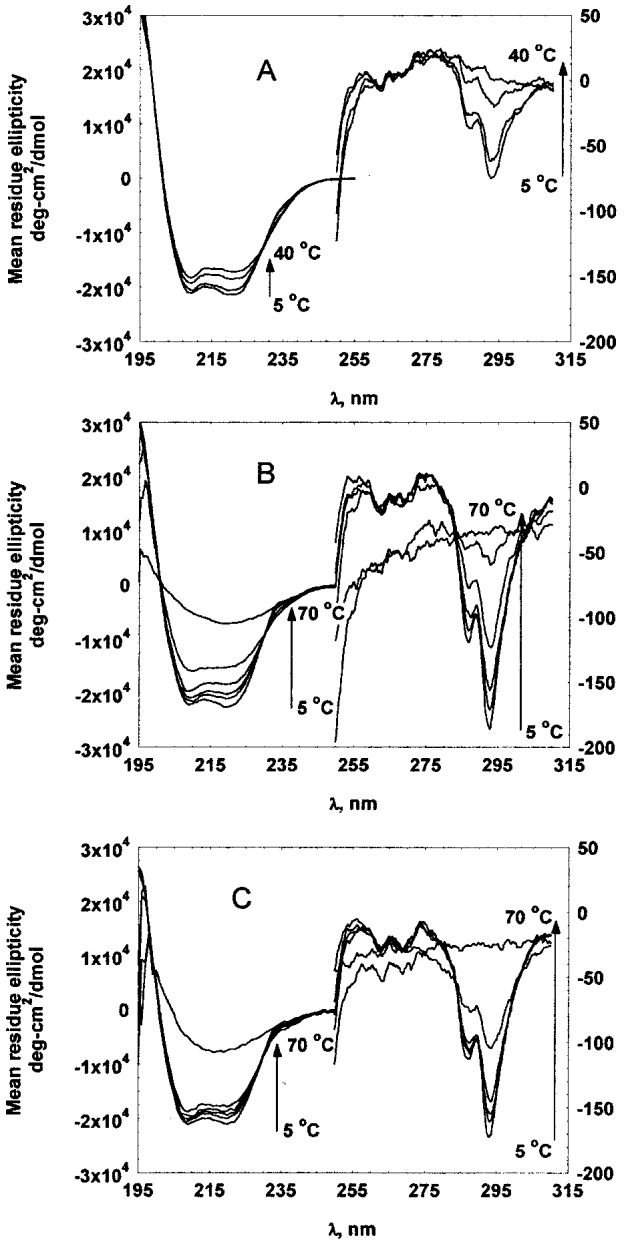

Fig. 6. Temperature dependence of far-UV and near-UV CD spectra of IFNα2a at various solution pH values. The temperature was increased from 5°C (bottommost spectrum) to 70°C (uppermost spectrum), and the spectra were recorded at 5°C, 30°C, 40°C, 50°C, 60°C, and 70°C for pH 4.0 and 5.0, and at 5°C, 15°C, 30°C and 40°C for pH 3.5. The left y-axis corresponds to the far-UV CD spectra, and the right y-axis corresponds to the near-UV CD spectra. (A) pH 3.5, (B) pH 4.0, and (C) pH 5.0.

the most up to several minutes only (31). For example, for another type of interferon, interferon-γ, the half-life of unfolding was observed to be about 34 min at 45°C (32). In the present studies, no change in the fluorescence spectra, intensity, or CD spectra were observed up to several hours of keeping the solution, especially under the solution conditions where partially unfolded states of IFNα2a were observed. This indicated that the partially unfolded states that were observed in the present studies were stable intermediates and not kinetic intermediates.

Differential Scanning Calorimetry

DSC is a commonly used technique to probe conformational changes in proteins with temperature and to obtain information about the thermodynamic stability of proteins. DSC studies were conducted on IFNα2a, and the results were compared to those obtained from fluorescence and CD studies. Figure 7 shows the DSC scans for IFNα2a at pH 3.0, 4.0, 5.0, and 7.4 at a fixed solution ionic strength of 140 mM. At pH 7.4 and 5.0, the onset of unfolding was observed around 55°C and 60°C, respectively. This was similar to the T_d observed in thermal fluorescence studies (Fig. 2A). However, no change in the baseline was observed before the onset of unfolding in DSC thermograms that could correspond to the initial curvature observed in the fluorescence studies at these pHs. This indicated that DSC was not sensitive to the subtle changes in the structure that occurred at moderate temperatures and were observed by fluorescence and CD studies. A clear Tm (midpoint of the unfolding transition) could not be observed because of aggregation of the protein before the completion of the unfolding transition. The aggregation of the protein inside the DSC cell resulted in a sharp decrease in the DSC signal in all solutions, and as a result, thermodynamic analysis of these scans could not be carried out.

At pH 4.0, the DSC scan showed an initial upward slope starting around 30°C, which continued throughout the temperature range until the signal dropped because of aggregation. The initial slope was related to the changes in IFNα2a conformation that occurred at temperatures much lower than the temperatures at which changes were observed for pH 5.0 and 7.4. Similar behavior was also observed in CD studies (Fig. 6B). At pH 3.0, the DSC scan showed changes throughout the temperature range with aggregation finally occurring around 55°C. This was also consistent with the CD results because near-UV CD studies indicated that IFNα2a has significant loss in tertiary structure at pH 3.5 and below, and a slight change in temperature induced significant changes with a complete loss of the tertiary structure around 40°C.

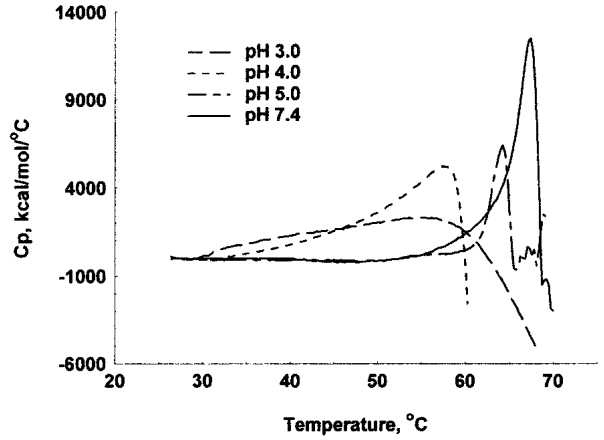

Fig. 7. DSC scans of IFNα2a in solutions of different pHs at a fixed ionic strength of 140 mM.

Overall, DSC studies indicated that the subtle changes that could take place under moderate conditions in the conformation of a protein, such as in the case of IFNα2a, might not induce changes in the heat capacity sensitive enough to be picked up by a microcalorimeter. The spectroscopic techniques such as fluorescence or CD, when used carefully, could help to produce a better understanding of these types of conformational changes.

CONCLUSIONS

From the present studies it can be concluded that IFNα2a has a tendency to attain multiple partially unfolded states whose conformation depends on the solution pH and temperature. Partial unfolding of the protein was observed even at moderate temperatures at all solution pH values studied, and the extent of unfolding was greater at higher temperatures and at lower pH. The partially unfolded states of IFNα2a were easily identified and characterized by use of fluorescence spectroscopy, especially fluorescence quenching studies and CD studies. At lower pH (pH 3.0 and 4.0), the changes in the tertiary and secondary structure of IFNα2a at moderate temperatures, as seen by spectroscopic studies, were indicated by an increase in heat capacity over a large temperature range in the DSC studies. However, DSC was insensitive to the subtle changes that were observed in the tertiary structure of IFNα2a at pH 5.0 and 7.4 in the lower temperature range (15–50°C).

Practical Implications

One of the major problems during formulation of protein therapeutics in solution form is protein aggregation. Prediction of the long-term aggregation of proteins presents a big challenge to the protein formulation scientist. Attempts have been made to correlate protein aggregation with the Tm of proteins obtained through DSC studies (33,34). These studies, however, do not necessarily extrapolate to aggregation below the temperature of onset of thermal unfolding of the protein, denoted as T_d in this paper. This could be mainly because below T_d, long-term changes in protein structure that lead to aggregation may not relate to the structural changes that take place during the unfolding transition in a DSC scan.

Aggregation that takes place above T_d mainly results from the interaction of protein molecules in the unfolded state with another unfolded protein and/or with protein molecules in the native state. Because the population of protein molecules in the native and unfolded states is related to Tm (50% each in a two-state folding transition), a change in Tm can be correlated with the change in aggregation behavior. However, this may not be true for aggregation observed below T_d, as changes in the protein conformation below T_d do not get reflected in Tm. This becomes more important when subtle changes in structural conformation of protein take place in changing solution conditions as observed in the case of IFNα2a in the present study. During our literature search, the authors observed that IFNα2a was previously formulated

with human serum albumin (HSA) in order to increase its long-term stability (35). However, aggregation was observed in these formulations on storage, which possibly resulted in immunogenicity in human subjects. The formulation was later modified by including Tween 80 instead of HSA (36). It is possible that IFNα2a formed partially unfolded states during storage that further formed aggregates with HSA and/or with itself. Tween 80 could have stabilized the partially unfolded states by screening the hydrophobic sites on partially unfolded protein. This mechanism of Tween 80 in preventing protein aggregation has been speculated in case of certain other proteins as well.

The presence of several partially unfolded states as observed for IFNα2a may not be a phenomenon unique to this protein and could be exhibited by other globular proteins as well. Hence, it is the responsibility of a protein formulation scientist to carefully investigate subtle structural changes in the protein conformation with changes in solution conditions so that better strategies can be designed for long-term stability of protein formulations in aqueous solutions.

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