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# Thermodynamic parameters associated with guanidine HCl- and temperature-induced unfolding of bFGF

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**Abstract**

*Purpose*: The thermodynamics and kinetics associated with either thermal- or chaotrope-induced unfolding of recombinant, human basic fibroblast growth factor (bFGF) was characterized using several analytical techniques. *Method*: Changes in the three-dimensional conformation of bFGF were detected using fluorescence spectroscopy and heparin affinity chromatography, and the observed data analyzed using a simple two-state [N↔D] model to estimate the value of several thermodynamic parameters. *Results*: Treatment of thermal denaturation data yielded mean values for the $T_m$ (temperature when $\Delta G = 0$) of $322 \pm 21$ K ($49.3 \pm 3.2$°C), $\Delta H_m$ (change in enthalpy when $\Delta G = 0$) of $214 \pm 23.8$ kJ/mol, and $\Delta S$ (change in entropy) of $664 \pm 40.2$ J $mol^{-1}$ $K^{-1}$. Treatment of the GnHCl-induced denaturation data yielded mean values of the $\Delta G_{(H_2O)}$ (change in free energy in the absence of the denaturant) of $13.0 \pm 0.67$ kJ/mol and the $GnHCl_{1/2}$ (concentration of GnHCl when $\Delta G = 0$) of $1.3 \pm 0.09$ M. Sucrose (0.26 M) was found to be an effective stabilizer against thermal-induced denaturation of bFGF. Renaturation of bFGF which had previously been denatured with 3 M GnHCl was demonstrated following dialysis. However, renaturation of bFGF following heat-induced denaturation was significantly inhibited. *Conclusions*: Using fluorescence spectroscopy, we have estimated the value of several thermodynamic parameters associated with bFGF unfolding induced by either chemical or thermal means. 

*Keywords:* Protein; Denaturation; Basic fibroblast growth factor; Fluorescence spectroscopy; Chaotrope; Heparin affinity chromatography; Renaturation; Sugars

## 1. Introduction

Preservation of a protein's chemical and structural stability is important during their production and storage. The response of a protein's

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conformation to structural perturbants such as denaturants and heat is a means to probe conformational stability, and is particularly useful when generating mutants with enhanced conformational stability. The thermodynamic parameters associated with such perturbations relate to the forces which hold together the native secondary structure of proteins as well as the packing of the hydrophobic core. These forces include electrostatic interactions such as ion pairs, hydrogen bonds, weak polar interactions and Van der Waals forces (Privalov and Kechinashvii, 1974). The small marginal difference between attractive and repulsive forces yield very small free energy changes associated with conformation ($\Delta g_{\text{conformation}}$) in the range of 8–60 kJ $mol^{-1}$.

Analytical techniques such as calorimetry and spectroscopy (Privalov and Kechinashvii, 1974) have been used to evaluate the conformational stability of proteins which arise from different physical insults. Among spectroscopic techniques, fluorimetry and circular dichroism have been used most frequently (Kokate et al., 1991; Sluzky et al., 1994). Our objective in this study was to evaluate the physical stability of bFGF, a mitogenic growth factor that is prone to precipitation (Eberlein et al., 1994; Wang et al., 1996). Since denaturation often occurs as a prelude to precipitation (Manning et al., 1989), we evaluated the thermodynamics of bFGF unfolding in response to either a chaotrope (guanidine HCl = GnHCl) or heat using fluorescence spectroscopy and heparin affinity chromatography. Data so obtained was then used to estimate various thermodynamic parameters associated with bFGF unfolding. The three assumptions made in the present study were that (1) the denaturation reaction could be regarded as a two-state process so that $\Delta G$, $\Delta S$ and $\Delta H$ could be calculated, (2) proteins are stable over a defined temperature range so that the calculated value of $\Delta G$ would be a positive number, and (3) the change in heat capacity ($\Delta C_p$) was constant, but had a positive value (Gekko and Timasheff, 1981a). The most important thermodynamic parameter characteristic of a two-state order–disorder transition in protein denaturation is a large positive change in $C_p$ which has invariably been observed to accompany a two-state reaction (Privalov and Kechinashvii, 1974). This has been suggested to result from the exposure of hydrophobic residues in the process of unfolding due to contact with water (Pace et al., 1990). Privalov and Kechinashvii (1974) established that $\Delta C_p$ of protein unfolding is typically quite large and has a positive value, and that within experimental error, it may be assumed to be a constant for a given protein ($\sim 5$ kJ $mol^{-1}$ $deg^{-1}$).

A protein's secondary structure is generally more resistant to environmental insults compared to the associated tertiary structure. Perturbations in the secondary structure of a protein may be studied with the use of circular dichroism (CD). One additional objective in the present study was to evaluate the changes, if any, in the CD spectra of bFGF as a function of time when a bFGF solution was subjected to thermal stress. Using fluorescence spectroscopy, the effect of sucrose on the kinetics of thermal-induced denaturation of bFGF was also assessed since sucrose has been documented to be a stabilizer for proteins (Gekko and Timasheff, 1981a,b). Lastly, the potential for refolding of bFGF following either thermal stress or GnHCl treatment was investigated.

## 2. Materials and methods

### 2.1. Reagents

Recombinant human bFGF was generously supplied by Scios, Inc. (Mountain View, CA) and used as received. GnHCl was obtained from ICN (Aurora, OH). Citric acid anhydrous, sucrose and sodium chloride were obtained from Fisher Biotech (Fairlawn, NJ) and were of enzyme grade. Disodium EDTA was also obtained from Fisher Biotech and was of electrophoresis grade.

### 2.2. Heparin affinity chromatography

To determine the percent of bFGF that remained in the native three-dimensional conformation, heparin affinity chromatography was used (Sluzky et al., 1994). A Toso Haas heparin TSK-gel column (7.5 mm i.d × 7.5 cm length) was purchased from Supelco (Bellefonte, PA). Experi-

ments were conducted using a Beckman Gold™ high-pressure liquid chromatographic system (Fullerton, CA) equipped with a model 166 UV detector, a model 128 solvent module, and Gold Nouveau™ software processed by a model 350-P90 IBM personal computer. The injecter used was a model 7725i Rheodyne injecter (Cotati, CA) with a sample loop volume of 20 $\mu$l. The column was equilibrated with 100 mM potassium phosphate buffer containing 1 mM $Na_2EDTA$ and maintained at pH 6.5 (mobile phase A). Mobile phase B contained 3 M NaCl in the same buffer. The flow rate was maintained at 1 ml/min. Mobile phase B was gradually increased from 0 to 80% of the total flow rate from 1 through 14 min. Composition of mobile phase B was decreased from 80 to 0% from 14 through 16 min. The detection wavelength used for all analyses was 215 nm.

### 2.3. Fluorescence spectroscopy

To determine the extent of bFGF unfolding, fluorescence spectroscopy was used (Sluzky et al., 1994). The excitation wavelength was set at 277 nm. Emission spectra from 290 to 360 nm were obtained using a Perkin-Elmer LS 50B fluorimeter (Beaconsfield, Buckinghamshire, UK). The slit width used for excitation and emission of the bFGF solution (3.5 $\mu$g/ml) was 5 nm. The ratio of emission intensities at 350 and 307 ($F_{350/307}$) were determined. Duplicate scans were performed and the results presented as the mean value of $F_{350/307}$ versus time. The conformation associated with bFGF's native state consistently resulted in an $F_{350/307}$ value of $0.25 \pm 0.02$ which reliably increased in value during the unfolding process. The scan speed was 99 nm/min. Background intensity due to buffer components (20 mM citrate, 1 mM EDTA, 9% sucrose at pH 5.0) was subtracted from the intensity values obtained following a scan of the bFGF, bFGF/GnHCl, and bFGF/GnHCl/sucrose solutions. For all experiments that involved heat as the stimulus to denature bFGF, the cell sample holder of the fluorescence spectrophotometer was equilibrated to the desired temperature using a model 1157 Polyscience programmable circulating water bath (Niles, IL).

### 2.4. Thermal- or GnHCl-induced bFGF unfolding studies

Using both heparin affinity chromatography and fluorescence spectroscopy, experiments were conducted to determine the degree of bFGF unfolding both as a function of increasing GnHCl concentration (Fig. 1) and as the temperature of a bFGF solution was increased from 298 to 333 K (Fig. 2). Three milliliters of a bFGF solution (3.5 $\mu$g/ml containing 20 mM citrate, 1 mM EDTA, and 9% sucrose; pH 5.0) was heated in the quartz cuvette contained in the sample compartment of the fluorimeter using the programmable circulating water bath. The buffer composition for bFGF was selected because it had previously been reported (Eberlein et al., 1994; Wang et al., 1996) to be the most stable solution medium for the protein. In the first set of experiments (Fig. 1), the concentration of GnHCl was increased from 0.0 to 3.0 M. A 20-$\mu$l solution sample was removed and immediately injected onto the heparin affinity column. At the same time, the $F_{350/307}$ value was determined as described above using the fluorimeter. The following experiment was performed in triplicate and the results expressed as the mean percent bFGF bound to the column versus the concentration of GnHCl. Similarly, the data acquired using fluorescence spectroscopy was expressed as the mean value of the $F_{350/307}$ value versus the concentration of GnHCl in the bFGF solution. The second set of experiments (Fig. 2) were similar to the experiments conducted of bFGF unfolding as a function of GnHCl concentration in the bFGF solution. However, the second set of experiments employed temperature as the stimulus to induce bFGF unfolding. At 298, 303, 308, 313, 318, 323, 328, and 333 K, a 20-$\mu$l solution sample was removed and immediately injected onto the heparin affinity column. The bFGF solution was maintained at each temperature for 5 min. At the same time, the $F_{350/307}$ value was determined as described above. The following experiment was performed in triplicate and the results expressed as the mean percent bFGF bound to the column versus the temperature of the bFGF solution. Similarly, the data acquired using fluorescence spectroscopy was expressed as

the mean value of the $F_{350/307}$ value versus the temperature of the bFGF solution.

The last set of experiments employed temperature as a stimulus to unfold bFGF in either the absence or presence (0.26 M) of sucrose in the protein solution as a function of time. In these experiments (data not shown; resultant kinetic parameters are listed in Table 2), only the $F_{350/307}$ values were determined every 5 min from 0 to 60 min for bFGF solutions (3.5 $\mu$g/ml) either with (0.26 M) or without sucrose at 298, 310, 315 and 323 K. Data were expressed as the mean $F_{350/307}$ value versus time at each temperature, and treated as described in Section 2.7.

### 2.5. Circular dichroism of bFGF solutions

Experiments which assessed potential changes in the secondary structure of bFGF were conducted with a Jasco J-720 spectropolarimeter (Easton, MD). Aqueous solutions of bFGF (36 $\mu$g/ml) were analyzed at 323 K over a 1-h period. A circulating water bath was used to thermostat the cell compartment during the scans. Scans of ellipticity in mdeg versus time were performed in duplicate over a wavelength range of 190–260 nm using a slit width of 3 nm. The scans obtained from the control solution were subtracted from the scans of the test bFGF solutions. The volume of the circular quartz cell used to analyze the bFGF solutions was 3 ml.

### 2.6. bFGF refolding following dialysis

To assess the potential of bFGF to refold to the native state when diluted into an infinite sink, an experiment was conducted which utilized both fluorescence spectroscopy and heparin affinity chromatography to monitor potential changes in the tertiary structure of bFGF prior to and following dialysis. Basic FGF (72 $\mu$g/ml) was intentionally denatured by incubating the protein in 3 M GnHCl for 50 min. Previous work had demonstrated that a maximum value of $F_{350/307}$ is obtained at this concentration of GnHCl (Johnston et al., 1998). At 0, 30 and 50 min and then at 25 min following dialysis against a pH 7.2 PBS solution using Slide-A-Lyzer® cassettes (Pierce, Rockford, IL), the $F_{350/307}$ ratio and the percent of bFGF bound to the heparin affinity column were determined. The solution volume removed at each time point was 40 $\mu$l; 20 $\mu$l for analysis using fluorimetry and 20 $\mu$l for heparin affinity chromatography. The method to determine the percent of bFGF which remained in the native state using heparin affinity chromatography is briefly described below.

The heparin affinity column allowed only the native conformation of bFGF to bind to the stationary phase. A commercially available concentrating solution for use with the Slide-A-Lyzer® technique was not necessary since the internal volume of the cassette did not increase over the course of the 25-min dialysis period due to the dilute concentration of bFGF used. Similarly, removal of three, 40-$\mu$l samples throughout the experiment represented only 4% of the total bFGF solution volume (amount) and, for the purpose of these experiments, was assumed to be negligible. Peak areas associated with bFGF samples collected at 30 and 50 min during the incubation and 25 min following the dialysis procedure were compared to the mean value of the peak area obtained at time $t = 0$ min and expressed as the percent of bFGF in the native state conformation (Fig. 4).

### 2.7. Data analysis

The resulting $F_{350/307}$ versus time profiles for bFGF control and test solutions which contained sucrose were compared using the trapezoidally integrated area-under-the-curve (AUC) values from time $t = 0$ to 60 min. The mean values of the AUCs (indicative of the amount of bFGF denatured) with sucrose present were then compared for statistical significance relative to corresponding mean AUC values when sucrose was absent using the Student's $t$-test (Ary and Jacobs, 1976). For each $F_{350/307}$ versus time profile (data not shown), a first-order rate constant associated with denaturation ($k_D$) and its corresponding half-life ($0.693/k_D$) were calculated. The values for the first-order rate constants were obtained by an exponential fit of the experimental data using RSTRIP (MicroMath, Inc.). The maximum value

of $F_{350/307}$ ($F_{350/307\,max}$) obtained during the experiment was also recorded. As stated above, the value of $F_{350/307\,min}$ was typically observed to be $0.25 \pm 0.02$. For experiments which utilized either GnHCl (Fig. 1) or temperature (Fig. 2) to induce bFGF unfolding, the maximum value of $F_{350/307}$ ($F_{350/307\,max}$) as well as the minimum value ($F_{350/307\,min}$) obtained during the experiment were also recorded.

Various parameters were determined by the method of Pace et al. (1990) assuming a two-state model of protein unfolding where $f_u$ represents the fraction of bFGF molecules in the unfolded conformation and $f_f$ designates the fraction of protein molecules in the folded conformation. The fraction of the bFGF molecules in the folded conformation, $f_f$, was obtained using the data obtained in the temperature-induced bFGF unfolding studies. In brief, for the thermal-induced unfolding data, $f_f$ was calculated using the following equation;

$$f_f = (F_{350/307\,max} - F_{350/307\,Temp.}) / (F_{350/307\,max} - F_{350/307\,min}),$$

where $F_{350/307\,Temp.}$ is the $F_{350/307}$ value at a given temperature. For the temperature-mediated unfolding of bFGF, $F_{350/307\,max}$ was equal to 1.45, whereas the $F_{350/307\,min}$ was equal to 0.25. The value of $f_u$ was then calculated at each temperature as; $f_u = 1 - f_f$. The equilibrium constant, $K$, was subsequently calculated at each temperature using the equation $K = f_u/f_f$. Knowing the value of $K$ at each temperature permitted the calculation of $\Delta G$ at each temperature using the relationship $\Delta G = -RT\ln K$. In order to calculate the value of $T_m$ (temperature when $\Delta G = 0$), $\Delta G$ was plotted as a function of the absolute temperature and the value of $T_m$ determined graphically at $\Delta G = 0$ (Pace et al., 1990). The change in entropy ($\Delta S$) associated with thermal-induced bFGF unfolding is the slope of a plot of $\Delta G$ versus $T$, i.e. $\Delta G = \Delta H$-T$\Delta$S (Pace et al., 1990). $\Delta H_m$ or the change in enthalpy when $\Delta G = 0$ can be calculated using the standard relationship $\Delta G = \Delta H - T\Delta S$ which yields $\Delta H_m = T_m \Delta S$ when $\Delta G = 0$. Thus, an estimate for $\Delta H_m$ is equal to the product of $T_m$ and the slope ($\Delta S$) of the regression line for the $\Delta G$ versus temperature graph (Pace et al., 1990). The change in enthalpy was also calculated by using the slope of two individual points near the point where $\Delta G = 0$ (Pace et al., 1990).

For calculation of $GnHCl_{1/2}$ and $\Delta G_{(H_2O)}$ for GnHCl-induced unfolding of bFGF, the equilibrium constant, $K$, was calculated as described above for the treatment of thermal-mediated bFGF unfolding data. Again, fluorescence data acquired in these experiments were used to calculate $f_f$ as above, with the exception that each mean value of $f_f$ was now calculated at different solution concentrations of GnHCl ranging from 0.0 to 3.0 M rather than as a function of temperature. In the GnHCl-induced bFGF unfolding experiments, the $F_{350/307\,max}$ was equal to 1.68, whereas the $F_{350/307\,min}$ was equal to 0.23. The mean values of $K$ were then used to calculate the mean values of $\Delta G$ as above. However, it should be noted that the temperature, $T$, used to calculate each value of $\Delta G$ through the relationship $\Delta G = -RT\ln K$ was constant and equal to 298 K for the bFGF unfolding studies which employed GnHCl as the denaturant. $GnHCl_{1/2}$ may be determined from the equation resulting from a linear regression of a plot of $\Delta G$ versus GnHCl concentration at $\Delta G = 0$ (Pace et al., 1990).

## 3. Results

### 3.1. Thermal- and GnHCl-mediated bFGF unfolding studies

Figs. 1 and 2 illustrate the relationship between the $F_{350/307}$ values and the percent of bFGF bound to the heparin affinity column at specific GnHCl concentrations (Fig. 1) or temperatures (Fig. 2). The $F_{350/307}$ versus either GnHCl concentration or temperature profiles were sigmoidal in shape with the minimum and maximum $F_{350/307}$ values ranging from 0.23 to 1.68 (GnHCl; Fig. 1) and 0.25 to 1.45 (temperature; Fig. 2). Inverse sigmoidal curves were observed for each type of denaturant when the percent bFGF bound to the heparin affinity column was plotted versus either the GnHCl concentration (Fig. 1) or the temperature (Fig. 2).

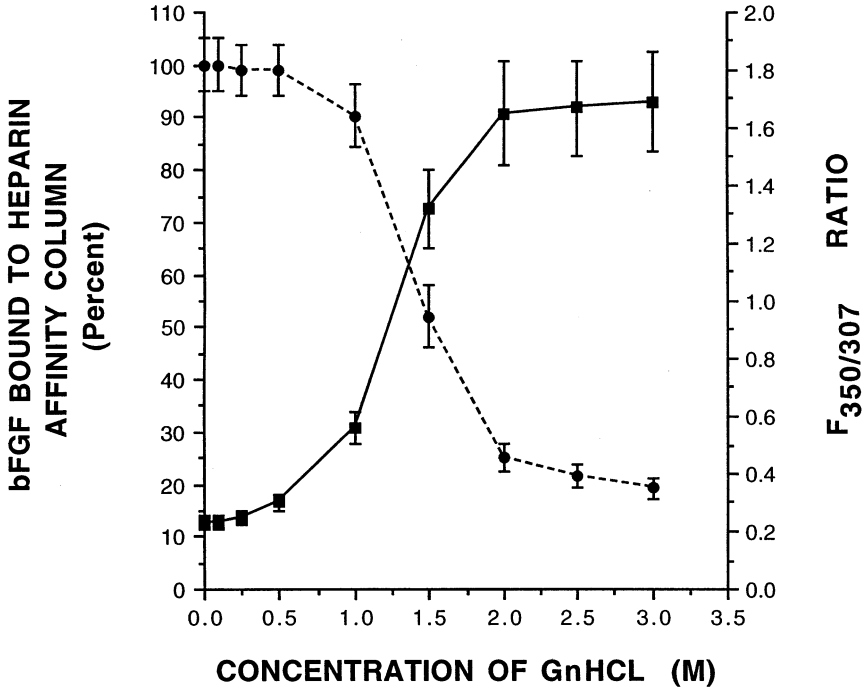


Fig. 1. Relationship of GnHCl-induced bFGF unfolding using fluorescence spectroscopy (■) and heparin affinity chromatography (●). All symbols represent the mean values ± standard deviation ($n = 3$).

### 3.2. Thermodynamic parameters

Parameters associated with GnHCl-induced denaturation, such as $\Delta G$ at a GnHCl concentration of zero [$\Delta G_{(H_2O)}$] obtained from the value for the $y$-intercept of the linear regression equation and $GnHCl_{1/2}$ (when $\Delta G = 0$) were $13.0 \pm 0.67$ kJ/mol and $1.3 \pm 0.09$ M, respectively (Table 1). Parameters associated with thermal-induced denaturation, such as $T_m$ (at $\Delta G = 0$), $\Delta H_m$, and $\Delta S$ were $322 \pm 21$ K ($49.3 \pm 3.2$°C), $214 \pm 23.8$ kJ/mol, and $664 \pm 40.2$ J mol$^{-1}$ K$^{-1}$, respectively. The values of $\Delta H$ and $\Delta S$ were $494 \pm 61.1$ kJ/mol and $770 \pm 121$ J mol$^{-1}$ K$^{-1}$ by calculating the slope between two data points near the point where $\Delta G = 0$ (Pace et al., 1990). As assessed using fluorescence spectroscopy, a modified van't Hoff plot ($\ln K$ versus $1/T$, where $K$ = equilib. constant = $f_u/f_f$) for thermal-induced bFGF unfolding was observed to be nonlinear and is shown in Fig. 3.

### 3.3. Effect of sucrose on thermal-induced denaturation of bFGF

As can be noted in Table 2, thermal-induced unfolding (denaturation) of bFGF in the absence of sucrose increased significantly ($p < 0.05$) with an increase in temperature compared to $F_{350/307\,max}$ values determined at 298 K. The mean value of $F_{350/307\,max}$ increased from $0.30 \pm 0.06$ at 298 K to $1.01 \pm 0.06$ at 323 K (Table 2). The associated mean AUC value increased from $16.7 \pm 1.8$ to $39.9 \pm 0.56$ from 298 to 323 K, respectively (Table 2). The rate of bFGF denaturation or unfolding increased significantly ($p < 0.05$), as reflected by the reduction in the mean values of the half-life associated with the unfolding phase of the $F_{350/307}$ versus time profiles (Table 2). Similar overall trends in the mean values of the parameters listed in Table 2 were observed for bFGF solutions with sucrose, relative to bFGF solutions without sucrose, as the temperature was

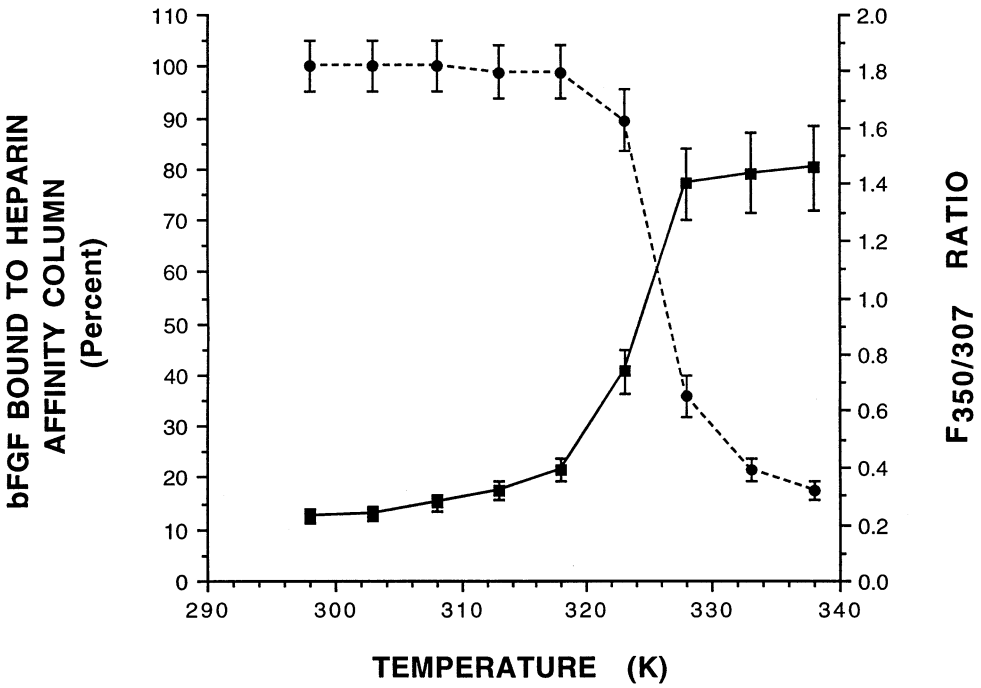


Fig. 2. Relationship of thermal-induced bFGF unfolding using fluorescence spectroscopy (■) and heparin affinity chromatography (●). All symbols represent the mean values ± standard deviation ($n = 3$).

increased from 298 to 323 K. The presence of sucrose (0.26 M) appeared to provide significant advantage in terms of conformational stability for bFGF when subjected to thermal stress (Table 2).

### 3.4. Effect of temperature on the secondary structure of bFGF

Studies using circular dichroism demonstrated a minima at 202 nm (data not shown). Time-dependent, thermal-induced denaturation of bFGF at 323 K resulted in a slight shift of the 202 nm peak to a wavelength of approximately 204–205 nm. In addition, the broad peak maxima at approximately 225 nm observed at time $t = 0$ min appeared to shift to a slightly negative value of ellipticity at 60 min. However, these changes in the CD spectrum when a bFGF solution was subjected to a temperature of 323 K were not statistically different from control.

### 3.5. Refolding of GnHCl-treated bFGF

As shown in Fig. 4, both fluorescence spectroscopy ($F_{350/307}$ ratios) and heparin affinity chromatography were utilized to demonstrate refolding of the bFGF molecule. Approximately $16 \pm 1.6\%$ of the bFGF remained in the native conformation 30 min following exposure of the protein to 3 M GnHCl. The corresponding $F_{350/307}$ ratio was $1.78 \pm 0.15$ at the 30-min sampling time point. At 50 min, analysis of bFGF solution samples demonstrated that the mean values of the $F_{350/307}$ ratio and the percent native bFGF bound were not significantly different than values determined at 30 min. Following dialysis, results using heparin affinity chromatography demonstrated that approximately $74 \pm 6.1\%$ of the bFGF existed in the native state or native three-dimensional conformation, whereas data from fluorescence measurements indicated an $F_{350/307}$ ratio in close agreement to the value normally observed for the native state ($0.25 \pm 0.02$).

### 3.6. Renaturation of bFGF following thermal denaturation

As shown in Fig. 5, the step-wise, incremental

Table 1
Relevant thermodynamic parameters for GnHCl and thermal-induced denaturation of bFGF

| Denaturant | $T_m$ (K) | [a]$\Delta H_m$ (kJ mol$^{-1}$) | $\Delta S$ (J mol$^{-1}$ K$^{-1}$) | $\Delta G_{(H_2O)}$ (kJ mol$^{-1}$) | $GnHCl_{1/2}$ (M) |
|---|---|---|---|---|---|
| GnHCl (298 K) | — | — | — | [b]$13.0 \pm 0.67$ | $1.3 \pm 0.09$ |
| Heat | $322 \pm 21$ | $214 \pm 23.8$ | $664 \pm 40.2$ | — | — |

[a] Subscript m designates the change in enthalpy when $\Delta G = 0$.
[b] Mean value $\pm$ standard deviation.

temperature ramping program which increased the temperature of a bFGF solution over a 100-min test period resulted in a dramatic increase in the fluorescence ratio between 313 and 323 K (Fig. 5). However, subsequent lowering of temperature did not result in a significant reduction in the $F_{350/307}$ ratio when compared to the value of the $F_{350/307\,max}$ (Fig. 5). Thus, the fluorescence ratio never attained the mean value of $F_{350/307}$ normally associated with the native conformation and thus would suggest irreversible unfolding (denaturation) of bFGF when heated to 323 K.

## 4. Discussion

In the bFGF molecule, a single amino acid (trytophan), located near the receptor binding site enables one to examine the state of the protein (native versus unfolded) through fluorescence spectroscopy. The fluorescence excitation spectrum of bFGF shows one asymmetric component that exhibits a peak at 277 nm. Excitation of native bFGF at this wavelength results in a broad emission spectrum at 306–308 nm due to the seven tyrosine residues, while the tryptophan emission arising at 350 nm is quenched. As bFGF unfolds, the emission from the trytophan residue is no longer quenched and emission at 350 nm increases such that the ratio of the emission intensity at 350 nm to that at 307 nm, i.e. $F_{350/307}$, can be used to assess the extent of protein unfolding. Basic FGF in the native state has a mean value of $F_{350/307}$ of approximately $0.25 \pm 0.02$. Tyrosine and trytophan fluorescence characteristics of this protein are highly conformation dependent and suggest their usefulness as sensitive probes for bFGF conformation in solutions. However, it should be noted that fluorescence emission of bFGF may report only a local conformational change particularly since quenching may be due to close proximity with a charged residue.

With GnHCl-induced denaturation of bFGF, it has been suggested that quenching of the emission intensity at 307 nm associated with tyrosine residues occurs simultaneously with an increase in the intensity of emission for tryptophan at 350 nm. The selection of a low concentration of bFGF (3.5 $\mu$g/ml) in the present study was to eliminate the possibility of protein aggregation which has been observed following an increase in temperature (Wang et al., 1996). Inclusion of EDTA in the bFGF stock solution also helped to prevent any aggregation from occurring due to possible formation of disulfide linkages (Wang et al., 1996).

Interaction of bFGF with our heparin affinity column relied on the maintenance of bFGF's tertiary structure. It should be noted, however, that this analytical technique may not be selective for minor conformational changes in the bFGF molecule since the interaction of bFGF with heparin involves the bringing together of proximal basic residues in the amino acid sequence (Wang et al., 1996). The strong binding affinity of FGFs with heparin can be disrupted with high concentrations of NaCl (Sluzky et al., 1994). It should also be noted that at either a high (3.0 M) concentration of GnHCl (Fig. 1) or an elevated temperature (338 K) (Fig. 2), approximately 17–19% of the bFGF remained in the folded state, as determined using heparin affinity chromatography. This may potentially result from some refolding of bFGF during elution of the protein on the heparin affinity column and suggests the impor-

Table 2
The effect of sucrose on thermal-induced denaturation of bFGF

| Parameter | Without sucrose | | | | With sucrose (0.26 M) | | | |
|---|---|---|---|---|---|---|---|---|
| | 298 K | 310 K | 315 K | 323 K | 298 K | 310 K | 315 K | 323 K |
| $k_D$ ($min^{-1}$) | $0.015 \pm 0.0045$[a] | $0.019 \pm 0.0056$ | $0.021 \pm 0.0035$ | $0.067 \pm 0.0028$* | $0.0059 \pm 0.0012$** | $0.0080 \pm 0.0023$** | $0.015 \pm 0.0041$*,** | $0.037 \pm 0.011$*,** |
| $t_{1/2D}$ (min) | $46.2 \pm 12.7$ | $37.1 \pm 10.4$ | $33.5 \pm 5.15$ | $10.3 \pm 0.42$* | $117.0 \pm 20.7$** | $90.8 \pm 27.2$** | $47.1 \pm 13.7$*,** | $19.4 \pm 5.85$*,** |
| $F^{b}_{(350/307)max}$ | $0.296 \pm 0.057$ | $0.667 \pm 0.13$* | $0.765 \pm 0.081$* | $1.01 \pm 0.062$* | $0.267 \pm 0.045$ | $0.643 \pm 0.026$* | $0.808 \pm 0.021$* | $0.90 \pm 0.17$* |
| $AUC^{c}_{0-60}$ | $16.7 \pm 1.8$ | $25.8 \pm 0.44$* | $31.2 \pm 2.7$* | $39.9 \pm 1.9$* | $15.8 \pm 2.8$ | $21.3 \pm 0.95$*,** | $24.6 \pm 1.4$*,** | $29.3 \pm 5.3$*,** |

[a] Mean value $\pm$ standard deviation.
[b] Maximum $F_{350/307}$ value obtained with the first-order, exponential fit of the data (data not shown in text, as described in Section 2).
[c] Arbitrary parameter selected to reflect the extent of bFGF unfolding over 1 h.*Indicates a significant ($p < 0.05$) difference compared to the mean value at 25°C within each group. **Indicates a significant ($p < 0.05$) difference when compared to the mean value at the same temperature for samples without sucrose.

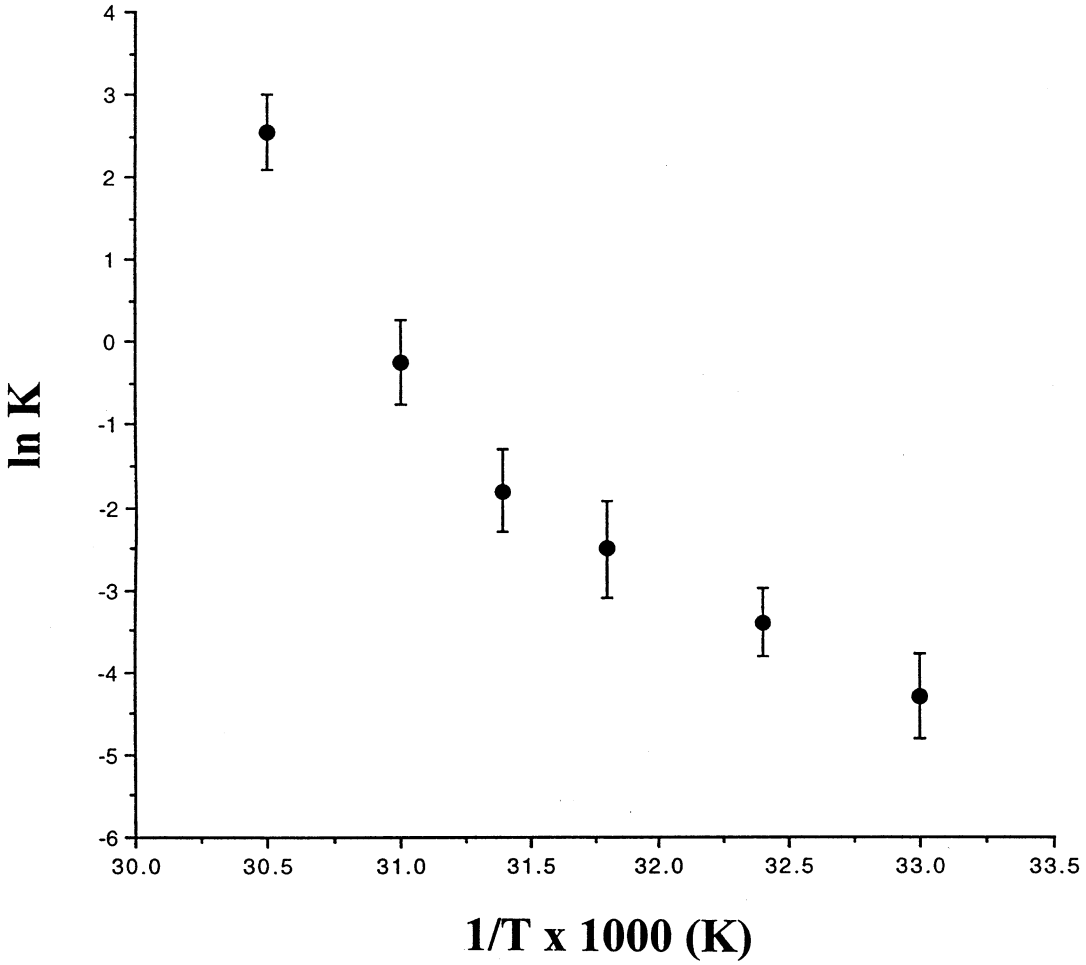


Fig. 3. van't Hoff plot demonstrating nonlinearity in the rate of thermal-induced unfolding of bFGF. (●) The mean values ± standard deviation ($n = 3$) of the first-order rate constants associated with the rate of bFGF unfolding as determined using $F_{350/307}$ ratios obtained by fluorescence spectroscopy.

tance of using more than one analytical technique to assess the physical stability of a protein drug.

The change in free energy ($\Delta G$) associated with bFGF unfolding varied linearly ($r = 0.9895$) with an increase in the concentration of GnHCl (data not shown). Similar results with chaotropes such as urea have been observed with several other proteins (Greene and Pace, 1974; Ahmad and Bigelow, 1982). However, changes in the enthalpy associated with protein unfolding as presented in van't Hoff plots are typically found to be non-linear. Using the analysis described by Pace et al. (1990) in which the natural logarithm of the equilibrium constant ($K$) was plotted versus $1/T$ (Fig. 3), a distinctly nonlinear profile was observed. This would suggest that $\Delta H$ varied with temperature and that the heat capacity of the products was different from the heat capacity of the reactants (Jaenicke, 1991). Although thermodynamic contributions are important to thermal-mediated protein unfolding, a thorough knowledge of the kinetics associated with the folding and unfolding process for a protein can greatly assist in the understanding of the underlying mechanisms (Jaenicke, 1991). The values of the thermodynamic parameters estimated in the present study

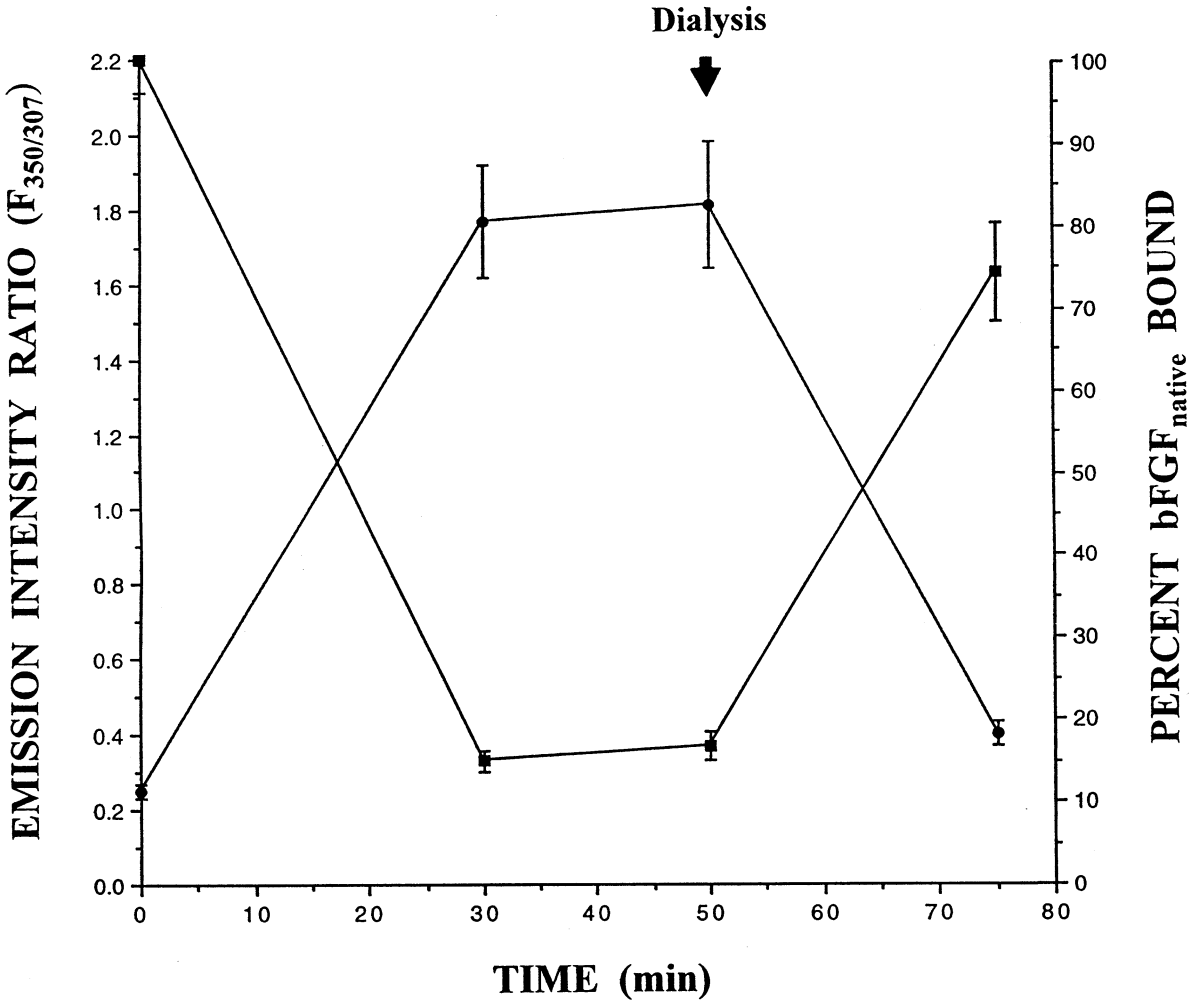


Fig. 4. Relationship of GnHCl-induced bFGF unfolding and refolding following dialysis using fluorescence spectroscopy and heparin affinity chromatography. (●) Mean values ± standard deviation ($n = 3$) of the $F_{350/307}$ ratio; (■) the mean percent ± standard deviation ($n = 3$) of bFGF in the native conformation bound to the heparin affinity column.

for GnHCl- and thermal-mediated denaturation of bFGF are of the same order of magnitude as those calculated for other proteins. Pace (1986) using RNase and urea (denaturant) calculated the $\Delta G_{(H_2O)}$ to be 23.4 kJ/mol and the $urea_{1/2}$ (when $\Delta G = 0$) equal to 4.6 M. The values of $\Delta S$, $\Delta H_m$, and $T_m$ (at $\Delta G = 0$) for RNase following thermal-induced denaturation were 1236 J $mol^{-1}$ $K^{-1}$, 397 kJ/mol, and 322 K (49.2°C), respectively (Pace, 1986). Using GnHCl as the denaturant, it has been reported that the values of $\Delta G_{(H_2O)}$ and $GnHCl_{1/2}$ (when $\Delta G = 0$) for myoglobin and lysozyme were 50.2 and 52.1 kJ/mol and 1.7 and 3.1 M, respectively (Pace et al., 1990).

Although a two-state model consisting of native and unfolded states is often used to characterize protein denaturation, partially unfolded intermediates are found in the conformational transition of many proteins. Analytical techniques such as UV spectroscopy and especially circular dichroism (CD) have proven invaluable in the detection of partially unfolded intermediates. Using both UV absorbance and CD, GnHCl-induced unfolding of bovine growth hormone (bGH) demonstrated that

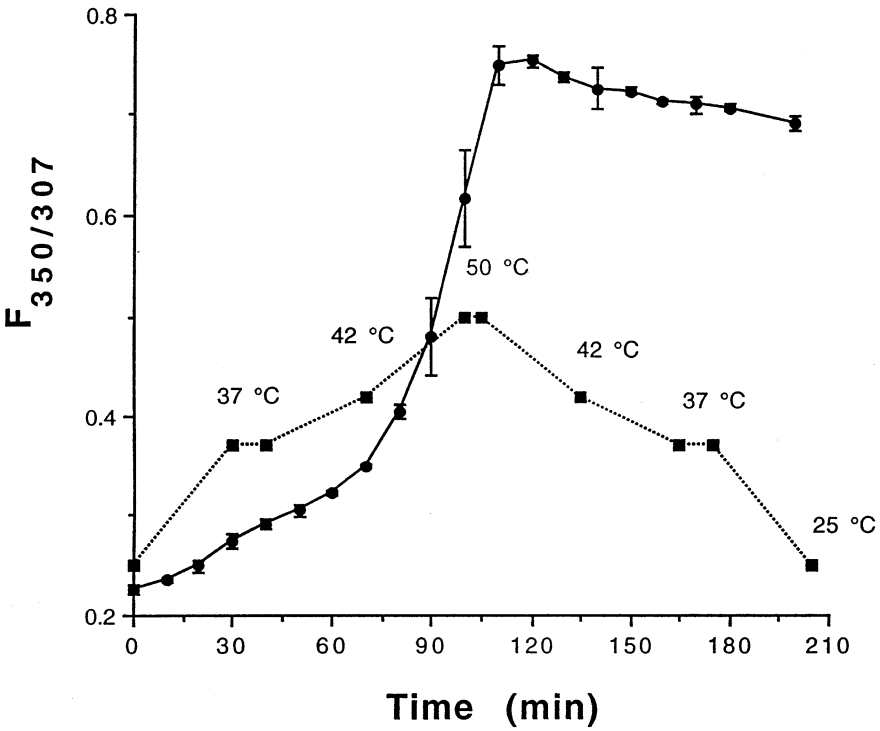


Fig. 5. Thermal-induced unfolding of bFGF as assessed using fluorescence spectroscopy. (●) Mean values of the $F_{350/307}$ ratio ± the standard deviation ($n = 3$); (■) the temperature ramping program used.

disruption of aromatic hydrophobic interactions (tertiary structure) and folded-state quenching occurred with partially unfolded bGH intermediates (Holladay et al., 1974; Holzman et al., 1990). In addition, CD analysis of GnHCl-treated bGH demonstrated significant attenuation in the magnitude of the 222-nm peak suggesting disruption of secondary structure and the presence of partially unfolded intermediates (Holladay et al., 1974; Holzman et al., 1990). Analysis of the secondary structure of bFGF using CD and FTIR have revealed that it is rich in distorted antiparallel $\beta$-sheet and devoid of $\alpha$-helical structure (Prestrelski et al., 1991). In the present study, while subtle changes in the CD spectrum were noted for a bFGF solution maintained at 323 K for 1 h, these were not statistically different ($p > 0.05$) from the mean values obtained for the control bFGF solution maintained at 295 K. Hence, there is no clear indication of partially unfolded species.

Reversible denaturation is defined as protein unfolding caused by an increase in temperature which can be reversed by subsequent lowering of the temperature. In the present investigation, it was desired to determine whether an analogous refolding process for bFGF would occur if a chaotrope were gradually removed from the incubation medium. In most cases, denaturation is reversible if an unfolded protein can return to its native structure when the denaturant is removed (Manning et al., 1989; Rozema and Gellman, 1996). However, in some instances, a protein can recover only its activity but not its original structure after the denaturant has been removed (Wong and Parasrampuria, 1997). Reactivation of enzyme activity upon removal of a denaturant has been observed by others (McVittie et al., 1977; Horowitz and Simon, 1986; Tandon and Horowitz, 1986, 1987, 1988; Wetlaufer and Xie, 1995). Refolding of bFGF following denaturation with GnHCl and subsequent dialysis to remove GnHCl demonstrated a fluorescence ratio in close agreement to the mean value normally observed for the native state. Using heparin affinity chro-

matography, we determined that approximately $74 \pm 6.1\%$ of bFGF refolded to the native state after having been denatured (unfolded) by GnHCl and then dialyzing the bFGF/GnHCl solution to remove GnHCl. Thus, removal of the denaturant (GnHCl) will result in a significant fraction of the bFGF molecules refolding to the native state. Presumably, biological activity of bFGF would also be regained. However, residual bioactivity would have to be experimentally verified using cell culture studies and will be the subject of future investigations. The remaining fraction of bFGF unrecovered on the heparin affinity column ($\sim 26\%$) following renaturation could potentially be soluble aggregates of bFGF, especially disulfide-linked aggregates under denaturing conditions. This possibility would also have to be confirmed perhaps by conducting turbidity measurements at, for example, 320 nm to demonstrate scattering due to the soluble aggregates.

Aggregation can also interfere with the renaturation process (Goldsberg et al., 1991) and has been shown to be a concentration-dependent process (Goldsberg et al., 1991). In our experiments designed to assess the reversibility of thermal-mediated unfolding of bFGF at a concentration of 70 $\mu$g/ml (Fig. 5), aggregation was observed at elevated temperatures which presumably interfered with the refolding process. Aggregation of proteins may occur well below the value of $T_m$ at which an equal fraction of the folded and unfolded protein exists. However, as Gombotz et al. (1994) has noted, typically additives which induce an elevation in the $T_m$ are generally protein stabilizers while additives that decrease the value of $T_m$ are protein destabilizers. In the present study, while the value of $T_m$ was not determined for a bFGF solution which contained sucrose, the sugar nevertheless was an effective stabilizer with regard to the rate of bFGF unfolding at 298, 310, 315 and 323 K. Moreover, the extent of bFGF unfolding, as reflected by AUC values in Table 2, was also significantly reduced at 310, 315, and 323 K when sucrose was present and supports previous work by others (Gekko and Timasheff, 1981a,b; Pace et al., 1990) which has demonstrated the protein-stabilizing effects of sucrose against thermal-mediated denaturation. Gombotz et al. (1994) using differential scanning calorimetry demonstrated that low concentrations ($< 1.0\%$, w/v) of poly[vinylpyrrolidone] (PVP) increased the stability of IgM monoclonal antibody. Higher concentrations of PVP (5 and 10%, w/v) resulted in a decrease in the experimentally determined values of $T_m$ and was dependent on the molecular weight of the PVP selected (Gombotz et al., 1994).

In the present investigation, we have quantitated the kinetic and thermodynamic parameters associated with thermal- and GnHCl-induced unfolding of bFGF. Fluorescence spectroscopy, CD, and heparin-binding affinity chromatography were useful analytical techniques with which to assess conformational stability of bFGF. However, it should be noted that the major limitation when using thermodynamic measurements to assess protein stability is the failure of such parameters to account for kinetic effects which can often lead to aggregation (Schein, 1990). Moreover, one must realize that for the vast majority of therapeutic proteins undergoing formulation and scale-up in the pharmaceutical/biotechnology industries, stability testing normally reveals that most proteins do not follow Arrhenius kinetics, as observed for many conventional drug substances. Accepting these limitations therefore necessitates more extensive stability testing with therapeutic peptides and proteins, especially to ensure residual biological activity during storage. Fluorescence spectroscopy, together with the complimentary technique of heparin affinity chromatography, are two such techniques that may be predictive for residual biological activity of bFGF when the protein formulation is subjected to either modest elevations in temperature or various additives that may act as potential denaturants.

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