Effect of Polyanions on the Unfolding of Acidic Fibroblast Growth Factor

Carl J. Burke,* David B. Volkin, Henryk Mach, and C. Russell Middaugh

Department of Pharmaceutical Research, WP78-302, Merck Research Laboratories, P.O. Box 4, West Point, Pennsylvania 19486

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ABSTRACT: The urea-induced unfolding of acidic fibroblast growth factor (aFGF) in the presence and absence of various polyanions has been quantitatively examined by fluorescence spectroscopy. In the absence of a stabilizing polyanion, the apparent free energy of unfolding of aFGF is 6.5 kcal mol^{-1}. The presence of equimolar or greater amounts of heparin stabilizes aFGF from unfolding by more than 2.5 kcal mol^{-1} and slows the rate of unfolding by greater than 2000-fold. The ability of heparin to stabilize aFGF is critically dependent upon many factors including the number of aFGF molecules bound to the heparin chain, ionic strength, temperature, and the extent of sulfation of the polysaccharide. The presence of similar amounts of other polyanions such as sulfated β-cyclodextrin or heparan sulfate also stabilizes aFGF to a similar extent as heparin. Additional experiments demonstrate that increasing charge density enhances the ability of polyanions such as sulfated β-cyclodextrins, phosphorylated inositols, and modified heparins to protect aFGF from urea-induced unfolding.

The heparin-binding growth factors are structurally related proteins distinguished by their strong interaction with heparin and the mitogenic effect on a wide variety of cell types (Gospodarowicz et al., 1987; Burgess & Maciag, 1989; Gospodarowicz, 1990; Baird & Bölen, 1990). Acidic fibroblast growth factor (aFGF), a prototypic member of this family, is dependent upon heparin and related polyanions for both its structural stability (Gospodarowicz & Cheng, 1986; Copeland et al., 1991; Volkin et al., 1993) and its mitogenic activity (Gimenez-Gallego et al., 1986). The fibroblast growth factors are found *in vivo* primarily bound to basement membranes and heparan sulfate proteoglycans in the extracellular matrix (ECM) (Weiner & Swain, 1989; Klagsbrun, 1990). It has been suggested that these proteins are stored in this form as latent complexes and their activity is regulated by heparanase-induced release from the ECM (D'Amore, 1990; Vlodavsky et al., 1991). Recent studies also suggest that the biologically active form of a similar growth factor, basic fibroblast growth factor (bFGF), is a bFGF/heparin complex in which multiple FGF molecules bind a sulfated polysaccharide (Ornitz et al., 1992). The resultant proximity of the FGF molecules might induce receptor aggregation and a consequent series of intracellular events leading to mitogenesis. Further support for the biological importance of growth factor/heparin complexes comes from recent observations that heparin or heparan sulfate proteoglycans must be bound to the heparin-binding domain of the cell-surface FGF receptor in order for aFGF and bFGF to bind (Kan et al., 1993).

At physiological temperatures and salt concentrations, purified recombinant human aFGF appears to be partially unfolded *in vitro* in the absence of polyanions (Dabora et al., 1991). In the presence of heparin or a variety of other polyanions such as sulfated polysaccharides and polynucleotides as well as low molecular weight sulfated and phosphorylated compounds, the thermal stability of aFGF dramatically increases (Volkin et al., 1992, 1993). The structural features of these polyanions necessary for their stabilizing activity appear to be relatively nonspecific with the only obvious requirement being a high negative charge density. Using chemical modification, mutagenesis, and stability studies in conjunction with the crystal structure of bovine aFGF containing bound anions (Harper & Lobb, 1988; Lobb, 1988; Burgess et al., 1991; Zhu et al., 1991; Eriksson et al., 1991; Zhang et al., 1991), the location of at least part of the polyanion-binding site in aFGF has been tentatively identified as a cluster of basic residues on the protein surface (Lys-112, Lys-118, and Arg-122) although the complete elucidation of the binding site awaits high-resolution structural analysis of aFGF/polyanion complexes (Volkin et al., 1993).

A few quantitative features of the aFGF/heparin interaction *in vitro* have been recently described (Mach et al., 1992, 1993). It has been shown that a single 16-kDa heparin chain is capable of binding up to 14–15 molecules of aFGF, with about 10 of these bound relatively uniformly to high-affinity sites. The dissociation constants of the latter sites appear to be of the order of 100 nM under physiological conditions. Acidic FGF protects a highly sulfated heparin tetrasaccharide from digestion by a mixture of heparin lyases. This same tetrasaccharide is the minimum heparin fragment necessary to induce significant thermal stabilization of the protein (Mach et al., 1993). The combination of the above results suggests that one aFGF molecule is present every four to five polysaccharide units on an aFGF-saturated heparin polymer.

Appropriate stabilizing polyanions permit unfolded aFGF to refold into its native state *in vitro* under physiological conditions (Dabora et al., 1991). Interestingly, no stabilizing anion is required for correct refolding between 10 and 30 °C, and the presence of any such anion seems to have little effect on the kinetics of refolding over this temperature range. In contrast, aFGF absolutely requires anions to refold above 30 °C, and the energetics of this process are strongly dependent on the nature of the stabilizing entity. Thus, the protein seems to initially refold without the requirement of an anionic ligand, but once the correct tertiary structure is formed, the binding of anions to a complementary polycationic site on aFGF appears to dramatically stabilize the growth factor.

Thus, the *in vitro* refolding, stabilization, and mitogenic activity of aFGF are all dramatically influenced by the presence of polyanions. The biological activities of aFGF may be potentially controlled through its complexation with polyanions as well (Vlodavsky et al., 1991; Ornitz et al., 1992). It is of

* To whom correspondence should be addressed.

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significant interest, therefore, to define the thermodynamic parameters of the stability of the protein in the presence and absence of polyanions. To this end, we have quantitatively examined the effect of heparin and other polyanions on the urea-induced equilibrium and kinetic unfolding of aFGF.

MATERIALS AND METHODS

Materials. Highly purified (>99%) recombinant human aFGF was expressed in transformed *Escherichia coli* cells and isolated by a combination of ion-exchange, affinity, and reverse-phase chromatography as previously described (Thomas et al., 1984; Linemeyer et al., 1987). This form of aFGF consists of 141 residues with the sequence numbering system Met-Phe(1)-Asn(2)-Leu(3)-...-Ser(138)-Ser(139)-Asp-(140). The mitogenic activity as well as the heparin and receptor binding of the amino-truncated aFGF is similar to the natural 154-amino acid form (Burgess, 1992). Protein concentration was determined spectrophotometrically using an $E^{0.1\%}_{280nm} = 1.2$.

Heparin (~16 kDa) was purchased from Hepar (Franklin, OH). Inositol, inositol hexaphosphate (phytic acid), and heparan sulfate (~7.5 kDa) were obtained from Sigma (St. Louis, MO). Low molecular weight heparin (~4.8 kDa) and inositol triphosphate were purchased from Calbiochem (San Diego, CA). The chemically modified (partially and completely desulfated) heparins and differentially sulfated β-cyclodextrins were obtained from Seikagaku America (Rockville, MD) and American Maize (Hammond, IN), respectively. Sequanal-grade urea (Pierce, Rockford, IL) was used for all experiments. The molar and weight ratios of aFGF (16.0 kDa) to 16-kDa heparin are approximately the same and are cited in the text without distinction.

Equilibrium Unfolding. Unless otherwise noted, samples contained 100 μg/mL aFGF in phosphate-buffered saline (PBS) containing 50 mM sodium phosphate, 1 mM EDTA, and 0.1 M NaCl at pH 7.2. The polyanions were present in a 3-fold weight excess relative to aFGF unless otherwise specified. All samples were incubated at 4 °C for 48–72 h prior to analysis to ensure equilibrium had been established (see Results). No evidence for carbamylation of aFGF was seen over this period as judged from isoelectric focusing experiments. Samples were incubated for only 24 h at higher temperatures (10–40 °C) to minimize potential oxidation and aggregation of unfolded aFGF (Volkin et al., 1993).

Fluorescence data were collected using an SLM 8000 spectrofluorometer in a photon counting mode. Samples were excited at 280 nm using a 4-nm band-pass, and the fluorescence was monitored through slits allowing band-passes of 8 and 16 nm for the monochromator and photomultiplier tube, respectively. The spectra were collected using a 1-s averaging time, and the single-wavelength values (300 and 350 nm) used for analysis of the unfolding reaction are 10-s averages. All samples were kept in a thermostated cell holder at the incubation temperature during data collection (4 °C unless otherwise indicated).

Assuming two-state behavior, the equilibrium denaturation profiles were analyzed using the linear extrapolation method (Santoro & Bolen, 1988). The ratio of the fluorescence intensities at 350 to 300 nm (I_{obs}) was fitted to the following equation using a nonlinear least-squares method from the RS/1 software package (BBN Software Products):

$$I_{obs} = \frac{I_n + m_n[U] + (I_u + m_u[U])e^{-(\Delta G + m[U])/RT}}{1 + e^{-(\Delta G + m[U])/RT}} \quad (1)$$

where I_n and I_u are the y intercepts of the respective base lines before and after unfolding, m_n and m_u are the respective slopes of the pre- and posttransition base lines, [U] is the molar concentration of urea, m is the slope of the linearized data in the transition region (from a plot of ΔG *vs* [urea]), ΔG is the apparent free energy of unfolding in the absence of urea, R is the gas constant, and T is the absolute temperature. Although eq 1 was fit to the ratioed data for the determination of ΔG and m, the denaturation curves are presented as the fraction of protein unfolded (F_u; eq 2) *vs* urea concentration to facilitate comparisons among polyanions.

$$F_u = \frac{I_{obs} - m_n[U] - y_n}{(m_u - m_n)[U] + y_u - y_n} \quad (2)$$

Circular dichroic spectra were collected with an AVIV 62DS spectropolarimeter. Samples were placed in a 0.1-cm quartz cuvette, and the ellipticity was recorded with a 5-s averaging time at 0.5-nm intervals employing a 1.5-nm bandwidth. Samples were again kept in a thermostated cell holder at the incubation temperature during collection of the spectral data.

If a significant change in heat capacity (ΔC_p) exists between folded and unfolded conformations, values for the apparent changes in enthalpy (ΔH_{app}) and entropy (ΔS_{app}) at 4 °C can be estimated by the following relation (Becktel & Schellman, 1987) if a two-state transition is assumed:

$$\Delta G = \Delta H - T\Delta S + \Delta C_p[T - 277.15 - T\ln(T/277.15)] \quad (3)$$

The free energy data with respect to temperature were fit to eq 3 as described above.

Kinetic Measurements. Unfolding kinetic studies were performed with a Hitachi 2000 spectrofluorometer by mixing 0.67 mL of a 300 μg/mL solution of aFGF in PBS containing 5 mM Na_2SO_4 with 1.33 mL of 6.6 M urea in PBS containing the desired amounts of 16-kDa heparin. The samples were continuously stirred and maintained at 8 °C with a jacketed cuvette holder. Both emission and excitation bandwidths were 10 nm. After an initial mixing period (ca. 10 s), the fluorescence spectra were recorded at 1-min intervals by monitoring the emission from 290 to 400 nm at a rate of 1200 nm/min.

Stopped-flow experiments were performed with a sequential stopped-flow spectrofluorometer (Applied Biophysics, Leatherhead, U.K.) which has a dead time of less than 5 ms. An excitation wavelength of 280 nm was used. Equal volumes (approximately 100 μL) of 1 mg/mL aFGF and 8.8 N urea in supplemented PBS buffer (as described above) were mixed, and the fluorescence signal was monitored at 350 nm for 20 s. The results were analyzed using a single-exponential model.

RESULTS AND DISCUSSION

Equilibrium Studies. Although the thermal unfolding of aFGF has been previously examined in the presence of various stabilizing ligands, thermodynamic parameters could not be determined due to the irreversible aggregation that occurs at high temperatures during unfolding. The use of chaotropic agents often avoids this complication by increasing the solubility of the structurally disrupted protein. Frequently, the use of strong denaturants results in two-state unfolding behavior, and the relevant thermodynamic parameters can be determined. Urea is often used to induce the reversible unfolding of proteins (Pace, 1975) and was selected instead of an ionic chaotrope such as guanidine hydrochloride since it should not perturb the ionic strength and alter the electrostatic interactions that are involved in the binding of polyanions to aFGF (Matsubara et al., 1992; Volkin et al.,

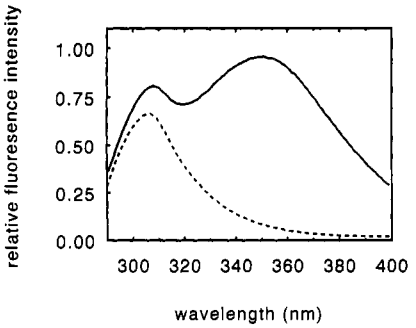

FIGURE 1: Fluorescence spectra of the aFGF/heparin complex in the presence and absence of urea. Both samples contain 100 μg/mL aFGF, 3× heparin (w/w), 0.1 M NaCl, 1 mM EDTA, 50 mM sodium phosphate at pH 7.2, and either no urea (dashed line) or 6 M urea (solid line). The samples were excited at 280 nm after equilibration at 4 °C.

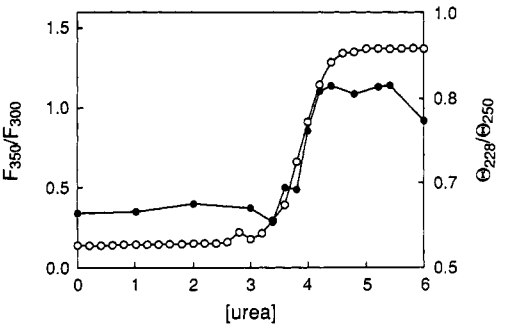

FIGURE 2: Circular dichroic (●) and fluorescence (○) urea-induced unfolding profiles of aFGF in the presence of heparin. Data from both techniques are presented as a ratio of the signals at the indicated wavelengths. The experimental conditions for the fluorescence spectra are given in the legend to Figure 1, except that the temperature is 15 °C. The CD spectra were collected under the same experimental conditions using 3-s averaging and a 1.5-nm bandwidth.

1993). Also, urea is preferred since the thermodynamic parameters of its binding to proteins do not significantly vary from linearity with respect to denaturant concentration (Makhatadze & Privalov, 1992) and the unfolding parameters are of similar magnitude to those obtained using a variety of different methods and denaturing agents (Hu et al., 1992; Santoro & Bolen, 1992).

The unfolding of aFGF can easily be followed by changes in the intrinsic Trp fluorescence spectrum of the protein. The fluorescence of the single Trp residue in native aFGF is quenched (Copeland et al., 1991), and the spectrum is dominated by multiple Tyr emission at 305 nm (Figure 1). As aFGF unfolds, the indole side chain becomes more solvent-exposed as the tertiary structure induced quenching is relieved, thus causing an increase in fluorescence at 350 nm. The 305-nm emission of the Tyr residues does not exhibit a significant change in intensity since these fluorophores are less sensitive to perturbations in their environment. Thus, the Tyr fluorescence can serve as a convenient reference point for the changing indole emission.

To ensure that changes in the Trp emission are not simply reflecting some local unfolding event, circular dichroism was utilized to monitor changes in the secondary structure upon exposure to urea. The far-UV CD spectrum of native aFGF possesses positive ellipticity at 228 nm and an intense negative peak at 205 nm (Copeland et al., 1991) characteristic of type β-II proteins (Wu et al., 1992). Circular dichroic spectra monitored at 215 and 228 nm as a function of urea concentration manifest superimposable transitions with the fluorescence data (Figure 2). The presence of urea does not permit CD measurements at lower wavelengths. Although indole rings give rise to an intrinsic CD band near 228 nm (Adler et al., 1973), the strength of this signal appears to primarily reflect the presence of secondary structure in aFGF since thermal CD transitions at 205, 215, and 228 nm are all superimposable. This conclusion is confirmed by FTIR studies of the spectral changes in the amide I region of the aFGF spectrum (not illustrated). Thus, these various spectral measurements indicate that aFGF bound to heparin displays cooperative two-state unfolding behavior in the presence of urea similar to other protein/ligand complexes (Schwartz, 1988; Shrake & Ross, 1988). The two-state unfolding transition is also a reasonable assumption given that the crystal structure of aFGF (and homologous bFGF) consists of a series of tightly coupled antiparallel β-strands (Zhu et al., 1991; Eriksson et al., 1991; Zhang et al., 1991). Disruption of one or more of these regions may destabilize the entire β-barrel, resulting in the observed two-state unfolding process.

Fluorescence was selected as the routine method to analyze aFGF unfolding processes since higher signal/noise data could be obtained, resulting in more precise thermodynamic parameters. The ratio of the fluorescence intensities at 350 and 300 nm was employed since lower residuals were obtained when the data were fit to eq 1 and compared to the use of intensity values at 350 nm. Similar values were obtained, however, employing either method. The use of a two-state model to analyze the unfolding process assumes that equilibrium has been attained. For highly stabilized aFGF (with 3× heparin) at 4 °C, equilibrium was reached within 1 h in the presence of 4 M urea. However, approximately 48 h was required before equilibrium was established at lower urea concentrations (data not shown). As a result, solutions were equilibrated for 72 h prior to spectral analysis. Using this experimental protocol, aFGF in the presence and absence of polyanions is observed to unfold between 2 and 4 M urea with linear pre- and posttransition regions (Figures 3–5). The shape of the transition regions is similar in most cases with unfolding usually complete within a 1 M urea range. The initial and final base lines of the ratioed data are similar in most of the experiments. The posttransition intensity (i.e., fluorescence of unfolded aFGF) slightly decreases with an increase in temperature or NaCl concentration. These changes are probably due to collisional and chloride quenching, respectively. In both sets of experiments, the intensity decreases by less than 11% compared to the protein at 4 °C in 100 mM NaCl. Regardless, the magnitude of the posttransition fluorescence will not affect the determination of the thermodynamic parameters since only the unfolded form of aFGF significantly contributes to the fluorescence transition signal.

It has been previously demonstrated that aFGF is stabilized by direct interaction with heparin and other polyanions (Volkin et al., 1993). The degree of stabilization is consequently dependent on the relative amounts of polyanion and aFGF (Figure 3A). For example, unliganded aFGF unfolds at a $[urea]_{1/2}$ of 2.3 M with the value increasing to 3.6 M in the presence of a 10-fold excess of heparin. Furthermore, as the relative amount of heparin is increased, both the apparent free energy of unfolding (ΔG_{app}) and the concentration of urea needed to induce 50% unfolding ($[urea]_{1/2}$) generally increase (Table I). The ΔG_{app} value of 6.5 kcal mol^{-1} obtained from the unfolding of the protein in the absence of polyanions is not unexpected based on the molecular weight and melting temperature of aFGF (Spolar et al., 1992). The addition of increasing amounts of heparin to aFGF (from 0.3× to 1× heparin) results in a stabilization of approximately 0.5 and 2 kcal/mol, respectively, relative to the unliganded protein.

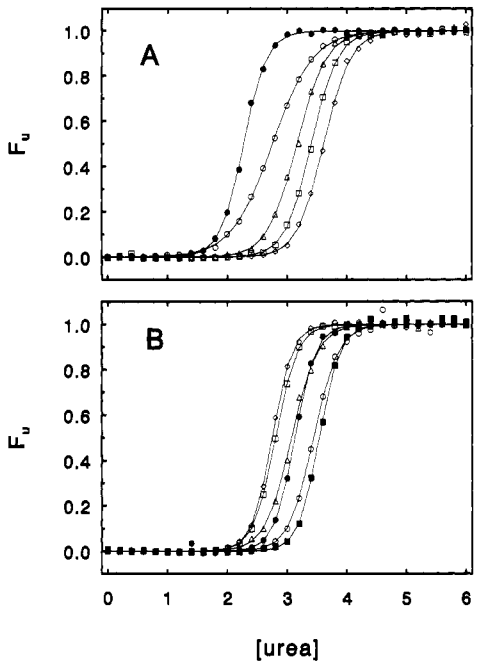

FIGURE 3: Effect of heparin and ionic strength on aFGF unfolding. Fluorescence data are represented as the fraction unfolded (F_u). Panel A illustrates the unfolding profiles of aFGF at various concentrations of heparin: 0× (●), 0.10× (○), 0.33× (△), 1.0× (□), and 10× (◇). These experiments were performed in 50 mM phosphate buffer, pH 7.2, in the presence of 1 mM EDTA and 0.1 M NaCl. The effect of varying ionic strength is shown in panel B with NaCl concentrations of 0 (●), 0.10 (○), 0.25 (△), 0.5 (□), 1.0 (◇), and 2.0 M (■). The latter set of experiments was performed in the presence of a 3× (w/w) excess of heparin using 50 mM phosphate buffer, pH 7.2. Both data sets were collected at 4 °C employing an aFGF concentration of 100 μg/mL. All curves displayed were fitted to the data by eq 1.

This effect appears to saturate at higher levels of heparin, with 3× and 10× polysaccharide excesses inducing ca. 2.5 kcal/mol of stabilization.

The only exception to these generalizations occurs at the lowest ratio of heparin to aFGF examined (0.1×), where the value of ΔG_{app} is 2 and 4 kcal/mol lower than that observed in the absence of heparin and at higher concentrations, respectively. Since the transition slope (m) is also much smaller than the slopes observed at greater heparin ratios, unfolding appears to proceed in a somewhat different manner. At equimolar heparin or greater, sizing analysis shows that on average one aFGF is bound per heparin chain (Mach et al., 1992, 1993). Thus, the more gradual transition slope at 0.1× heparin may be attributed either to the presence of some unliganded aFGF due to saturation of heparin or to interaction between bound aFGF molecules at high protein binding densities. Intermolecular interactions between bound aFGF molecules could then result in the observed decrease in stabilization through either enhanced dissociation or an intrinsic decrease in the stability of the associated form. The presence of unliganded (and therefore intrinsically unstable) aFGF is not expected to significantly contribute to the observed unfolding since free protein should not be present in large amounts at a 0.1× heparin/aFGF ratio. Therefore, the value of ΔG_{app} cannot be meaningfully interpreted since the assumption of two-state behavior is clearly violated under these conditions.

The interaction of aFGF with heparin is thought to be predominantly ionic since the protein dissociates from heparin affinity columns at about 1 M NaCl (Klagsbrun, 1989). This binding is proposed to be mediated by the sulfate groups of

Table I: Thermodynamic Parameters for the Two-State Urea Denaturation Equilibrium of aFGF[a]

conditions[b]	ΔG_{app} (kcal mol^{-1})[c]	m (kcal mol^{-1} M^{-1})[d]	$[urea]_{1/2}$ (M)[e]
0× heparin	6.5 ± 0.2	−2.88 ± 0.08	2.3
0.1× heparin	4.5 ± 0.1	−1.63 ± 0.04	2.8
0.3× heparin	7.1 ± 0.2	−2.24 ± 0.07	3.2
1× heparin	8.6 ± 0.2	−2.52 ± 0.06	3.4
3× heparin	9.2 ± 0.5	−2.66 ± 0.13	3.5
10× heparin	8.9 ± 0.8	−2.47 ± 0.24	3.6
0 M NaCl	7.7 ± 0.3	−2.52 ± 0.08	3.1
0.1 M NaCl	9.2 ± 0.5	−2.66 ± 0.13	3.5
0.25 M NaCl	9.8 ± 0.3	−3.13 ± 0.09	3.1
0.5 M NaCl	8.4 ± 0.1	−2.99 ± 0.04	2.8
1 M NaCl	8.9 ± 0.2	−3.24 ± 0.07	2.7
2 M NaCl	11.1 ± 0.9	−3.15 ± 0.26	3.5
1.5% S heparin	7.2 ± 0.2	−3.19 ± 0.09	2.3
4–7% S heparin	6.8 ± 0.2	−2.74 ± 0.07	2.5
8–10% S heparin	7.2 ± 0.2	−2.70 ± 0.07	2.7
13.5% S heparin	9.2 ± 0.5	−2.66 ± 0.13	3.5
low MW heparin	8.9 ± 0.5	−2.51 ± 0.14	3.5
heparan sulfate	9.7 ± 0.6	−2.74 ± 0.17	3.5
BCD	7.0 ± 0.2	−3.11 ± 0.07	2.3
8% S BCD	6.5 ± 0.1	−2.91 ± 0.04	2.2
14% S BCD	9.1 ± 0.2	−2.68 ± 0.05	3.4
inositol	6.5 ± 0.1	−2.89 ± 0.06	2.2
inositol-TP	7.5 ± 0.2	−2.82 ± 0.06	2.7
inositol-HP	7[f]	−1[f]	7[f]
4 °C	9.2 ± 0.5	−2.66 ± 0.13	3.5
15 °C	10.5 ± 0.3	−2.70 ± 0.09	3.9
25 °C	9.3 ± 0.2	−2.44 ± 0.05	3.8
35 °C	9.0 ± 0.9	−2.55 ± 0.26	3.5
40 °C	7.5 ± 0.2	−2.58 ± 0.06	2.9

[a] Unless otherwise specified, ligands are present in a 3-fold weight excess with 0.1 M NaCl and 50 mM sodium phosphate buffer, pH 7.2 at 4 °C. Where a ligand is not specified, 3× heparin is present. The concentration of aFGF is 100 μg/mL. The uncertainties given are the standard error. [b] Only exceptions to the conditions described in footnote *a* are noted. Abbreviations: S, sulfated; BCD, β-cyclodextrin; TP, triphosphate; HP, hexaphosphate. [c] In the absence of urea. [d] Slope of the linearized transition region (or cooperativity parameter). [e] Calculated as $\Delta G_{app}/-m$. [f] Values estimated; see text for further details.

heparin and a cluster of surface-exposed charged basic side chains on aFGF (Zhu et al., 1991; Eriksson et al., 1991; Zhang et al., 1991; Volkin et al., 1993). To investigate the ionic strength dependence of unfolding, the extent of equilibrium unfolding was monitored in the presence of increasing NaCl concentrations and a constant amount (50 mM) of sodium phosphate. In general, increasing the ionic strength tends to lower the transition midpoint (Figure 3B) but does not significantly alter ΔG_{app} up to 1.0 M NaCl (Table I). In the absence of NaCl with only 50 mM sodium phosphate present, aFGF unfolds at lower urea concentrations than protein containing 0.1 M NaCl. The smaller ΔG_{app} at low ionic strength is probably caused by the lack of Debye–Hückel charge shielding which may decrease the stability of free aFGF. At high ionic strength (2 M NaCl), aFGF itself is strongly stabilized against urea-induced unfolding even though it is presumably not associated with heparin because of the extensive charge shielding. The presence of 2 M NaCl (and 3× heparin) protects aFGF from unfolding by 4.6 kcal/mol, which is, in fact, greater than the stabilization induced by heparin in 0.1 M NaCl. This surprising result is supported by CD thermal melting experiments which find that 2 M NaCl in the absence of heparin raises the unfolding temperature of aFGF by 20 °C, approximately the same degree of stabilization induced by heparin (not illustrated). Thus, compensating processes appear to account for the unexpected changes in aFGF stability under different conditions. In-

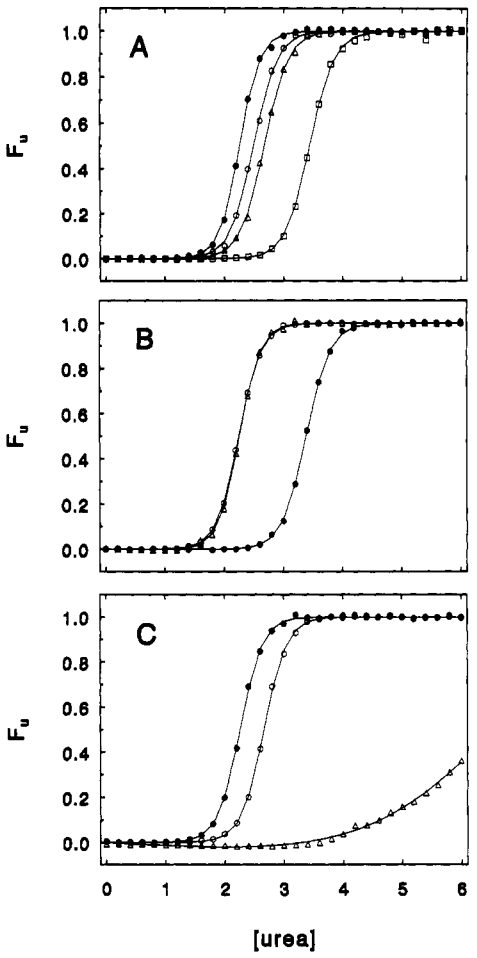

FIGURE 4: Effect of ligand charge density on the urea-induced unfolding of aFGF. The dependence of the degree of sulfation of heparin is shown in panel A with the sulfur percentage represented by 1.5% (●), 4.5–7% (○), 8–11% (△), and 13% (□). Panel B illustrates aFGF unfolding in the presence of variably sulfated β-cyclodextrins: 0% (○), 8% (△), and 14% (●) sulfur content. The denaturation of aFGF in the presence of inositol with various phosphorylation degrees is shown in panel C. Data are given for inositol with 0 (●), 3 (○), and 6 (△) phosphate groups. All ligands were present in a 3× weight excess. Experimental conditions are described in the legend to Figure 1. All curves displayed were fitted to the data by eq 1.

creasing the ionic strength will tend to decrease the extent of heparin binding and, therefore, destabilize the protein. Conversely, high NaCl concentrations enhance the intrinsic stability of aFGF. Although chloride anion is predicted to be neutral with respect to protein-stabilizing ability based on the Hofmeister lyotropic series (Collins & Washbaugh, 1985), high concentrations of Cl^- clearly stabilize aFGF from unfolding, perhaps by binding to residues in the polyanion ligation site. In the absence of NaCl, the unfolding parameters probably reflect the intrinsic instability of aFGF since the protein was also observed to be much less thermostable under conditions of low ionic strength (not illustrated).

The role of ionic interactions in the stabilization of aFGF was further probed by varying the charge on the polyanion itself. As shown in Figure 4A, decreasing the number of sulfate groups on heparin by chemical modification diminishes its ability to stabilize aFGF against urea denaturation. The midpoint of the unfolding transition and the value of ΔG_{app} dramatically decrease upon reduction of the sulfur content of heparin from 13.5% to 4–7% (Table I, Figure 4A). Further desulfation of heparin (1.5% S) nearly eliminates its ability to stabilize aFGF from unfolding. This completely desulfated heparin appears to bind only weakly, if at all, to aFGF as evidenced by the ΔG_{app} and $[urea]_{1/2}$ values which are similar to those obtained in the absence of polyanion. Although protein binding can be mediated by carboxylate groups in heparin-like regions of heparan sulfate (Lortat-Jacob & Grimaud, 1992), our findings support previous work (Volkin et al., 1993) which clearly establishes a primary role for sulfate groups in mediating aFGF binding to polyanions. Also, the data agree with the observation that low-sulfate oligosaccharides are not as effective in releasing bFGF from the extracellular matrix compared to highly sulfated oligosaccharides (Ishai-Michaeli et al., 1992). Interestingly, decreasing the size of fully sulfated heparin to 4 kDa (low molecular mass heparin) does not appear to affect its ability to stabilize aFGF since, in the presence of a 3-fold weight excess of this smaller heparin, the thermodynamic parameters are almost identical as those obtained in the presence of the 16-kDa form (Table I).

A variety of other sulfated polysaccharides have been found to stabilize aFGF against thermal unfolding (Volkin et al., 1992, 1993), including sulfated β-cyclodextrin (BCD). Like heparin, BCDs sulfated to varying extents appear to display threshold behavior in their ability to stabilize aFGF. As shown in Figure 4B, desulfated and 8% sulfur containing BCD (corresponding to zero and one sulfate group per monomer, respectively) provided no additional stabilization when compared to aFGF in the absence of ligand (Table I). Only the 14% sulfur form (approximately two sulfate groups per monosaccharide) displays the ability to stabilize aFGF to the same degree as fully sulfated heparin (Figure 4B, Table I). This result probably arises from the spatial constraints imposed on the sulfates by the heptasaccharide ring structure. For long-chain polyanions such as heparin, random removal of sulfate groups should leave a significant number of binding sites relatively unaltered as discussed above. On the other hand, sulfated BCD possesses only two potential aFGF-binding sites per molecule (unpublished results). Thus, a single modification will disrupt at least half of the total binding capability since random removal of ca. 40% of the sulfate groups should disrupt a substantial portion of both binding sites on BCD.

To further investigate the charge requirements for polyanionic stabilization of aFGF, a series of lower molecular weight compounds, the phosphorylated inositols, were examined. Both phosphorylated and sulfated inositols have been previously demonstrated to stabilize aFGF against thermal denaturation with the effect directly dependent upon the number of charged groups on the inositol ring (Volkin et al., 1993). As shown in Figure 4C and Table I, increasing the number of inositol phosphate groups provides protection against urea-induced denaturation as well. Nonphosphorylated inositol offers no protection against unfolding, and inositol triphosphate provides only a modest stabilization as reflected by the small increase in ΔG_{app}. In contrast, inositol hexaphosphate dramatically protects aFGF against unfolding as manifested by a transition midpoint near or above 7 M urea. Since only a part of the unfolding transition is observed, the values of the parameters shown in Table I for inositol hexaphosphate were estimated by using the posttransition base line obtained from experiments with the other inositols. The base lines before and after the transition are similar for all ligands examined, so these parameters would seem to be reasonable approximations. Using these estimates, it appears that despite the large increase in $[urea]_{1/2}$, the apparent free energy of unfolding is close to that of the uncomplexed protein. The slope in the transition region is, however, substantially

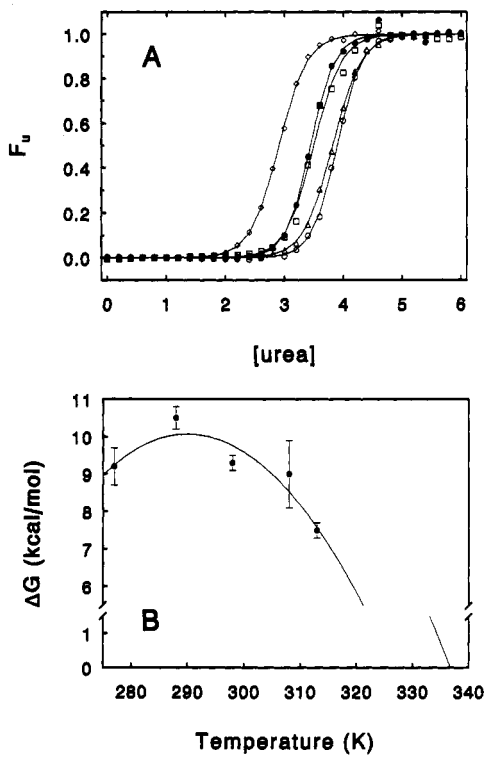

FIGURE 5: Temperature dependence of the unfolding of aFGF. Panel A shows the urea denaturation profiles of aFGF in the presence of 3× (w/w) heparin at the following temperatures: 4 (●), 15 (○), 25 (△), 30 (□), and 40 (◇) °C. All curves displayed were fitted to the data by eq 1. The dependence of ΔG on temperature is illustrated in panel B. The line represents the fit to eq 3. The experimental conditions are similar to those described in the legend to Figure 1.

lower than those observed for the various ligands and suggests a significant deviation from two-state behavior. Thus, the value of ΔG_{app} in this instance, like that seen in the presence of 0.1× heparin, is probably not physically meaningful. Although the apparent free energy of unfolding of aFGF in the presence of inositol hexaphosphate (IHP) cannot be directly compared to the other data because of deviations from two-state behavior, the large shift in the transition midpoint suggests that IHP binds unusually tightly to aFGF. Strong binding is also supported by thermal experiments in which it is found that the IHP/aFGF complex unfolds at a slightly higher temperature than heparin/aFGF (Volkin et al., 1993). The decreased transition slopes seen in the presence of IHP may indicate reduced solvent accessibility of the unfolded conformation and suggest that partially unfolded intermediates may be present under these particular equilibrium conditions (Pace, 1975; Pace et al., 1992). It is also possible that the decreased slope values result from a contraction of the denatured state as electrostatic repulsion between surface charges is relieved (Pace et al., 1990). This again suggests that IHP could protect against complete denaturation by binding to partially unfolded conformations of aFGF.

In addition to the amount and type of ligand, temperature also affects the ability of polyanions to protect aFGF against urea-induced unfolding (Mach et al., 1992). Increasing the incubation temperature of aFGF in the presence of 3× heparin between 4 and 40 °C, one finds that the stability of the protein is maximum near 15 °C as reflected by maximum values of both ΔG_{app} and $[urea]_{1/2}$ (Figure 5, Table I). The deviation from linearity of ΔG_{app} seen at low temperature may arise primarily from the reduced stability of aFGF in the cold as observed for many proteins in this temperature range (Privalov, 1990), suggesting a significant contribution from the heat

Table II: Rate Constants for Urea-Induced Unfolding of aFGF in the Presence of Polyanions[a]

ligand	k (s^{-1}) × 10^5
none	20000 ± 1000
0.3× heparin	65 ± 1
1× heparin	8.7 ± 0.1
3× heparin	5.3 ± 0.1
10× heparin	3.7 ± 0.1
3× heparan sulfate	5.7 ± 0.1
3× sulfated β-cyclodextrin	20 ± 1
3× inositol hexaphosphate	97 ± 2

[a] First-order parameters were obtained for unfolding in 4.4 M urea in 0.1 M NaCl and 50 mM sodium phosphate, pH 7.2 at 8 °C, with a protein concentration of 100 μg/mL. The uncertainties given are the standard error.

capacity (ΔC_p) term of eq 3. The data were therefore fitted to eq 3, and the following values were obtained: $\Delta H_{app} = -26 \pm 20$ kcal mol^{-1}, $\Delta S_{app} = -130 \pm 70$ cal mol^{-1} K^{-1}, and $\Delta C_p = 2.8 \pm 1.1$ kcal mol^{-1} K^{-1}. Although the five data points in Figure 5B only allow these parameters to be estimated, the resultant values are in reasonable agreement with those obtained with other proteins of similar size [see Murphy and Freire (1992)]. Reassuringly, a value of $\Delta G_{app} \sim 0$ at 64 °C is obtained from the curve fit, which agrees with the value observed in thermal unfolding experiments (Copeland et al., 1991; Volkin et al., 1993). This suggests that the ΔG_{app} of unfolding over this temperature range is comprised of similar enthalpic and entropic contributions. Interpretation of these values is not straightforward, however, since they reflect the effect of temperature on the stability of aFGF itself as well as the binding interaction between polyanion and protein.

Kinetic Studies. Another approach to quantitatively investigate aFGF–polyanion interactions is to examine the rate of urea-induced unfolding of aFGF. As shown in Table II, increasing the heparin/aFGF ratio decreases the rate of unfolding of aFGF in 4.4 M urea. The most dramatic effect on unfolding is seen below equimolar heparin/aFGF ratios compared to further increases in heparin which are able to slow the unfolding rate only slightly. The presence of 0.3× heparin inhibits the unfolding rate by approximately 300-fold relative to aFGF alone. When heparin is present in an equimolar amount, the rate of unfolding is decreased by greater than 2000 times that of unliganded aFGF. In the presence of 10× heparin, the unfolding rate is slowed slightly greater than a factor of 2.

At heparin/aFGF ratios greater than or equal to 1, only one aFGF molecule is, on the average, bound to any liganded heparin molecule (Mach et al., 1993). Therefore, further increases in the amount of heparin are not expected to produce additional stabilization, and similar thermodynamic unfolding parameters are indeed seen at higher heparin levels (Table I). Nevertheless, the decrease in the unfolding rates suggests that there is an additional stabilization of aFGF in the presence of molar excesses of heparin. One potential explanation for this observation is that large amounts of heparin may slow the unfolding of the growth factor by weakly binding to nonspecific secondary cationic sites on aFGF. As well as the proposed primary heparin-binding site, aFGF possesses other basic regions that may bind additional anions (Volkin et al., 1993). The ability of high concentrations of NaCl to stabilize aFGF and the finding that the bivalent polyanion suramin can extensively cross-link the growth factor (Middaugh et al., 1992) are consistent with this view. A second explanation simply postulates an increase in the rate of rebinding of aFGF

to heparin molecules due to their increased number. A contribution of both mechanisms to the phenomenon cannot be excluded.

A 3-fold weight excess of heparan sulfate and sulfated BCD also slows the unfolding rate similarly to heparin. These results parallel those observed in the equilibrium unfolding experiments and suggest that binding of aFGF to heparan sulfate and sulfated BCD results in comparable stabilization of the growth factor. On the other hand, the unfolding rate of aFGF in the presence of 3× IHP is significantly less than 3× heparin or BCD although aFGF/IHP requires a much higher urea concentration to induce unfolding. The increased unfolding rate suggests that IHP may bind more strongly to partially unfolded forms of aFGF than to the native molecule. The IHP-bound state of aFGF, however, appears to be similar to native aFGF bound to other polyanions since the unfolding rate is comparable to that in the presence of 0.3× heparin. Furthermore, the fluorescence and CD spectra of the various polyanion-complexed forms of aFGF are equivalent (Volkin et al., 1993) and both heparin and IHP protect aFGF to a similar extent against trypsin digestion (Wiedlocha et al., 1992).

CONCLUSIONS

The ability of heparin and various polysaccharides to stabilize aFGF against unfolding has been quantified using urea-induced structural perturbation. The stabilizing ability of the polyanions depends on the extent of sulfation or phosphorylation and, at least for heparin, on ionic strength, temperature, and the number of aFGF molecules bound to the heparin chain. Since heparan sulfate appears to impart the same stabilization to aFGF as heparin and is proposed to be composed of heparin-like regions (Gallagher et al., 1992), the parameters obtained under various conditions with heparin should be at least roughly applicable to growth factor binding in the extracellular matrix or by other cell-surface proteoglycans. Thus, by analogy to the stabilization conferred by heparin and heparan sulfate, aFGF bound to low-affinity physiological receptors *in vivo* (i.e., sulfated proteoglycans in the extracellular matrix) should be stabilized by approximately 8 kcal mol^{-1} at physiological temperatures relative to the unfolded form of the protein. Release from the stabilizing polyanion would destabilize the protein by about 2.5 kcal mol^{-1}. The additional stabilization is probably crucial for growth factor stability *in vivo* since free aFGF appears to be partially unfolded at physiological temperatures (Dabora et al., 1991). Thus, interaction with stabilizing polyanions allows aFGF to maintain its stable, active form *in vivo*.

Maintenance of the native conformation may also play a role in compartmentalization of the growth factor. Although aFGF lacks a signal sequence, recent studies suggest that a more disordered form of the protein can be translocated across membranes (Weidlocha et al., 1992). As a result, stabilizing glycosaminoglycans may inhibit aFGF translocation by both anchoring the growth factor and raising the energy required to convert the protein to a translocation-competent state.

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