Journal of Protein Chemistry, Vol. 10, No. 4, 1991

Conformational Changes of Recombinant Human Granulocyte-Colony Stimulating Factor Induced by *p*H and Guanidine Hydrochloride

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Received November 9, 1990

Fluorescence and circular dichroism were used to follow the *p*H-dependent conformational changes of granulocyte colony stimulating factor (G-CSF). Tryptophan fluorescence of the spectra monitored at 344 nm, or after deconvolution of the emission spectra, at 345 nm, showed a decrease in intensity on going from *p*H 7 to 4, with a midtransition *p*H of 5.8. On the other hand, tyrosine fluorescence measured either by the ratio of intensity at 308 nm to that at 344 nm, or by the fluorescence intensity at 303 nm after deconvolution of the spectra, increased in intensity as the *p*H was changed from 6 to 2.5, with a midtransition *p*H of 4.5. Near UV circular dichroic spectra also showed changes between *p*H 7.5 and 4.5, which correlated with the transition monitored by the tryptophan fluorescence. The guanidine hydrochloride-induced conformational changes of G-CSF at five *p*H values from 2.5 to 7.5 were also studied. Circular dichroic and fluorescence spectra revealed minor conformational changes by the addition of 1 or 2 M guanidine HCl at all *p*H values examined, while the major conformational transition occurred between 2 and 4 M guanidine hydrochloride. The secondary structure of the protein was most stable between *p*H 3.3 and 4.5. The guanidine HCl-induced denaturation of G-CSF involved more than a two-state transition, with detectable intermediate(s) present, and the structure of the intermediate(s) appeared to depend on the *p*H used. These results are consistent with the *p*H dependence of the structure described above, and demonstrate the complex conformational properties of G-CSF.

KEY WORDS: Granulocyte-colony stimulating factor; conformational changes; circular dichroism; guanidine-induced denaturation.

1. INTRODUCTION

Granulocyte colony stimulating factor (G-CSF)[3] is a protein responsible for the specific generation of granulocytic colonies (Metcalf, 1984, 1985; Zsebo *et al.*, 1986; Cohen *et al.*, 1987). Several laboratories have reported on biological and structural characteristics of recombinant G-CSF, derived from *E. coli* (Cohen *et al.*, 1987; Kuga *et al.*, 1989; Komatsu *et al.*, 1987; Wingfield *et al.*, 1988). From CD analysis, the purified and refolded protein possesses 66% α-helix at *p*H 7.5 (Wingfield *et al.*, 1988), while a higher value was obtained at *p*H 4.5 by Lu *et al.*, 1989, suggesting that the secondary structure depends on the solvent *p*H. Nevertheless, these CD results reveal that G-CSF assumes a typical α-helical conformation. Lu *et al.* (1989) also reported that at *p*H 4.5 the near UV CD spectrum had several aromatic transition signals, suggesting that the protein is folded into a distinct tertiary structure. Wingfield *et al.* (1988) showed that G-CSF is denatured by urea at *p*H 7.5, with a midtransition urea concentration of 5.3 M at *p*H 7.5, indicating that recombinant G-CSF

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[3] Abbreviations used: G-CSF, granulocyte colony stimulating factor; CD, circular dichroism; FWHM, full width half maximum; HP-GPC, high-performance gel permeation chromatography; Tris, Tris[hydroxymethyl] aminomethane; CHES, 2-[N-Cyclohexylamino]-ethanesulfonic acid; GdnHCl, guanidine hydrochloride.

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is a stable protein comparable to most globular proteins. This paper extends these earlier investigations and describes effects of *p*H on the conformation of this therapeutically important molecule, and the *p*H dependence of GdnHCl-induced denaturation of the protein using CD and fluorescence spectroscopy.

2. MATERIALS AND METHODS

Recombinant human G-CSF was obtained as described previously (Zsebo *et al.*, 1986). The protein concentration was spectrophotometrically determined using an extinction coefficient of 0.86 at 280 nm and a 1 cm path for a 0.1% protein solution. The buffers used were 20 mM glycine (*p*H adjusted with HCl), 20 mM acetate (*p*H adjusted with NaOH), 20 mM citrate (*p*H adjusted with NaOH), 20 mM Tris (*p*H adjusted with HCl), and 20 mM CHES (*p*H adjusted with NaOH), all containing 100 mM NaCl.

CD spectra were determined on a Jasco J-500C spectropolarimeter equipped with an Oki Model 30 computer. The spectra were taken at room temperature using a 1 cm cuvette for the near UV region and a 0.02 cm cuvette for the far UV region. The data were expressed, after subtraction of solvent spectrum, as the mean residue ellipticity, $[\theta]$, calculated using the mean residue weight of 107. Fluorescence spectra were determined on a Perkin-Elmer LS-5 fluorescence spectrophotometer at 25°C using a 0.5 cm cuvette. The slit widths were adjusted at 10 nm for excitation and 3 nm for emission. Samples were diluted from the stock solution into 1 ml of buffer, or buffered GdnHCl, for a final protein concentration of 1.5 mg/ml for CD analysis and 0.21 mg/ml for fluorescence analysis, and incubated at room temperature for 10–15 min prior to recording the spectra.

High-performance gel permeation chromatography was performed as previously described (Watson and Kenney, 1988).

3. RESULTS AND DISCUSSION

G-CSF contains two tryptophan and three tyrosine residues per monomer, and hence is amenable to fluorescence studies.

The fluorescence spectra of G-CSF, determined at *p*H 7.5 in 20 mM Tris, 100 mM NaCl, are shown in Fig. 1A. When the protein is excited at 280 nm (solid line), the spectrum appears symmetrical with

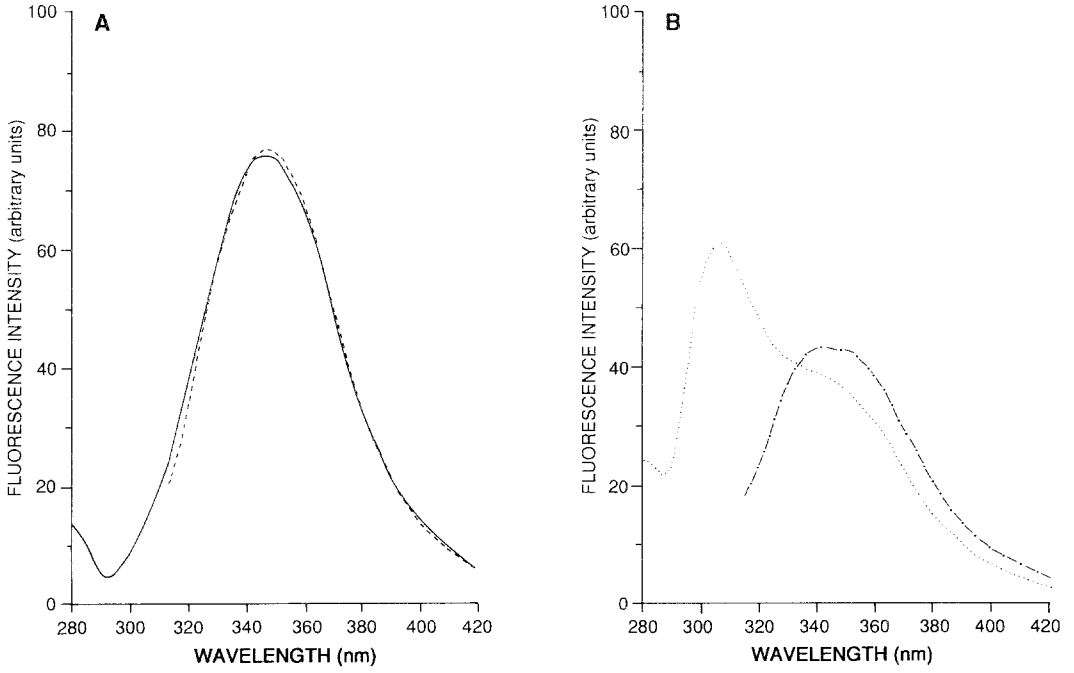

Fig. 1. Fluorescence spectra of G-CSF at *p*H 7.5 (A) and *p*H 2.5 (B). A: Excited at 280 nm, ——; excited at 300 nm, – – – –. The fluorescence intensity 100 for 280 nm excitation corresponds to 28.6 for 300 nm excitation. B: Excited at 280 nm, · · · ·; excited at 300 nm, — · —. The intensity 100 for 280 nm excitation corresponds to 20 for 300 nm excitation. G-CSF concentration was 0.21 mg/ml.

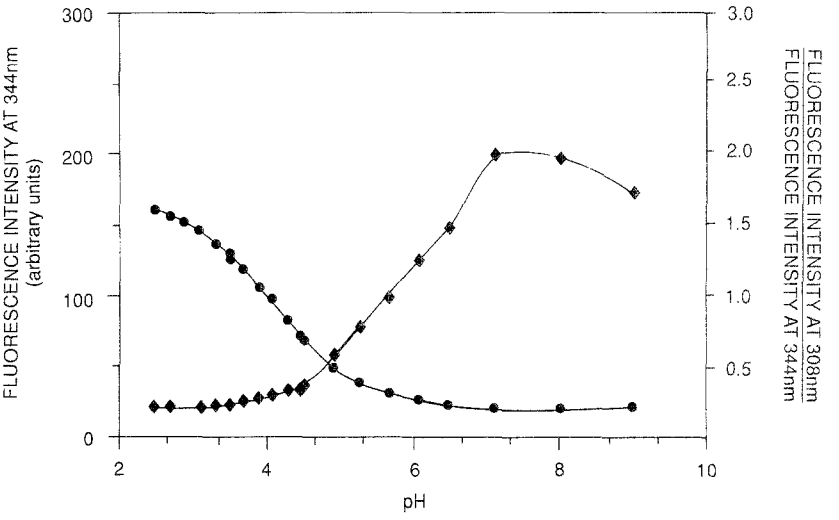

Fig. 2. *p*H dependence of fluorescence intensity at 344 nm (●) or of ratio of fluorescence intensity at 308 nm to that at 344 nm (◆). The fluorescence spectra of G-CSF were determined at 0.21 mg/ml. The buffers used were as follows: 20 mM glycine, 100 mM NaCl for *p*H 2.5 to 3.5, 20 mM sodium citrate, 100 mM NaCl for *p*H 3.5 to 4.5; 20 mM sodium citrate, 100 mM NaCl for *p*H 4.5 to 7.1; 20 mM Tris, 100 mM NaCl for *p*H 7.5 to 8.5; and 20 mM CHES, 100 mM NaCl for *p*H 8.5 to 9.5. Identical spectra were obtained using different buffers at the same *p*H.

peak maximum at 344 nm and FWHM of 66 nm, suggesting that the two tryptophans are in a similar environment, or that only one tryptophan is fluorescent. This peak maximum indicates that the fluorescent tryptophan(s) are only partially solvent-exposed, since free tryptophan has a fluorescence maximum of around 350 nm (Neinryb and Steiner, 1971). Tyrosine fluorescence appears to be completely quenched by tryptophan, as is usually the case for globular proteins containing both tryptophan and tyrosine residues. The absence of tyrosine fluorescence is further supported by the spectrum obtained upon excitation at 300 nm, which excite only tryptophans. This spectrum (broken line) is essentially superimposable on that obtained using 280 nm excitation.

At *p*H 2.5, an entirely different fluorescence spectrum is observed when the protein is excited at 280 nm (Fig. 1B, dotted line). The spectrum is characterized by a peak at 308 nm and a shoulder at 344 nm. Furthermore, the fluorescence intensity is considerably lower at *p*H 2.5 than at *p*H 7.5. Thus, at least two spectral features at *p*H 2.5 are different from those seen at *p*H 7.5; the appearance of tyrosine fluorescence, and the quenching of tryptophan fluorescence. These spectral changes suggest an altered conformation in acidic *p*H, relative to that at *p*H 7.5, which

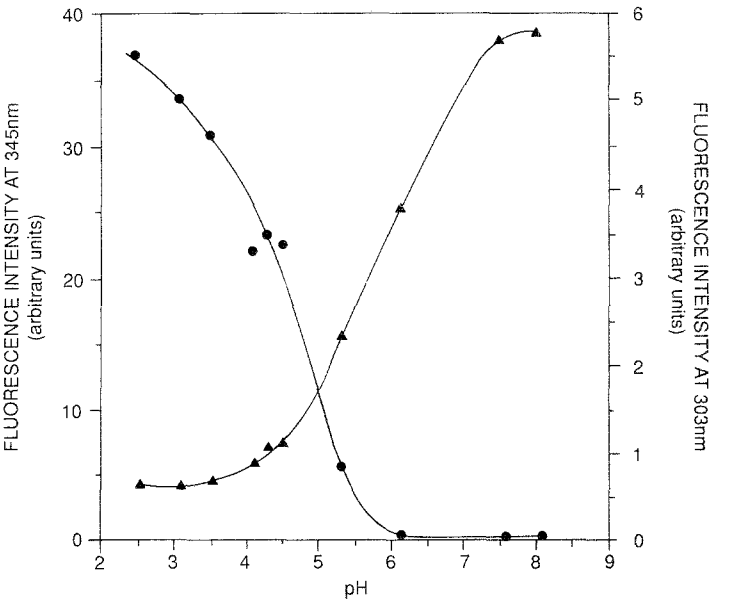

Fig. 3. *p*H dependence of fluorescence intensity of deconvoluted peaks. The fluorescence intensity at 345 nm (●) or 303 nm (▲) after deconvolution of the spectra were plotted vs. *p*H.

diminishes energy transfer from tyrosine to tryptophan and renders tyrosine fluorescent. The presence of tyrosine fluorescence is more evident by comparing the emission spectrum of G-CSF when excited at 280 nm with that resulting from excitation at 300 nm. The latter spectrum lacks the peak observed at 308 nm when the protein was excited at 280 nm, hence eliminating the possibility that the 308 nm peak is due to tryptophan residues.

The observed fluorescence spectral changes were further studied by following changes in the fluorescence spectra as a function of *p*H. In Fig. 2, a plot of the fluorescence intensity at 344 nm vs. *p*H obtained at 0.21 mg G-CSF/ml (diamond) is shown. Comparable results were obtained at 0.7 mg/ml, although inner-filter effects were more apparent at the higher protein concentration. The fluorescence intensity remains constant between *p*H 7 and 9, and decreases with *p*H until it becomes constant below approximately *p*H 4. The ratio of fluorescence intensity at 308 nm to that at 344 nm, plotted in Fig. 2 (circle), remains constant between *p*H 6 and 9.5, and increases as the *p*H is lowered. Comparison of the *p*H dependence of this ratio with that of the 344 nm intensity suggests that the fluorescence above *p*H 6 is due to tryptophan, and the tryptophan is gradually quenched as the *p*H is decreased. Fluorescence from tyrosine becomes evident (increase in 308 nm/344 nm ratio) at *p*H 6 and the further quenching of tryptophan fluorescence occurs in parallel with the increasing intensity of tyrosine fluorescence between *p*H 6 and 4. Below *p*H 4, the tryptophan is maximally quenched while the tyrosine fluorescence is still enhanced as the *p*H is decreased.

To further clarify this interpretation, several spectra were deconvoluted into gaussian distributions. In all deconvolutions attempted, two components were observed: one characterized by a peak at 345 nm with FWHM of about 66 nm (due to tryptophan), and the other by a peak at 303 nm with FWHM of about 22 nm (due to tyrosine). The fluorescence maximum at 345 nm is different from that for a tryptophan residue in a denaturing environment (350 nm), since this maximum is strongly dependent on the environment of the tryptophan(s) within G-CSF. On the other hand, the tyrosine fluorescence maximum is comparable to that for free tyrosine in solution, as is expected from the fact that the wavelength of tyrosine

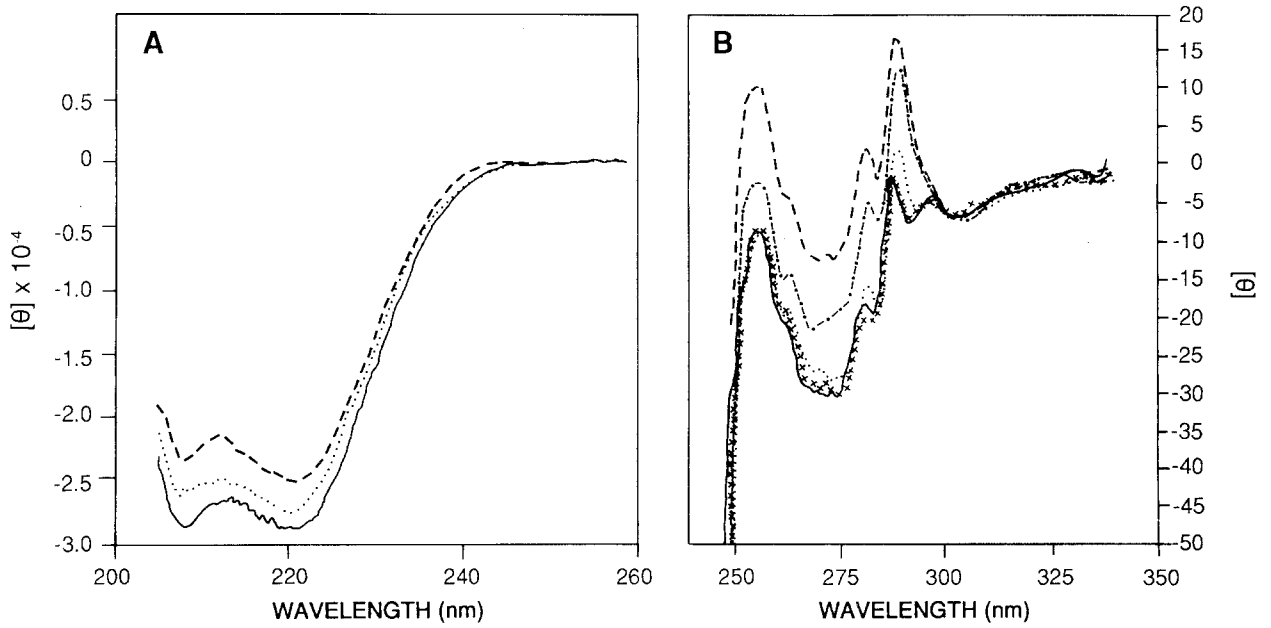

Fig. 4. Circular dichroic spectra of G-CSF. A: Far UV CD spectra: *p*H 2.5 (20 mM glycine + 100 mM NaCl), ——; *p*H 4.5 (20 mM sodium acetate + 100 mM NaCl), · · · ·; *p*H 7.5 (20 mM Tris + 100 mM NaCl), – – – –. The conditions were: protein concentration = 0.7 mg/ml, cuvette = 0.02 cm, and scan speed = 2 nm/min. The ordinate corresponds to $[\theta]$ in deg. cm^2/decimole. B: Near UV CD spectra: *p*H 2.5 (20 mM glycine + 100 mM NaCl), ——; *p*H 3.2 (20 mM glycine + 100 mM NaCl), ××××; *p*H 4.5 (20 mM sodium acetate + 100 mM NaCl), · · · ·; *p*H 6.1 (20 mM sodium citrate + 100 mM NaCl), — · —; *p*H 7.5 (20 mM Tris + 100 mM NaCl), – – – –. The conditions were: protein concentration = 1.5 mg/ml, cuvette = 1 cm, and scan speed = 1 nm/min. The spectra shown are the average of three scans. The ordinate corresponds to $[\theta]$ in deg. cm^2/decimole.

fluorescence is insensitive to its environment (Neinryb and Steiner, 1971).

Figure 3 presents the fluorescence intensity of the two deconvoluted peaks, at 303 and 345 nm as a function of *p*H. The *p*H profile for the 345 nm peak (triangle) closely resembles that of the 344 nm intensity (Fig. 2) and that for the 303 nm peak (circle) is similar to the 308 nm/344 nm ratio, supporting the previous conclusions that the tryptophan quenching occurs between *p*H 7 and 4 and the tyrosine fluorescence appears below *p*H 6 and increases as the *p*H is decreased. The midtransition *p*H for the tryptophan quenching is approximately 5.8, while the conformational changes characterized by tyrosine fluorescence have a midtransition *p*H of 4.5. These results suggest that conformational changes induced by *p*H involve at least two reactions: one leading to tryptophan quenching with an apparent pK_a value of

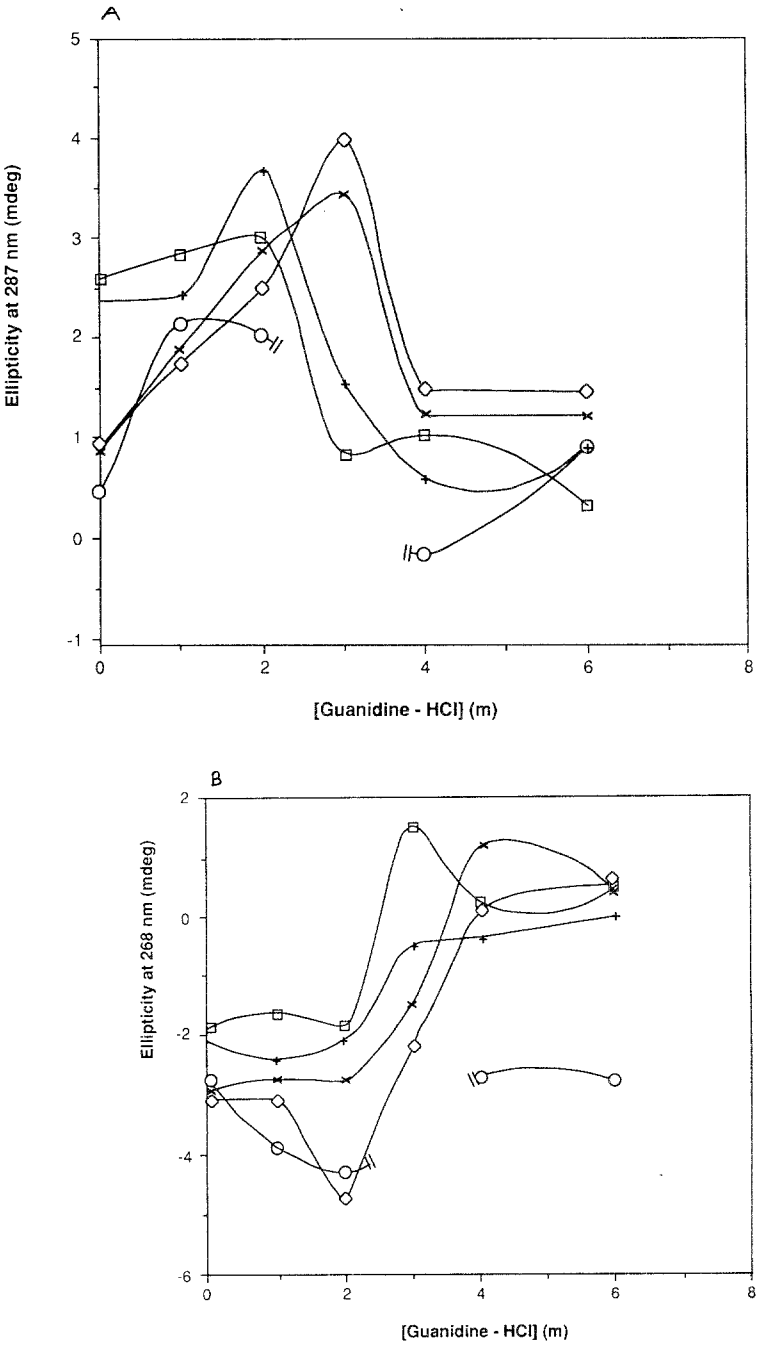

Fig. 5. Effect of GdnHCl concentration on the structure of G-CSF at several *p*H's. Samples of stock solution were diluted into buffered GdnHCl, allowed to incubate at 24°C for 10–15 min, and the CD spectra were then recorded. The ellipticities at the indicated wavelength were then determined. Symbols are: *p*H 7.5, (□); *p*H 6.3, (+); *p*H 4.5, (×); *p*H 3.3, (◇); *p*H 2.5, (○). A, ellipticity at 287 nm; B, ellipticity at 268 nm; C, ellipticity at 222 nm.

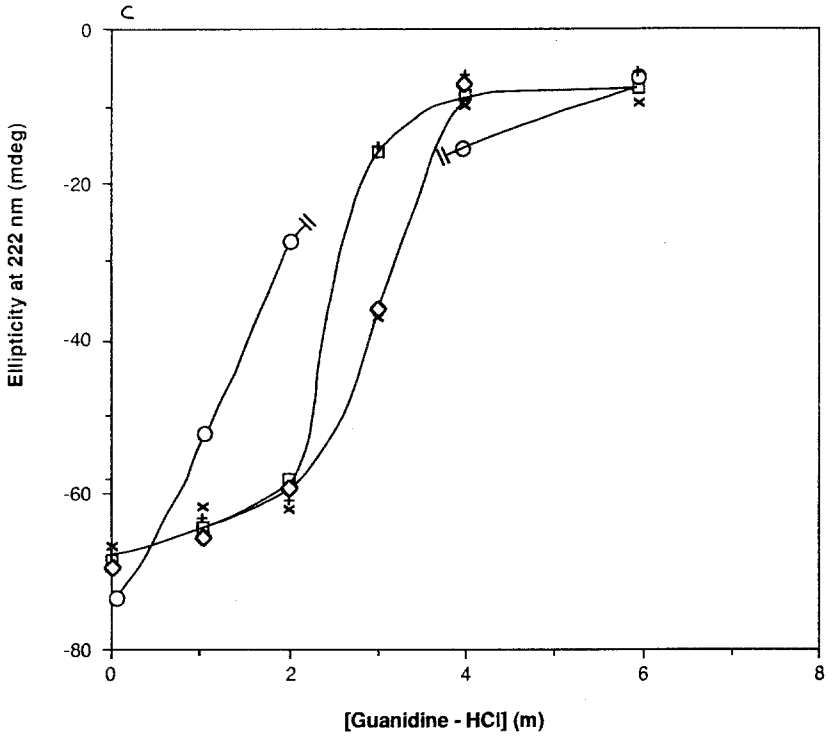

Fig. 5. Continued.

5.8, and the other leading to enhancement of tyrosine fluorescence with an apparent pK_a value of 4.5. These apparent pK_a values suggest the possible involvement of histidyl and carboxyl residues, respectively.

HP-GPC experiments were performed at protein concentrations used for fluorescence analyses. Between *p*H 7.1 and 2.5 (the *p*H range tested), the protein gave a single peak corresponding to its monomeric size; hence, interchain interactions would not explain the fluorescence changes observed.

To further assess the conformational change of the protein, near and far UV CD spectra of the protein were determined (Fig. 4A and B). The far UV spectra (panel A) show increases in the ellipticity between 208 and 222 nm as the *p*H is lowered, with the apparent α-helicity of the protein increasing from 66% α-helix at *p*H 7.5, to 75% α-helix at *p*H 4.5, to 84% α-helix at *p*H 2.5, in agreement with earlier reports (Wingfield *et al.*, 1988; Lu *et al.*, 1989). The near UV spectra, shown in Fig. 4B, also indicate slight changes in the conformation of the protein. The CD intensities become gradually more negative as the *p*H is decreased from 7.4 to 4.5 with little change between *p*H 4.5 and 2.5. However, fine structures for aromatic residues remain unchanged in these spectra, indicating no gross conformational changes of the protein. Thus, the conformational change is local and related to an alteration of the environment for a limited number of aromatic groups.

Comparison of the above results with those given in Fig. 3 shows a similarity to the tryptophan quenching profile, but not to the tyrosine fluorescence change. This suggests that the CD spectral change in the near UV region might correlate with the alteration in the tryptophan environment.

Since the conformation of G-CSF is *p*H-dependent, the addition of denaturant might result in either a different course of denaturation at different *p*H values, or could cause the protein to first converge into the same structure followed by a common major conformational transition. To test this, we followed the course of GdnHCl-induced denaturation of G-CSF at *p*H 2.5, 3.3, 4.5, 6.3, and 7.5 using both CD and fluorescence.

The near UV CD spectra of G-CSF goes through several *p*H-dependent changes before tertiary structure is lost. This is illustrated in Fig. 5, where the ellipticity at two different wavelengths (panels A and B) has been plotted vs. GdnHCl concentration for each *p*H. The ellipticity at 287 nm (panel A) corresponds to the first maxima in the CD spectra. At *p*H 7.5, there is a slight increase in ellipticity as the GdnHCl concentration is increased from 0–2 M, and then a decrease in the signal as the concentration of

denaturant is further increased. At *p*H 6.5, the increase in ellipticity at 2 M GdnHCl is larger while the same trends are followed. At *p*H 4.5, the ellipticity continues to increase with the addition of 3 M GdnHCl and then decreases rapidly with 4 M GdnHCl. At *p*H 3.3, the maximum ellipticity is even more clearly shifted to 3 M GdnHCl. At *p*H 2.5, the protein precipitated at intermediate GdnHCl concentrations, but the initial curve appears to be different than that seen at the other *p*H's. The ellipticity at 267 nm (the minima in the native CD spectra, panel B) shows similar *p*H-dependent trends in the GdnHCl-induced spectra. From these results it appears that the course of GdnHCl-induced denaturation of G-CSF is very complex and *p*H-dependent.

The course of GdnHCl-induced denaturation of the secondary structure of G-CSF at the five *p*H values was also studied. Panel C in Fig. 5 shows the change in ellipticity at 222 nm with GdnHCL concentration. The loss of secondary structure appears to be a cooperative event, and the protein appears to be most stable at *p*H 3.3–4.5. The GdnHCl concentrations at which half of the structure remains are 2.4 M at *p*H 7.5 or 6.3, 2.9 M at *p*H 4.5 or 3.3, and less than

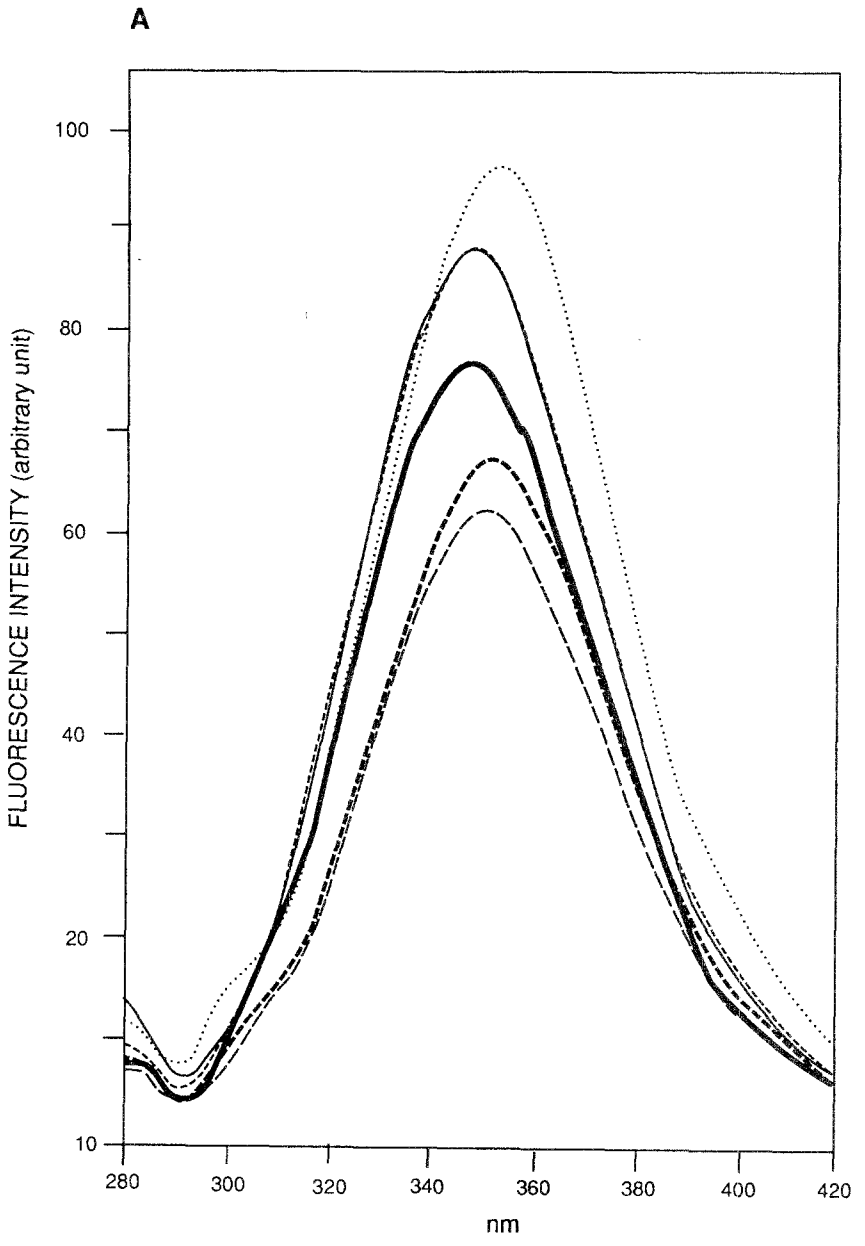

Fig. 6. Fluorescence spectra of G-CSF as a function of GdnHCl concentration. Aliquots of stock solution were diluted to 0.21 mg/ml in buffered GdnHCl, incubated at 24°C for 10 min. The sample was excited at 280 nm and the emission-spectrum recorded. Lines are: ——,0 M GdnHCl; – – – –, 1 M; — —, 2 M; — · —, 3 M; ■ ■ ■, 4 M; · · · ·, 6 M.A, *p*H 7.5; B, *p*H 3.3.

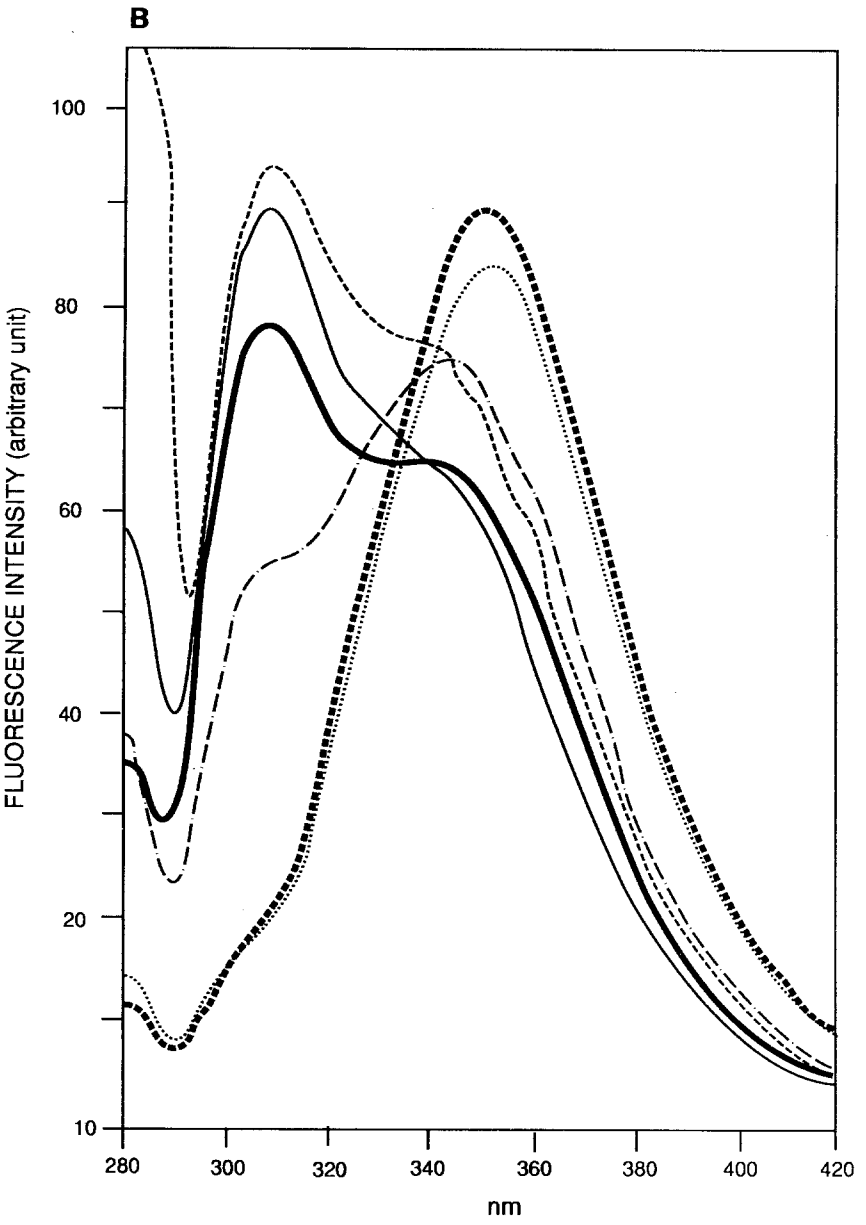

Fig. 6. Continued.

2 M at *p*H 2.5. Identical results are obtained when the ellipticity at 208 nm is monitored. This is consistent with the near UV CD results; at *p*H 4.5 and 3.3 significant ellipticity is present at 3 M GdnHCl, while there was a decreased signal at this concentration of denaturant at *p*H 7.5 and 6.3, and at *p*H 2.5 the protein had denatured and precipitated. Thus, while the loss of tertiary structure is complicated and appears to involve more than a two-step reaction, the loss of secondary structure appears to be much simpler.

The effect of GdnHCl on the fluorescence spectra of G-CSF was determined in order to confirm that the GdnHCl-induced denaturation of G-CSF involves *p*H-dependent intermediate structures. Figure 6 shows the changes induced in the spectra at *p*H 7.5 (A) and 3.3 (B). Again, it appears that intermediates are involved. At *p*H 7.5, addition of 2 M GdnHCl results in slight enhancement of the intensity of fluorescence at 344 nm, while the addition of 3 M GdnHCl results in a shift in emission maximum to 348 nm, indicating that the fluorescent tryptophan(s) are more solvent-exposed. Similar results were obtained at *p*H 6.3. At *p*H 3.3, the addition of 1 or 2 M GdnHCl caused a small enhancement in the tyrosine fluorescence, and a

concomitant decrease in the tryptophan fluorescence. Strong enhancement of the tryptophan fluorescence accompanied by the disappearance of the tyrosine fluorescence occurs as the GdnHCl concentration is increased, until at 4 M GdnHCl the spectrum resembles that seen at *p*H 7.5. Similar trends were seen at *p*H 4.5 and 2.5. These results confirm those obtained by the CD, and indicate that both the fluorescent tryptophan(s) and tyrosine(s) are in different environments in the unfolding intermediates than in the native molecule.

In conclusion, G-CSF appears to undergo *p*H-dependent changes in local conformation, leading to fluorescence changes for either tryptophan, tyrosine, or both. Taken together, the *p*H-dependent changes in fluorescence and CD intensities indicate that at least two events are occurring: one which is detectable by the fluorescence and CD of tryptophan and has a midtransition *p*H of 5.8; and a second, occurring with a midtransition *p*H of 4.5, detectable by tyrosine fluorescence.

The GdnHCl-induced denaturation of G-CSF is a complex event involving a transition into a partially denatured structure at low GdnHCl concentrations (1 or 2 M), followed by a major conformational change involving loss of secondary and tertiary structure at GdnHCl concentrations above 2 M. The tertiary structures of the intermediate forms appear to be *p*H-dependent, and involve changes in both the tryptophan and tyrosine environments. G-CSF appears to be more stable to the major conformational change (as indicated by loss of secondary structure) at *p*H 4.5 and 3.3 than at either higher or lower *p*H values.

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