
pH Dependence of structural stability of interleukin-2 and granulocyte colony-stimulating factor

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Abstract

After a cytokine binds to its receptor on the cell surface (pH ~7), the complex is internalized into acidic endosomal compartments (pH ~5–6), where partially unfolded intermediates can form. The nature of these structural transitions was studied for wild-type interleukin-2 (IL-2) and wild-type granulocyte colony-stimulating factor (G-CSF). A noncoincidence of denaturation transitions in the secondary and tertiary structure of IL-2 and tertiary structural perturbations in G-CSF suggest the presence of an intermediate state for each, a common feature of this structural family of four-helical bundle proteins. Unexpectedly, both IL-2 and G-CSF display monotonic increases in stability as the pH is decreased from 7 to 4. We hypothesize that such cytokines with cell-based clearance mechanisms *in vivo* may have evolved to help stabilize endosomal complexes for sorting to lysosomal degradation. We show that mutants of both IL-2 and G-CSF have differential stabilities to their wild-type counterparts as a function of pH, and that these differences may explain the differences in ligand trafficking and depletion. Further understanding of the structural changes accompanying unfolding may help guide cytokine design with respect to ligand binding, endocytic trafficking, and, consequently, therapeutic efficacy.

Keywords: Protein folding; protein stability; equilibrium denaturation; folding intermediates; endocytic trafficking; IL-2; G-CSF

Interleukin-2 (IL-2) and granulocyte colony-stimulating factor (G-CSF) are members of the four-helical bundle family of proteins, having a structure similar to that of human growth hormone (hGH), granulocyte-macrophage colony-stimulating factor, erythropoietin, leptin, interferon- β , megakaryocyte growth and development factor (MGDF), and other cytokines (Brandhuber et al. 1987; Hill et al.

1993). For some of these proteins, physical stability is a problem for long-term storage and delivery. Precipitation and loss of protein during refolding, purification, storage, or delivery has been previously studied for many of these proteins, including IL-2 (Marston 1986; Brems 1988; Schein 1990; DeYoung et al. 1993; Vlasveld et al. 1993; Prestrelski et al. 1995; Tzannis et al. 1996). Conformational studies of G-CSF, hGH, and MGDF as a function of pH and denaturant concentration have shown the presence of folding intermediate states (Narhi et al. 1991; Defelippis et al. 1993; Hamburger et al. 1998). Understanding the mechanism of the structural changes that accompany protein unfolding and precipitation may lead to improved formulations for similar therapeutic proteins.

The structure, stability, and interaction of the IL-2 molecule and its receptor are also critical factors in the regulation of cellular trafficking. The IL-2 receptor is composed of

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α , β , and γ subunits, and mutational analysis of the ligand has identified that residues are involved in receptor subunit binding (Berndt et al. 1994; Chang et al. 1995). The α -subunit binding site is located in the short 3_{10} helix of the ligand, the β subunit is bound by the amino-terminal helix, and the γ -subunit binding site is in the carboxy-terminal helix (Fig. 1A). Cellular trafficking studies have shown that the α subunit of the receptor is recycled from endosomal compartments, whereas the $\beta\gamma$ subunit complex is routed to the lysosomes for degradation. Mutations in the ligand that disrupt binding to the $\beta\gamma$ subunits or increase the affinity to the α subunit can result in greater ligand recycling and enhanced mitogenic potency without changes to either the extracellular binding affinity or the internalization rate relative to wild-type IL-2 (Collins et al. 1988; Hemar et al. 1995; Fallon et al. 2000). One of these analogs has mutations L18M and L19S located in the amino-terminal helix near a site known to be essential for binding to the β subunit

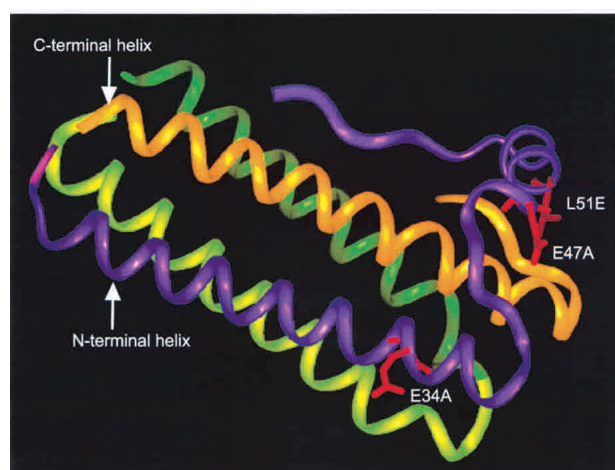
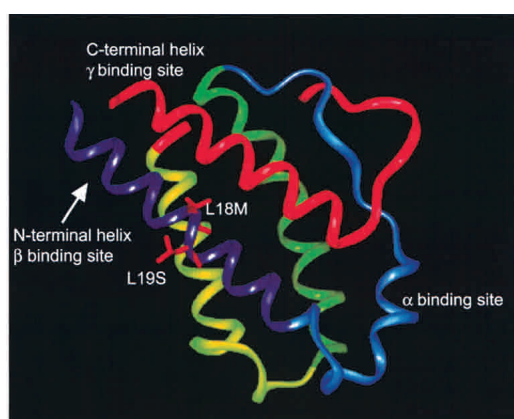


Figure 1. (A) Ribbon diagram of IL-2. Note the binding site of the α subunit of the receptor to the first loop region, the β subunit to the amino-terminal helix, and the γ subunit to the carboxy-terminal helix. IL-2 analog mutations L18M and L19S, which are found in the β -subunit binding site, are also shown. (B) Ribbon diagram of G-CSF. Note the locations of the mutation E34A in the amino-terminal helix A, and E47A and L51E in the 3_{10} helix segment in the long AB loop just after helix A.

of the receptor (Fig. 1A; Collins et al. 1988). This mutant may have a greater endosomal affinity for the recycled α subunit of the receptor or a lower intracellular affinity for the $\beta\gamma$ receptor complex, which undergoes degradation in the lysosomes (Hemar et al. 1995).

Conformational studies of G-CSF have shown that the molecule undergoes structural changes induced by pH, heat, and guanidine hydrochloride (Narhi et al. 1991; Kolvenbach et al. 1993, 1997). Over a pH range from 2.0 to 7.0, G-CSF retains its native-like helical structure. Although the tertiary structure differs at pH 2.0, 4.0, and 7.0, the maintenance of a distinct tertiary structure and the compactness at pH 2.0 suggest that the molecule does not adopt a partially unfolded “A” state in acid (Kolvenbach et al. 1997). At pH 4.0 and 7.0, G-CSF loses its secondary and tertiary structures at the same point during thermal denaturation, and at pH 2.0 a portion of the helical structure unravels at a slightly lower temperature than the cooperative unfolding of the remaining secondary structure and tertiary structure.

Several mutational studies have identified certain amino acids in G-CSF that are involved in receptor binding and dimerization, as well as overall bioactivity (Reidhaar-Olson et al. 1996; Li et al. 1997; Young et al. 1997; J.S. Philo, T. Arakawa, L.O. Narhi, T.S. Li, K. Lowenhaupt, M. Mann, T. Osslund, D. Yui, D.A. Lauffenburger, and T. Horan, unpubl.). Some of the single site G-CSF mutants have different binding and trafficking properties from wild type, resulting in altered potencies in vitro (Sarkar et al. 2002, 2003). For example, mutant L51E binds the G-CSF receptor with lower affinity than wild-type G-CSF, yet elicits a better cellular response, suggesting that the mutant elicits enhanced signaling or reduced degradation. Another mutant, E47A binds roughly the same as wild-type G-CSF, yet elicits a significantly worse cellular response, acting as a partial agonist. Conversely, mutant E34A also binds similarly to wild-type G-CSF and elicits roughly the same cellular response at high ligand concentrations, yet elicits a twofold increase in cellular response at ligand concentrations near the K_d , which suggests enhanced trafficking properties of this mutant. The mutation E34A is located in the amino-terminal helix A, and E47A and L51E are in the 3_{10} helix segment in the long AB loop just after helix A (Fig. 1B). This 3_{10} helix segment is thought to be involved in receptor binding (Li et al. 1997).

For cytokines that have primarily cell-based clearance mechanisms, the extent of endosomal ligand sorting to recycling versus degradation may be controlled by structural or stability properties at low pH. Previous work in evaluating the thermodynamic stability of G-CSF at neutral pH was performed to identify mutations with enhanced stability (Bishop et al. 2001). This series of equilibrium denaturation experiments investigated the unfolding transitions of IL-2 and G-CSF as a function of pH to characterize structural or stability properties that may alter trafficking within the pH range encountered in the cellular compartments.

Results

Because IL-2 and G-CSF encounter a pH environment ranging from the neutral extracellular space to the acidic endosomal compartments, the structure and stability of these proteins were examined as a function of pH. Equilibrium denaturation studies were performed to characterize the structure and stability of IL-2 and G-CSF. Circular dichroism (CD) and intrinsic fluorescence were monitored as a function of pH or denaturant concentration to provide information on changes in structure or stability. Comparison of the denaturation transitions, as measured by CD and fluorescence, may elucidate the nature of these structural changes.

Structural analysis as a function of pH

The secondary structures of IL-2 and G-CSF were analyzed by CD. Far-UV CD data indicated that the proteins displayed similar helical content across a pH range of 4 through 7 (data not shown). No apparent changes in secondary structure were found as a function of pH within the physiological pH range of cytokine trafficking. Tertiary structure was also studied as a function of pH using intrinsic fluorescence as a conformational probe. For IL-2, an increase in intrinsic fluorescence occurred below pH 4 (Fig. 2A). No shift in the fluorescence emission wavelength maximum was observed, and therefore, only the intensity of the fluorescence emission was reported. Additional data showed that this transition was accompanied by an increase in hydrophobicity as measured by 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence.

In contrast, G-CSF displayed a significant decrease in intrinsic fluorescence with decreasing pH (Fig. 2B). The pH-induced change in intrinsic fluorescence was accompanied by increased near-UV CD signal as a function of pH (data not shown). This could be due to local charge changes near the tryptophan residues as the neighboring histidine residues are titrated (H156–W58, H79–W118).⁶ In addition, slight tertiary structural conformational changes may contribute to the altered fluorescence and near-UV CD signals as a function of pH (Narhi et al. 1991; Kolvenbach et al. 1993).

Equilibrium denaturation of IL-2

The stability of IL-2 was determined by measuring the secondary structural signal as a function of guanidine hydrochloride (GuHCl) concentration. The far-UV CD signal at 222 nm was followed as a measure of helical structure during titrations of GuHCl (Fig. 3A). These titrations were performed at pH values between 7 and 4 to mimic the trans-

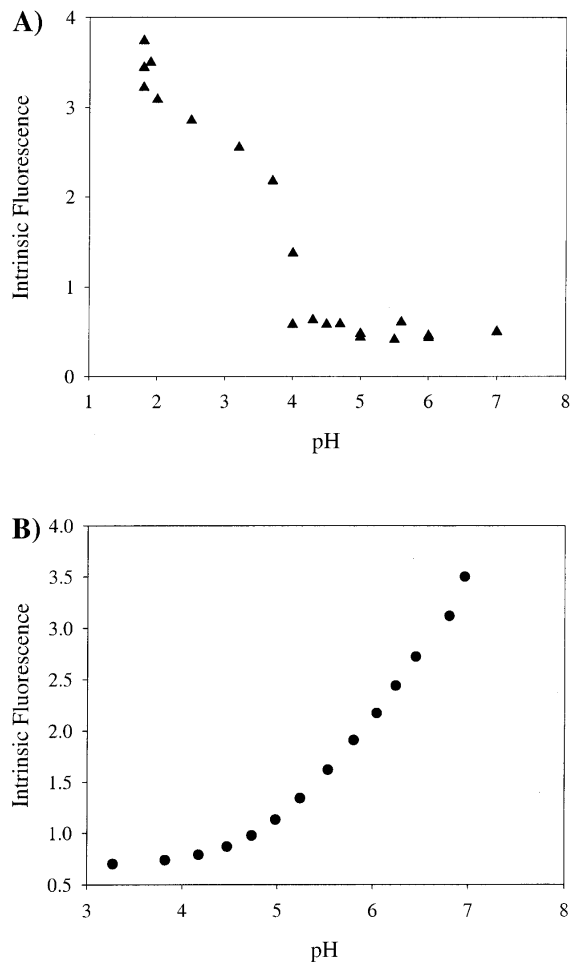


Figure 2. (A) Intrinsic fluorescence of IL-2 (triangles) as a function of pH. A pH titration was performed over a pH range from 2 to 7 at a protein concentration of 0.05 mg/mL, and the fluorescence intensity was recorded at an excitation wavelength of 280 nm and emission wavelength of 320 nm. (B) Intrinsic fluorescence of G-CSF (circles) as a function of pH. A pH titration was performed over a pH range of 3 to 7 at a protein concentration of 0.025 mg/mL, and the fluorescence intensity was recorded at an excitation wavelength of 280 nm and emission wavelength of 350 nm.

port of IL-2 from the cell surface to the lysosomes. IL-2 displayed greater secondary structural stability at low pH.

In addition, tertiary structural transitions were characterized for IL-2 by monitoring intrinsic fluorescence as a function of denaturant concentration. The emission maximum was chosen as a means of following the tertiary structural transition rather than the intensity of fluorescence. IL-2 displayed a sloped baseline of increased intrinsic fluorescence at low GuHCl concentrations due to the solvent effects. As a function of increased denaturant levels, a pronounced non-linear increase or peak in fluorescence intensity occurred at moderate denaturant levels (3.5–4.5 M GuHCl), suggesting the presence of a folding intermediate of greater fluorescence than the folded or unfolded states. At relatively high concentrations of denaturant, the fluorescence intensity de-

⁶Note N-terminal methionine not counted in numbering residues.

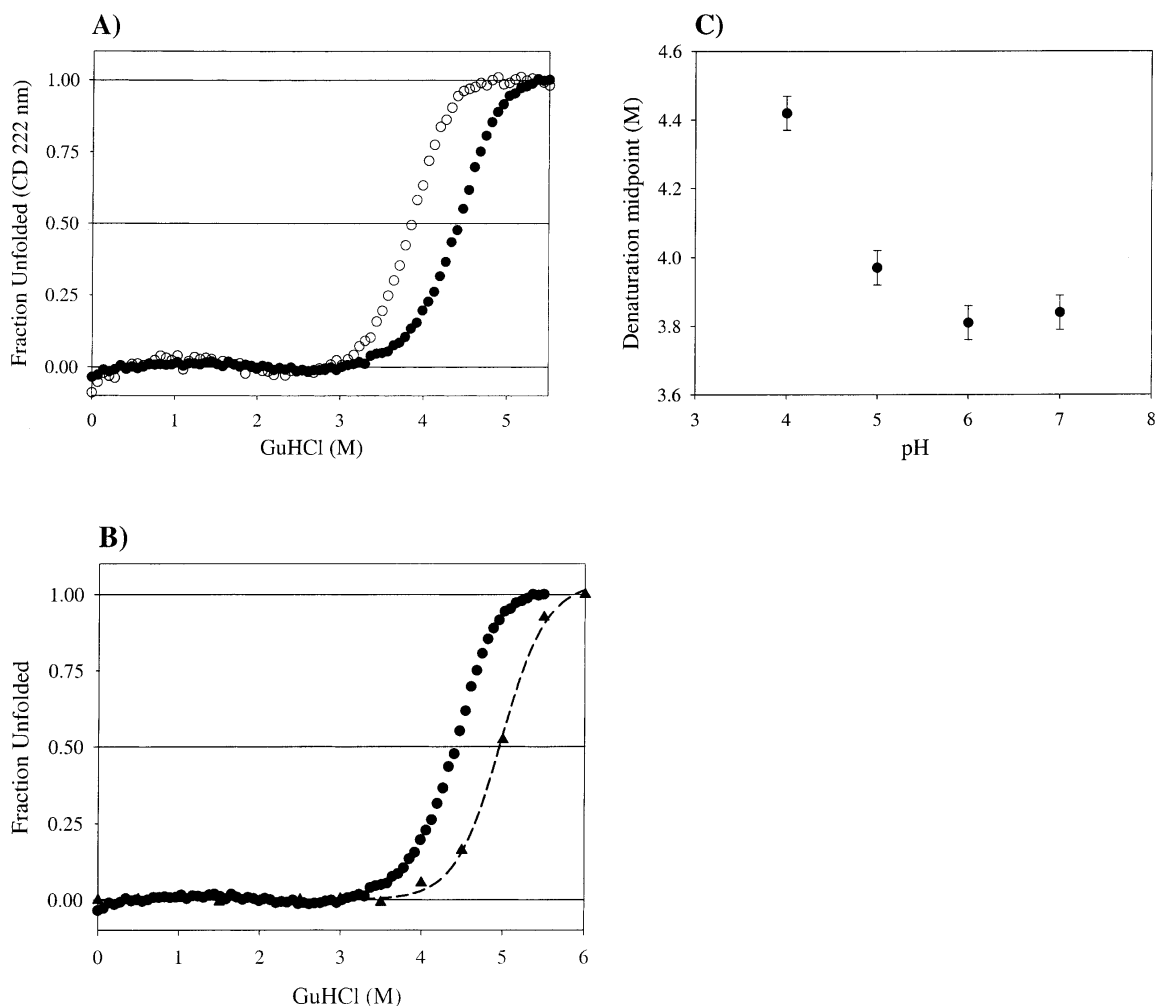


Figure 3. (A) Secondary structural transitions as determined by equilibrium denaturation, monitoring the far-UV CD signal at 222 nm as a function of GuHCl concentration for IL-2. IL-2 was at a protein concentration of 0.05 mg/mL at pH 4 (filled circles) through pH 7 (open circles). (B) Far UV-CD data at 222 nm (filled circles) and intrinsic fluorescence (triangles) of wild-type IL-2 as a function of GuHCl. All samples were at a protein concentration of 0.05 mg/mL in pH 4 buffer. (C) The CD denaturation midpoints for wild-type IL-2 (filled circles) for pH 4 through 7. Error bars represent the reproducibility of the denaturation transitions for multiple experiments.

creased as the emission maximum shifted to longer wavelengths. This red shift in emission maximum was consistent with the solvent exposure of the lone tryptophan residue (W121) from within the buried hydrophobic core. The fraction unfolded, based on emission maximum wavelengths, was plotted versus GuHCl concentration for this cytokine (Fig. 3B).

Comparison of the secondary and tertiary structural transitions revealed a noncoincidence of signals (Fig. 3B), and the loss of helical structure occurred at a lower denaturant concentration than the exposure of the hydrophobic core. Therefore, under partially denaturing conditions, a folding intermediate was populated that had an intact hydrophobic core and disrupted helical structure. This suggested that the unfolding occurred through a multistate transition, which was a pathway more complicated than a two-state (native \leftrightarrow denatured) model.

Analysis of the denaturation midpoints from the CD data indicated that the secondary structural transition for IL-2 was dependent on pH, with increased stability under acidic conditions (Fig. 3C).

Equilibrium denaturation of G-CSF

Equilibrium denaturation studies with G-CSF were also completed at pH values ranging from 4 to 7. Secondary structural transitions monitored by far-UV CD signal at 222 nm are shown for pH 4 and pH 7 data in Figure 4A. Like IL-2, G-CSF displayed increased stability under acidic conditions.

Figure 4B shows the intrinsic fluorescence data for the equilibrium denaturation transitions at pH 4 and pH 7. Similar to the secondary structural data, the tertiary structure was significantly more stable at acidic pH. Measurements at pH 6 and pH 7 were more difficult to make because of protein

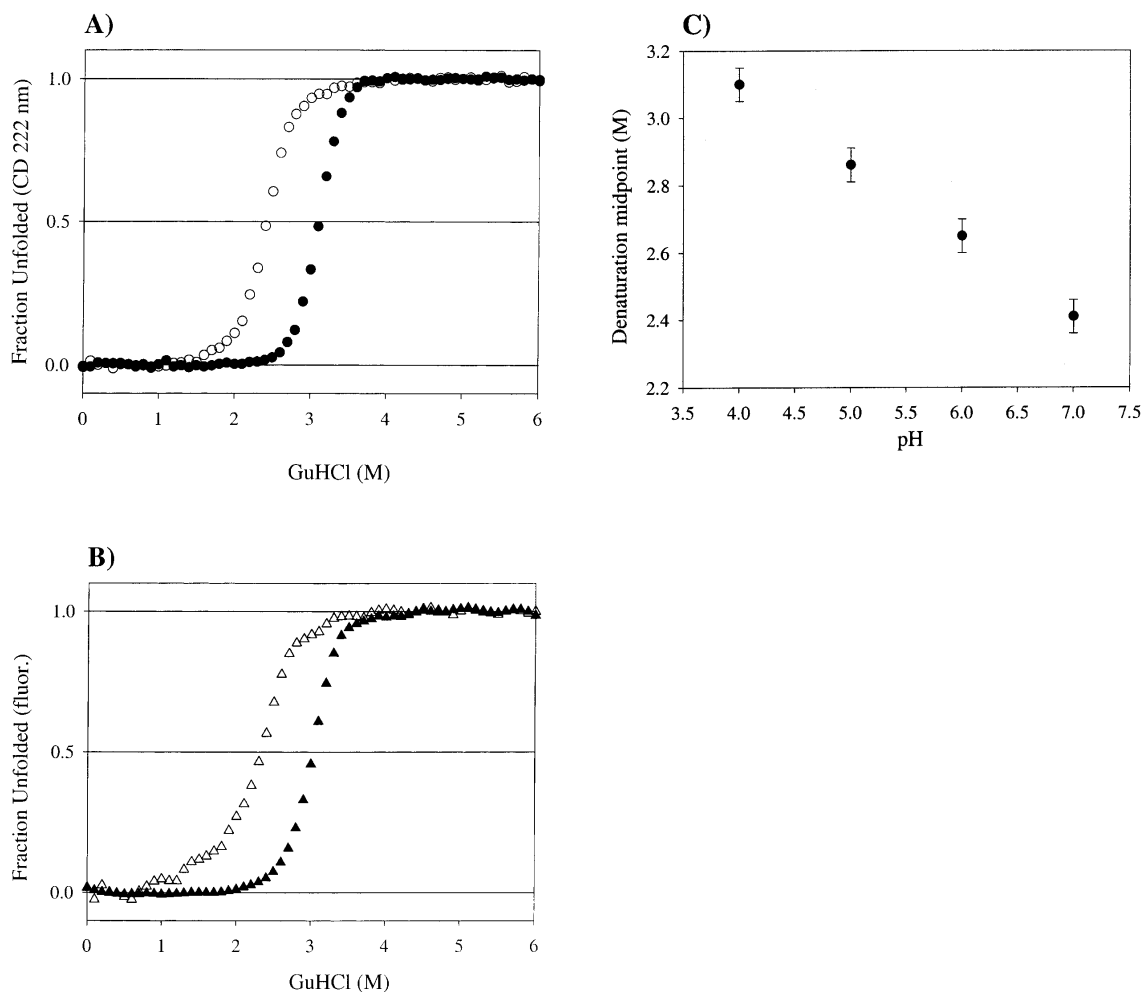


Figure 4. (A) Secondary structural transitions as determined by equilibrium denaturation, monitoring the far-UV CD signal at 222 nm as a function of GuHCl concentration for G-CSF. G-CSF was at a protein concentration of 0.025 mg/mL at pH 4 (filled circles) through pH 7 (open circles). (B) Equilibrium denaturation of G-CSF followed by intrinsic fluorescence (excitation 280 nm, emission 350 nm), at pH 4 (filled triangles) through pH 7 (open triangles). (C) The CD denaturation midpoints for G-CSF at pH 4 through pH 7.

instability and baseline artifacts. The CD and fluorescence-detected transitions (Fig. 4A versus 4B) were similar and within the error of the measurement. Generally, the denaturation midpoint by fluorescence was slightly lower but within 0.05 M GuHCl units of the CD denaturation midpoint. Both the CD and fluorescence data indicated that G-CSF had increased structural stability at low pH (CD data shown in Fig. 4C).

Effect of mutations

Structural studies were also conducted on mutants of IL-2 and G-CSF with altered ligand depletion and trafficking properties (Fallon et al. 2000; Sarkar et al. 2003). The IL-2 analog chosen for these studies had mutations L18M and L19S in the amino-terminal helix near a site known to be essential for binding to the β subunit of the receptor (see

Fig. 1A; Collins et al. 1988). This mutant was found to have similar extracellular receptor binding affinity, but reduced affinity to the β subunit of the receptor in endosomal compartments, resulting in increased ligand recycling and longer half-life in culture (Fallon et al. 2000).

In fluorescence measurements as a function of pH, the pH-induced transition in tertiary structure was similar for both wild-type IL-2 and the double mutant. No observable secondary structural changes occurred for either protein in the pH range of interest between the extracellular pH of 7 and the lysosomal pH of 4 (wild-type data in Fig. 2A).

In GuHCl equilibrium denaturation studies at neutral pH, the secondary structural transition for the wild-type IL-2 was similar to that of the IL-2 analog. However, at pH 4, the secondary structural transition midpoint occurred at a lower denaturant concentration with corresponding lower apparent ΔG of unfolding for the mutant IL-2 compared to that of the

wild-type molecule (Table 1, IL-2 CD data). Therefore, the analog had comparable stability to wild-type IL-2 at neutral pH, but was less stable than wild-type IL-2 under acidic conditions. For the mutant IL-2 at pH 4, the tertiary structural transition measured by fluorescence was not coincident with the CD signal transition (midpoints of 4.5 M and 4.0 M GuHCl, respectively), similar to the wild-type data shown in Figure 3B (midpoints of 4.8 M and 4.4 M GuHCl, respectively). In effect, the difference in CD and fluorescence-detected transitions for wild-type IL-2 was maintained and shifted to lower GuHCl concentrations for the IL-2 analog. Although denaturation midpoints for fluorescence were estimated based on the shift in wavelength maximum, no values for ΔG of unfolding were reported because the fluorescence intensity data were more complex than a simple two-state model required for thermodynamic analysis.

For G-CSF, structural analysis was conducted on several single-point mutants having altered depletion profiles in endocytic trafficking studies (Sarkar et al. 2003). The single-site mutants examined included E34A, E47A, and L51E. The mutants displayed differences in the pH-induced change in intrinsic fluorescence (wild-type data shown in Fig. 2B). The result suggested that the local charge effects or conformational changes occurred to different extents in the wild type and mutants as a function of pH.

In GuHCl equilibrium denaturation studies, the mutants displayed a progressive increase in secondary structural stability under acidic conditions, similar to the wild-type data shown in Figure 4C (Table 1, G-CSF CD data). There were slight differences in stability among the various analogs at each pH, and generally the ranking of relative stabilities among the analogs was fairly consistent at each pH (4, 5, 6, 7).

Likewise, fluorescence measurements indicated that the mutants displayed increased tertiary structural stability un-

der acidic conditions compared to neutral pH (Table 1, G-CSF fluorescence data). Like the CD data suggested, minor differences in fluorescence-detected stability existed among the different analogs, and the ranking of relative stabilities was generally consistent across the full pH range and was compatible with the CD data.

Discussion

Increased stability at low pH is an unusual property for small monomeric proteins. In fact, many such proteins undergo conformational destabilization under acidic conditions. Previous studies of ~20 monomeric proteins have indicated that these proteins fall in distinct categories displaying certain acid unfolding transitions that depend on the type of protein and the buffer conditions (Fink et al. 1994). In particular, a partially unfolded conformer may exist at pH 3–4, and lower pH values may populate the acid unfolded “A” state, which is a looser structure of the molten globule state. The populations of these conformers and the nature of the acid denaturation transitions are affected by salt, denaturant, and temperature.

Role of intermediates in protein folding

During the early kinetic stages of refolding of many small globular proteins, a hydrophobic collapse occurs as the extended polypeptide chain protects its hydrophobic residues from exposure to the aqueous environment (Kim and Baldwin 1990). In the equilibrium denaturation of IL-2, the partially unfolded state is similar to this collapsed form with an intact hydrophobic core but without defined native-like structure. Thus, the denaturation experiments may identify equilibrium conditions that populate species that resemble kinetic intermediates in the refolding pathway, such as the

Table 1. Denaturation midpoint and apparent ΔG of unfolding at pH 4 and 7 calculated from CD data (222 nm) for wild-type IL-2 and L18M/L19S mutant, CD data (222 nm) for wild-type G-CSF and analogs E34A, E47A, and L51E, and intrinsic fluorescence data (excitation 280 nm, emission 350 nm) for G-CSF and analogs

Data	Denaturation midpoint (M) ^a				ΔG_{unf} (kcal/mole) ^a			
IL-2 CD								
pH	wt	L18M/L19S			wt	L18M/L19S		
4	4.4	4.0			9.9	9.5		
7	3.8	3.7			9.3	9.3		
G-CSF								
pH	wt	E34A	E47A	L51E	wt	E34A	E47A	L51E
4	3.1	3.0	3.0	2.9	11.9	9.8	9.8	9.6
7	2.4	2.4	2.3	2.3	7.8	8.6	6.5	8.3
G-CSF fluorescence								
pH	wt	E34A	E47A	L51E	wt	E34A	E47A	L51E
4	3.0	2.9	3.0	2.8	10.1	10.7	10.0	8.4
7	2.3	2.5	2.4	2.3	5.1	7.0	5.9	6.8

^a Multiple replicates and data fitting methods demonstrate error of measurement ± 0.05 M for denaturation midpoint and ± 0.5 kcal/mole for apparent ΔG_{unf} values.

hyperfluorescent IL-2 intermediate at moderate denaturant levels in the equilibrium denaturation studies. The location of the single tryptophan in IL-2 appears to be conveniently located to provide key spectral information regarding the hydrophobic core of an important equilibrium intermediate and possibly a critical early kinetic intermediate.

Structural studies on related cytokines have frequently identified a molten globule intermediate with partial disruption of tertiary structure and intact native-like secondary structure (Brems and Havel 1989; Ptitsyn et al. 1990; Cleland and Wang 1991; Hamburger et al. 1998). A difference that was evident for the IL-2 folding intermediate but less apparent for G-CSF was that the secondary structural transition occurred at a lower denaturant concentration than the disruption of tertiary structure, as indicated by the noncoincidence of the CD and fluorescence transitions. For the mutant IL-2 at pH 4, the CD and fluorescence-detected transitions were also noncoincident and occurred at lower denaturant concentrations than the respective wild-type transitions. Because the noncoincidence of structural transitions was maintained and shifted to lower GuHCl concentrations for the mutant, this suggested that the IL-2 analog had decreased stability in acid compared to the wild-type molecule in both the native and intermediate states.

In comparing changes in secondary and tertiary structure, the exact location of the tryptophan moieties is critical. The probe for monitoring tertiary structure of IL-2 is a single tryptophan residue in the hydrophobic core. The sole tryptophan residue may reflect the environment of a highly stable core region that is more stable than certain helical segments. In addition, subtle tertiary structural changes that are undetected by the fluorescence of the sole tryptophan residue may occur at a lower denaturant concentration than that for the disruption of helices. For G-CSF, there are two tryptophan residues, each of which resides in close proximity to histidines (H156–W58, H79–W118). Subtle tertiary structural changes or local charge changes as the histidine residues titrate near neutral pH may affect the intrinsic fluorescence and near-UV CD spectral properties.

Role of acid stability in cellular trafficking

Both G-CSF and IL-2 display increased stability at low pH, perhaps because these molecules have significant cell-based clearance mechanisms. In contrast, this increase in stability is not observed for hGH, a 4-helical-bundle cytokine that does not have a dominant cell-based clearance mechanism *in vivo*. Increased structural stability at low pH ensures that these molecules remain bound to the receptor (intact G-CSF receptor or $\beta\gamma$ -subunit complex of the IL-2 receptor) within the acidic endosomal environment. The ligand therefore gets degraded in the lysosomes as part of an optimized cell-based clearance mechanism.

However, for applications as therapeutic proteins, the native ligands may be improved by engineering for superior endocytic trafficking properties (Lauffenburger et al. 1998; Fallon et al. 2000; Sarkar et al. 2002, 2003). The role of pH in endocytic trafficking of proteins within cells is critical for many systems; the dependence of receptor-ligand affinity on pH balances the amounts of ligand and receptor that are recycled to the extracellular fluid, degraded in the lysosomes, or transported across the plasma membrane (Maier and Steverding 1996). These processes are contingent on exquisite recognition between the ligand and receptor, and even slight differences in conformational stability may have significant effects on trafficking. In a system where the number of ligands per cell is not limiting (French and Lauffenburger 1997), mutants with lower stability relative to wild type may dissociate from the receptor complex or from certain receptor subunits under acidic conditions, and thereby reduce exposure to the lysosomal proteases and enhance recycling to the extracellular space. For G-CSF, this would involve disruption of the 2:2 G-CSF:receptor complex by mutating residues that lie at the major binding interface (Aritomi et al. 1999). For IL-2, the binding site to the β subunit of the receptor should be disrupted in the endosomal compartments.

Possible role for structural stability in altering endocytic trafficking

Analysis of the effects of mutations on stability and trafficking properties can elucidate the role of conformational stability in endocytic trafficking. The set of mutants in these studies does not conclusively prove the direct correlation between stability and trafficking, but the equilibrium denaturation results provide supporting data consistent with the correlation between increased conformational stability in acid and clearance.

Of all the mutants examined in these structural stability studies, the IL-2 analog L18M/L19S had the most pronounced difference in stability from the wild-type molecule as a function of pH. The mutations in the IL-2 analog did not cause any identifiable structural changes, but rather a decrease in stability compared to wild-type IL-2 in acid. The analog had a lower denaturation midpoint of unfolding in acid, resulting in an altered equilibrium between the folded and unfolded forms of the molecule. The differences in stability between wild-type IL-2 and the mutant as a function of pH may be related to the modified trafficking.

Although differences in stability were observed between the wild type and mutants for IL-2 and G-CSF, the molecular mechanism by which the mutations confer altered stability across a pH range is unknown. The L18M/L19S analog of IL-2 does not introduce any ionizable residues that would disrupt ligand binding to the receptor subunits when the complex encounters the acidic endosomal compart-

ments. Likewise, most of the G-CSF mutations involve alanine substitutions, except L51E. Although glutamate is ionizable (pK_a of ~ 4.5), it is located on the side of the molecule opposite the receptor-binding site, and therefore, titration of this group does not directly affect binding affinity. However, detailed binding affinity measurements must be performed to determine the effect of the acidic environment on the binding of the wild-type molecules and the analogs to the corresponding receptor or receptor subunit.

In addition to binding differences, structural perturbations at low pH may also affect the susceptibility to proteolysis in the lysosomes. Because the IL-2 analog L18M/L19S and G-CSF single site mutants L51E and E34A were less stable than the wild-type molecules at low pH, one would expect the variants to be more susceptible to proteolysis. However, data indicate that these analogs elicit greater recycling and reduced degradation of ligand compared to the wild-type molecules (Fallon et al. 2000; Sarkar et al. 2003). The other G-CSF analog having lower stability than wild type (E47A) at endosomal conditions also had lower stability at extracellular pH, suggesting potentially greater depletion of protein from the medium that may temper any beneficial effects of lower stability at endosomal pH. Correspondingly, we observed that E47A acts as a partial agonist in eliciting a reduced cellular response (Sarkar et al. 2003). Slight differences in the free energy of unfolding of the mutants in acid can lead to significant changes in membrane interactions and receptor-ligand binding affinity. Because only stability differences rather than structural changes in acid were observed, it is likely that interactions with either receptor subunits or other cellular components play a role in protecting the analog from degradation.

The dramatic effect of specific amino acid mutations on protein folding and stability has been documented for several proteins (Brems and Havel 1989; Ptitsyn et al. 1990; Cleland and Wang 1991). Minor changes in the stability and solubility of partially folded polypeptide chains can have significant effects on the global properties of the protein. The IL-2 and G-CSF results described in these studies underscore the specificity of mutations in regulating protein stability, receptor-ligand interactions, and trafficking of cytokines. Slight differences in stability among the mutants are conserved across the pH range, and these differences may contribute to the differences in trafficking. The overall increase in stability at low pH may allow sufficient structural stability to preserve binding to the receptor leading to degradation in the lysosomes. If acid unfolding were to occur, disruption of the receptor-ligand complex may result in the free ligand being recycled to the cell surface. For proteins that have primarily cell-based clearance mechanisms, the wild-type molecule may be designed for optimal lysosomal degradation. Mutants that tweak the balance between lysosomal degradation and recycling may display significant improvements in overall local pharmacokinetics.

By engineering therapeutic proteins, the resulting analogs may prove to have greater stability, solubility, or efficacy.

Materials and methods

Materials

Recombinant human IL-2 (C125A) and G-CSF were produced in *Escherichia coli* at Amgen, Inc. Additional C125A IL-2 (termed wild-type IL-2) and IL-2 analog L18M/L19S were produced and purified as previously described (Berndt et al. 1994). The purified IL-2 was stored in a formulation buffer of 10 mM sodium acetate at pH 4. The G-CSF variants that were studied included wild type and single-site mutants E34A, E47A, and L51E. (Note that residue numbering includes the additional N-terminal methionine associated with *E. coli* expression.) Purified wild-type G-CSF was stored in 0.58 mM HCl at pH 3.0, and the G-CSF mutants were in a buffer of 10 mM sodium acetate, 100 mM NaCl at pH 5.0.

The buffer for the circular dichroism wavelength scans was 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate at pH 4 through 7. For the denaturation studies, the buffer was 50 mM Tris, 20 mM sodium acetate, 20 mM MES at pH 4 through 7. Guanidine hydrochloride was obtained from Pierce, Fluka, and ICN.

CD

Circular dichroism was obtained using an Aviv CD spectrometer (model 62A DS) with a titrator (Microlab 500 series). The far-UV signal was measured at 222 nm, and the spectrum was recorded between 300 nm and 180 nm. The pH titration was accomplished by adding 1 μ L aliquots of 1 N HCl to a 5-mL stock solution of IL-2 at 0.050 mg/mL in 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate, and spectral measurements were recorded at approximately every 0.5 pH unit from pH 8.3 to pH 3.4. The equilibrium denaturation studies were done by preparing stock protein solutions at 0.025–0.050 mg/mL in 50 mM Tris, 20 mM sodium acetate, 20 mM MES with 0 M or greater than 6 M GuHCl at each given pH. After each CD measurement, the titration apparatus removed a set volume of solution from the cuvette and injected an equivalent volume of denatured protein, increasing the concentration of GuHCl by 0.1 M each time. Mixing and equilibration times were incorporated into the sequence between each measurement.

Near-UV CD measurements were made on a Jasco spectropolarimeter (model J-720). Wavelength scans were recorded between 320 nm and 250 nm using 0.5 mg/mL protein solutions, and the near-UV signals at 291 nm and 280 nm were used as measures of tertiary structure at each pH.

Fluorescence

For the IL-2 studies, a PTI fluorometer was used to measure intrinsic fluorescence at 280 nm, and emission scans were performed at wavelengths from 300 nm to 400 nm. A manual pH titration from pH 2 to 7 was accomplished by removing a given volume of protein solution and mixing in an equivalent volume of pH 7 protein solution. Stock solutions of IL-2 were at a protein concentration of 0.050 mg/mL in 50 mM Tris, 20 mM sodium acetate, 20 mM MES at pH 7 and pH 2. For the IL-2 equilibrium denaturation studies, separate samples across a range of denaturant concentrations were prepared individually at 0.050 mg/mL protein concen-

tration in 50 mM Tris, 20 mM sodium acetate, 20 mM MES, at pH 4.

For the G-CSF fluorescence studies, an Aviv automated titrating differential/ratio spectrofluorometer (model ATF105) with a titrator was used, and equilibrium denaturation studies were done by preparing stock protein solutions at 0.025 mg/mL in 50 mM Tris, 20 mM sodium acetate, 20 mM MES with 0 M or greater than 6 M GuHCl at each pH. Samples were excited at either 280 nm or 295 nm, and wavelength emission scans were recorded from 300 nm to 400 nm. Manual pH titrations from pH 3 to pH 7 for wild type and the mutants were performed in the same way as experiments for IL-2. Equilibrium denaturation studies using fluorescence were accomplished similarly to those described for CD. Each sample was excited at 280 nm and the fluorescence signal was monitored at 350 nm (the emission maximum).

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