Articles

Equilibrium Denaturation of Recombinant Murine Interleukin-6: Effect of pH, Denaturants, and Salt on Formation of Folding Intermediates†

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ABSTRACT: The equilibrium denaturation of an *Escherichia coli*-derived recombinant murine interleukin-6 (mIL-6) was studied using fluorescence and circular dichroism spectroscopy. The urea-induced unfolding of mIL-6 at pH 4.0 can be described by a two-state unfolding mechanism based on the superimposibility of the CD and fluorescence unfolding transitions. Assuming a two-state mechanism and a linear dependence of the free energy of unfolding on denaturant concentration, a value of 6.9−9.0 kcal/mol was calculated for the free energy of unfolding in the absence of denaturant [$\Delta G_U(H_2O)$]. However, when GuHCl was used as a denaturant at pH 4.0, a biphasic unfolding transition was observed. This unfolding transition has a distinct midpoint occurring at 2.5 M GuHCl, which is indicative of the formation of stable folding intermediates. Similar intermediate folded species were also observed at pH 7.4 when either urea or GuHCl were used as denaturants. The intermediate folded states of mIL-6 exhibited a tendency to aggregate, as judged by the concentration dependence of their fluorescence characteristics. The fluorescence emission maximum of mIL-6 at pH 7.4 in the presence of 1.5 M GuHCl, for example, was blue-shifted from 343 nm at a protein concentration of 50 μg/mL to 336 nm at 500 μg/mL. Intermediate formation at pH 4.0, using 10 mM sodium acetate buffer and urea as the denaturant, was facilitated by the addition of 0.4 and 0.8 M salt, where the salt was either NaCl or GuHCl. These data, together with the pH-dependent fluorescence characteristics, suggest that ionic effects and the charged state of mIL-6—particularly in the region of Trp36—play an important role in the formation of folding intermediates and aggregation.

Interleukin-6 (IL-6),[1] a multifunctional cytokine which plays a central role in host defense, stimulates or inhibits cellular proliferation and differentiation depending on the nature of the target cells [for reviews, see Van Snick (1990), Akira *et al.*, (1993), and Hirano, (1994)]. Activities attributed to IL-6 include the ability to induce late B-cell development (Hirano *et al.*, 1985, 1986), growth and differentiation of T cells (Garman *et al.*, 1987), regulation of the acute-phase response (Andus *et al.*, 1987; Gauldie *et al.*, 1987), induction of the proliferation of haematopoietic progenitor cells (Ikebuchi *et al.*, 1987), and differentiation of megakaryocytes (Ishibashi *et al.*, 1989). Dysregulation of IL-6 expression has been associated with autoimmune diseases such as rheumatoid arthritis, chronic proliferative diseases such as psoriasis (Grossman *et al.*, 1989), and a number of malignancies including plasmacytomas, myelomas, and plasma cell leukemias (Hirano *et al.*, 1990). The selective inhibition of IL-6 activity may have clinical significance in the treatment of IL-6 associated diseases.

A detailed understanding of the three-dimensional structure of IL-6 and the manner by which IL-6 interacts with its specific, low affinity, cell surface receptor (IL-6R) and the high affinity converter and signal transducing subunit, gp-130 (Taga & Kishimoto, 1992), may facilitate the rational design of IL-6 antagonists. As a first step toward designing an IL-6 antagonist, we have undertaken a detailed structure−function study of a recombinant murine IL-6 (mIL-6). Recently, we described a large-scale purification strategy for isolating bacterially expressed mIL-6 (Zhang *et al.*, 1992). This mIL-6 preparation, as well as a number of mIL-6 mutants, has been subjected to a detailed physiochemical study to assess the conformational state of the molecule using techniques such as CD and fluorescence spectroscopy (Ward *et al.*, 1993a,b; Hammacher *et al.*, 1994).

To gain further insight into the forces that contribute to the conformational stability of mIL-6, we have undertaken equilibrium denaturation studies of the recombinant protein. Typically, these studies are analyzed by assuming a two-state denaturation/renaturation mechanism in order to estimate the conformational stability of the protein (Pace, 1986). The two-state model assumes that the unfolding of a protein is a highly cooperative process and that only the fully folded and denatured states of the protein are significantly populated

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[1] Abbreviations: bGH, bovine growth hormone; CD, circular dichroism; λ_{max}, fluorescence emission maximum; GuHCl, guanidine hydrochloride; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; mAb, monoclonal antibody; mIL-6, recombinant murine interleukin-6; pGH, porcine growth hormone; UV, ultraviolet.

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at equilibrium. We show here that the denaturant-induced unfolding of mIL-6 under some solution conditions is not described adequately by a cooperative two-state mechanism. The presence of intermediates in the unfolding pathway was demonstrated to be dependent on pH, the presence of salt, and the type of denaturant utilized.

MATERIALS AND METHODS

Chemicals. Urea was purchased from Bio-Rad (Richmond, CA). Guanidine hydrochloride (8 M stock solution) was obtained from Pierce (Rockford, IL). All buffers were prepared with deionized water purified by a tandem Milli-RO and Milli-Q system from Millipore.

Preparation of Recombinant Murine Interleukin-6. Recombinant mIL-6 was expressed in *Escherichia coli* (strain JM101) as a β-galactosidase fusion protein using the *lac* operon inducible plasmid pUC9 (Simpson *et al.*, 1988b) and purified according to the procedure of Zhang *et al.* (1992). The first eight amino acids of the recombinant mIL-6 are from the N-terminus of bacterial β-galactosidase and the polylinker region of pUC9 (Thr-Met-Ile-Thr-Pro-Ser-Leu-Ala), while the remaining 176 residues correspond to residues Thr12−Thr187 of native mIL-6 (Simpson *et al.*, 1988a). The specific activity of mIL-6 (2×10^8 units/mg) using the murine hybridoma growth factor assay (Van Snick *et al.*, 1986) is comparable to that of natural mIL-6.

Protein Estimation. The concentration of the mIL-6 was determined spectrophotometrically using the absorption coefficient $A_{280nm}^{0.1\%} = 1.07$. The absorption coefficient was calculated by measuring the absorption at 280 nm of an aqueous solution of mIL-6 (in 0.1% trifluoroacetic acid) of known concentration. This concentration was determined by amino acid analysis using a Beckman amino acid analyzer (model 6307) equipped with a model 7000 integrator (Simpson *et al.*, 1986).

Denaturant-Induced Equilibrium Unfolding. The unfolding of mIL-6 as a function of denaturant (GuHCl or urea) concentration was monitored by both fluorescence and far-UV CD spectroscopy. Lyophilized, recombinant mIL-6 was dissolved in water, and then diluted with either 10 mM sodium acetate buffer, pH 4.0, or 10 mM Tris-HCl buffer, pH 7.4, containing denaturant as indicated. The final protein concentration of these solutions was100 μg/mL. Solutions were incubated at 25 °C for at least 2 h prior to measurement. In most cases samples were equilibrated for 16 h; however, similar results were obtained if a 2 h incubation was used (data not shown). Reversibility experiments were performed by incubating concentrated mIL-6 in 6 M GuHCl or urea for at least 2 h before diluting the solution to the indicated denaturant concentrations. Concentration-dependent experiments were performed using the indicated final concentrations of mIL-6.

Spectroscopic Methods. The CD signal at 222 nm was measured using an Aviv model 62DS circular dichroism spectrometer, in a 0.1 cm cell, at 25 °C. Fluorescence emission spectra were measured with a Perkin Elmer LS 5 Luminescence Spectrometer at 25 °C in 0.5-cm pathlength cells. Fluorescence intensities were determined by measuring the emission at 345 nm. Values of λ_{max} were estimated from the emission spectra by scanning over the wavelength range 310−400 nm. In all cases the excitation wavelength was 295 nm, and slit widths of both monochromators were 5 nm.

Data Analysis. The structural stability of mIL-6 was monitored by analyzing the extent of unfolding of the protein as a function of denaturant concentration. The folding transitions were analysed by assuming a two-state transition between the folded (N) and the unfolded (U) conformations. The equilibrium constant for this process is described by the following relation (Pace, 1986):

$$K_U = \exp(-\Delta G_U/RT) = f_U/f_N = (S - S_N)/(S_U - S) \quad (1)$$

where f_U and f_N are the fractions of protein present in the unfolded (U) and native (N) states, respectively. R is the gas constant and T the absolute temperature. S is the measured property of the protein at a particular denaturant concentration. S_N and S_U are the values of S corresponding to the native and denatured states, respectively. The free energy of unfolding in water in the absence of denaturant, $\Delta G_U(H_2O)$, is empirically related to ΔG_U by an essentially linear relationship (Pace, 1986)

$$\Delta G_U = \Delta G_U(H_2O) - m[D] \quad (2)$$

where D is the denaturant and m is the slope of the curve in the transition region which provides a measure of the cooperativity of unfolding. If there is a two-state unfolding mechanism and no pre- or posttransitional dependence of the signal on denaturant concentration, estimates of $\Delta G_U(H_2O)$, m, and $[D]_{50\%}$, the denaturant concentration at the midpoint of the unfolding transition can be obtained by fitting the whole data set to (Santoro & Bolen, 1988)

$$S = \frac{S_N + S_U \exp A}{1 + \exp A} \quad (3)$$

Where $A = \{m[D] - \Delta G_U(H_2O)\}/RT = m([D] - [D]_{50\%})/RT$. When there are linear pre- and posttransitional dependencies on denaturant concentration, data can be fitted to (Santoro & Bolen, 1988)

$$S = \frac{S_N + a_N[D] + (S_U + a_U[D]) \exp A}{1 + \exp A} \quad (4)$$

where a_N and a_U are the slopes of the pre- and posttransition baselines.

Equations 3 and 4 assume that the folding/unfolding mechanism is a highly cooperative process with only native and denatured forms of a protein being significantly populated at equilibrium. If this assumption is not met and intermediate species are present, large underestimates of $\Delta G_U(H_2O)$ can be obtained (Pace, 1986). Data for unfolding transitions were fitted to the above equations using the nonlinear regression analysis program KaleidaGraph (version 3.0 Synergy Software; PCS Inc).

RESULTS

Far-UV CD Studies of the Equilibrium Unfolding of mIL-6. The equilibrium unfolding of mIL-6 was initially studied at pH 7.4 using GuHCl as the denaturant, using far-UV CD spectroscopy, as shown in Figure 1A.

When fitted to eq 3, these data yielded a $\Delta G_U(H_2O)$ value of 1.8 ± 0.3 kcal/mol, which is lower than the often-quoted range of conformational stability for small globular proteins

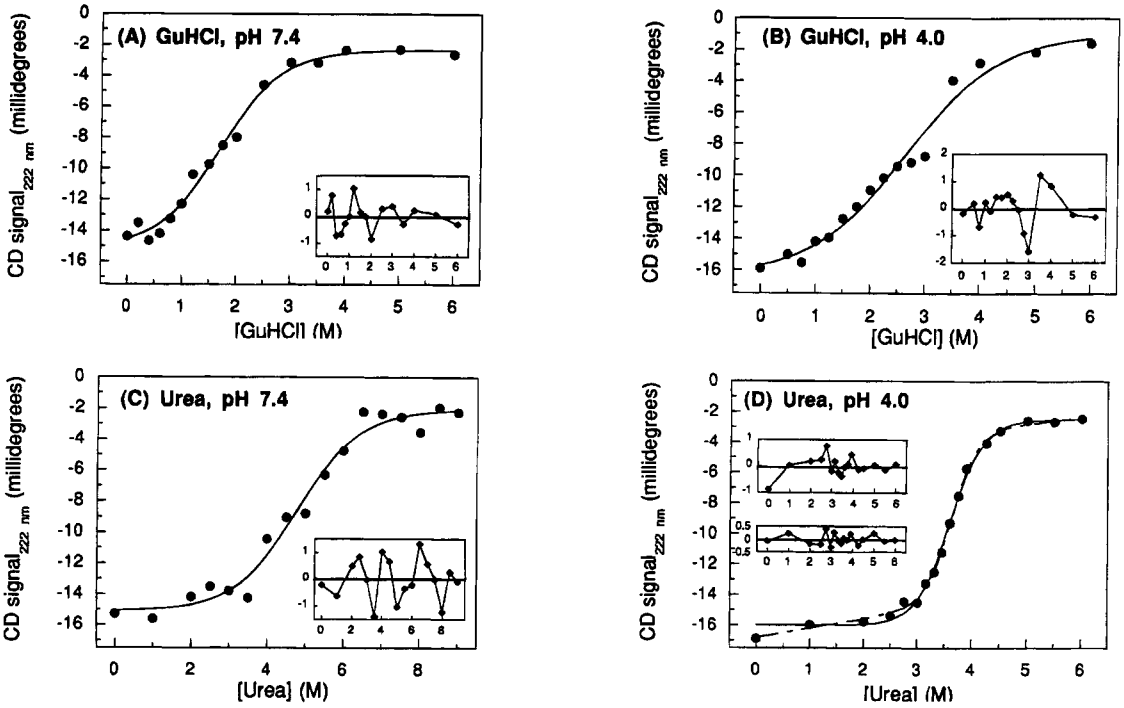


FIGURE 1: Denaturant-induced equilibrium unfolding of mIL-6 monitored by far-UV CD spectroscopy. The concentration of protein in all assays was 100 μg/mL. The main panels show the uncorrected CD signal at 222 nm plotted against the concentration of denaturant. Data points shown are the average of three measurements. The insert panels show the residuals resulting from the fitting of the data to eqs 3 and 4, with the axes of the insert panels being the same as the main panels. All main data sets are fitted to eq 3 (—). The data in panel D are additionally fitted to eq 4 (– - –), with residuals shown in the bottom insert panel. Panel A shows the inital GuHCl-induced denaturation of mIL-6 at pH 7.4; panel B shows GuHCl-induced denaturation of mIL-6 at pH 4.0; panel C shows urea-induced denaturation at pH 7.4; and panel D shows urea-induced denaturation at pH 7.4. Buffers used were 10 mM sodium acetate, pH 4.0, and 10 mM Tris-HCl, pH 7.4.

Table 1: Analysis of Denaturant-Induced Unfolding of mIL-6 Monitored by Far-UV CD[a]

buffer[b]	denaturant	$\Delta G_U(H_2O)$[c] (kcal/mol)	m[c] (kcal mol^{-1} M^{-1})	[denaturant]$_{50\%}$[c] (M)
1	urea	9.0 ± 0.7[d]	2.5 ± 0.2[d]	3.63 ± 0.02[d]
1	urea	6.9 ± 0.5[e]	1.9 ± 0.1[e]	3.60 ± 0.02[e]
2	urea	3.4 ± 0.6[e]	0.7 ± 0.1[e]	4.8 ± 0.2[e]
1	GuHCl	1.8 ± 0.4[e]	0.7 ± 0.1[e]	2.7 ± 0.1[e]
2	GuHCl	1.8 ± 0.3[e]	1.0 ± 0.2[e]	1.7 ± 0.1[e]

[a] Tolerances shown are curve fitting errors. [b] Buffer 1: 10 mM sodium acetate, pH 4.0; buffer 2: 10 mM Tris-HCl, pH 7.4. [c] The values of $\Delta G_U(H_2O)$, m, and [denaturant]$_{50\%}$ were obtained by a nonlinear regression fit of the data, according to eq 3 (no pre- and posttransition baseline dependence of CD signal on denaturant concentration) and eq 4 (assuming linear pre- and posttransition baseline dependence of CD signal on denaturant concentration), assuming a two-state denaturation/renaturation model. [d] Data fitted according to eq 4. [e] Data fitted according to eq 3.

(5−15 kcal/mol; Pace, 1990). The dependence of this value on solution conditions and type of denaturant was investigated.

As with GuHCl at pH 7.4 (Figure 1A), when using GuHCl at pH 4.0 (Figure 1B) or urea at pH 7.4 (Figure 1C), the unfolding transitions are relatively broad. This is reflected in m values of 1.0, 0.7, and 0.7 kcal mol^{-1} M^{-1}, respectively (Table 1). In contrast, when using urea as the denaturant at pH 4.0 (Figure 1D), the unfolding transition was considerably more cooperative, with higher values of m (1.9 kcal mol^{-1} M^{-1}) and $\Delta G_U(H_2O)$ (6.9 kcal/mol) being observed (Table 1). All comparisons were performed using eq 3. Although there appears to be some pre- and posttransitional baseline dependence of the CD signal at 222 nm on denaturant concentration, some of these baselines are too short to allow their reliable estimation by eq 4.

A possible explanation for these data would be that at pH 4.0, in urea, the unfolding transition for mIL-6 follows a two-state transition. Under the other solution conditions, however, this is not the case. Unfolding intermediates appear to accumulate at intermediate denaturant concentrations leading to less cooperative transitions and lower estimates of $\Delta G_U(H_2O)$. A closer approximation to a two-state unfolding transition at pH 4.0 using urea as the denaturant (Figure 1D) is also supported by the better statistical fit observed under these conditions (Figure 1, insert panels) and the consequent smaller errors obtained for estimates of [D]$_{50\%}$ (Table 1). When the data obtained at pH 4.0, using urea as the denaturant, are fitted to eq 4 (Figure 1D, lower insert panel; Table 1), even higher estimates of m (2.5 kcal mol^{-1} M^{-1}) and $\Delta G_U(H_2O)$ (9.0 kcal/mol) are obtained.

Ideally, when there are well-populated intermediate states at equilibrium, it should be possible to use three-state (or higher) equations to estimate values of $\Delta G_U(H_2O)$ for the various unfolding transitions (Morjana *et al.*, 1993). In practice, however, the large numbers of variable parameters involved mean that unreliable estimates can be obtained. This was the case where data sets for the unfolding of mIL-6 under non two-state conditions were fitted to a three-state equation (data not shown), although it appeared that estimates of $\Delta G_U(H_2O)$ thus calculated would be much higher than those estimated by eq 3.

Fluorescence Studies of the Equilibrium Unfolding of mIL-6. It has previously been demonstrated for bovine growth hormone (bGH) (Brems *et al.*, 1985) that the presence of equilibrium intermediates results in the noncoincidence of the denaturation transitions when monitored by UV and CD

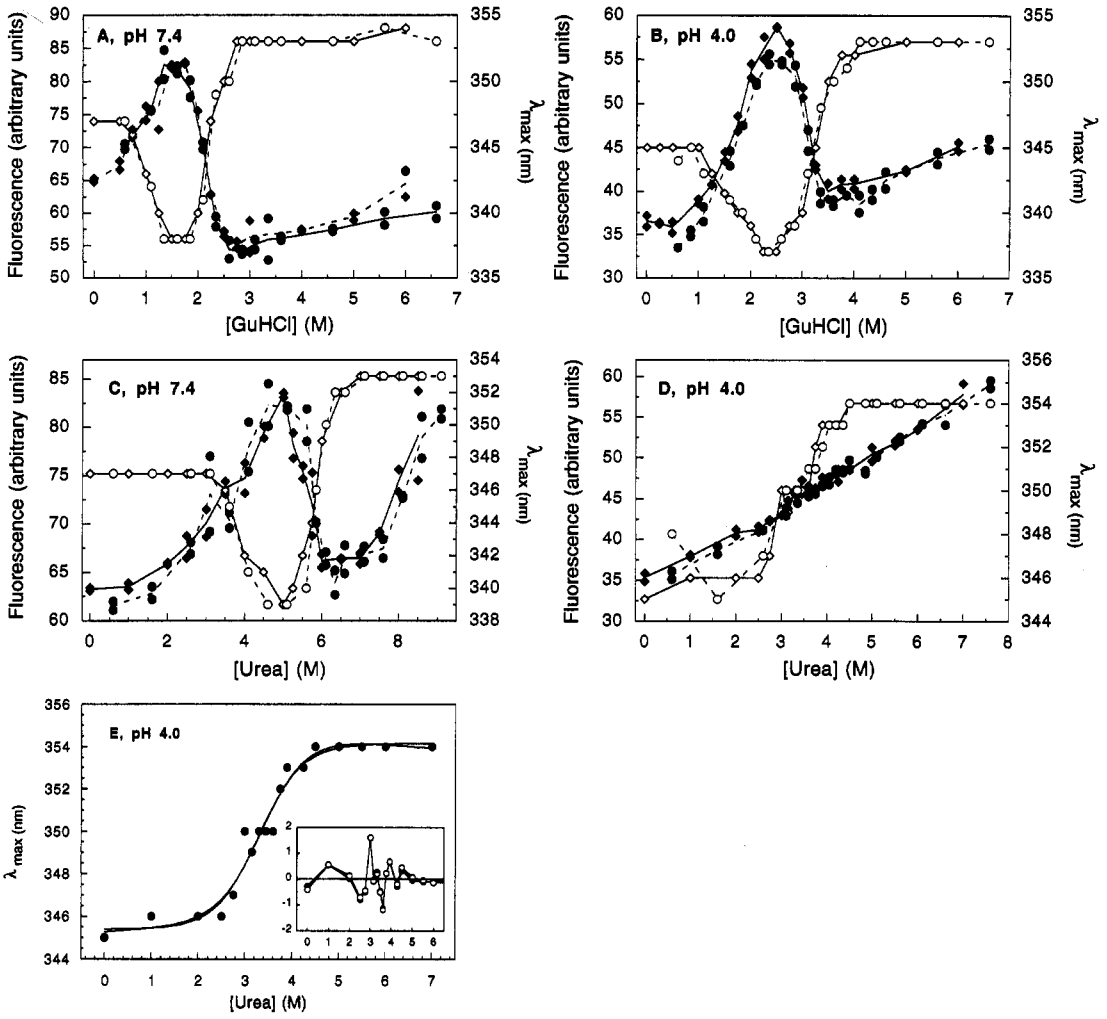


FIGURE 2: Effect of pH on urea- and GuHCl-induced equilibrium unfolding of mIL-6 monitored by fluorescence spectroscopy. The relative fluorescence emission at 345 nm (closed symbols) and λ_{max} (open symbols) in the region 310−400 nm were plotted against denaturant concentration. The excitation wavelength was 295 nm in all cases. Fluorescence measurements were performed using a protein concentration of 100 μg/mL. The unfolding equilibria (native starting material) are shown as diamonds and the refolding equilibria (denatured starting material) are shown as circles. Measurements at pH 7.4, 4.0, and 4.0 using urea as a denaturant are shown in panels C, D, and E, respectively. Measurements at pH 7.4 and 4.0 using GuHCl as a denaturant are shown in panels A and B, respectively. Panel E shows the fitting of the λ_{max} data from panel D to eqs 3 and 4 with the insert panel showing the residuals resulting from the fitting of the data to eqs 3 (open circles) and 4 (closed circles), with the axes of the insert panel being the same as the main panels. Buffers used were 10 mM sodium acetate, pH 4.0, and 10 mM Tris-HCl, pH 7.4.

spectroscopy. For this reason, the denaturant-induced equilibrium unfolding of mIL-6 was investigated by fluorescence spectroscopy. The change in the fluorescence characteristics of mIL-6 as a function of denaturant (urea or GuHCl) at pH 4.0 and 7.4 is shown in Figure 2. The fluorescence experiments support the interpretation of the CD experiments in that under some solution conditions (at pH 7.4 and at 4.0 when using GuHCl as the denaturant) the equilibrium unfolding of mIL-6 is not described adequately by a cooperative two-state system.

Using the same buffer systems and denaturants where the presence of intermediate species of mIL-6 were implicated from the CD measurements (pH 7.4, GuHCl; pH 4.0, GuHCl; pH 7.4, urea; see Figure 1A−C), highly unusual dependencies of fluorescence emission intensities on denaturant concentrations were observed (Figure 2A−C). Taking GuHCl-induced denaturation at pH 4.0 as an example (Figure 2B), there was an increase in fluorescence intensity upon going from 0−2.5 M denaturant, a decrease from 2.5−4.0 M, and an increase above 4 M GuHCl. Presumably, the decrease in fluorescence intensity between 2.5 and 4.0 M GuHCl corresponds to a transition to the denatured form of mIL-6 since it is accompanied by a corresponding red-shift in λ_{max} from 339 to 353 nm. This is consistent with the exposure of the indole side-chain of tryptophan residues to bulk solvent in the denatured states (Lakowicz, 1983). The blue-shift of the λ_{max} of mIL-6 from 345 to 340 nm in the pretransition region (0−2.75 M GuHCl) indicates the presence of a conformational state of mIL-6 at intermediate denaturant concentrations that differs from the native or denatured states (Lakowicz, 1983).

The maximum in fluorescence emission and corresponding minimum in the λ_{max} at 2.5 M GuHCl (Figure 2B) correspond to the midpoint in the biphasic dependency of the CD signal at 222 nm on concentration of denaturant (Figure 1B). Taken together, the CD and fluorescence data indicate that, upon partial unfolding, mIL-6 assumes a conformational state in which one or both of its tryptophan residues are less solvent exposed.

While the CD data for urea-mediated denaturation, pH 4.0, are consistent with a two-state unfolding process, the

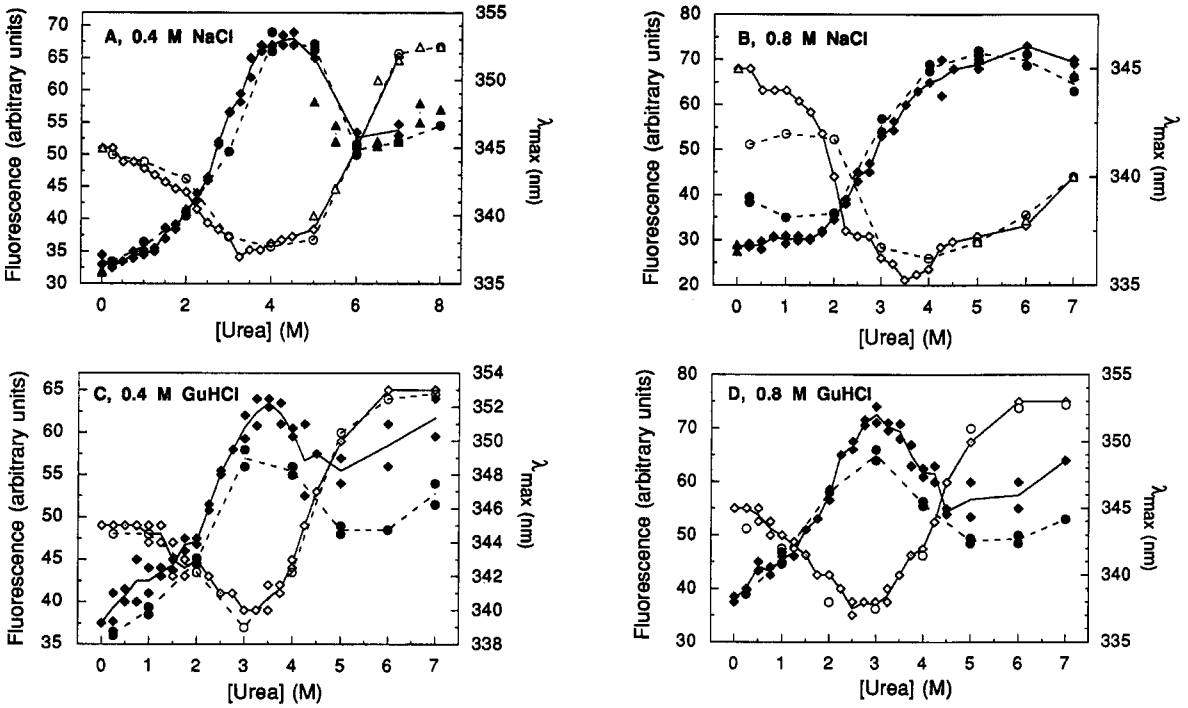


FIGURE 3: Effect of salt on the urea-mediated denaturation of mIL-6 at pH 4.0 as monitored by fluorescence spectroscopy. The relative fluorescence emission at 345 nm (closed symbols) and λ_{max} in the region 310−400 nm (open symbols) are plotted against denaturant concentration. The excitation wavelength was 295 nm in all cases. Fluorescence measurements were performed using a protein concentration of 100 μg/mL. The unfolding equilibria (native starting material) are shown as diamonds or triangles (data from different experiments) and the refolding equilibria (denatured starting material) are shown as circles. All solutions contained 10 mM sodium acetate buffer, pH 4.0. Panel A shows the effect of 0.4 M NaCl; panel (B), 0.8 M NaCl; panel C, 0.4 M GuHCl; panel D 0.4 M GuHCl.

fluorescence data are less clear. For example, the λ_{max} exhibits a red shift from 345 to 354 nm with a fairly sharp transition region (Figure 2D). The range of λ_{max} values is too small to allow an accurate estimation of m and ΔG_U-(H_2O), however, when fitted to eqs 3 and 4 (Figure 2E), these data give $[D]_{50\%}$ values of 3.3 ± 0.1 and 3.4 ± 0.2, respectively. This is consistent with the apparent two-state unfolding transition seen by CD (Figure 1D). The fluorescence emission data, however, is more difficult to interpret because of the large values of the slopes of the pre- and posttransition baselines.

Reversibility of Unfolding Transition by Fluorescence. The reversibility of the folding/unfolding transition for mIL-6 was examined using fluorescence. Solutions of native mIL-6 (in H_2O) and denatured mIL-6 (in 6 M denaturant) were diluted to give buffered solutions containing denaturant at the indicated concentrations. This was performed using both GuHCl and urea as the denaturing agent at pH 4.0 and 7.4 (Figure 2). Near identical transitions were obtained, indicating that the unfolding of mIL-6 is readily reversible under the conditions employed here.

Effect of Salt on the Unfolding Transition. As shown in Figures 1 and 2, an apparent two-state unfolding transition at pH 4.0 was observed for urea-mediated denaturation but not for GuHCl-mediated denaturation. Both of these denaturants act by solubilizing the polypeptide chain (Pace, 1986), effectively stabilizing the unfolded state relative to the folded state of the protein. The major difference between the two denaturants, other than that GuHCl is the more efficient denaturant, is that, unlike urea, GuHCl is a salt.

To determine whether ionic effects were causing this deviation from apparent two-state behavior in the presence of GuHCl, the urea-mediated denaturation experiments at pH 4.0 were also carried out in the presence of NaCl and GuHCl.

As described above, the reversibility of the mIL-6 unfolding transition was established by using both native and denatured mIL-6 as the starting material. In all cases the unfolding transition monitored by changes in λ_{max} was shown to be fully reversible. When monitored by fluorescence emission, the folding transition was fully reversible in the presence of NaCl, but in some cases lower fluorescence intensities were seen in the presence of GuHCl, when denatured mIL-6 was used as the starting material. In Figure 3 it can be seen that, at concentrations of 0.4 and 0.8 M NaCl or GuHCl, the fluorescence behavior of mIL-6 was similar to that observed at pH 7.4 (using either GuHCl or urea as the denaturant, Figure 2A,C) or at pH 4.0 using GuHCl as the denaturant (Figure 2B).

The fluorescence properties of mIL-6 in the presence of 0.4 and 0.8 M NaCl and GuHCl are not identical. As would be expected with addition of a known denaturant, equivalent fluorescence behavior occurs at lower urea concentrations in the presence of GuHCl than in the presence of NaCl (and at lower urea concentrations for 0.8 M compared to 0.4 M GuHCl). Deviations from two-state behavior are more marked for the higher salt concentrations in both cases. With all of these experiments, fluorescence emission shows an initial increase in intensity, followed by a decrease and finally a second increase in intensity at high urea concentrations. Similar deviations from apparent two-state behavior upon the addition of NaCl were observed using far-UV CD (data not shown).

Concentration Dependence of λ_{max} and Fluorescence Intensity. A possible explanation of the blue shift in λ_{max} at intermediate concentrations is the self-association of partially

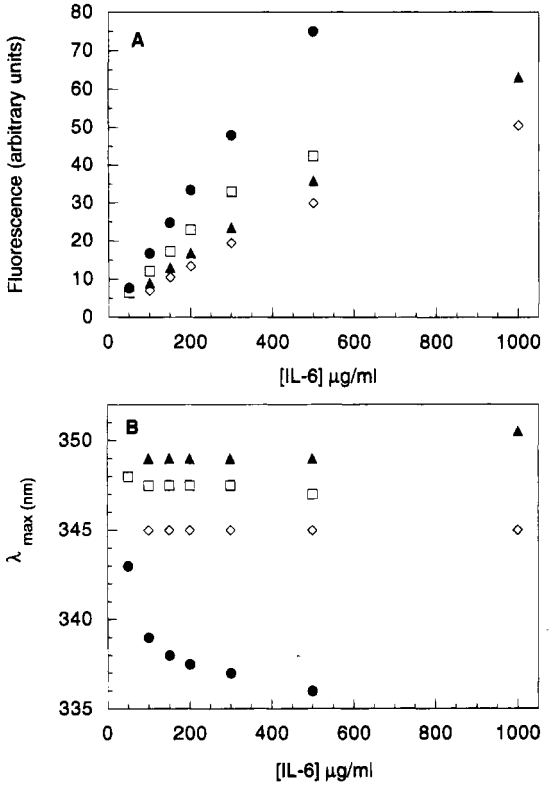


FIGURE 4: Concentration dependence of mIL-6 fluorescence emission spectrum. Panel A shows fluorescence emission at 345 nm, and panel B shows λ_{max} in the range 310−400 nm plotted against protein concentration. Excitation is at 295 nm. Both panels: 10 mM sodium acetate, pH 4.0, no added salt (◇), 3.5 M urea (▲); 10 mM Tris-HCl, pH 7.4, no added salt (□), 1.5 M GuHCl (●).

folded forms of the protein. To probe this, the fluorescence emission spectrum of mIL-6 was studied as a function of protein concentration. These experiments were performed over the protein concentration range 50−1000 μg/mL at pH 4.0 in the absence and presence of 3.5 M urea and at pH 7.4 in the absence and presence of 1.5 M GuHCl (Figure 4). These denaturant concentrations were chosen since they represent the midpoints of the unfolding transitions at the appropriate pH and denaturing conditions as determined by CD (Figure 1A,D). Under these conditions, the fluorescence emission is maximal in the presence of 1.5 M GuHCl, at pH 7.4, where the signal intensity is approximately twice that of mIL-6 at identical protein concentrations but under different conditions (Figure 4A; pH 4.0, 0 M GuHCl; pH 4.0, 0 and 3.5 M urea).

The fluorescence emission intensity showed an essentially linear dependence on mIL-6 concentration (Figure 4A), with only slight deviations from linearity apparent at the higher concentrations, which could be attributed to inner filter effects (Ward, 1986). However, it is clear that in the presence of 1.5 M GuHCl at pH 7.4, the quantum yield of tryptophan is significantly increased relative to that observed in the absence of denaturant. The relative increase in fluorescence intensity is concentration dependent. For example, at 100 μg/mL the increase is of the order of 1.3-fold, while at 500 μg/mL it is of the order of 1.9-fold (Figure 4A). Concentration dependence of λ_{max} was critically dependent on solution conditions (Figure 4B). In the absence of denaturant, the λ_{max} at pH 4.0 remains constant at 345 nm. However, at pH 7.4 there is only a slight blue-shift (348 to 347 nm). In 3.5 M urea, pH 4.0, there is a small red-shift from 349 nm (at 500 μg/mL) to 350.5 nm (at 1000 μg/mL), while in 1.5 M GuHCl at pH 7.4 there is a significant, nonlinear blue shift from 343 nm at 50 μg/mL to 336 nm at 500 μg/mL.

These data are consistent with a concentration dependent association of mIL-6 in intermediate concentrations of GuHCl at pH 7.4, in which tryptophan residue(s) would be exposed to a less polar environment resulting in a subsequent blue-shift in the fluorescence emission spectrum.

DISCUSSION

In this study the stability of mIL-6 was investigated by determining its sensitivity to unfolding by denaturant. The folding/unfolding transition of mIL-6 was readily reversible as evidenced by the identical transitions observed when using folded versus totally denatured protein as the starting material. These data agree with results obtained by urea-gradient gel electrophoresis where identical transitions were observed irregardless of whether mIL-6 was applied to the gel in native or denatured configurations at pH 4.0, 8.0, or 10.2 (Ward *et al.*, 1993).

The recombinant mIL-6 used in this study was a fusion protein comprising eight amino acids of β-galactosidase and the polylinker region of pUC9 and the N-terminally truncated form of mIL-6 starting at Thr12. The N-terminal truncation does not influence the activity or folding properties of mIL-6 as 22 N-terminal amino acids can be deleted from mIL-6 without affecting biological activity (Ward *et al.*, 1993b; Hammacher *et al.* 1994) or the $\Delta G_U(H_2O)$ of unfolding at pH 4.0, using urea as the denaturant (Ward, unpublished data).

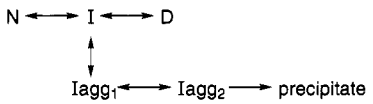
When mIL-6 was unfolded at pH 4.0, using urea as the denaturant, the coincidence of CD and fluorescence λ_{max} data were consistent with a simple two-state denaturation/renaturation model (Tanford, 1970; Kim & Baldwin, 1990). When such a mechanism and a linear dependence of ΔG_U on the concentration of denaturant were assumed (Pace *et al.*,1986), a value of $\Delta G_U(H_2O) = 6.9 \pm 0.5$ or 9.0 ± 0.5 kcal/mol was calculated, depending on which equation was used to fit the data. This value is in reasonable agreement with that of 6.0 kcal/mol calculated previously in this laboratory using urea-gradient gel electrophoresis at pH 4.0 (Zhang *et al.*, 1992).

Under other solution conditions, namely, at pH 7.4 using urea or GuHCl as the denaturant or at pH 4.0 when using GuHCl as the denaturant, the biphasic nature of both the fluorescence and CD denaturation curves were indicative of intermediates in the unfolding pathway. For some proteins the presence of more than one step in the denaturation curve has been demonstrated to be due to the presence of more than one domain in the protein (Azuma *et al.*, 1972; Rudolph *et al.*, 1990). This does not seem likely for a small globular protein such as mIL-6.

The folding of single domain proteins has long been thought to be described adequately by a two-state unfolding mechanism (Privalov, 1979). Most small proteins do not demonstrate equilibrium folding intermediates. Presumably such intermediates are unstable under conditions that disrupt the native state and thus are not highly populated [reviewed by Creighton (1985)]. However, in recent years there have been increasing reports of conformational states of proteins

intermediate between the fully folded and unfolded forms [for reviews, see Kuwajima (1989), Kim and Baldwin (1990), and Christensen and Pain (1991)]. It has been proposed that proteins containing regions of the polypeptide chain with a high propensity to form secondary structural elements in the absence of long-range interactions may not conform to a two-state folding model (Dobson, 1992).

On the basis of structure-prediction algorithms (Bazan, 1990; Parry *et al.*, 1991), IL-6 has been postulated to belong to a family of 4-α-helical bundle type proteins (e.g., granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, growth hormone, interleukins-2, −4, and −11, leukemia inhibitory factor). This is consistent with the high α-helical content of mIL-6 (52%) as judged by CD (Zhang *et al.*, 1992). Conformational states intermediate between the native and denatured states have been observed during the folding of other members of this 4 α-helical bundle family including bGH (Brems *et al.*, 1986), pGH (Bastiras & Wallace, 1992), and granulocyte-colony stimulating factor (Narhi *et al.*, 1991). The properties of the intermediate forms of mIL-6 described here resemble those of bGH. Extensive studies on bGH have led to the following model for the equilibrium unfolding of growth hormone (Lehrman *et al.*, 1991).



At intermediate denaturant concentrations (i.e., 2−3 M GuHCl) bGH appears to partially unfold to a state (I) with a high propensity to aggregate (Havel *et al.*, 1986; Brems *et al.*, 1986; Brems, 1988). The unfolding has been implied to involve exposure of the hydrophobic face of the α-helix in the region of residues of 109−133 (Brems *et al.*, 1986; Gooley et al., 1988). This partially folded state (I) has been noted to possess many of the properties of the "molten globule" state (Brems & Havel, 1989). The molten globule describes a conformational state with a hydrodynamic volume between that of native and unfolded states, which contains secondary but little or no tertiary structure, and it has been postulated to be similar to a kinetic intermediate in the folding pathway of proteins (Ptitsyn *et al.*, 1990).

The propensity of mIL-6 to form folding intermediates was critically dependent on the pH and the type of denaturant used. For example, at pH 4.0, evidence for folding intermediates was only observed when GuHCl was used as the denaturant. However, the formation of intermediates could be induced during urea-mediated unfolding if low concentrations of GuHCl or NaCl were included in the incubation mixture. The proportion of intermediates accumulating was also concentration dependent. Taken together these data suggest that the differential behavior of GuHCl and urea results from the ionic nature of GuHCl. The high ionic strength of the denaturing solution masks the high charge on mIL-6 at pH 4.0 (estimated molecular charge = 16) which, in turn, facilitates aggregation of the folding intermediates. Similarly, NaCl facilitates the aggregation at pH 4.0 if urea is used to unfold the protein. The dependence of ionic strength is not as significant at pH 7.4 due to low charge on mIL-6 at this pH (estimated molecular charge = 0).

The increased fluorescence quantum yield and blue-shift of λ_{max} of the aggregated form of mIL-6 relative to the folded molecule suggests a decrease in the efficiency of fluorescence quenching of one or both of the tryptophan residues and also transfer of the tryptophan residue to a more hydrophobic environment. We have previously demonstrated that the fluorescence of mIL-6 is quenched 40% upon decreasing the pH, the midpoint of the transition occurring at pH 6.9 (Ward et al., 1993a). This quenching was shown to be predominantly due to interaction of the protonated form of His33 with Trp36 (Ward et al., 1993a). These residues are predicted to form part of the exposed surface of helix A in the recently constructed model of mIL-6 (Hammacher *et al.*, 1994). It is possible that the increased quantum yield of the aggregated intermediate results from a change of orientation of His33 with Trp36 and that the blue-shift is due to the burial of Trp36 within the protein−protein contacts of the aggregated intermediate. This hypothesis is currently under investigation using a site-directed mutagenesis approach where the role of Trp36 and His33 in the unusual unfolding behavior of mIL-6 is being investigated.

The results of these studies have a number of practical applications and help explain the "ageing" of mIL-6 that we have observed under some conditions. For example, during the course of NMR studies of mIL-6, we observed that at high protein concentrations (15−20 mg/mL) there is a time-dependent decrease in signal, presumably due to aggregation of mIL-6 (Morton *et al.*, 1994; Ward *et al.*, 1993b). These aggregates appear to be the same as those intermediates observed in this study, as judged by their identical spectroscopic properties (Ward, unpublished). The rate of aggregate formation in the NMR studies was increased at neutral pH rather than at acidic pH. Also, the rate of aggregation was more rapid for those mutant proteins of mIL-6 that were less stable to unfolding by urea at pH 4.0 and less rapid for the more stable mutant proteins (Ward *et al.*, 1993b). Taken together, these data indicate that the ageing and time-dependent aggregation of mIL-6 is due to partial unfolding of mIL-6, where this form has a strong tendency to aggregate. This parallels the equilibrium unfolding properties of mIL-6 in the presence of denaturant. The ageing process can therefore be delayed by either changing the folding equilibrium N ↔ I in favor of the fully folded form (e.g., increasing the stability of mIL-6) or reducing the aggregation phenomenon I ↔ I_{agg1} (e.g., pH, ionic strength). In summary, these studies demonstrate that under some solution conditions intermediate folded forms of mIL-6 accumulate upon unfolding with denaturant.

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