ADDED IN PROOF

We recently isolated a β cDNA clone with a longer 5′ extension. Compared to that in Figure 2, the derived amino acid sequence differs only from position −54 to position −51. The revised sequence MKNS encompasses a strong translation initiation signal at methionine −54, thus suggesting that β has a leader sequence of 54 amino acids. The sequence preceding residue −50 in Figure 2 is now considered artifactual.

ACKNOWLEDGMENTS

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Conformation, Stability, and Folding of Interleukin 1β[†]

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ABSTRACT: Recombinant human interleukin 1β has been studied in solution with respect to its conformation, stability, and characteristics of unfolding and refolding. It is an all-β-type, stable globular protein with a high cooperativity under conditions where refolding is reversible. The tryptophan residue is approximately 40% exposed to solvent, and the four tyrosines are 50% exposed. The fluorescence of the single tryptophan residue is quenched at pH 7.5 but dequenched by high salt, by titration to lower pH with a pK of 6.59, and by denaturants, resulting in an unusual biphasic change in fluorescence on unfolding. Both histidine and thiol residues have been excluded as being responsible for the pH dependence of fluorescence by site-directed mutagenesis and by chemical modification, respectively. The likely candidate is an aspartate or glutamate.

The family of interleukin 1 proteins have a wide range of biological effects in vivo both local at the site of production by macrophages and also in distant tissues (Duff, 1985; Feldmann, 1985). These biological effects range from prostaglandin E production to stimulation of the acute phase response.

Preparations of authentic human IL-1β[1] are contaminated by other cytokines so it is therefore important to be able to

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[1] Abbreviations: IL-1β, interleukin 1β; CD, circular dichroism; UV, ultraviolet; Gdn-HCl, guanidine hydrochloride; MES, 2-(N-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NATA, N-acetyltryptophanamide; $\Delta G'_{H_2O}$, free energy of stabilization in the absence of denaturant; EDTA, ethylenediaminetetraacetic acid.

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produce IL-1β by recombinant techniques. Human IL-1β is a small protein of 153 residues with a M_r of 17 377 derived from a M_r 31 000 precursor (March et al., 1985). It contains no disulfide bonds but has two solvent-inaccessible sulfydryl groups (Wingfield et al., 1986) and four tyrosine residues, one of which is adjacent to the single tryptophan residue at position 120 in the molecule. The human gene encoding this protein has been cloned, and pure recombinant protein has been produced that is chemically identical with authentic IL-1β (Wingfield et al., 1986).

In this paper the stability and conformation of native recombinant human IL-1β are characterized by circular dichroism, absorbance, and fluorescence. In addition, the unfolding and refolding properties of the molecule have been studied.

Materials and Methods

Chemicals were of Analar grade (BDH, Poole, U.K.). MES, Tris, and NATA were from Sigma and ultrapure Gdn-HCl and urea from BRL. Ethylene glycol was supplied by Pierce as a 50% aqueous solution. Buffers were 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA adjusted to pH 6.5 with HCl and 0.01 M Tris and 0.05 M NaCl adjusted to pH 7.8 with HCl.

Recombinant IL-1β was prepared and purified by the method of Wingfield et al. (1986) and assayed as described.

Mutagenesis of IL-1β. The changes of the histidine codon were introduced by oligonucleotide-directed mutagenesis according to the double-primer method (Norris et al., 1983; Zoller & Smith, 1984). An 1100-bp DNA fragment spanning the coding region for mature IL-1β inserted into a derivative of phage M13mp8, named M13mp108 (which carries an additional *NcoI* site next to the multiple cloning site), was used as template.

The oligonucleotides carrying the mutations were 17 bases long with double mismatches. Mutant phage was detected by plaque hybridization (Wallace et al., 1980) and its sequence confirmed by the dideoxy method (Sanger et al., 1977). The mutated IL-1β gene was subsequently cloned into an expression vector derived from pPLC24 (Buell et al., 1985) in which IL-1β is expressed under the control of bacteriophage λ's p_L promoter and ribosome binding site of the bacteriophage Mu *ner* gene (Wingfield et al., 1986).

Circular dichroism spectra were measured on a Jobin-Yvon Dichrographe IV. Spectra are averages of 4–10 scans with the base line subtracted. Protein concentrations were measured with a calculated value of $A^{1\%}_{1\,cm} = 5.92$ at 280 nm. All solutions were filtered through 0.22-μm filters (Millipore) before use.

Additions of Gdn-HCl were made from a concentrated stock solution in the appropriate buffer. The equilibrium unfolding data were analyzed by the linear extrapolation method (Pace, 1975). A plot of ln K_{eq} vs. [Gdn-HCl] was extrapolated to [Gdn-HCl] = 0 to obtain a value of the apparent free energy change for the transition at zero concentration of denaturant $\Delta G'_{H_2O}$. ln K_{eq} was plotted against ln [Gdn-HCl] to obtain C_M, the midpoint of the transition of $G'_{H_2O} = 0$, the the slope n, which is a measure of the cooperativity of the transition.

Unfolding and refolding kinetics were measured as described in the legends.

Absorbance spectra and protein concentrations were measured on a Cary 210 spectrophotometer with thermostated cell holders. Difference spectra were recorded on a Hewlett-Packard 8450 UV/vis spectrophotometer interfaced to a Hewlett-Packard 9895A flexible disc memory. Difference spectra were directly recorded in matched quartz 2 × 10 mm pathlength tandem cuvettes or were generated by electronic subtraction of separately recorded spectra. For solvent perturbation measurements, 91 μM IL-1β in 100 mM Tris-HCl, pH 7.4, was used. The perturbant was 20% (w/v) ethylene glycol. Calculation of fractional exposure of tryptophanyl and tyrosyl residues was according to the method of Herskovits and Sorensen (1968). Solvent perturbation at denaturing concentrations of 4 M Gdn-HCl was carried out similarly. The molar absorbance change used for tryptophan exposure was $\Delta(\epsilon)_{291-293} = -1600$, and that for tyrosine was $\Delta(\epsilon)_{285} = -700$ (Donovan, 1969). Absorbance values at 291 and 286 nm were corrected for positive absorbance changes due to solvent perturbation of exposed chromophores. The molar absorbances for this effect in 4 M Gdn-HCl were calculated from model amino acid studies and were $\Delta(\epsilon)_{291-293} = 492$ and $\Delta(\epsilon)_{285-287} = 255$ for tryptophan and $\Delta(\epsilon)_{285} = 117$ for tyrosine.

Fluorescence measurements were made on a Perkin-Elmer MPF-3 spectrofluorometer with a thermostated cell holder, and solutions were filtered through 0.22-μm filters (Millipore) before use.

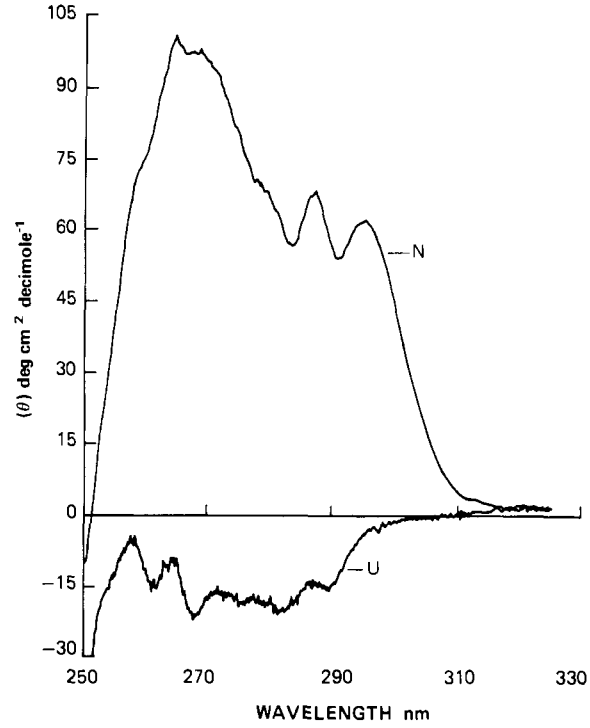

FIGURE 1: Near-UV circular dichroism spectra of native and unfolded interleukin 1β. Spectra are an average of 10 scans with the base line subtracted. Protein concentration was 1.782 and 2.36 mg/mL in 0 and 2 M Gdn-HCl, 0.01 M Tris, and 0.05 M NaCl, pH 7.8, respectively. Spectra were recorded at 25 °C with a 1-cm path-length cell and a 1-nm bandwidth.

Results and Discussion

Circular Dichroism of Interleukin 1β. The near-UV CD spectrum of IL-1β at pH 7.8 is shown in Figure 1. The spectrum shows intense positive ellipticity with a major peak at 266 nm and minor peaks at 287 and 295 nm. The spectrum of IL-1β over the same wavelength range at pH 6.5 is indistinguishable from that in Figure 1, demonstrating no dependence of native conformation over that pH range. Also shown is the spectrum of IL-1β unfolded in 2 M Gdn-HCl, showing the loss of asymmetry of the aromatic groups in the unfolded molecule. The single tryptophan residue at position 120 makes a major contribution to the near-UV CD spectrum.

The far-UV CD spectrum of native IL-1β is shown in Figure 2. The spectrum is not very intense and has minima at 205

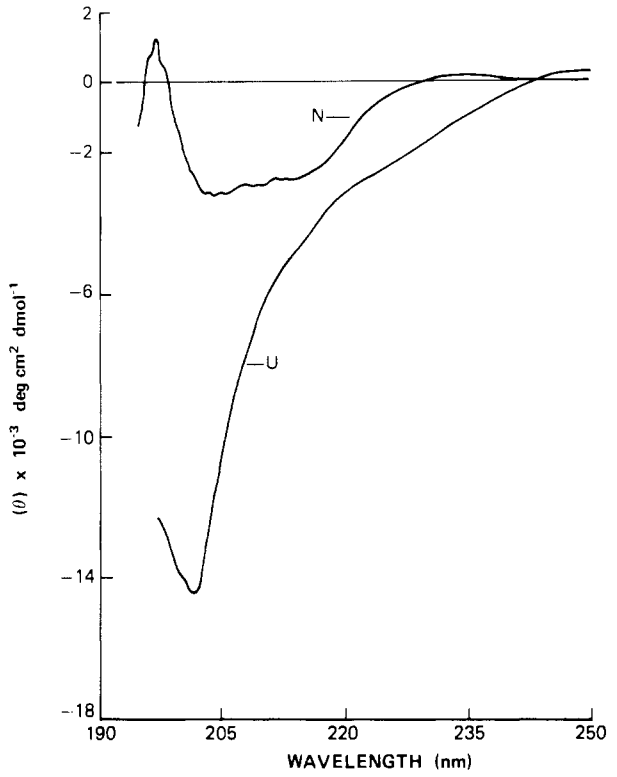

FIGURE 2: Far-UV circular dichroism spectra of native and unfolded interleukin 1β. Spectra are an average of 10 scans with the base line subtracted. Protein concentration was 1.782 and 2.36 mg/mL in 0 and 2 M Gdn-HCl, 0.01 M Tris, and 0.05 M NaCl, pH 7.8, respectively. Spectra were recorded at 25 °C with a 0.01-cm path-length cell and a 2-nm bandwidth.

Table I: Solvent Perturbation Difference Spectroscopy of IL-1β[a]

perturbant	wavelength of major peaks (nm)	molar absorbance, $\Delta(\epsilon)$	fraction exposed Trp	fraction exposed Tyr
ethylene glycol (20%)	291	+176	0.47	
	285	+260		0.49
4 M Gdn-HCl	292	-778 (-1086)	0.32	
	285	-1109 (-1187)		0.58

[a] Ultraviolet difference spectroscopy was carried out as described under Materials and Methods with 91 μM IL-1β. The molar absorbances, based on a IL-1β M_r of 17400, were calculated from the absorbance indicated at peak maxima (ethylene glycol) and peak minima (Gdn-HCl). The Gdn-HCl-derived molar absorbances at 292 and 285 nm have been corrected for the solvent effect of 4 M Gdn-HCl, and in addition, the 285-nm absorbance has been adjusted for the negative Trp contribution resulting from exposure on unfolding at this wavelength. The corrected molar absorbances are given in parentheses. The content of buried and exposed chromophores was calculated from the indicated $\Delta(\epsilon)$ values for native protein in ethylene glycol (Herskovits & Sorensen, 1968) and for the unfolded protein in Gdn-HCl (Donovan, 1969). The fractional expossure of the chromophore is estimated on the basis of one Trp and four Tyr residues per molecule.

and 214 nm, suggesting that there is little α-helix structure present. Analysis of the spectrum with the CONTIN program (Provencher, 1982; Provencher & Gloeckner, 1981) for secondary structure yields estimates of 0% helix, 59% β, and 41% remainder. The far-UV CD spectrum of IL-1β unfolded in 2 M Gdn-HCl also shown in Figure 2 is characteristic of a random-coil structure and emphasizes the low intensity of the native ellipticity.

Exposure of Aromatic Residues in IL-1β. The results of solvent perturbation spectroscopy of IL-1β are shown in Table I and Figure 3. From the red shifts in ethylene glycol and the blue shifts in 4 M Gdn-HCl, the single tryptophan residue is approximately 40% exposed to solvent while 50% of the four

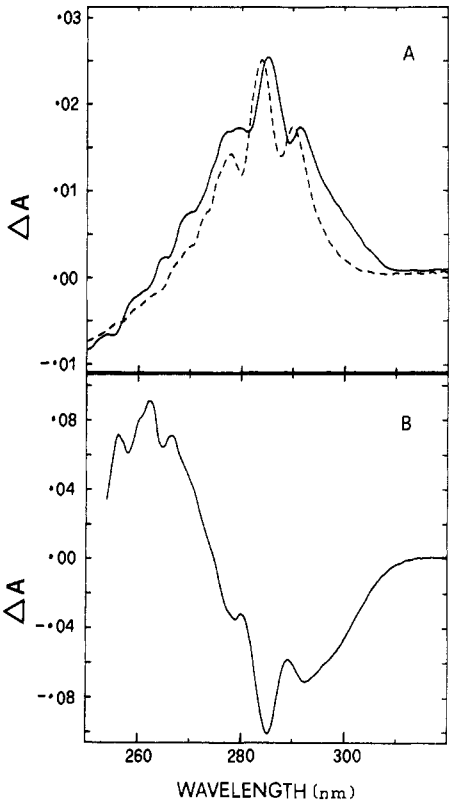

FIGURE 3: Solvent perturbation difference spectra of IL-1β. (A) The solid line is the difference spectrum observed for native protein, 91 μM in tryptophanyl and 364 μM in tyrosyl residues. The dashed line is the spectrum observed for 46 μM *N*-acetyltryptophanamide and 145 μM *N*-acetyltyrosinamide. The solvent perturbant was 20% ethylene glycol. (B) The spectrum is that observed for unfolded protein in 4 M Gdn-HCl, which contains 91 μM tryptophanyl and 364 μM tyrosyl residues. All protein and model chromophore solvents contained 50 mM Tris-HCl, pH 7.4.

tyrosine residues are exposed.

Reversibility of Unfolding. Initial unfolding experiments were carried out in 2, 4, and 6 M Gdn-HCl, 0.01 M Tris, 0.05 M NaCl, pH 7.8. The protein was found to be fully unfolded by near- and far-UV CD spectra at 2 M Gdn-HCl and above. Reversibility soon after unfolding as judged by dilution to low concentrations of Gdn-HCl at the same pH was found to be only 50% as measured by recovery of near-UV ellipticity. The yield of refolded protein was found to decrease to zero if the protein was allowed to stand in concentrations of Gdn-HCl between 2 and 6 M at pH 7.8, indicating that the protein aggregates even in high concentrations of Gdn-HCl. Removal of the denaturant by dialysis resulted in aggregation of all the protein as measured by absorbance.

Similar lack of reversibility was found on unfolding IL-1β in 8 M urea at pH 7.8. Unfolded β-lactamse from *Staphylococcus aureus* can be refolded in the presence of denaturing concentrations of urea simply by addition of ammonium sulfate to the solution (Mitchinson & Pain, 1985). Addition of ammonium sulfate to a concentration of 1.2 M to IL-1β unfolded in 8 M urea at pH 7.8 however led to complete aggregation.

Unfolding of IL-1β in 4 M Gdn-HCl, pH 7.8, and refolding in 0.4 M Gdn-HCl, pH 7.8, in the presence of 1% β-mercaptoethanol increased the yield of refolded protein to 85%, demonstrating that the lack of reversibility of unfolding and aggregation of the unfolded state is associated under these conditions primarily with intermolecular disulfide bond formation.

In order to diminish disulfide bonding and improve yields without the necessity of adding reductant, refolding was at-

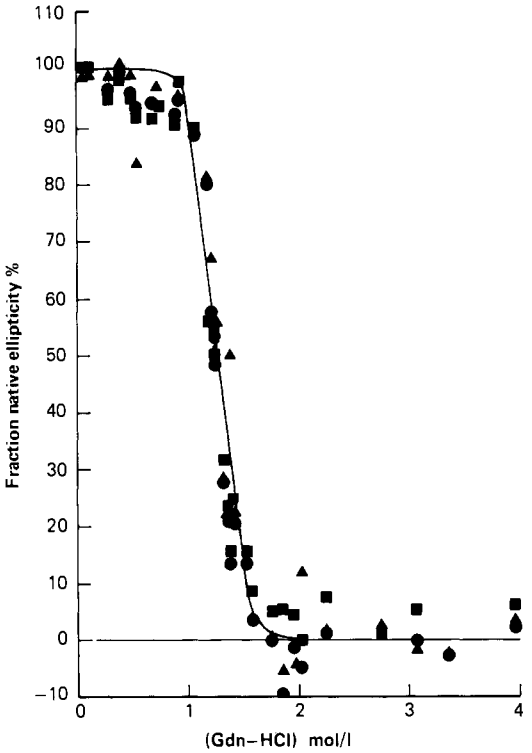

FIGURE 4: Reversible unfolding transition of interleukin 1β in Gdn-HCl measured by near-UV CD. Protein concentration was 0.64–0.89 mg/mL in 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5. Protein solution was titrated with small weighed additions of a concentrated stock solution of Gdn-HCl made up in the same buffer. The circular dichroism at 266 (▲), 287 (●), and 295 nm (■) was measured after overnight incubation to reach equilibrium at 25 °C. Unfolding data from each wavelength are normalized and pooled for the calculation of thermodynamic parameters. The solid line is the theoretical curve calculated from the midpoint of the transition.

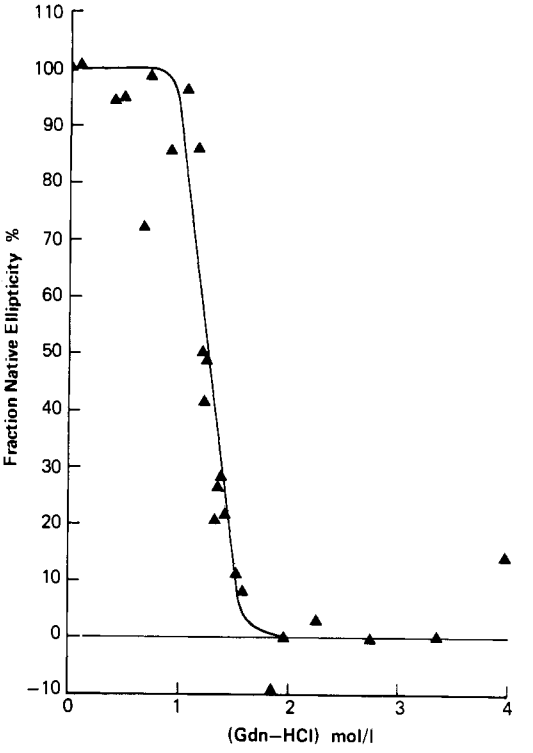

FIGURE 5: Reversible unfolding transition of interleukin 1β measured by far-UV CD. Protein concentration was 0.64–0.89 mg/mL in 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5. Protein solution was titrated with small weighed additions of a concentrated stock solution of Gdn-HCl made up in the same buffer. The circular dichroism at 205 nm with a 0.005-cm path-length was measured after overnight incubation at 25 °C to reach equilibrium. The solid line is the theoretical curve calculated from the near-UV unfolding transition (Figure 4).

tempted at a lower pH in the presence of EDTA. It was found that when IL-1β was unfolded in 4 M Gdn-HCl, 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5, followed by dilution to 0.8 M Gdn-HCl in the same buffer, reproducible yields of 90–100% refolded protein were obtained as judged by near-UV ellipticity.

Equilibrium Unfolding by Gdn-HCl. Conditions for reversible unfolding having been established, the equilibrium unfolding curves shown in Figures 4 and 5 were constructed. The unfolding was followed at 205 nm in the far-UV and at 266, 287, and 295 nm in the near-UV CD. The values of ellipticity at all three near-UV wavelengths change simultaneously (Figure 4).

The near-UV CD data were analyzed as described under Materials and Methods assuming a two-state transition, and the thermodynamic parameters obtained are $\Delta G'_{H_2O}$ = 29.4 ± 2.2 kJ/mol, d ln K_{eq}/d ln [Gdn-HCl] = 12.2 ± 0.8, and C_M = 1.26 ± 0.15 mol/L.

The midpoint of 1.26 mol/L was used to calculate a theoretical unfolding curve, which is plotted as a solid line through the data in Figures 4 and 5. The data show that IL-1β is a thermodynamically stable protein with a high cooperativity of unfolding as shown by the value of d ln K_{eq}/d ln [Gdn-HCl]. Figure 5 demonstrates the coincidence of unfolding of the secondary structure in the far-UV CD with that of the tertiary structure in the near-UV CD, which underlines the cooperativity of the unfolding process. It demonstrates the absence of any structured equilibrium intermediates in the process, which justifies the two-state assumption.

Kinetics of Unfolding. A comparison of the apparent first-order rate constants for the unfolding of IL-1β in Gdn-HCl monitored by the decrease in ellipticity at 266 nm is given for pH 6.5 and 7.8 in Table II. The results show that the protein unfolds faster at the higher pH. The first-order kinetic plots are monophasic; extrapolating to the native value of $[\theta]_{266nm}$ at zero time and between 2 and 6 M Gdn-HCl, there is a linear relationship between ln k'_{app} and Gdn-HCl concentration.

Table II: Kinetics of Unfolding for IL-1β in Gdn-HCl Measured by Circular Dichroism and Fluorescence Emission[a]

[Gdn-HCl] (mol/L)	$k'_{app} \times 10^4$ (s^{-1}) pH 6.5	pH 7.8
	(a) Circular Dichroism	
2.0	3.5 ± 0.3	7.3 ± 0.1
4.0	30 ± 2.0	58 ± 6.0
6.0	250 ± 20	
	(b) Fluorescence	
2.0		8.0 ± 0.4
4.0	34 ± 4.0	74 ± 3.0

[a] Kinetics were measured at 25 °C at the pH and Gdn-HCl concentration stated above. Protein concentrations were (a) 0.47–1.3 mg/mL and (b) 0.15–0.19 mg/mL in 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5, or 0.01 M Tris and 0.05 M NaCl, pH 7.8. The decrease in ellipticity at 266 nm was measured with a 1-cm path-length cell and 2-nm bandwidth. The decrease in fluorescence emission at 340 nm (5.5-nm bandwidth) was measured with a 1-cm path-length cell and excitation at 291 nm (5-nm bandwidth).

When the unfolding of IL-1β at pH 6.5 is followed by the change in fluorescence at the wavelength of maximum emission, 344 nm (λ_{ex} = 295 nm), an initial increase in fluorescence to 160% of that of the unperturbed molecule occurs within the dead time for measurement. This is followed by a first-order

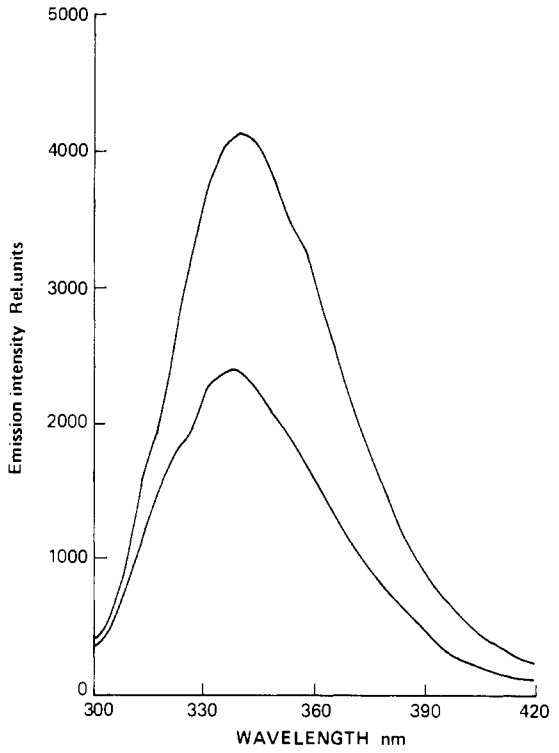

FIGURE 6: Effect of NaCl on fluorescence emission spectrum of IL-1β. Spectra have base lines subtracted; values were calculated as relative fluorescence. Protein concentration was 0.033 and 0.036 mg/mL in the absence (lower spectrum) and presence (upper spectrum) of 2.5 M NaCl in 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5. Protein was excited at 291 nm with a 5-nm bandwidth and 1-cm path length, and the intensity of emission was measured at a 5.5-nm bandwidth.

Table III: Fluorescence Emission Studies on IL-1β[a]

IL-1	concn (μg/mL)	pH	emission λ_{max} (nm)	corrected relative intensity/50 μg/mL
native	50	7.5	344.5	96
unfolded	50	7.5	352.5	69
native	50	5.5	345.0	164.17
unfolded	50	5.5	353.5	78.69
native (alk)[b]	44	7.5	340.0	124.25
native (alk)[b]	44	5.5	343.5	181.68
NATA[c]	50[d]	7.5	356.0	154.2

[a] Fluorescence was measured as described under Materials and Methods. [b] IL-1β alkylated with iodoacetamide (see text). [c] *N*-Acetyltryptophanamide. [d] NATA concentration equivalent to 50 μg/mL IL-1β.

decay of fluorescence with kinetic rate constants similar to those measured by circular dichroism and which therefore reflects the unfolding of the protein. A similar rapid increase is induced by 2.5 M NaCl, pH 6.5 (Figure 6), and also by lowering the pH in 0.1 ionic strength buffer to pH 5.5. Titration of the fluorescence occurs with a pK_a of 6.59 to minimum fluorescence emission at higher pH (Figure 7). The enhanced fluorescence is close to that of *N*-acetyltryptophanamide (NATA) at the same molar concentration (Table III). It is suggested that the tryptophan fluorescence is quenched at higher pH in IL-1β by the presence of a neighboring group whose position relative to the tryptophan residue depends on the interactions of a group having a pK of 6.59. Protonation of this group, charge shielding by high salt concentrations, or subjection to Gdn-HCl each modify the interactions of the group and hence its spatial location. Both quenched and unquenched forms of the protein exhibit the

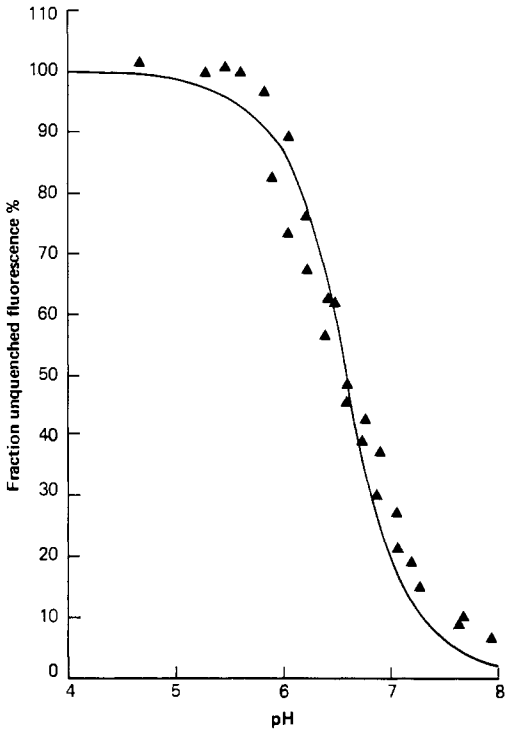

FIGURE 7: Effect of pH on fluorescence emission intensity of IL-1β at 340 nm. Protein initially at 0.14 mg/mL in 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5, was titrated with small amounts of HCl and NaOH. The protein was excited at 291 nm with a 5-nm bandwidth, and the intensity of emission at 340 nm was measured with a 5-nm bandwidth and a 1-cm path-length cell.

same emission maximum wavelength of 344 nm, which shifts on complete unfolding in Gdn-HCl to 353 nm, close to that of NATA at 356 nm (Table III). This supports the conclusion from difference spectra that the tryptophan residue is partly buried in IL-1β, independent of the pH within this range.

Unfolding the protein by 8 M urea at pH 6.5 showed the same phenomenon with a large, rapid increase in fluorescence emission followed by a slow decrease, indicating unfolding with a rate constant 6×10^2 times that in 4 M Gdn-HCl. This supports the suggestion that quenching may be due to rapid separation of the quenching group from the tryptophan residue as a result of a minor conformational change. Extrapolation of the first-order kinetic plots obtained from unfolding both in Gdn-HCl and in urea and monitored by circular dichroism show that no equivalent fast change in ellipticity occurs. Similarly, 3.3 M NaCl and 2.4 M ammonium sulfate have little or no effect on the near-UV CD spectrum (Figure 8). These results suggest that the quenching group moves relative to the tryptophan without disturbing the environmental polarity of the latter. Confirmation of the detailed mechanism will have to await the three-dimensional structure of IL-1β.

The pK value of 6.59 might most likely be assigned to histidine, thiol, or carboxyl residues or to the amino-terminal amino group. Protonation of the indole ring of tryptophan causes quenching but with a pK of less than 2 (White, 1959). Two mutants of IL-1β were constructed in which the single histidine residue at position 30 is replaced by asparagine and arginine, respectively. The near- and far-UV CD spectra, fluorescence emission spectra, and high-resolution NMR spectra (A. Gronenborn, personal communication) were closely similar to those of IL-1β. Both mutants show a similar pH dependence of fluorescence to that of IL-1β with pK values of 6.63 and 6.51 for the IL-1β(Asn-30) and IL-1β(Arg-30), respectively. The possible direct or indirect involvement of one of the two thiol groups was similarly excluded by the fact

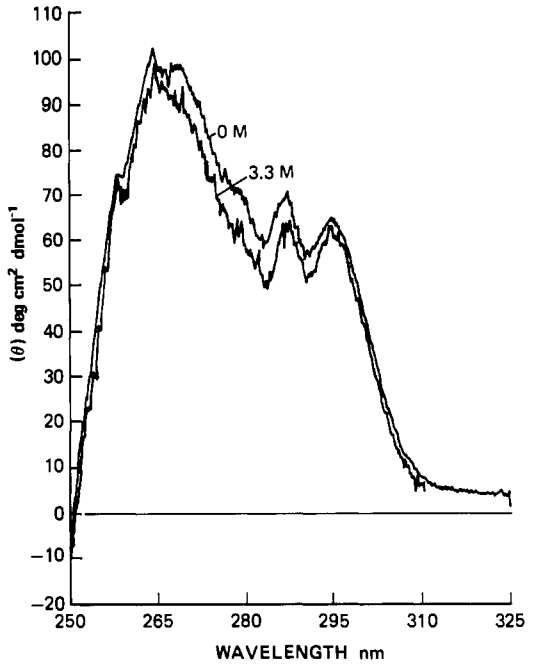

FIGURE 8: Effect of NaCl on near-UV CD spectrum of IL-1β. Spectra are an average of four scans with the base line subtracted. Protein concentration was 0.88 and 1.7 mg/mL in 0 and 3.3 M NaCl, 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5, respectively. Spectrum was recorded at 20 °C with a 1-cm path-length cell and a 1-nm bandwidth.

that IL-1β alkylated with iodoacetamide (Wingfield et al., 1986) showed the same pH dependence of fluorescence as native IL-1β (Table III). For an NH_2-terminal α-amino group to exhibit a pK of 6.6, it would have to be in a constrained environment. This preparation of IL-1β consists of 80% of a form with NH_2-terminal alanine and 20% methionylalanyl protein. The latter can be separated from the non-methionylated form (Wingfield et al., 1987) and is found to show the same pH dependence of fluorescence as the non-methionylated form. Any constraint on the NH_2-terminal amino group would have therefore to be unaffected by the insertion of an additional peptide residue. Further, the NH_2-terminal methionine is rapidly removed by the specific methionine amino peptidase, indicating that the group is readily accessible and not buried. Thus it is unlikely that the terminal amino group can account for the pH-dependent quenching of tryptophan fluorescence. This leaves a carboxyl group as the most likely candidate, with an anomalous pK induced by salt bridge or hydrogen-bond interactions.

Refolding Kinetics of IL-1β. The kinetics of refolding of IL-1β measured by changes in near-UV ellipticity at various concentrations of Gdn-HCl at pH 6.5 are summarized in Table IV. The first-order plots are biphasic with the slow measurable phase preceded by a fast phase ($t_{1/2} < 1$ s). The slow phase decreases from 50% of the total amplitude at 0.8 M Gdn-HCl to a constant value of 35% below 0.4 M Gdn-HCl.

Two types of mechanism may be advanced to explain the biphasic kinetics. These mechanisms are (a) proline isomerization giving rise to both fast and slow refolding populations of unfolded and partially folded molecules (Brandts et al., 1975; Kim & Baldwin, 1982) and (b) a sequential mechanism of a type similar to that described by Kim and Baldwin (1982). In this latter refolding scheme, a fast reaction would be followed sequentially by a slower reaction, in each of which there is an increase in ellipticity.

In order to test for the first possibility, a double-jump experiment of the type described by Brandts et al. (1975) was used. Samples of IL-1β freeze-dried from water were taken up at 10 mg/mL in 7.65 M Gdn-HCl, 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5, and unfolded at 20 °C for 40 and 3300 s, respectively. Under these conditions it was shown that the near-UV ellipticity at 266 nm coincides with that of unfolded IL-1β after 40 s. After these times the protein was diluted to 0.38 M Gdn-HCl in the same buffer, and the kinetics of regain of ellipticity at 266 nm were followed. The apparent first-order refolding rate constants obtained after 40- and 3300-s unfolding were $k'_{app} \times 10^3 = 5.7 \pm 0.5$ and 5.5 ± 0.8 s^{-1}, respectively. The kinetics in each case were biphasic as described above for refolding at 25 °C with the same amplitudes for the fast and slow phases as measured in those experiments.

Table IV: Kinetics of the Slow Refolding Phase of IL-1β[a]

[Gdn-HCl] (mol/L)	$k'_{app} \times 10^3$ (s^{-1})	amplitude (%)
0.2	15.2 ± 3.2	33 ± 6
0.4	7.6 ± 0.7	35 ± 2
0.8	2.8 ± 0.4	53 ± 2

[a] Kinetics were measured at 25 °C. IL-1β was unfolded in 4 or 6 M Gdn-HCl, 0.01 M MES, 0.09 M NaCl, and 1 mM Na_2EDTA, pH 6.5, and was diluted in the same buffer to the stated final concentration of Gdn-HCl giving protein concentrations of 0.25–0.6 mg/mL. The increase in ellipticity at 266 nm on dilution to refolding conditions was measured with a 1-cm path-length cell and a 2-nm bandwidth.

These results demonstrate that the slow kinetic phase in the refolding of IL-1β is not a consequence of isomerization of proline or other residues to nonnative conformers in the unfolded molecule.

Conclusions

The major fraction (80%) of the recombinant IL-1β used in these experiments is chemically identical with authentic IL-1β (the remaining 20% differs only in having an NH_2-terminal methionine, so that the results of this investigation apply also to naturally occurring IL-1β.

The secondary structure estimates for IL-1β obtained from the far-UV circular dichroism spectrum place this protein in the all-β class (Levitt & Chothia, 1976). Within the folded structure of the protein the fluorescence of the single tryptophan is dependent on the state of a group, probably a carboxyl, with pK = 6.59. The fluorescence emission can be dequenched by high ionic strength and low pH and also by addition of denaturants.

Interleukin 1β is a thermodynamically stable globular protein with $\Delta G'_{H_2O}$ = 29.4 kJ/mol, which demonstrates a complete and fully reversible unfolding transition in Gdn-HCl at pH 6.5. The reversibility of refolding is pH-dependent, the yield of refolded protein decreasing at higher pH with the onset of aggregation. The protein exhibits a highly cooperative two-state unfolding transition with the absence of any stable unfolding intermediates in the transition region.

The unfolded molecule refolds with biphasic kinetics. The biphasic refolding is not caused by isomerization of proline or other residues, and the results are suggestive of a sequential mechanism in which some tertiary structure is formed in each phase.

There is growing evidence from a number of proteins that exhibit biphasic refolding transitions that the first phase in folding is a rapid formation of secondary structure within a collapsed, compact globular state (Creighton, 1979; Creighton & Pain, 1980; Mitchinson & Pain, 1985; Craig et al., 1985; Dolgikh et al., 1985). This initial fast phase is followed by a slow rearrangement of the compact globule to form the native state of the protein. The rearrangement step involves shuffling

of the structural units with formation and breaking of moderate intramolecular forces until the contacts and interfaces characteristic of the native state are formed. The results presented here on the refolding of IL-1β would be consistent with this type of mechanism.

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Comparative Phosphorescence and Optically Detected Magnetic Resonance Studies of Fatty Acid Binding to Serum Albumin†

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ABSTRACT: The binding of free fatty acid to bovine serum albumin (BSA) and human serum albumin (HSA) was studied by phosphorescence and optical detection of triplet-state magnetic resonance spectroscopy in zero applied magnetic field. We have found that oleic acid perturbs the excited triplet state of Trp-134 but not that of Trp-212 in BSA. The assignment is made by comparing the BSA results with those obtained from oleic acid binding to HSA. The phosphorescence 0,0 band as well as the zero-field splittings of Trp-134 undergoes significant changes upon binding of oleic acid to BSA. Shifts of the 0,0-band wavelength and of the zero-field splittings point to large changes in the Trp-134 local environment which accompany the complex formation. The shifts are progressive until 3-4 mol of oleic acid is added. The spectroscopic changes may be attributed to Stark effects caused by a protein conformational change near Trp-134 in the BSA–oleate complex. Oleic acid binding has a minimal effect on the triplet-state properties of the single Trp-214 of HSA. The binding specificity with regard to chain length and unsaturation is reflected by the differences in the Trp environment when BSA forms complexes with various fatty acids.

Albumin is the most abundant protein in mammalian plasma. Albumin's most important physiological function is to bind and transport fatty acids, but it also carries many other hydrophobic ligands such as lysolecithin, bilirubin, tryptophan, steroids, and drugs (Goldstein, 1949; Peters, 1975). Serum albumin's structure and function, including fatty acid metabolism and binding sites, have been recently reviewed by Peters (1985). Serum albumin is a single peptide chain of about 580 residues; it is cross-linked by 17 disulfide bonds. The amino acid sequences of bovine serum albumin (BSA)[1] and human serum albumin (HSA) have been compared in some detail by Brown (1977). The general structural details of these are essentially the same; the differences in sequence

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; BSA, bovine serum albumin; HSA, human serum albumin; ODMR, optical detection of triplet-state magnetic resonance; SDS, sodium dodecyl sulfate.

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