Analysis of the Conformation and Stability of *Escherichia coli* Derived Recombinant Human Interleukin 4 by Circular Dichroism

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ABSTRACT: The conformation and stability of *Escherichia coli* derived recombinant human interleukin 4 (rhuIL-4) have been examined by circular dichroism (CD). Protein unfolding was detected by ellipticity changes at 222 nm with increasing concentrations of guanidine hydrochloride (GdnHCl). The unfolding midpoint ($[GdnHCl]_{1/2}$) was 3.7 M, the free energy of unfolding, ($\Delta G_D^{H_2O}$), was 5.9 kcal/mol and the dependence of ΔG_D on the GdnHCl concentration (m) was 1.6 (kcal/mol)/M. This unfolding was demonstrated to be reversible upon removal of the GdnHCl by dialysis. Analysis of the far-UV CD spectrum indicated the presence of a high percentage of α-helical structure (ca. 73%). A small change in ellipticity was noted over the pH range 1.9–9.6, suggesting that the protein undergoes a minor conformational change with an apparent pK_a of 4.17. Virtually complete biological activity, measured in vitro in a T-cell proliferation assay, was recovered following exposure to extreme values of pH (i.e., pH 3 and 10). An analysis of the near-UV CD spectrum indicated that the single tryptophan residue at position 91 was unconstrained and most likely exposed to the solvent. Titration with 4,4′-dithiodipyridine and 2-nitrothiosulfobenzoate established that the six cysteine residues in rhuIL-4 were involved in intramolecular disulfide linkages. These data support that rhuIL-4 has a highly stable three-dimensional structure.

A cDNA sequence coding for human interleukin 4 (huIL-4),[1] originally known as B-cell growth factor I or B-cell stimulating factor 1, has been isolated on the basis of homology with the cDNA of murine IL-4 (Yokota et al., 1986). Expression of the cDNA for huIL-4 in both mammalian and bacterial hosts has provided material for physicochemical and biological evaluation (van Kimmenade et al., 1988; Le et al., 1988, Sonoda et al., 1988). RhuIL-4 elicits pleiotropic biological activities on various cell types of both lymphoid and myeloid origin [reviewed by J. Banchereau (1990)]. In particular, rhuIL-4 has been shown to costimulate the proliferation of both preactivated B cells and T cells (Yokota et al., 1986; DeFrance et al., 1987; Spits et al., 1987), act as a differentiating factor for activated B-cells to produce various immunoglobulin subclasses (DeFrance et al., 1988), and induce class II major histocompatibility antigens (Rousset et al., 1988) as well as lymphocyte function adhesion antigens (Rousset et al., 1989). Thus, rhuIL-4 may play a fundamental role in B-cell differentiation and growth as well as antigen presentation. In addition, rhuIL-4 enhances the differentiation and functional activities of myeloid cells (Grace et al., 1989). Of special interest is the ability of rhuIL-4 to inhibit the interleukin 2 induced proliferation of chronic lymphocytic leukemic B cells (Karray et al., 1988). On the basis of these diverse properties, rhuIL-4 is predicted to have value in a variety of clinical indications, including B-cell leukemias and lymphomas as well as immunodeficiency diseases.

Although a significant number of biological activities have been elucidated for rhuIL-4, there are little data on the physicochemical properties of the purified protein. In this report we characterize aspects of the secondary and tertiary structure of rhuIL-4 on the basis of circular dichroism studies. Stability studies demonstrated that rhuIL-4 can undergo reversible conformational changes upon exposure to high concentrations of guanidine hydrochloride (GdnHCl) or extreme values of pH. This reversibility may be related, at least in part, to the presence of three disulfide bonds. We conclude that rhuIL-4 has a three-dimensional structure that is highly stable under various solvent conditions.

Materials and Methods

Materials. RhuIL-4 derived from *Escherichia coli* was purified, refolded, and oxidized by using chromatography and refolding methods similar to those reported by van Kimmenade et al. (1988). *E. coli* derived rhuIL-4 contains an additional initiator methionine at the N-terminus.

Spectroscopy. All absorption spectra were recorded by using a dual-beam Cary 118C spectrophotometer. Samples were prefiltered with a 0.22-μm GV millipore filter and spectra were obtained by using a 1-cm path length cell at room temperature. Buffer blanks were subtracted from all spectra. CD spectra were recorded on an IBM-interfaced Jasco 500C spectropolarimeter. Spectra were recorded at room temperature by using 0.02-, 0.1-, and 1.0-cm path length cells for protein concentrations of 0.2, 0.1, and 1.0 mg/mL, respectively. Each spectrum is the average of 10 scans. A solvent spectrum was subtracted from each protein spectrum. Reported spectra are expressed as mean residue ellipticity $[\theta]_{MRW}$ and calculated by using a mean residue weight of 115. CD data in the near-UV region are also expressed as the molar circular dichroic extinction coefficient, $\Delta\epsilon = [\theta]_{MRW}N/3300$, where $N = 130$ is the total number of amino acids.

Protein Concentration Determination. Protein concentration was first determined from the absorbance of the peptide backbone at 205 nm, as described by Scopes (1974). Utilizing this method, we determined the molar extinction coefficient of rhuIL-4 at 278 nm, the absorbance maximum, which was

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[1] Abbreviations: IL-4, interleukin 4; rhuIL-4, recombinant human IL-4; huIL-4, human IL-4; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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used for determining protein concentrations in all of the experiments. Samples used in determining the extinction coefficient were dialyzed into 5 mM potassium phosphate, pH 7.2, and 50 mM potassium sulfate and filtered with a 0.22-μm GV millipore filter. Control experiments using bovine serum albumin and ribonuclease A indicated that the calculated extinction coefficients from Scopes' method were within 6% of reported values (Whitaker & Granum, 1980). The molar extinction coefficient of rhuIL-4 at 278 nm was determined to be 9370 M^{-1} cm^{-1} (or $^{0.1\%}\epsilon_{278}$ = 0.625 mg^{-1} cm^2). This value is only 9% larger than the theoretically determined molar extinction coefficient, 8550 M^{-1} cm^{-1}, which was calculated by using the individual extinction coefficients for tryptophan, tyrosine, phenylalanine, and cystine (Fasman, 1976). The value of the measured extinction coefficient was confirmed by quantitating the concentration of tryptophan residues from spectral changes arising from oxidation of tryptophan using N-bromosuccinimide (Spande & Witkop, 1967). The extinction coefficient determined by this method was within 4% of the measured value (data not shown). Amino acid analysis further confirmed the calculated value for the extinction coefficient.

Unfolding Experiments. Unfolding of rhuIL-4 (50 mM sodium phosphate, pH 7.2) in a final volume of 250 μL was achieved by adding increasing volumes of 8 M GdnHCl (ultrapure, Schwartz/Mann, Cambridge, MA) and 50 mM sodium phosphate, pH 7.2. Samples were equilibrated at room temperature for 30 min prior to recording the CD spectra. This incubation time was sufficient for the unfolding process to reach equilibrium based on the absence of further changes in the ellipticity at either the near- or far-UV wavelength region. The far-UV CD spectrum of rhuIL-4 was also measured as a function of pH. Concentrated rhuIL-4 (1.2 mg/mL) in water was diluted 10-fold into a buffer containing 0.05 M potassium sulfate and 5 mM of either sodium citrate, sodium acetate, sodium phosphate, or Tris-HCl adjusted to the indicated pH values. Spectra were recorded at room temperature 30 min after dilution. The pH of each sample was recorded at both the beginning and the end of each spectrum. The apparent pK_a was calculated by nonlinear least-squares analysis.

Cysteine Quantitation. Reduction of rhuIL-4 (0.5 mg/mL) was achieved by incubating with 3 mM DTT for 2.5 h at 37 °C in the presence of 0.25 M Tris-HCl, 2 mM EDTA, and 4 M GdnHCl, pH 8.5, under nitrogen. After reduction, the protein was S-carboxymethylated with the addition of iodoacetate (7.5 mM) and incubated at 37 °C in the dark for 30 min. Unreacted iodoacetate was removed by desalting on a Sephadex G-25 column equilibrated with 50 mM sodium phosphate, pH 7.0. Quantitation of the carboxymethylcysteine derivatives was performed by amino acid analysis.

Chemical Analysis of Free Sulfhydryl and Disulfide Content. The sulfhydryl content was determined by using 4,4'-dithiodipyridine (Grassetti & Murray, 1967). Control experiments using bovine serum albumin yielded 0.78 ± 0.01 sulfhydryls per mole of protein, which is similar to the value of 0.687 reported by Grassetti and Murray (1967). The disulfide content was determined by using disodium 2-nitro-5-thiosulfobenzoate in the presence of excess sodium sulfite (Thannhauser et al., 1984; Damodaran, 1985). Control experiments using lysozyme and ribonuclease, which contain four disulfide bonds each, yielded values of 4.05 ± 0.14 and 4.04 ± 0.31 cystines per mole of protein, respectively. Data are expressed per mole of rhuIL-4 (molecular weight 14963) and are the mean of three or four replicates ± SEM.

Bioactivity. The in vitro biological activity of rhuIL-4 was determined with a T-cell proliferation assay employing human peripheral blood mononuclear cells stimulated with phytohemagglutinin (Yokota et al., 1986).

Results

CD Spectral Analysis. Near-UV CD spectra can provide valuable information regarding the tertiary structure of aromatic residues. The near-UV CD spectrum of *E. coli* derived rhuIL-4 is shown in Figure 1A. The low-magnitude positive ellipticity observed at 292 nm is most likely the result of an optical rotation arising from the ^1L_b resonance of the indole ring from the single tryptophan present in rhuIL-4 (Trp-91) (Strickland, 1974). The magnitude of the ellipticity at this wavelength is relatively low and may be due to a lack of constraint on the tryptophan residue as a result of its high degree of exposure to the solvent (Strickland, 1974). No significant ellipticity or fine structure is observed in the region between 270 and 285 nm, where optical activity arising from the two tyrosine residues (Tyr-56, Tyr-124) would be expected. The remaining region of the spectrum contains two negative ellipticity peaks at 263 and 268 nm, which most probably arise from one or more of the six phenylalanine residues present in rhuIL-4 (Strickland, 1974). An interesting feature of the phenylalanine ellipticity is that the peak positions are negatively offset from the origin. The increasing negative ellipticity between 270 and 250 nm most likely arises from the three disulfide linkages present in rhuIL-4 (see below).

The far-UV CD spectrum of *E. coli* derived rhuIL-4 has prominent negative ellipticities at 208 and 222 nm and a large positive ellipticity at 192 nm (Figure 1B). The shape and magnitude of the ellipticity spectrum suggest that the secondary structure of rhuIL-4 is highly ordered. The mean residue ellipticity spectrum of rhuIL-4 was fit by the linear least-squares procedures of Provencher and Glockner (1981) and Chang et al. (1978). The predicted fractions of secondary structure determined from these routines are respectively $f_{\alpha\text{helix}}$ = 0.73, $f_{\beta\text{sheet}}$ = 0.12, $f_{\text{remainder}}$ = 0.15 and $f_{\alpha\text{helix}}$ = 0.75, $f_{\beta\text{sheet}+\beta\text{turn}}$ = 0.25, $f_{\text{remainder}}$ = 0.0.

It is evident from the spectrum in Figure 1B and the above calculations that rhuIL-4 is predominantly α helical. This result is also predicted from secondary structure calculations based on the primary sequence of the protein (Chou & Fasman, 1978; Garnier et al., 1978).

Protein Structural Stability. a. Unfolding by GdnHCl. Protein structural stability is frequently determined through an analysis of the extent of unfolding as a function of GdnHCl or urea concentration. Unfolding of proteins using these denaturants has been shown to be reversible and involve a two-state equilibrium mechanism, N $\rightleftharpoons$ D (Tanford, 1968, 1970). A measure of structural stability is afforded from the value of the free energy change of unfolding, ΔG_D. This value is readily obtained from a protein secondary structure unfolding curve (Greene & Pace, 1974; Pace, 1975) if a two-state unfolding mechanism is assumed. Under this condition the equilibrium constant, K_D, is expressed as $K_D = f_D/f_N = e^{-\Delta G_D/RT}$ where f_D and f_N represent the fractions of protein present in the denatured (D) and native (N) states, respectively.

We have used GdnHCl to evaluate the structural stability of rhuIL-4. The far-UV CD spectrum for the fully unfolded protein in the presence of 6.0 M GdnHCl is shown in Figure 1B. This spectrum is similar to that previously observed for nonstructured, unfolded polypeptides (Greenfield & Fasman, 1969). The extent of unfolded secondary structure was followed by measuring the change in ellipticity at 222 nm with

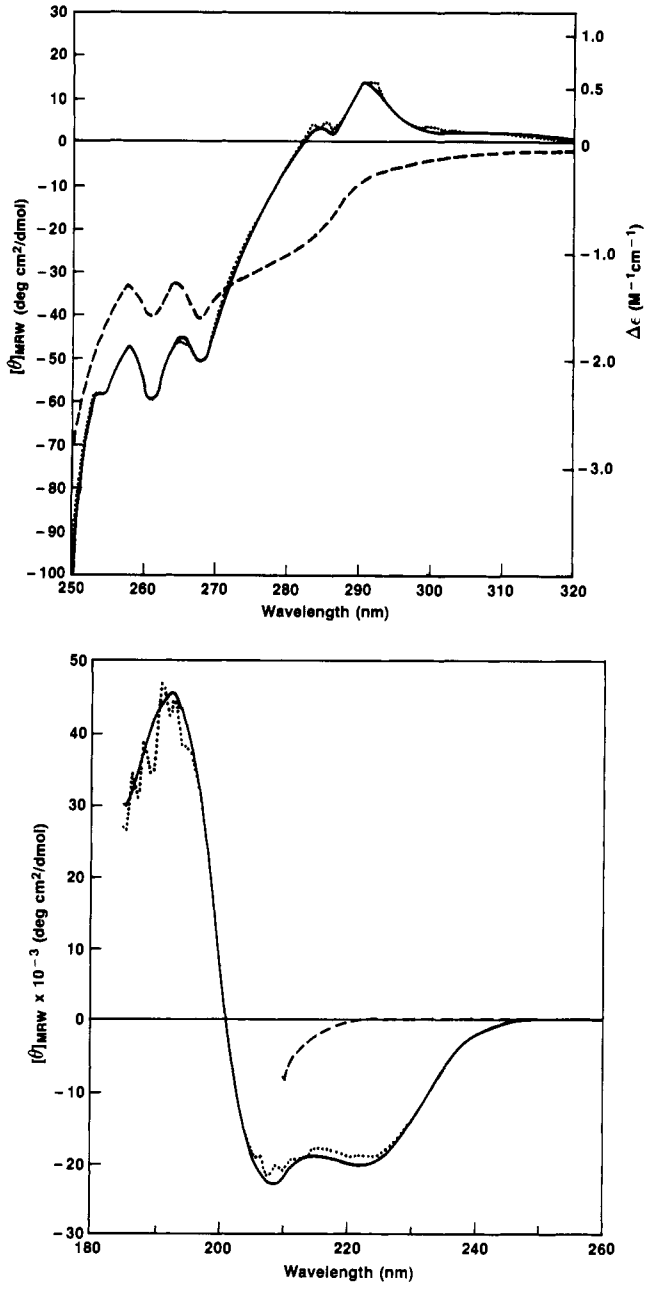

FIGURE 1: (A, Top) Near-UV CD spectrum of *E. coli* derived rhuIL-4 ± GdnHCl. (—) Native rhuIL-4 in the absence of denaturant; (---) rhuIL-4 in the presence of 6.0 M GdnHCl; (···) refolded rhuIL-4 following removal of GdnHCl by dialysis against 50 mM sodium phosphate, pH 7.2. The left-hand scale is given in terms of the mean residue ellipticity, $[\theta]_{MRW}$, and the right-hand scale is in terms of the differential molar circular dichroic extinction coefficient, $\Delta\epsilon$ (see Discussion). In all cases, the protein concentration was 1.0 mg/mL and the buffer was 50 mM sodium phosphate, pH 7.2. (B, Bottom) Far-UV CD spectrum of *E. coli* derived rhuIL-4 ± GdnHCl. (—) Native rhuIL-4 in the absence of denaturant; (---) rhuIL-4 in the presence of 6.0 M GdnHCl; (···) refolded rhuIL-4 following removal of GdnHCl by dialysis against 50 mM sodium phosphate, pH 7.2. In all cases the protein concentration was 0.2 mg/mL and the solvent was 50 mM sodium phosphate, pH 7.2, ± 6.0 M GdnHCl.

respect to increasing GdnHCl, Figure 2. The midpoint of unfolding, $[GdnHCl]_{1/2}$, occurred at 3.7 M GdnHCl, which is an unusually high value for globular proteins (Pace, 1975; Saito & Wada, 1983). The shallowness of the curve may arise from either a low cooperativity of unfolding or the presence of intermediate species, a condition that would invalidate the two-state assumption (Pace, 1975).

To obtain an estimate of the stability of rhuIL-4 in the absence of denaturant, the free energy of unfolding in water,

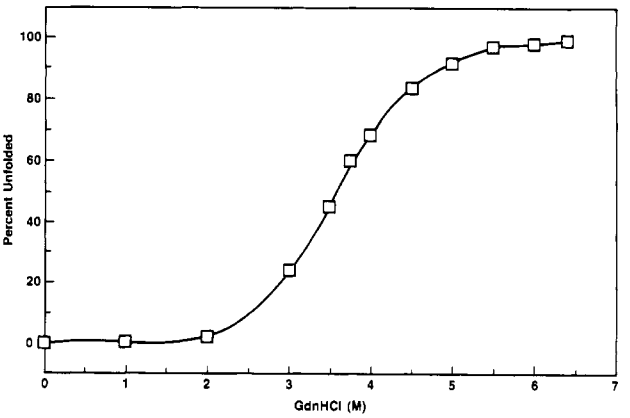

FIGURE 2: GdnHCl-induced protein unfolding curve of rhuIL-4. The relative change in $[\theta]_{MRW}$ at 222 nm is plotted with respect to increasing concentration of GdnHCl. Protein concentrations were 0.2 mg/mL and the solvent was 50 mM sodium phosphate, pH 7.2.

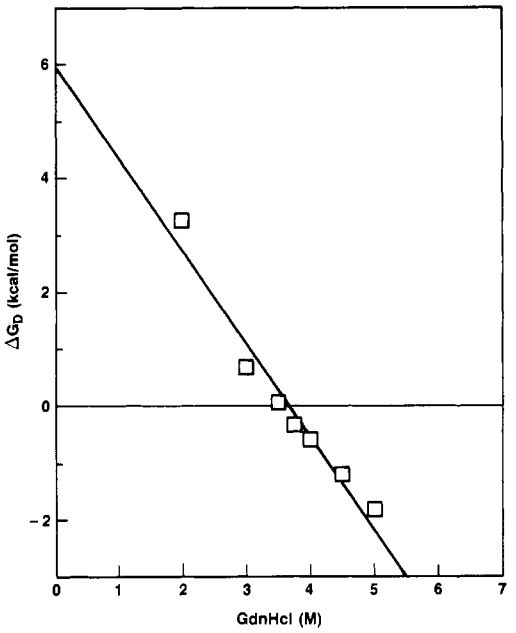

FIGURE 3: Free energy of unfolding versus [GdnHCl]. Appropriate data taken from Figure 2 were used to calculate ΔG_D and replotted with respect to increasing concentration of GdnHCl. The slope and intercept calculated from a least-squares fit provides the value of m and $\Delta G_D^{H_2O}$, respectively.

$\Delta G_D^{H_2O}$, was obtained through extrapolation of a plot of ΔG_D versus GdnHCl to 0 M GdnHCl. The following equation has been suggested by Greene and Pace (1974) to estimate $\Delta G_D^{H_2O}$:

$$\Delta G_D = \Delta G_D^{H_2O} - m[GdnHCl] \quad (1)$$

where m, the slope of the curve, indicates the ability of a denaturant to unfold a protein and [GdnHCl] is the concentration of denaturant. The relevant data in Figure 2 have been replotted in Figure 3 according to eq 1. A linear least-squares analysis (Pace, 1975) of the dependence of ΔG_D on denaturant concentration yielded values for $\Delta G_D^{H_2O}$ and m as summarized below:

$\Delta G_D^{H_2O}$	m	$[GdnHCl]_{1/2}$
5.9 kcal/mol	1.6 (kcal/mol)/M	3.7 M

The near-UV CD spectrum of rhuIL-4 in the presence of 6.0 M GdnHCl is indicative of a fully unfolded tertiary structure (Figure 1A). The weak ellipticity due to the tryptophan residue is completely eliminated, as expected for a random coil polypeptide. The phenylalanine residues provide

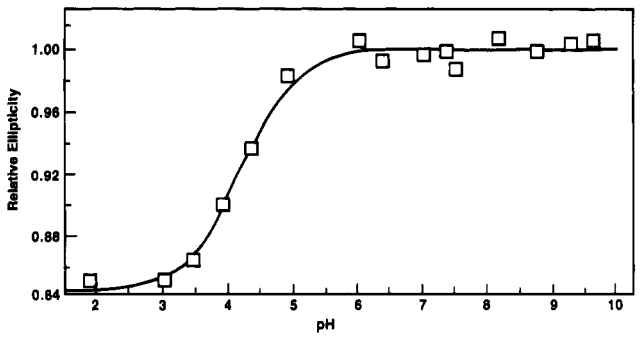

FIGURE 4: pH-induced protein unfolding curve of rhuIL-4. The relative change in $[\theta]_{MRW}$ at 222 nm is plotted with respect to increasing pH. The solvents employed were 5 mM sodium citrate, 5 mM sodium acetate, 5 mM sodium phosphate, or 5 mM Tris-HCl, each containing 50 mM potassium sulfate. A common stock solution of rhuIL-4 in water was diluted into the appropriate buffers to a final concentration of 0.1 mg/mL. The pH was measured following each spectrum. Samples were equilibrated for 30 min at room temperature prior to acquiring the spectra. A pK_a of 4.17 for the transition was calculated by using a nonlinear least-squares routine.

optical activity at 263 and 268 nm, which is not uncommon for this type of aromatic residue (Strickland, 1974). The intact disulfide bonds also appear to continue to generate a negative ellipticity in this wavelength region.

Refolding of rhuIL-4 is completely reversible. RhuIL-4 in the presence of 6.0 M GdnHCl was refolded from the denatured state by either dialyzing the sample overnight into 50 mM sodium phosphate, pH 7.2, in the absence of GdnHCl, or diluting the sample 10-fold to give a final GdnHCl concentration of 0.60 M. By either method the far-UV CD spectrum was identical with the native spectrum in the absence of denaturants (Figure 1B). Complete refolding of the secondary structure occurred within 30 min following a 10-fold dilution of the denaturant. The near-UV CD spectrum of the unfolded sample became virtually identical with the spectrum of the native protein following dialysis to remove the denaturant (Figure 1A). Thus, the aromatic residues are capable of refolding into their unique local conformations following removal of the denaturant. These results indicate that the physical properties of rhuIL-4, stemming from its unique primary sequence and disulfide bonds, constrain the total number of folded states and favor the native state.

b. pH Stability. The structural stability of rhuIL-4 was examined by far-UV CD over a wide range of pH values (data not shown). No significant spectral changes occurred between pH values of 9.6 and 5.5. Decreases in the far-UV ellipticity occurred below pH 5.5, reaching a minimum at pH 3.0. It is clear from the relative change in ellipticity at 222 nm with respect to pH (Figure 4) that a transition from one state to another occurred over a relatively narrow pH range. An apparent pK_a of 4.17 was calculated for the amino acid residue(s) contributing to the conformational changes. Although there is clearly a conformational change occurring at acidic pH, the predominantly α-helical state is maintained. No visible precipitate was observed at any pH. We conclude that rhuIL-4 is stable with respect to extreme values of pH.

Effect of pH and GdnHCl on T-Cell Proliferation. Experiments have also been performed to examine the in vitro biological activity following exposure to extreme buffer conditions. Protein samples were dialyzed overnight from 50 mM sodium phosphate, pH 7.0, into buffers of varying pH (i.e., pH 3, 4, 5, 6, 7, 8, 9, and 10) or GdnHCl concentrations (i.e., 0, 3, 5, and 6 M), followed by dialysis into the original solvent. It was observed that the specific activities in an in vitro T-cell proliferation assay of samples exposed to different concentrations of GdnHCl were within 10% of the control. Samples exposed to different pHs exhibited specific activities within 25% of the control. Thus, rhuIL-4 is stable upon exposure to a wide range of buffer conditions.

Cysteine/Disulfide Content. Based on the cDNA of the mature rhuIL-4 there are six cysteine residues at positions 3, 24, 46, 65, 99, and 127 (Yokota et al., 1986). The cysteine content was confirmed by a procedure in which the protein was completely reduced under denaturing conditions (i.e., 6 M GdnHCl and 3 mM DTT) followed by alkylation with iodoacetate, as described under Materials and Methods. Quantitation of the carboxymethylcysteine derivatives by amino acid analysis confirmed the presence of six cysteine residues (data not shown). Reduced and alkylated rhuIL-4 had no detectable activity in the T-cell proliferation assay. The number of free cysteine residues in the fully active, native protein was determined with 4,4′-dithiodipyridine (Grassetti & Murray, 1967). On the basis of this chemical analysis the native protein was found to contain only trace amounts of free cysteine residues, 0.07 ± 0.02 sulfhydryls/mol of rhuIL-4 (see Materials and Methods), as also reported by van Kimmenade et al. (1988).

These data suggested that rhuIL-4 contains three disulfide bonds. Direct measurement of the cystine content by reaction with 2-nitro-5-thiosulfobenzoate (Thannhauser et al., 1984; Damodaran, 1985) indicated a disulfide content of 2.95 ± 0.14 disulfides/mol of rhuIL-4 (see Materials and Methods). This result further supports the absence of significant quantities of free sulfhydryl groups.

Discussion

The CD studies reported here were designed to elucidate aspects of the structure and stability of refolded, *E. coli* derived rhuIL-4. The data support that rhuIL-4 is a highly α-helical (i.e., 73–75% helical), highly stable protein. Stability was assessed by determining the sensitivity of rhuIL-4 to unfolding by increasing concentrations of GdnHCl. The observed value for $\Delta G_D^{H_2O}$ (5.9 kcal/mol) is typical for globular proteins under physiological conditions, which generally exhibit values of $\Delta G_D^{H_2O}$ in the range 5–15 kcal/mol (Tanford, 1970). Although this value implies that the native state of the protein is only marginally more stable than the unfolded state the relatively high GdnHCl unfolding midpoint ($[GdnHCl]_{1/2}$ = 3.7 M) supports the conclusion that the protein is more stable to unfolding by GdnHCl than most globular proteins (Pace, 1975; Saito & Wada, 1983). The value for the dependence of the free energy of unfolding on the concentration of GdnHCl, m, represents an additional measure of the stability of a protein to unfolding. This value [1.6 (kcal/mol)/M], which was also calculated on the basis of a two-state unfolding mechanism, indicates that rhuIL-4 has a relatively high resistance to unfolding to GdnHCl. Considered together, the values of $\Delta G_D^{H_2O}$, $[GdnHCl]_{1/2}$, and m indicate that the structural stability of rhuIL-4 is higher than that of the average globular protein. Refolding of rhuIL-4, following acid and GdnHCl unfolding, also appeared to be completely reversible, based on the fact that secondary and tertiary structure as well as in vitro biological activity was retained.

Analysis of the unfolding curve was based on a two-state unfolding mechanism in order to provide a basis for comparison with other proteins (Pace, 1975, 1986). It is possible, however, that one or more intermediates exist during the unfolding process since the cooperativity of unfolding is relatively low, based on the shallowness of the unfolding curve (Figure 2). This low degree of cooperativity of unfolding is reflected quantitatively in the low value of m, which, if not an inherent

property of the protein, can arise from either the presence of protein impurities or the existence of one or more intermediate states. The former alternative is unlikely since the rhuIL-4 employed in this study is greater than 98% pure, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. However, the existence of intermediates has not been excluded. On the assumption that rhuIL-4 contains intermediates upon unfolding, the measured free energy of unfolding would have been underestimated and the value of 5.9 kcal/mol would represent a lower limit of its thermodynamic stability (Pace 1975, 1986). Although unfolded intermediate species may exist for rhuIL-4 during the denaturation experiments, the kinetics of unfolding was relatively fast since complete unfolding or refolding occurred within 30 min of the perturbation.

We have shown that the secondary structure of rhuIL-4 is fairly insensitive to changes in pH. A small pH-dependent conformational change occurs between pH 3 and 5.5, as detected by a 15% change in ellipticity at 222 nm. The pH-induced conformational change has a corresponding pK_a of 4.17, which suggests the unfolding is mediated through the titration of glutamic and/or aspartic acid residues. The small decrease in α-helical content at low pH may arise from the elimination of stabilizing hydrogen bonds and electrostatic interactions as a result of the carboxyl groups being protonated. The lack of conformational change between pH 5.5 and 9.6 also suggests that the secondary conformation is not dependent on the state of ionization of the five histidine residues. There was no detectable protein precipitation between pH 1.9 and 9.6, which is consistent with the observation that rhuIL-4 has a pI equal to or greater than pH 10 (W. Windsor, unpublished observation).

Chemical modification studies have established that *E. coli* derived recombinant huIL-4 contains six cysteine residues that are oxidized in the folded state to form these disulfide bonds. The high stability of rhuIL-4 can be attributed, at least in part, to the presence of these three disulfide bonds. Intact disulfide bonds are also critical for in vitro T-cell proliferation activity since full reduction of the disulfide bonds leads to complete loss of biological activity. This loss in biological activity following reduction and alkylation of the disulfide bonds is most likely due to a large change in secondary structure, which has recently been observed in the far-UV CD spectrum for rhuIL-4 derived from Chinese hamster ovary cells (Windsor et al., 1990). Other biological activities of rhuIL-4, however, may not have the same dependence on the state of oxidation of the cysteine residues.

RhuIL-4 contains one tryptophan, two tyrosine, and six phenylalanine residues. Spectroscopic analysis of these aromatic residues can provide valuable information regarding solvent accessibility and extent of structural asymmetry or constraint, as determined by analysis of near-UV CD spectra. As Strickland (1974) points out, it is most appropriate to express ellipticity values from the near-UV region in terms of the differential molar circular dichroic extinction coefficient $\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are the molar absorptivities of left and right circularly polarized light. The conversion for $[\theta]_{MRW}$ to $\Delta\epsilon$ is given by $\Delta\epsilon = [\theta]_{MRW}N/3300$, where N is the number of amino acids per protein.

The shape of the rhuIL-4 CD spectrum between 280 and 295 nm is fairly nondescript, except for a small positive band at 292 nm. The position of this band is similar to that expected for the major ^1L_b electronic transition band arising from the single tryptophan in rhuIL-4 (Strickland, 1974). Interpretation of the ^1L_b band is straightforward since spectral contributions from other residues are not significant in this region. The value of $[\theta]_{MRW}$ at 292 nm is +13 deg cm^2 dmol^{-1} and is equivalent to $\Delta\epsilon = +0.5$ M^{-1} cm^{-1}/Trp. Expected $|\Delta\epsilon|$ values for the major ^1L_b band lie between 0.2 M^{-1} cm^{-1}/Trp for unconstrained and 2.5 M^{-1} cm^{-1}/Trp for structurally constrained tryptophan residues (Strickland, 1974). We conclude that the tryptophan residue in rhuIL-4 appears to reside in a relatively nonrigid environment most likely exposed to the solvent. This conclusion is consistent with the observations that (1) the emission fluorescence maximum of Trp-91 in rhuIL-4 is similar to that of L-tryptophan and (2) chemical modification of Trp-91 occurs readily with *N*-bromosuccinimide (W. Windsor and P. P. Trotta, unpublished observations; Windsor et al., 1990).

Unconstrained and constrained $|\Delta\epsilon|$ values for tyrosine at 275 nm lie between 0.15 and 2.5 M^{-1} cm^{-1}/Tyr, respectively (Strickland, 1974). The lack of any significant optical rotation between 270 and 285 in rhuIL-4 ($\Delta\epsilon_{275} = -0.41$ M^{-1} cm^{-1}/Tyr) suggests that tyrosine residues are relatively unconstrained. However, since tyrosine ellipticities can be positive or negative depending on structural constraints, the observed low ellipticity values could be due to a fortuitous cancellation effect of two different tyrosine CD spectra, each in a different direction with comparable magnitudes. In fact, recent chemical modification studies of the tyrosine residues using tetranitromethane indicate Tyr-56 is unreactive in the folded protein and most likely constrained, while Tyr-124 is readily modified (Windsor et al., 1990).

The expected range of $|\Delta\epsilon|$ for phenylalanine at 267 nm is between 0.05 M^{-1} cm^{-1}/Phe and 0.3 M^{-1} cm^{-1}/Phe for unconstrained and constrained residues, respectively (Strickland, 1974). RhuIL-4 has a $\Delta\epsilon$ at 267 nm of -0.34 M^{-1} cm^{-1}/Phe, which suggests that its six phenylalanines are highly constrained. However, part of the negative spectrum in this region arises from optical rotations derived from the three disulfide bonds in rhuIL-4. This negative contribution may lead to an overestimation of the extent of constraint for the phenylalanine residues (see Figure 1A).

In conclusion, the physical studies reported here on *E. coli* derived rhuIL-4 indicate that the protein is structurally stable with respect to a wide range of solvent conditions. This high degree of stability may be related, at least in part, to the presence of three disulfide bonds and the high content of α-helical structure. Although these studies were performed on the refolded *E. coli* derived form of rhuIL-4 containing an N-terminal methionine, nearly identical results for all of these experiments have also been obtained for the secreted, glycosylated form of rhuIL-4 derived from Chinese hamster ovary cells (W. Windsor, H. Le, and P. Trotta, unpublished observations). Further understanding of the relationship between structure and stability will require additional three-dimensional structural information, as, for example, by X-ray diffraction, which is currently being pursued.

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Scaffold-Attached Regions from the Human Interferon β Domain Can Be Used To Enhance the Stable Expression of Genes under the Control of Various Promoters†

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ABSTRACT: We have transfected DNA corresponding to the complete chromatin domain of human interferon β (huIFN-β) gene into mouse L cells. In this construct, which is flanked by scaffold-attached regions (SARs), the gene's transcription was enhanced 20-30-fold with respect to DNAs containing only the immediate regulatory elements. To elucidate the role of SAR elements in the transcriptional enhancement, their position was varied relative to several artificial promoter–gene combinations. It was found that SARs enhance general promoter functions in an orientation- and partially distance-independent manner; their effect is restricted to the integrated state of transfected templates. During the phase of transient expression, SAR elements were generally found to have an antagonizing effect.

The induction mechanism of the type I interferon (IFN) genes is understood in considerable detail [see Maniatis (1986) and Bode and Hauser (1990) for reviews]. All elements involved in this process have been localized within 200 and most of them within 100 base pairs of DNA upstream from the transcriptional start. This information about the immediate control region arises from "reverse genetics", which involves

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