Characterization of the Quaternary Structure and Conformational Properties of the Human Stem Cell Inhibitor Protein LD78 in Solution

Shilpa R. Patel,[‡] Steven Evans,[‡] Kevin Dunne,[‡] Graham C. Knight,[‡] Peter J. Morgan,[§] Paul G. Varley,[∥] and Stewart Craig*,[‡]

British Bio-technology Ltd., Cowley, Oxford OX4 5LY, U.K., National Centre For Macromolecular Hydrodynamics, University of Nottingham, LE12 5RD, U.K., and Department of Biochemistry and Genetics, University of Newcastle upon Tyne, NE2 4HH, U.K.

Received December 22, 1992; Revised Manuscript Received March 12, 1993

ABSTRACT: The human LD78 protein (sometimes referred to as human macrophage inflammatory protein-1α) has been shown to protect multipotential hemopoietic stem cells from the effects of cytotoxic agents. Administration of the recombinant stem cell inhibitor molecule LD78 as an adjunct to chemotherapy has potential clinical benefit in reducing or preventing the neutropenia associated with this treatment. At physiological ionic strength, the 8-kDa LD78 molecule exists as soluble, heterogeneous, multimeric complexes of mass ranging from 100 to >250 kDa. The hydrodynamic and structural properties of LD78 have been determined in various buffer solutions using analytical ultracentrifugation, circular dichroism, and fluorescence spectroscopy. The results demonstrate that defined, homogeneous monomer and tetramer forms of LD78 can be prepared which display distinct conformational properties. The combined use of hydrodynamic and spectroscopic analysis provides an insight into the pathway and molecular mechanics of LD78 self-association.

The diverse cells of the hemopoietic system are derived from multipotential stem cells by a process of sequential division and differentiation. The proliferation of the stem cell population is controlled in part by an inhibitory molecule produced by bone marrow macrophages (Lord et al., 1976). The murine hemopoietic stem cell inhibitor (SCI) has been shown to be an 8-kDa protein, macrophage inflammatory protein 1-α [MIP-1α (Graham et al., 1990)], a member of the intercrine family of cytokines. On the basis of sequence homologies, it was suggested (Schall, 1991; Dunlop et al., 1992) that the human tonsillar lymphocyte-derived 78 (LD78) gene product (Obaru et al., 1986) was the human equivalent to murine MIP-1α. The properties of these SCI proteins include protection of stem cells from the toxic effects of cell-cycle-specific cytotoxic agents (Lord & Wright, 1980; Lord et al., 1992; Dunlop et al., 1992). Stem cell inhibitors, therefore, have clinical potential as an agent to protect stem cells from the chemotherapy used in tumor treatment. Reduction or prevention of the neutropenia induced by administration of the cytotoxic agent would allow more aggressive and more frequent chemotherapy and result in more successful destruction of tumors.

A major problem shared by the homologous proteins MIP-1α and LD78, which limits their potential clinical utility, is that in physiologically relevant buffers they form large, soluble, heterogeneous multimeric complexes [see, for example, Wolpe et al. (1988) and Graham and Pragnell (1992)]. The soluble multimeric complexes show a broad heterogeneous mixture of molecular masses ranging from 100 kDa up to 2000 kDa. The function (if any) of this multimerization phenomenon is unknown, though it may be a natural mechanism for storage, acting as a "depot" of the protein. The principal consequence of the self-association behavior is that clinical administration of the protein as a heterogeneous preparation could lead to varying efficacy, impaired tissue penetration, and enhanced immunogenicity. For clinical administration, therefore, a homogeneous SCI preparation of defined molecular mass in a physiologically acceptable formulation is likely to be a prerequisite. The primary goal of our research on these molecules is to define the multimerization process and determine the relationship between size and biological activity.

A first step in approaching this problem is to define the pathway and elucidate the molecular mechanics of the self-association process. Preliminary reports have indicated that high-salt conditions "disaggregate" the murine SCI MIP-1α [Sherry quoted in Wolpe and Cerami (1989); Oh et al., 1991; Graham & Pragnell, 1992]. We have now quantitatively defined the quaternary structure and tertiary conformation of recombinant LD78 in a diverse range of solution conditions using analytical ultracentrifugation, size-exclusion chromatography (SEC), circular dichroism (CD), and steady-state and lifetime fluorescence emission spectroscopy. The results demonstrate that there are distinct spectral differences between the monomeric, tetrameric, and multimeric states of LD78. The observed spectral variations arise directly from specific changes in the environment of aromatic groups during the self-association process.

MATERIALS AND METHODS

Expression in Yeast. A synthetic gene for LD78 was constructed, optimized for codon usage, transformed, and expressed in *Saccharomyces cerevisiae* in a similar manner to that previously described for murine MIP-1α (Clements et al., 1992).

Recombinant LD78 was grown from a glycerol in 1-L shake-flask cultures containing 6.7 g of yeast nitrogen base (without amino acids), 3 g of glucose, 20 g of galactose, 20 mL amino acid stock solution, and 1 mL of sterile polypropylene glycol P2000. Flasks were incubated for 42 h at 30 °C in a shaking incubator. Prior to purification, cells were removed from the broth by centrifugation in a Sorvall RC-3B at 4500 rpm for 15 min.

* Address correspondence to this author at British Bio-technology Ltd., Watlington Rd., Cowley, Oxford, OX4 5LY, United Kingdom. Telephone: (0)865 748747. FAX: (0)865 717598.
‡ British Bio-technology Ltd.
§ University of Nottingham.
∥ University of Newcastle upon Tyne.

0006-2960/93/0432-5466$04.00/0

Purification. Cell-free supernatant was adjusted to pH 8 and batch-adsorbed onto preequilibrated (50 mM Tris, pH 8.0) Q-Sepharose (Pharmacia) overnight at 4 °C. Protein was eluted from the resin in 50 mM Tris/500 mM NaCl, pH 8.0, and dialyzed in a 3-kDa cutoff membrane against 10 volumes of 50 mM Tris, pH 8.0, at 4 °C. The sample was then adjusted to 20% acetonitrile (final concentration) and the pH adjusted to 3.0 with hydrochloric acid. The sample was then loaded onto a 20-mL VYDAC C-18 (10 μm) semipreparative reverse-phase column preequilibrated at 3 mL/min in 20% acetonitrile/0.1% TFA. LD78 was eluted at ≥98% purity with a linear gradient from 20% to 50% acetonitrile/0.1% TFA over 40 min. Eluting fractions were detected by UV absorbance at 280 nm, and pure LD78 was analyzed by SDS–PAGE (Laemmli, 1970), N-terminal sequence analysis, and electrospray mass spectroscopy as described in subsequent sections.

N-Terminal Sequence Analysis. Automated N-terminal sequencing was carried out using an Applied Biosystems 471A pulse liquid sequenator with a 140SDS gradient system for analysis of derivatized amino acids.

Mass Spectrometry. The point mass of purified recombinant LD78 was determined using a VG Bio-Q electrospray spectrometer. The sample was loaded via an Applied Biosystems 140 solvent delivery system.

Size-Exclusion Chromatography. Size-exclusion chromatography was carried out using an HR 10/30 Superdex 75 gel filtration column (Pharmacia) attached to an FPLC system (Pharmacia). Chromatography was carried out at 1 mL/min with LD78 reconstituted at 0.5 mg/mL in the buffer systems described in the text. Eluting protein was detected by the absorbance at 280 nm.

Sedimentation Equilibrium in the Analytical Ultracentrifuge. Low-speed sedimentation equilibrium studies (Creeth & Pain, 1967) were made using a Beckman Optima XL-A analytical ultracentrifuge at 20.0 °C. Multichannel centerpieces were used (Yphantis, 1964) to enable simultaneous measurements of LD78 reconstituted at 0.5 mg/mL in the solvent systems described in the text. Final solute distributions were recorded via their absorption at 278 nm. The final solute distribution ASCII data were captured and analyzed on the IBM 3084Q Phoenix mainframe at the University of Cambridge using the FORTRAN MSTARA program (Harding et al., 1992). Whole-cell weight-average molecular masses (M°_{w}) were extracted by using the limiting value at the cell base of the M^{*} function (Creeth & Harding, 1982). The partial specific volume of 0.725 mL g^{-1} was calculated from the known amino acid sequence according to the method of Traube (Cohn & Edsall, 1943).

Ultraviolet Absorbance Measurements. All absorbance measurements were carried out using either a Cary-210 (Varian) or a PU8740 (Pye-Unicam) spectrophotometer. Protein concentrations were determined using a calculated value of $A^{0.1\%}_{1cm} = 1.25$ at 280 nm. The absence of light scattering in samples for c.d. and fluorescence spectroscopy was confirmed by u.v. absorbance spectra in the 240–320-nm wavelength range with A_{320nm} <5% of A_{280nm} and the ratio of A_{280nm}/A_{250nm} >2.

Circular Dichroism Measurements. CD spectra were measured using a Jobin-Yvon Dichrographe 6. Spectra shown are an average of two scans with the base line subtracted and are displayed with no smoothing. Samples in the near- and far-UV wavelength range were scanned at 5 nm/min, with a 2-s time constant. The mean residue molecular mass of LD78 was calculated to be 111.8 Da using a molecular mass of 7712 Da. All CD data are expressed as mean residue ellipticity, $[\theta]_{mrw}$.

Steady-State Fluorescence Measurements. Steady-state fluorescence spectra were recorded using a Perkin-Elmer LS-50 fluorometer with the cell holder thermostatically controlled at 20 ± 0.1 °C. Intensity values were calculated as relative fluorescence. Samples were excited at 280 nm with a 2.5-nm bandwidth and 1-cm path length, and the intensity of emission was measured at 2.5-nm bandwidth. Sample concentration was 0.046 mg/mL in (A) 150 mM PBS, pH 7.4 (polymer), (B) 10 mM MES/500 mM NaCl, pH 6.4 (tetramer), and (C) 30% acetonitrile/0.1% TFA (monomer).

Fluorescence Lifetime Measurements. Fluorescence lifetime decays were measured by single photon counting with an Edinburgh Instruments 199T fluorometer. Exciting light was produced by a flashlamp operated at 56 kHz in 0.5-bar hydrogen, giving pulse widths of 1.5-ns duration. Excitation was at 295 nm with a bandwidth of 10 nm, and emission was monitored at 350 nm through a combination of cutoff and band-pass filters giving a bandwidth of 20 nm. Anisotropy decays were measured by collecting data through quartz Glan-Thompson prism polarizers. Protein concentration in 10 mM acetic acid, pH 3.2, or 10 mM MES/500 mM NaCl, pH 6.4, was 0.5 mg/mL.

Lifetime data were analyzed with an iterative reconvolution procedure as described by Dryden et al. (1992) with a lamp pulse being determined using a dilute scattering solution. Goodness of fit was judged by a final value of $\chi^2 = <1.2$ with residuals to the fitted function being random across the whole decay.

RESULTS

In order to make sufficient quantities of LD78 for study, a recombinant expression system in *Saccharomyces cerevisiae* was constructed in a similar manner to that previously described by us for the murine SCI MIP-1α (Clements et al., 1992). The fidelity and homogeneity of recombinant LD78 purified as described under Materials and Methods were confirmed by mass spectroscopy, N-terminal sequencing, and SDS–PAGE. The material was shown to be biologically active in the assays previously described for MIP-1α (Clements et al., 1992).

Characterization of the Molecular Mass of LD78 in Solution. From the primary sequence, the molecular mass of LD78 is calculated to be 7712 daltons. The purified protein was confirmed to have the correct mass by electrospray mass spectroscopy proving that no glycosylation or posttranslational processing occurs during yeast expression. Preliminary characterization of the molecular mass of LD78 in aqueous solution has been carried out using size-exclusion chromatography. The most accurate (and anomaly-free) method of absolute molecular mass determination in solution is sedimentation equilibrium in the analytical ultracentrifuge [see, for example, Yphantis (1964) and Harding et al. (1992)]. Within a population of protein molecules distributed at equilibrium through the rotor cell, approximate values for the lower molecular mass species in solution are obtained from the cell meniscus [$M_w(\xi=0)$] and for the larger molecular mass species from the cell base [$M_w(\xi=1)$]. This analysis can also provide the whole-cell weight-average molecular mass (M°_{w}) (i.e., the average molecular mass of solute distributed throughout the rotor cell). In this manner, the self-association properties of a protein molecule can accurately be determined from the measured dispersity in the observed mass ranges. Characteristically, polydisperse solutions show an upward

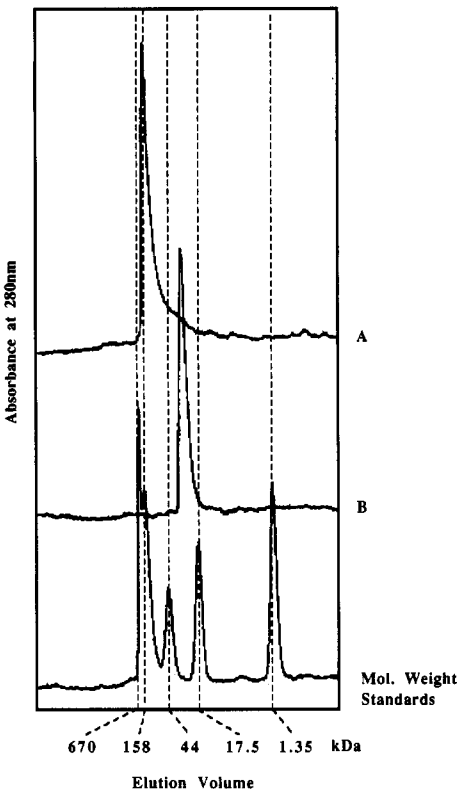

FIGURE 1: Size-exclusion chromatography elution profile in LD78 in different solution conditions. Size-exclusion chromatography of LD78 in (A) 150 mM PBS, pH 7.4, and (B) 10 mM MES/500 mM NaCl, pH 6.4, was carried out as described under Materials and Methods. The elution profile of molecular mass standards is included for reference. As detailed in Figure 2, solution conditions A give heterogeneous polymer species, and conditions B give defined homogeneous tetramers.

curvature when the logarithm of absorbance (ln A) is plotted as a function of the radial displacement parameter (ξ) in analyses of the type detailed by Creeth and Harding (1982). Proteins which exist in solution at a single defined mass (i.e., monodisperse population) should exhibit linear plots of ln A vs ξ. Under nonideal solution conditions, ln A vs ξ exhibits a downward curvature, and it is possible for the combined effects of polydispersity and nonideality to cancel each other and produce a linear plot (Creeth & Pain, 1967).

LD78 Is a Polydisperse Solution of High Molecular Mass Complexes in Physiological Saline. Standard 150 mM phosphate-buffered saline, pH 7.4 (PBS), is commonly regarded as equivalent to physiological buffer conditions. The SEC elution profile of LD78 on Superdex resin equilibrated in PBS (Figure 1) shows an asymmetric peak partially excluded from the resin trailing to lower molecular mass. The broad, asymmetric shape of the peak reflects a heterogeneous mixture of soluble multimeric complexes ranging in mass from approximately 20 to $\gg$ 160 kDa. The results of the sedimentation equilibrium analysis (Figure 2 and Table I) showed that LD78 exists as a polydisperse population of protein species ranging in mass from monomer approximately 10 kDa [$M_w(\xi=0)$] to 250 kDa [$M_w(\xi=1)$] with M°_w = 160 kDa. Under these physiological buffer conditions, LD78 exists predominantly as a range of large self-associated multimers, although smaller associated units originating from monomers are present. The fact that these large heterogeneous multimers are soluble suggests that some kind of order is present in the association process.

LD78 Is a Homogeneous Tetramer in 10 mM MES/500 mM NaCl, pH 6.4, Buffer. Previous publications have stated

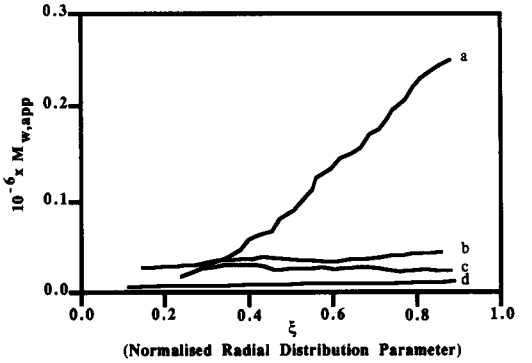

FIGURE 2: Sedimentation equilibrium analysis plot of ln A vs ξ for LD78 in different solution conditions. The figure represents the solute distribution of protein at equilibrium in the ultracentrifuge rotor. Protein concentrations were 0.5 mg/mL in (a) 150 mM PBS, pH 7.4, (b) 10 mM acetic acid, pH 3.2, and (c) 10 mM MES/500 mM NaCl, pH 6.4, at 8000 rpm. The solute distribution of protein at equilibrium in the ultracentrifuge rotor at 0.5 mg/mL in 30% acetonitrile/0.1% TFA (d) at 28 000 rpm has been overlaid in the figure to provide a comparative reference for the monomeric LD78 species. Eighty microliters of sample was loaded into the 12-mm path-length Yphantis (1964)-type multichannel centerpiece, and the centrifuge was run for 24–30 h to reach equilibrium. Other experimental details are described under Materials and Methods.

Table I: Sedimentation Equilibrium Molecular Mass Determination of LD78 in Different Solution Conditions[a]

buffer	weight-average molecular mass range (kDa)		
	M°_w	$M_w(\xi=0)$	$M_w(\xi=1)$
150 mM PBS, pH 7.4	160	10	250
10 mM MES/ 500 mM NaCl, pH 6.4	28	monodisperse	
10 mM acetic acid, pH 3.2	33	30	35
30% acetonitrile/0.1% TFA	7	monodisperse	

[a] M°_w = whole-cell weight-average molecular mass; $M_w(\xi=0)$ = smallest mass species present (at the meniscus); $M_w(\xi=1)$ = largest mass species present at the cell base; ξ = normalized radial displacement parameter calculated from the equation $(r^2 - a^2)/(b^2 - a^2)$ where r = radial displacement and $a + b$ are the corresponding values at the cell meniscus and cell base, respectively. Sedimentation equilibrium was measured and analyzed as described under Materials and Methods and Figure 2.

that "disaggregation" of the murine SCI protein MIP-1α occurs at high salt concentrations [Sherry quoted in Wolpe and Cerami (1989) and Oh et al. (1991)] though the disaggregated size has not been determined. The SEC elution profile of LD78 in 10 mM MES/500 mM NaCl, pH 6.4, contains a single symmetrical peak (Figure 1) centered on a mass of approximately 25 kDa. The symmetry of the peak shows that a homogeneous population is present, however, the apparent mass could represent either a trimeric (23 kDa) or a tetrameric (30.8 kDa) form of the molecule. The sedimentation equilibrium measurements prove (Figure 2 and Table I) the existence of a monodisperse population of mass 28 kDa, close to that of 30.8 kDa calculated for an LD78 tetramer.

The results demonstrate that 10 mM MES/500 mM NaCl, pH 6.4, can inhibit the self-association of LD78 to any structure greater than a tetramer. The electrostatic interactions broken by the high-salt environment are, therefore, key to the stabilization of intermolecular association to masses above tetramer. In light of our results, we predict that the tetramer is the basic unit for formation of the higher order LD78 multimeric complexes. Graham and Pragnell (1992) have observed apparently homogeneous species of mass 90 kDa which would correspond to LD78 dodecamers. It seems

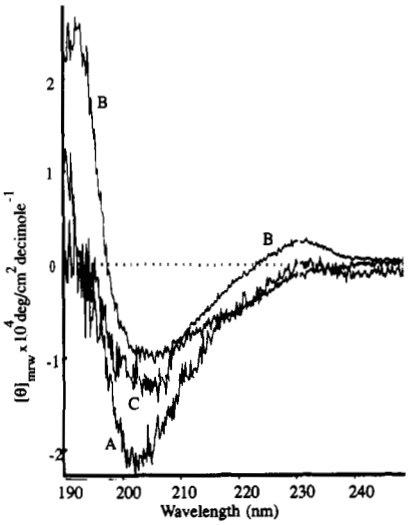

FIGURE 3: Far-UV CD spectra of different LD78 quaternary structures. Spectra are an average of two scans with the base line subtracted. Protein concentration was 0.2–0.35 mg/mL in (A) 150 mM PBS, pH 7.4 (polymer), (B) 10 mM MES/500 mM NaCl, pH 6.4 (tetramer), and (C) 30% acetonitrile/0.1% TFA (monomer). Spectra were recorded at 20 °C with a 0.02-cm path length and 2-nm bandwidth.

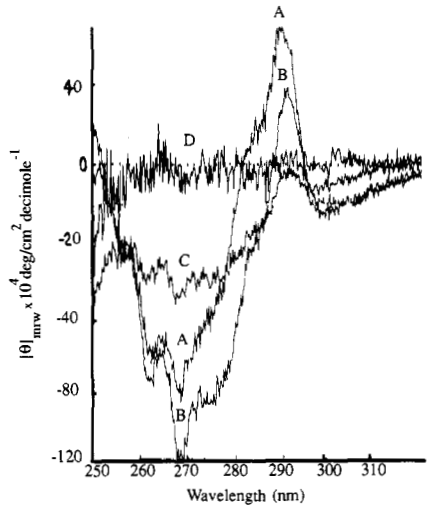

FIGURE 4: Near-UV CD spectra of different LD78 quatenary structures. Spectra are an average of two scans with the base line subtracted. Protein concentration was 0.2–0.35 mg/mL in (A) 150 mM PBS, pH 7.4 (polymer), (B) 10 mM MES/500 mM NaCl, pH 6.4 (tetramer), (C) 30% acetonitrile/0.1% TFA (monomer), and (D) 8 M Gdn-HCl, 150 mM PBS, and 10 mM DTT, pH 7.4 (unfolded/reduced). Spectra were recorded at 20 °C with a 1-cm path length and 2-nm bandwidth.

probable, therefore, that large multimeric complexes form initially by the association of tetramers to dodecamers and then to larger masses.

LD78 Is a Homogeneous Tetramer in 10 mM Acetic Acid, pH 3.2. We have previously described the characterization of MIP-1α at mild acidic pH (Clements et al., 1992), conditions in which the protein was found to be very soluble. Electrostatic interactions can also be disrupted at low pH via direct charge competition and by titration of charged side chains. We have, therefore, determined the molecular mass of LD78 in 10 mM acetic acid, pH 3.2. Acidic pH can cause shrinkage of SEC gels, leading to distorted chromatography. As a result, the mass could only be determined in the ultracentrifuge. Analysis of the sedimentation equilibrium data (Figure 2 and Table I) demonstrates the existence of a monodisperse population of species with a mass of 33 kDa. LD78, therefore, is a defined, homogeneous tetramer in 10 mM acetic acid, pH 3.2.

LD78 Is a Homogeneous Monomer in 30% Acetonitrile/0.1% TFA (pH 2). Mantel et al. (1992) have described the biological characterization of LD78 which is monomeric by SEC. The LD78 monomer is obtained in the reverse-phase solvent 30% acetonitrile/0.1% TFA. We have measured the sedimentation equilibrium behavior of LD78 in this solvent system, and the results (Figure 2 and Table I) confirm that a monodisperse population of mass 6.8 kDa (monomers) is present.

Far-UV CD Spectroscopy of LD78. The far-UV CD spectra of multimeric, tetrameric, and monomeric LD78 displayed in Figure 3 show that the shape and intensity of the spectra vary greatly for each quaternary state of the protein. The reason for these striking differences is unclear, though it is possible that the variations in near-UV CD due to changes in aromatic group asymmetry described below are also manifest in the far-UV wavelength band. The monomer spectrum demonstrates that the protein is still folded into regular structure.

The unusual shape and the differences in the spectra compromise the ability of the CONTIN analysis program (Provencher, 1982; Provencher & Gloeckner, 1981) to determine accurately the secondary structure content of the different LD78 association states. Fourier-transform infrared spectroscopy is now being examined to attempt accurate determination of regular structure content in the different solvent systems described in this paper.

Near-UV CD Spectroscopy of LD78. Circular dichroism spectra in the 250–320-nm wavelength range arise partly from disulfide bonds but mainly from the specific environment of aromatic groups such as tyrosine and tryptophan and to a lesser extent phenylalanine (Strickland, 1974). The near-UV CD range provides a sensitive spectroscopic probe of protein folding and association. LD78 contains a single tryptophan, three tyrosine, and four phenylalanine residues. The near-UV CD spectra of multimeric, tetrameric, and monomeric LD78 are shown in Figure 4. As observed with the far-UV CD spectra, each quaternary state of the molecule has a characteristic spectral shape and intensity in the 250–320-nm wavelength band.

The intense negative ellipticity at λ <285 nm in the multimeric and tetrameric LD78 spectra is characteristic of tyrosine with some phenylalanine superimposed at λ <270 nm. The intensity of the spectra in this range strongly suggests that coupling of transitions is occurring between a tyrosine and another aromatic residue and may in fact reflect coupling of a tyrosine residue and the single tryptophan residue (Trp-57). By analogy with the sequence-related bovine platelet factor 4 (PF-4) structure (St. Charles et al., 1989), Trp-57 may be at the first turn of a C-terminal helix, which could place the side chain of Tyr-61 within a favorable distance for aromatic ring coupling. Identification of the coupled side chains must await solving the three-dimensional structure.

The spectra of LD78 in 10 mM acetic acid and 10 mM MES/500 mM NaCl, pH 6.4 (Figure 5), are superimposable, demonstrating an identical conformation for the protein in these two solvents. Combined with the mass analysis above, this points to LD78 forming identical folded tetramers under these conditions. In fact, the spectrum of LD78 in 10 mM acetic acid is very similar to that previously described for its functional murine homologue muMIP-1α (Clements et al., 1992) in the same solvent. From this, we infer that the highly related sequence homologues have the same structural fold down to the level of tertiary packing of side chains.

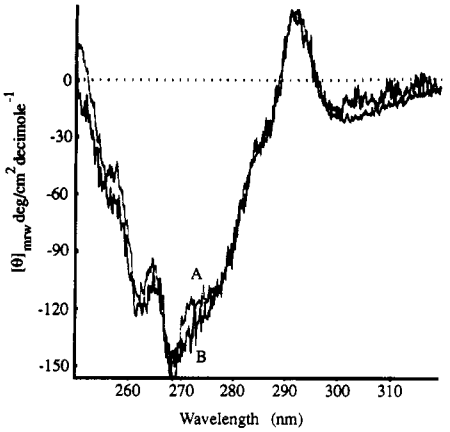

FIGURE 5: Near-UV CD spectra of tetrameric LD78. Spectra are an average of two scans with the base line subtracted. Protein concentration was 0.2–0.35 mg/mL in (A) 10 mM MES/500 mM NaCl, pH 6.4, and (B) 10 mM acetic acid, pH 3.2. Spectra were recorded at 20 °C with a 1-cm path length and 2-nm bandwidth.

The major difference in shape between the multimer and tetramer spectra is the 285–295-nm region where the asymmetric environment of the single tryptophan (Trp-57) appears to have changed. In addition, some change in the intense tyrosine asymmetry is evident, though it is possible that this is linked with the change in tryptophan contribution.

The monomer spectrum shows a reduction in intensity and a change in shape suggesting that the environment of Trp-57 and the coupled aromatics has changed. Considerable fine banding is present in the monomer compared to the unfolded molecule (Figure 4), confirming that complete unfolding of the molecule has not occurred. As discussed later, the effect of nonpolar acetonitrile on the surface solvation may in part be responsible for the changes.

Steady-State Fluorescence Emission of LD78. In order to probe the environment of Trp-57, the steady-state fluorescence emission spectrum was measured as a function of quaternary structure. The intensity of emission measured for tetrameric LD78 [relative intensity maximum (RI_{max}) = 529 units] suggests that the tryptophan is relatively unquenched by neighboring groups or interactions. The results show, however, that the emission of Trp-57 in the multimer (RI_{max} = 404 units) is quenched relative to the tetramer state (RI_{max} = 529 units). The λ_{max} emission of 335 nm is identical for both states, demonstrating a buried, hydrophobic environment for the tryptophan residue. It is concluded, therefore, that no gross conformational change has occurred to alter the degree to which residue Trp-57 is shielded from solvent.

As described above, we have established that electrostatic interactions stabilize the association of tetrameric LD78 to form high molecular weight multimeric complexes. The ability of electrostatic interactions to act as quenchers of fluorescence emission is well-known (Lackowicz, 1983). The observed decrease in emission energy of the multimer is, therefore, consistent with a charged side chain proximal to Trp-57 forming an electrostatic interaction upon association.

Comparison of the emission spectrum of tetrameric (RI_{max} = 529 units) and monomeric (RI_{max} = 148 units) LD78 reveals a distinct difference between the states. The emission energy is highly quenched, and a red shift of λ_{max} to 341 nm indicates a more exposed, polar environment for Trp-57 in monomeric LD78. Acetonitrile does not itself quench the fluorescence emission of *N*-acetyltryptophanamide (NATA), the standard reference compound (data not shown). The reduced intensity of the monomer spectrum does not, therefore, arise due to direct solvent quenching. It appears from these results that dissociation of tetramers to monomers has a direct effect on the protein conformation as evidenced by the near-UV CD and the fluorescence spectra.

Table II: Fluorescence Lifetimes of Tetrameric LD78[a]

buffer	τ_1 (ns)	amplitude (%)	τ_2 (ns)	amplitude (%)
10 mM acetic acid, pH 3.2	2.61 ± 1.3	7.9	6.39 ± 0.5	92.1
10 mM MES/500 mM NaCl, pH 6.4	1.4 ± 1.1	4.0	6.23 ± 0.1	96.0

[a] Fluorescence lifetime decays were measured and analyzed as described under Materials and Methods.

Lifetime Fluorescence Emission and Anisotropy of LD78. Fluorescence lifetime studies have demonstrated that individual tryptophan residues exhibit lifetime decay curves with more than one component [e.g., see Varley et al. (1991)]. The multiexponential decays are thought to originate from different configurations of the indole ring, allowing varying degrees of quenching by charge transfer. The exact mechanism, however, is far from clear, and interpretation of multiexponential decays in terms of structural information is complex.

Lifetime emission and anisotropy measurements have been carried out on the tetrameric LD78 molecule in 10 mM MES/500 mM NaCl, pH 6.4, and in 10 mM acetic acid, pH 3.2. Initial examination of the decay curves suggested that they are in fact single-exponential decays. Upon close examination, however, the best fit was obtained with two components (Table II) of 6.5 ns (95% of total) and 2 ns (5% of total). Since, however, the flashlamp in the machine has an accuracy of only 5% of the total intensity, the small fast component of the lifetime cannot be confirmed. It remains a strong possibility that Trp-57 has a single-exponential decay of fluoresence emission in the tetrameric state. The fact that the lifetime decay consists almost entirely (if not completely) of a single long lifetime confirms that Trp-57 is virtually unquenched by proximal groups in this quaternary state. These data correlate with the steady-state fluorescence emission of Trp-57 which strongly suggested that no quenching of emission energy was present in tetrameric LD78. Given that the multiexponential decays arise (at least partly) from tryptophan occupying various conformers in the excited state, the almost single-exponential behavior suggests that Trp-57 is held in a rigid environment within the protein interior. The LD78 decay system is highly unusual as very few proteins exhibit such monoexponential characteristics.

Fluorescence lifetime anisotropy decay data provide an estimate of the environmental rigidity of tryptophan residues in proteins. Analysis of the lifetime anisotropy decays of LD78 in acetic acid and 10 mM MES/500 mM NaCl with single-exponential fits gave correlation times of 14 and 10 ns for LD78 in acetic acid and MES/NaCl, respectively. These slow correlation times demonstrate that the Trp-57 side chain is rotating very slowly, confirming that it is environmentally constrained. Estimations of molecular mass can be made from these correlation times (Kouyama et al., 1989) for rigid tryptophans assuming that the molecule is spherical and exhibits hydration and viscosity typical for a globular protein. The estimated molecular masses for LD78 in acetic acid and MES/NaCl are 33 and 22 kDa, respectively, which, given the assumptions inherent in the calculation, are in good agreement with the value of 30 kDa for the tetrameric state defined experimentally above.

DISCUSSION

The results presented in this paper provide direct quantitation and structural characterization of previous observations of solvent effects on "disaggregation" of SCI molecules (Graham & Pragnell, 1992; Mantel et al., 1992). It is clear that intermolecular electrostatic interactions between tetramer units are key to the formation and stabilization of the high molecular weight multimeric complexes. One of the electrostatic interactions involved in this process has been shown to involve a charge group proximal to Trp-57 in the LD78 structure. Identification of the actual side chain involved must await the three-dimensional structure assignment.

The fact that further dissociation of tetramers to dimers or monomers is not observed in conditions which disrupt ionic interactions strongly suggests that the major stabilizing force for the tetramer unit is hydrophobic. This rationale is confirmed by the fact that 30% acetonitrile/0.1% TFA results in a monodisperse population of monomeric LD78. Nonpolar conditions alone may not be sufficient to disrupt the multimer complexes as the solvation effects will favor maintenance of ionic interaction. However, the acidic pH of 0.1% TFA will cause dissociation of multimers to tetramers in the same manner as 10 mM acetic acid described above. The nonpolar solvent acetonitrile can then disrupt the hydrophobic interactions stabilizing the tetramer. The observed monomerization of LD78 results from the simultaneous disruption of two different types of interactions stabilizing different quaternary structures of LD78.

It has not yet been demonstrated if a dimeric structure is present on the association pathway of LD78. It seems likely that if it exists, the dimer will also be stabilized by hydrophobic interaction.

The overall tertiary structure/conformation of LD78 is apparently very similar in the multimeric and tetrameric states except for changes in the electrostatic potential of the environment surrounding Trp-57. The electrostatic association of tetramers to dodecamers and higher order states does not, therefore, result in gross structural changes. The dissociation of tetramers to monomers, however, appears to cause some conformational perturbation especially in the environment of Trp-57 and the coupled tyrosine environment. It is possible that the intense near-UV CD ellipticity arises directly from interaction of Tyr-61 with Trp-57 in the C-terminal region of LD78. The changes in CD and fluorescence upon association/dissociation of states may be related directly to this specific environment.

The conditions used to achieve monomeric LD78 are, however, relatively harsh, and the preferential solvation effects (Timasheff & Arakawa, 1990) of 30% acetonitrile on the protein surface may contribute to the observed environmental change. Certainly the increased solvent exposure of Trp-57 in the monomer may result from surface solvation changes proximal to the group. That hydrophobic interactions are responsible for the initial association of monomer units means that some nonpolar side chains must be on the surface of the monomer. Attempts to obtain a monomer species in more aqueous solvent would, therefore, be expected to result in some side-chain rearrangement to shield the hydrophobic groups. As the monomeric form of LD78 appears to be active on transfer from the acidic acetonitrile into in vitro assay systems (Mantel et al., 1992), it is probable that the observed conformational changes reflect minor surface perturbations rather than gross disturbances.

These data clearly demonstrate that the environment of key aromatic groups is affected by the self-association of the LD78 molecule. These spectroscopic markers provide sensitive probes for the self-association of the human SCI protein LD78.

ACKNOWLEDGMENT

We thank Dr. R. Aplin and Dr. C. Robinson (Oxford) for carrying out the electrospray mass spectroscopy. We thank Professor R. H. Pain (Newcastle) for allowing us access to the CD and lifetime fluorescence equipment and Dr. S. E. Harding for access to the Optima XL-A. We also thank Carol Partlett for growing up the shake flask cultures.

REFERENCES

Clements, J. M., Craig, S., Gearing, A. J. H., Hunter, M. G., Heyworth, C. M., Dexter, T. M., & Lord, B. I. (1992) *Cytokine 4*, 76–82.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, Van Nostrand Rheinhold, New York.

Creeth, J. M., & Pain, R. H. (1967) *Prog. Biophys. Mol. Biol. 17*, 217–287.

Creeth, J. M., & Harding, S. E. (1982) *J. Biochem. Biophys. Methods 7*, 25–34.

Dryden, D. T. F., Varley, P. G., & Pain, R. H. (1992) *Eur. J. Biochem. 208*, 115–123.

Dunlop, D. J., Wright, E. G., Lorimore, S., *et al.* (1992) *Blood 79*, 2221–2225.

Graham, G. J., & Pragnell, I. B. (1992) *Dev. Biol. 151*, 377–381.

Graham, G. J., Wright, E. G., Hewick, R., Wolpe, S. D., Wilkie, N. M., Donaldson, D., Lorrimore, S., & Pragnell, I. B. (1990) *Nature (London) 344*, 442–444.

Harding, S. E., Horton, J. C., & Rowe, A. J., Eds. (1992) *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, Royal Society of Chemistry Press, Cambridge.

Kouyama, T., Kinosita, K., & Ikegami, A. (1989) *Eur. J. Biochem. 182*, 517–521.

Lackowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Publishers, New York.

Lord, B. I., & Wright, E. G. (1980) *Blood Cells 6*, 581–593.

Lord, B. I., Mori, K. J., Wright, E. G., & Lajtha, L. G. (1976) *Br. J. Haematol. 34*, 441–445.

Lord, B. I., Dexter, T. M., Clements, J. M., Hunter, M. G., & Gearing, A. J. H. (1992) *Blood 79*, 2605–2609.

Mantel, G., Kim, Y. J., Cooper, S., Kwon, B. S., & Broxmeyer, H. E. (1992) *Exp. Hematol. 20(368)*, 800.

Obaru, K., Fukuda, M., Maeda, S., & Shimada, K. (1986) *J. Biochem. 99*, 885–894.

Oh, K. O., Zhon, Z., Kim, K. K., *et al.* (1991) *J. Immunol. 147*, 2978–2983.

Provencher, S. W. (1982) *Comput. Phys. Commun. 27*, 229–242.

Provencher, S. W., & Gloeckner, J. (1981) *Biochemistry 20*, 33–37.

Schall, T. J. (1991) *Cytokine 3*, 165–183.

Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 133–175.

Timasheff, S. N., & Arakawa, T. (1990) *Protein Structure—A Practical Approach* (Creighton, T. E., Ed.) pp 331–345, IRL Press, Oxford.

Wolpe, S. D., & Cerami, A. (1988) *FASEB J. 3*, 2565–2573.

Wolpe, S. D., Davatelis, G., Sherry, B., Beutler, B., Hesse, D. G., Nguygen, H. T., Moldawer, L. L., Nathan, C. F., Lowry, S. F., & Cerami, A. (1988) *J. Exp. Med. 167*, 570–581.

Varley, P. G., Dryden, D. T. F., & Pain, R. H. (1991) *Biochim. Biophys. Acta 1077*, 19–24.

Yphantis, Y. (1964) *Biochemistry 3*, 297–317.