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Circular Dichroism Study on the Conformational Stability of the Dimerization Domain of Transcription Factor LFB1

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ABSTRACT: LFB1, a dimeric DNA binding protein, is a major determinant of hepatocyte-specific transcription. The thermal and chemical equilibrium unfolding of a 32-residue α-helical peptide comprising its dimerization domain (B1-Dim) was monitored by circular dichroism spectroscopy. The conformational stability of this peptide is shown to be concentration dependent, and the unfolding reaction is described as a two-state transition between folded dimers and unfolded monomers. The thermodynamic parameters associated with the unfolding reaction were determined under the two-state assumption by the van't Hoff procedure. The enthalpy of unfolding increases linearly with temperature, and the corresponding value of ΔC_p, the difference in heat capacity between the unfolded and the folded forms of the peptide, is estimated to be ca. 0.7 kcal mol^{-1} K^{-1}. The dimeric folded structure of the peptide is stabilized, at 25 °C, by a ΔG of about 11.5 kcal mol^{-1}, which is equivalent to a dimerization constant greater than 10^8 mol^{-1}. These results indicate that the dimerization domain of LFB1 can fold and dimerize independently of the rest of the protein, with a thermodynamic stability comparable to that of a small globular protein.

LFB1, also named hepatocyte nuclear factor 1 (Courtois et al., 1987), is a sequence-specific DNA binding protein that acts as a trans-activator of RNA polymerase II transcription of several liver-specific genes in higher eukaryotes (Monaci et al., 1988; Lichtsteiner & Schibler, 1989). Previous studies (Frain et al., 1989; Nicosia et al., 1990) showed that LFB1 contains a homeobox-like motif and binds to DNA as a dimer. Furthermore, in solution, the dimer exists in equilibrium with the monomer independently of DNA binding (Nicosia et al., 1990).

Many DNA binding proteins that trans-activate transcription in eukaryotes have been shown to form either homodimers or heterodimers with related factors: this feature is of great biological significance with respect to the question of how the activity of these proteins is in its turn regulated and modulated (Johnson & McKnight, 1989). The rôle of dimerization in determining the biological activity and/or specificity of transcriptional activators has been addressed in a recent review (Jones, 1990).

We characterized extensively the DNA binding and dimerization properties of LFB1 by site-directed mutagenesis (Nicosia et al., 1990). The portion of the protein sufficient to confer sequence-specific DNA binding activity spans amino acids 1-281. The DNA binding domain, as defined by mutational analysis, is in turn comprised of three distinct regions, the integrity of which is a prerequisite for functionality. One of these regions, corresponding to the N-terminal portion of the protein (the "A-domain"), turns out to be necessary and sufficient to guide the dimerization of LFB1. On the basis of sequence analysis and homology to a part of the myosin rod, this region was predicted to form a dimer of α-helices with the hydrophobic amino acids of the common interface packed in a coiled-coil fashion (Cohen & Parry, 1990). Such an arrangement of amphipathic α-helices provides the dimer interface for many DNA binding proteins containing a regular repeat of leucines or "leucine zipper" (Landschulz et al., 1988). However, the N-terminal region of LFB1 shows no sequence homology to the leucine zipper motif.

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In order to establish whether the A domain has the structural characteristics of a coiled-coil, a 32 amino acid peptide comprising the N-terminal region of LFB1 was synthesized. This synthetic peptide showed substantial helical content in aqueous solution and migrated during high-performance gel filtration chromatography with an apparent molecular weight consistent with that expected for a dimer. Furthermore, a molar excess of this peptide prevented dimerization of the intact protein through the formation of protein–peptide heterodimers (Nicosia et al., 1990).

In the present report, we describe a circular dichroism (CD)[1] study on this synthetic peptide and show that it contains sufficient information for dimerization and constitutes an autonomously folded structural domain. We also evaluate the thermodynamic parameters of the self-association.

Materials and Methods

Materials. The synthetic peptide used in this study (B1-Dim) had the following sequence: NH_2-MVSKL^5-SQLQT^{10}ELLAA^{15}LLESG^{20}LSKEA^{25}LIQAL^{30}GG-COOH. The glutamic acid present at position 32 in the protein sequence was substituted by a glycine. The peptide was synthesized and purified by Dr. H. Gausepohl of the Biochemical Instrumentation Programme at the European Molecular Biology Laboratory. The concentration of peptide stock solutions (10 g L^{-1}) was determined gravimetrically. Ultrapure GuHCl was obtained from BRL (Gaithersburg, MD). Urea (Merck, Darmstadt, FRG) was recrystallized from 95% ethanol. Only freshly prepared solutions of urea were used.

All CD measurements were carried out in a pH 7.5 buffer containing 10 mM NaH_2PO_4/Na_2HPO_4 and 150 mM NaF, unless otherwise stated. NaF was used instead of NaCl because of its higher transparency in the far-UV region.

CD Measurements. CD measurements were performed on a Jasco J-500A spectropolarimeter equipped with a thermostatically controlled cell holder stabilized by circulating water from a bath. The spectropolarimeter was interfaced to an IBM AT personal computer for data collection and manipulation. Rectangular cuvettes with 1-, 2-, or 10-mm path length were employed, and the instrument was calibrated with *d*-10-camphorsulfonic acid solution. Molar mean residue ellipticity (θ) values are expressed for all wavelengths as degrees centimeter squared per decimole and were calculated from the equation:

$$\theta = \theta_{obs}103/10dc$$

θ_{obs} is the ellipticity measured in degrees, 103 is the mean residue molar weight of B1-Dim, c is the peptide concentration in grams per milliliter, and d is the optical path in centimeters. All of these spectra were base line corrected. The relative content in α-helix was deduced by using the method of Yang and co-workers (Chang et al., 1978).

For chemical denaturation experiments, the peptide was incubated for at least 30 min at each GuHCl or urea concentration before taking spectra. Thermal denaturation was monitored by the changes in ellipticity at 222 nm. The spectra were recorded after the signal had not changed for at least 10 min at each temperature.

The molar fraction of unfolded peptide, U, was calculated as

$$U = (\theta_{222} - \theta_N)/(\theta_U - \theta_N)$$

where θ_N is the ellipticity of the peptide in the native state and θ_U that of the denatured peptide. θ_U was assumed equal to θ_{222} at 90 °C for the temperature experiments and to θ_{222} at 8 M GuHCl in the GuHCl experiments, respectively. θ_N was calculated according to the equation $\theta_N = -18\,580 + 32.2T$ (see Figure 1).

[1] Abbreviations: CD, circular dichroism; GuHCl, guanidine hydrochloride.

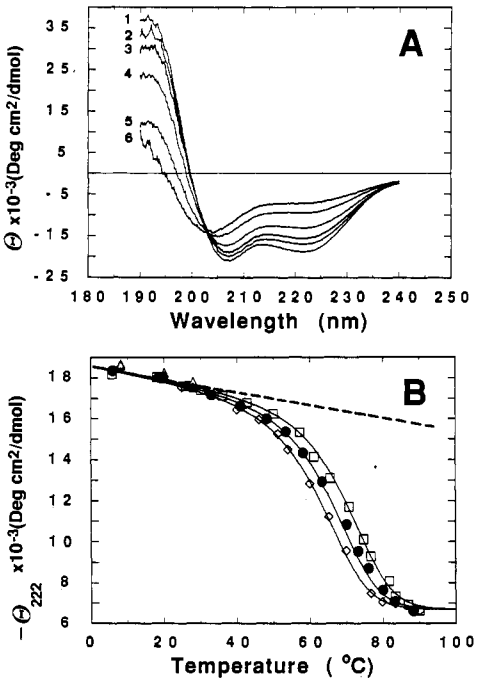

FIGURE 1: Concentration dependence of the thermal denaturation of B1-Dim. (A) CD spectra of B1-Dim recorded in 10 mM NaH_2PO_4/Na_2HPO_4 and 150 mM NaF, pH 7.5, at temperature values of 30 (1), 51 (2), 60 (3), 70 (4), 80 (5), and 90 °C (6). The peptide concentration was 30 μM. (B) Temperature dependence of the mean residue ellipticity (θ) at 222 nm. Peptide concentrations were 6 (open diamonds), 12 (filled circles), and 30 μM (open squares and triangles). The least-squares line corresponding to the pretransition region, obtained from independent measurements below 30 °C, has a slope of 32.2 deg cm^2 dmol^{-1} C^{-1} and an intercept of −18 580 deg cm^2 dmol^{-1}. Solid lines are computer-generated curves following eq 6 with the parameter values as indicated in the text.

Results

CD as a Probe of Conformation Stability. Different optical methods are available to monitor protein unfolding reactions (Schmid, 1990). We used CD because there are no chromophores suitable for UV or fluorescence spectroscopy in the amino acid sequence. Figure 1a shows the CD spectra of the 32-residue peptide derived from the dimerization domain of LFB1 (B1-Dim), recorded in physiological conditions of pH and ionic strength and at increasing temperature. The CD spectral changes seen upon thermal denaturation of the peptide reflect the transition from a native folded state to a more disordered one and are completely reversed by lowering the temperature back to the initial value. The ellipticity minima at 208 and 222 nm in the native spectrum are characteristic of a high degree of α-helical secondary structure, and the isodichroic point near 203 nm is that expected for a two-state helix-to-coil transition (Greenfield & Fasman, 1969). The α-helical content of the native state was estimated to be around 60%, and was not significantly affected by the ionic strength (0–4 M NaCl at 1 M intervals) or by the pH in the range 3–7.5 (data not shown).

Curves corresponding to a smooth cooperative transition are obtained by plotting the variations in the observed ellipticity at 222 nm vs the temperature (Figure 1B). The helical structure of the peptide is remarkably stable, with observed T_m values for the helix–coil transition (i.e., the temperature at which 50% of the peptide is in its unfolded form) ranging

from 63 to 71 °C depending on the peptide concentration.

Unfolding Reaction. The peptide corresponding to the N-terminal region of LFB1 has been previously proposed to be a dimer in conditions similar to those used for this study (Nicosia et al., 1990). The observation that the apparent T_m value increases with the peptide concentration is consistent with the formation of dimers and indicates that peptide self-association is at least partly responsible for the stabilization of the folded form of the peptide. Thus, the unfolding reaction must start with folded dimers (F_2) and end with two unfolded monomers (U). If the folded monomer (F) is sufficiently stable, then the overall reaction can be described by

$$F_2 \overset{K_1}{\rightleftharpoons} 2F \overset{K_2}{\rightleftharpoons} 2U \quad (1)$$

where

$$K_1 = [F]^2/[F_2] \text{ and } K_2 = [U]/[F] \quad (2)$$

The possibility that we are monitoring only the transition between folded and unfolded monomers, with the first part of the reaction not being observable as a CD change, is ruled out by the consideration that this latter transition should be unimolecular and therefore independent of the peptide concentration. The folded dimeric state must therefore be populated during the transition. On this basis, it seems reasonable to conclude that folding and dimerization occur through a concerted mechanism.

If we assume that the folded monomer is not present at significant concentrations in equilibrium, we can simplify the unfolding reaction to include only unfolded monomers and folded dimers:

$$F_2 \overset{K_u}{\rightleftharpoons} 2U \quad (3)$$

where

$$K_u = [U]^2/[F_2] = 2P_t[f_U^2/(1 - f_U)] \quad (4)$$

P_t is the total peptide concentration and f_U the molar fraction of unfolded peptide as judged from the ellipticity at 222 nm.

According to this model, K_u is equal to the dimer dissociation constant. This assumption does not exclude the presence of transiently folded monomers as kinetic intermediates. On the other hand, monomeric peptides of this length are not expected to be stably folded in aqueous solution because the formation of an isolated α-helix is usually thermodynamically unfavorable. According to the simplified two-state model, it is possible to calculate the value for K_u at each different temperature from the CD measurements in the transition zone. If the two-state model provides an appropriate description of the system at equilibrium during the transition, the calculated values of K_u and ΔG_u (Gibbs free energy variation associated with the unfolding reaction, calculated as $-RT \log K_u$) should be the same at a given temperature regardless of the peptide concentration. Figure 2 shows a van't Hoff plot obtained when the experimental data are analyzed on the dimer two-state model. The estimated K_u at each temperature is the same, within experimental error, when calculated from experiments carried out at three different peptide concentrations. The system is therefore well described in terms of two unfolded monomers that fold into a helical dimer.

The inset of Figure 2 shows that the van't Hoff enthalpy associated with the thermal unfolding of B1-Dim (i.e., calculated as the first derivative of the curve in Figure 2) is linearly related to the temperature with a positive slope. This behavior is expected for a two-state transition and has been observed for a variety of small globular proteins (Privalov, 1979). The observed slope of about 740 cal mol^{-1} K^{-1} of

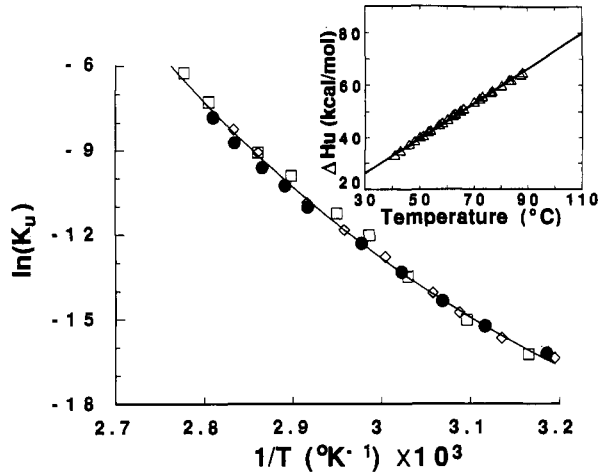

FIGURE 2: Thermodynamic parameters of the thermal unfolding of B1-Dim. van't Hoff plot of the thermal denaturation data for B1-Dim. The equilibrium constants were calculated according to eq 4 from the data shown in Figure 1B. Symbols are as in Figure 1B. The solid curve is the least-squares fit of the data points using a third-order polynomial equation. Inset: Dependence of the enthalpy of unfolding, ΔH_u, of B1-Dim on temperature. ΔH_u values were obtained from the first derivative of the van't Hoff plot. The slope of the least-squares line indicates the change in heat capacity associated with denaturation (ΔC_p = 0.74 kcal mol^{-1} K^{-1}).

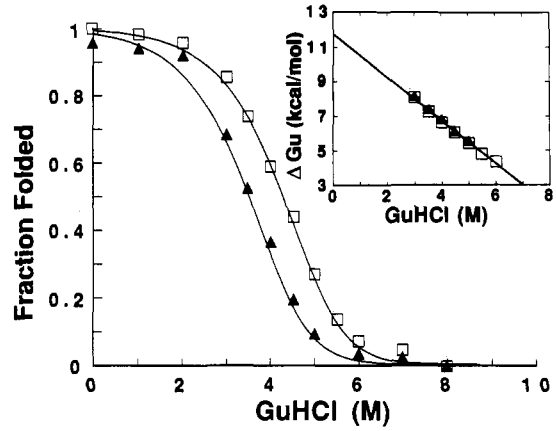

FIGURE 3: Concentration dependence of the GuHCl unfolding of B1-Dim. Equilibrium curves for the GuHCl unfolding of B1-Dim in 10 mM NaH_2PO_4/Na_2HPO_4 and 150 mM NaF, pH 7.5 at 28 °C, as followed by CD. Concentrations of peptide were 6 μM (filled triangles) and 30 μM (open squares). Solid lines are computer-generated curves according to eq 5 with the parameter values indicated below. Inset: Free energy associated with the unfolding of B1-Dim as a function of GuHCl concentration, as obtained from the data above. The least-squares line has a slope (m) of −1.3 kcal mol^{-1} M^{-1} and an intercept of $\Delta G_u^{H_2O}$ = 12 kcal mol^{-1}.

peptide dimer represents the difference in thermal capacity at constant pressure (ΔC_p) between the folded and the unfolded states of the molecule. The value of this parameter is directly related to the magnitude of the hydrophobic interactions in the folded state of a protein (the "hydrophobic effect"). The significance of these thermodynamic parameters will be addressed under Discussion.

Peptide Denaturation with GuHCl. The denaturation curves determined by monitoring the ellipticity of the peptide at 222 nm as a function of GuHCl concentration at 28 °C are displayed in Figure 3 for two different concentrations of peptide. With urea, only 30–40% of the unfolded form of the peptide could be obtained at 8 M denaturant (data not shown). As for temperature denaturation, the apparent stability of the peptide against GuHCl denaturation shows a strong concentration dependence. As expected, the free energies of unfolding, calculated at different concentrations of peptide as

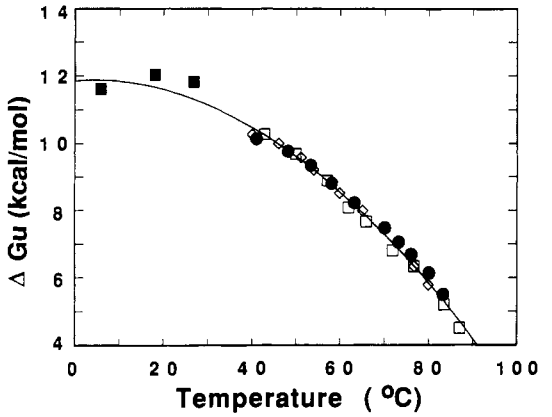

FIGURE 4: Unfolding free energies as a function of the temperature. The data above 40 °C are unfolding free energies measured from the temperature denaturation studies (symbols as in Figure 1B); solid squares represent the $\Delta G_u^{H_2O}$ values determined from the analysis of GuHCl unfolding curves measured at the different temperatures. The solid line is a computer-generated curve according to eq 6. The values of the different parameters are given in the text.

a function of GuHCl concentration (inset of Figure 3), are the same within experimental error when the two-state model illustrated in eq 3 is assumed. The value of ΔG_u in the absence of denaturant was estimated by linear extrapolation to zero GuHCl concentration (Schellman, 1978; Figure 3) according to the equation:

$$\Delta G_u^{obs} = \Delta G_u^{H_2O} + m[\text{GuHCl}] \quad (5)$$

The free energy associated with the unfolding process at this temperature was calculated to be about 12 kcal mol^{-1}.

Figure 4 summarizes the estimated unfolding free energy as a function of the temperature, calculated applying the dimer model to the data from temperature and GuHCl unfolding experiments run at different peptide concentrations. The data points below 40 °C are obtained by analysis of GuHCl denaturation curves at the respective temperatures. The solid line is obtained by computer simulation according to the equation:

$$\Delta G_u(T) = \Delta H_1 - (T/T_1)(\Delta H_1 - \Delta G_1) + \Delta C_p - T_1 - T \log (T/T_1) \quad (6)$$

which is derived from the assumption of a two-state transition and of a constancy of ΔC_p over the experimental range of temperatures (Privalov, 1979).

The constants ΔG_1 (8 kcal mol^{-1}) and ΔH_1 (50 kcal mol^{-1}) are experimental values at T_1 (338 K), an arbitrary temperature near the midpoint of the transition, and ΔC_p (0.74 kcal mol^{-1} K^{-1}) is calculated from the slope of the temperature dependence of ΔH_u shown in Figure 2. A folding free energy of around 11.5 kcal mol^{-1} at 25 °C is estimated from this equation, in good agreement with the values extrapolated from the GuHCl denaturation curves. This value would, according to our model, correspond to a dimerization constant of the order of 10^8 M.

Discussion

In recent years, emphasis has been given to the notion that globular proteins are assemblies of autonomously folded substructures. A consequence of this "modular assembly" model is that suitable protein fragments, corresponding to domains in the native globular structure, might fold independently of the rest of the polypeptide chain. Growing experimental evidence indicates that some proteins can actually be divided into individual modules which often retain a well-defined biological function. These modules correspond to autonomous folding units and are often much smaller than structural domains recognizable by visual inspection of three-dimensional structures of globular proteins. Studies on synthetic analogues of these protein modules have been of enormous help in understanding structure–function relationships in a number of proteins, including the DNA binding proteins GCN4 (O'Shea et al., 1989a) and FOS/JUN (O'Shea et al., 1989b) and the retroviral trans-activator TAT (Frankel et al., 1988).

In the present study, we have shown that a synthetic peptide derived from the N-terminal domain of LFB1 is folded in aqueous solution as a stable dimer. We therefore believe that B1-Dim mimics the dimerization domain of the intact protein. We originally proposed that this domain is folded as a dimer of α-helices, arranged in a coiled-coil fashion (Nicosia et al., 1990).

The stability of a coiled-coil protein molecule is achieved mainly by the packing of the hydrophobic side chains, which lie all on one face of the α-helix, these being the dimer interface in the cases of tropomyosin and of the leucine zipper. The shielding from the surrounding water of the bulky hydrophobic residues packed into the dimer interface provides enough energy to overcome the intrinsic instability of long α-helices.

As discussed below, the values of the thermodynamic parameters estimated from our study are consistent with a hydrophobic stabilization of the peptide dimer. The ΔH_u determined from the van't Hoff plot increases linearly with the temperature: this behavior has been observed for a number of small globular proteins and is indicative of a two-state transition. Privalov (1979) pointed out that despite the individual differences found in the dependence of the enthalpy of unfolding with the temperature, the values of ΔH_u per unit of protein mass extrapolated to 110 °C tend to be the same (ca. 13 cal g^{-1}) for a variety of different globular proteins. This has been interpreted as an index of the compactness of a globular structure. The value of ΔH_u, extrapolated at 110 °C for the B1-Dim peptide, is in excellent agreement with this value [12.6 cal g^{-1} for a dimer with relative mass (M_r) of 6600].

The linear increase of ΔH_u with temperature reflects a positive value of ΔC_p, the difference in the thermal capacity between the unfolded and the folded state of the peptide. Positive heat capacity changes are the hallmark of processes that expose nonpolar surface to water and are related to the anomalous heat capacity displayed by aqueous solutions of hydrocarbons (Sturtevant, 1977). The formation of cages of structured water around the apolar residues could be the cause of the increase in the measured heat capacity of the solution (Tanford, 1970).

The magnitude of ΔC_p observed upon protein denaturation is therefore correlated with the contribution of the burial of nonpolar side chains (hydrophobic effect) to the stability of a globular protein. Our value of 0.74 kcal mol^{-1} K^{-1} (11 cal g^{-1} K^{-1}) is in the range observed for several globular proteins, whose conformational stability is mainly due to hydrophobic interactions (Privalov, 1979). Tanford (1970) proposed a figure of 80 cal mol^{-1} K^{-1} for the ΔC_p of the transition between a fully buried and a fully exposed leucine side chain. This value drops to about 30 cal mol^{-1} K^{-1} if one corrects for the relative extent of side-chain exposure in the native and in the random-coil conformations. In the following paper, we show that there are eight leucines and one isoleucine residue in each monomer of the peptide under study that could be engaged in hydrophobic contacts (Pastore et al., 1991). One could estimate the contribution to the observed ΔC_p of burying these

residues as about 540 cal mol^{-1} K^{-1}. The excellent agreement between the calculated and the estimated value indicates that burial of the bulky chains of the leucine and isoleucine residues may actually provide the major contribution to the hydrophobic stabilization of the peptide dimer.

The curve of ΔG_u as a function of temperature exhibits marked flattening at low temperature. This is predicted to be a consequence of the positive value of ΔC_p (Becktel & Schellman, 1987). We estimate from this curve and from the GuHCl denaturation experiments that the free energy of unfolding should be around 11.5 kcal mol^{-1} at 25 °C. This value is higher than those determined by similar methods for the unfolding of small globular proteins, such as lysozyme (8.9 kcal mol^{-1}), myoglobin (7.6 kcal mol^{-1}), and ribonuclease A (7.5 kcal mol^{-1}) (Ahmad & Bigelow, 1982; Pace, 1975), and comparable to the unfolding free energy of the dimeric Arc repressor protein (11 kcal mol^{-1}; Bowie & Sauer, 1989). An unfolding free energy of 11.5 kcal mol^{-1} corresponds in our model to a dimer dissociation constant of ca. 10^{-8} M, whereas the intact protein was shown to be dimeric at a much lower concentration (10^{-11}–10^{-12} M; Nicosia et al., 1990). It is thus possible that the domain we are studying provides for most, but not all, of the contacts in the protein dimer. Alternatively, the N-terminal domain of LFB1 could be at least partially buried in the native dimer and gain extra stability by excluding from the solvent some of the hydrophobic residues that are not at the helix–helix interface. Burying an extra leucine in the protein dimer would contribute with a free energy of around 2.4 kcal mol^{-1} to the dimer stability (1.2 kcal mol^{-1} of monomer; Guy, 1985). A leucine side chain seems to be in fact exposed to the solvent in the dimeric peptide, as from the nuclear magnetic resonance data (Pastore et al., 1991).

All the thermodynamic parameters described above are obtained from the analysis of the experimental data assuming two-state behavior. The equilibrium unfolding of the peptide under study is very well described by an "all-or-none" transition between folded dimers and unfolded monomers. The agreement between the Gibbs free energies calculated according to this model from experiments run at different concentration together with the observation that the enthalpy of unfolding increases monotonically with the temperature argues in favor of the two-state model. However, in the absence of detailed calorimetric measurements of the enthalpy of unfolding, the presence of intermediates (such as a partially folded monomer) cannot be excluded.

To summarize, in the present study we show that a synthetic peptide corresponding to the N-terminal region of LFB1 can fold into a stable dimeric structure. Strikingly, the conformational stability displayed by this relatively short peptide is that typical of a compact globular protein that buries a maximum of hydrophobic surface area within its fold. Therefore, the region of LFB1 corresponding to the B1-Dim peptide must be regarded as an independent domain, in a structural as well as a functional sense.

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Registry No. B1-Dim, 130495-63-5.

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