Synthesis, physicochemical characterization, and crystallization of a putative *retro*-coiled coil

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Abstract

An artificial HIV enhancer-binding polypeptide has recently been dimerized by covalently linking it to the leucine zipper motif of the yeast transcriptional activator GCN4 (Liu N et al., 1997, *Eur Biophys J* 25:399–403). Although it seemed that the dimerization of this peptide could be best achieved by the use of the *retro* sequence of the leucine zipper, this approach was not implemented in the original construct. As the first step toward the synthesis of a basic region-*retro* leucine zipper HIV enhancer-binding fusion protein, we have now prepared the *retro* version of the leucine zipper (*r*-LZ35) and performed initial physicochemical characterization. Circular dichroism and sedimentation equilibrium studies showed that, at concentrations <100 μ M, the *retro* peptide was an unstructured monomer. At higher concentrations, however, the monomer was in equilibrium with a tetramer and, at 1 mM, the *retro* peptide was almost fully helical. N-terminal extension of the *retro* peptide by the tripeptide Cys-Gly-Gly resulted in a 38-residue polypeptide that could be covalently dimerized by forming a disulfide bond between two chains to give the peptide (*r*-LZ38)₂. Even in the low micromolar concentration range peptide (*r*-LZ38)₂ formed a stable, noncovalent, helical dimer as revealed by circular dichroism and sedimentation equilibrium in the presence and absence of guanidinium chloride. (*r*-LZ38)₂ has been crystallized and X-ray structural analysis is under way. The disulfide-crosslinked *retro*-leucine zipper may lend itself to interesting protein structural studies, including protein design. The present work also highlights the structural and functional potential of *retro* proteins in general.

Keywords: circular dichroism; coiled coil; *retro*-peptide; sedimentation equilibrium

An artificial 42-residue HIV-1 enhancer-binding polypeptide, R42, based on the X-ray structure of the repressor-operator complex of bacteriophage 434 (Anderson et al., 1987; Aggarwal et al., 1988), was able to suppress HIV enhancer-controlled transcription in HeLa cell nuclear extracts (Hehlhans et al., 1993; Städler et al., 1995; Caderas, 1997; Liu et al., 1997) and in living cells (Caderas, 1997). R42 is monomeric, but most natural regulatory DNA-binding proteins are dimers or tetramers. Many of the dimers recognize short nucleotide sequences that are displayed on both stands of the DNA double-helix in dyad symmetry. This mode of protein–DNA interaction enhances the affinity and specificity of binding and hence the biological activity.

The dimerization interfaces of many transcription factors are formed by coiled coil structures, which, in the molecular biological world, are often referred to as leucine zippers (Landschulz et al., 1988), although this term does not correctly represent the interaction between the amino acid residues in the core of the system. The packing of α -helices to form coiled coils was first proposed by Crick (1953), and the α -helical coiled coil has been found to be a widespread protein structural motif (Cohen & Parry, 1990). It is found in long, filamentous structural proteins and in α -helical bundles in globular and membrane proteins. Its presence in transcriptional regulators has revived interest in the structural features that control the stoichiometry of DNA binding of these proteins. The simplest description of the coiled coil is that of a heptad of amino acids, conventionally labelled **a-g**, that is repeated at least four times. Two or more strands interact to form a rope-like structure, hydrophobic residues at the **a** and **d** positions of the constituent chains interacting strongly to form an oligomerization interface, while charged residues at the **e** positions form ion pairs with residues at the **g** positions of other contributory helices. As the coiled coil is relatively simple to model and contains a variety of interactive sites that define and stabilize the multimeric system, it lends itself to protein structural and folding studies and also to being used as a transplantable dimerization module for natural as well as artificial proteins (Riley et al., 1996; Liu et al., 1997). The most

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Abbreviations: CD, circular dichroism; GCN4-p1, C-terminal 33-residue leucine zipper moiety of yeast transcriptional activator GCN4; GuHCl, guanidine hydrochloride; HIV-1, human immunodeficiency virus type 1; *r*-LZ35 and *r*-LZ38, 35- and 38-residue *retro*-leucine zippers based on the sequence of GCN4-p1; (*r*-LZ38)₂, disulfide crosslinked *r*-LZ38; SMC2 “structural maintenance of chromosomes” protein from yeast; TFA, trifluoroacetic acid; $M_{w,app}$, apparent relative molecular mass.

highly characterized leucine zipper is the 33-residue p1 moiety of the yeast transcriptional activator GCN4 (O'Shea et al., 1989; Rasmussen et al., 1991). Detailed structural studies on the native sequence and its analogues have revealed many of the features that control stability and oligomerization in this peptide (O'Shea et al., 1989, 1991; Harbury et al., 1993, 1994; Lovejoy et al., 1993; Betz et al., 1995; Gonzalez et al., 1996a, 1996b). The determination of molecular mass and, hence, interaction stoichiometry, of coiled coils in dilute aqueous solution is best performed by sedimentation equilibrium experiments in the analytical ultracentrifuge. Estimates of oligomerization equilibrium constants as well as the determination of other thermodynamic parameters are also accessible to this technique. At the same time, coiled coil peptides, due to their structural regularity, are an ideal model for the formulation and testing of both practical and analytical methods for the investigation of the hydrodynamics of associative systems (Thomas et al., 1995, 1997).

There are two sites in the enhancer region of the HIV-1 genome that are largely symmetrical (Fig. 1A) and whose geometry would allow adjacent binding of two molecules of the designed HIV enhancer-binding peptide R42 stabilized by a covalently linked leucine zipper moiety. Theoretically, R42 could be dimerized in the proposed HIV enhancer binding mode (Anderson et al., 1987; Städler et al., 1995) by linking it to a leucine zipper in which the N- and C-termini were reversed, i.e., a *retro*-leucine zipper (Fig. 1B). As it seemed that use of the GCN4-p1 *retro* peptide offered a novel route to protein oligomerization, this approach has

Fig. 1. Dimerization of an artificial HIV-1 enhancer-binding peptide. **A:** Sequences of the two, largely symmetrical sites (underlined) in the HIV-1 enhancer region. **B:** Leucine zipper moieties of natural DNA-binding proteins form the C-terminal end of the corresponding polypeptide chains. In an attempt to dimerize the artificial polypeptide R42 (Hehlhans et al., 1993) in the proposed HIV enhancer-binding mode, it was fused to the COOH-terminus of a *retro*-leucine zipper chain (C_{LZ}), i.e., a leucine zipper chain in which the N- and C-termini are reversed (C_{LZ} becomes N_{LZ} and N_{LZ} becomes C_{LZ}).

been pursued systematically. As a first step, we have prepared the *retro*-leucine zipper moiety, *r*-LZ35, of the fusion protein shown in Figure 1B as well as an elongated version, *r*-LZ38, that can be crosslinked by a disulfide bond (Fig. 2). Although the three-dimensional structure of the *retro*-peptide to be described here has yet to be solved, the assumption that it, too, forms a coiled coil has been taken as a working hypothesis. This is based on the observation that the *retro* sequence itself maintains the spacing of hydrophobic residues seen in the idealized heptad (Fig. 2), although residues found at **a** positions in the parent sequence are found at **d** positions in the *retro* counterpart. Although the equivalent transposition applies to the other heptad positions, the reversal of the nature of the **a** and **d** residues may have significant consequences, especially in the determination of the oligomeric state (Harbury et al., 1993).

Here we report on the preparation, purification of the *retro*-peptides and their physicochemical characterization by circular dichroism spectroscopy and conventional sedimentation equilibrium.

Results

Quality and solubility of the synthetic peptides

The synthetic *retro*-leucine zipper peptides *r*-LZ35, *r*-LZ38, and (*r*-LZ38)₂ were of high purity, as determined by high-performance liquid chromatography, amino acid analysis, and electrospray ionization mass spectrometry. Calculated and observed molecular mass of the disulfide peptide were 8908.3 and 8908.0 Da, respectively. The solubility of (*r*-LZ38)₂ depended strongly on the pH of the buffer; it was >1 mM at pH 5 but <5 μM at pH 7.2. The net charge of (*r*-LZ38)₂ was +4, and its calculated pI was 8.42. The peptide sequences used in this study are shown in Figure 2.

Circular dichroism and binding energies

The formation of stable secondary structure of *r*-LZ35 was strongly concentration dependent. In 30 μM solution the helix content of *r*-LZ35 was only 12.5%, whereas it approached 90% at 1 mM peptide concentration (Fig. 3). At 500 μM, the mid-point of unfolding in GuHCl was at 1.73 M (data not shown) and the free energy change, ΔG_u, was calculated to be approximately 32 kJ mol⁻¹. In contrast, (*r*-LZ38)₂ was already 85% helical by a concentration of 30 μM, the helix content in dilute solution thus being comparable to that of acetylated GCN4-p1, measured at 0 °C (Lumb

	d	a	d	a	d	a	d	a
GCN4-p1	R	M	K	Q	L	E	D	K
<i>r</i> -LZ35	R	E	G	V	L	K	K	L
<i>r</i> -LZ38	C	G	G	R	E	G	V	L

Fig. 2. Polypeptide sequences used in the present study. **GCN4-p1:** C-terminal 33-residue dimerization module of yeast transcriptional activator GCN4. ***r*-LZ35:** 35-residue *retro*-leucine zipper based on the sequence of GCN4-p1. ***r*-LZ38:** 38-residue *retro*-leucine zipper obtained by the N-terminal extension of *r*-LZ35 by the tripeptide Cys-Gly-Gly; air oxidation gave the disulfide peptide (*r*-LZ38)₂. All sequences are drawn in the normal way with the N-terminus at the left. Apart from the additional C-terminal dipeptide Gln-Leu the sequence of *r*-LZ35 when read from right to left is identical to that of GCN4-p1. As can be seen, the relative positioning of hydrophobic residues at the **a** and **d** (bold print) positions of the heptads of GCN4-p1 and the *retro*-peptides is conserved.

Fig. 3. Concentration dependence of the CD spectrum of *r*-LZ35 (··· 30 μ M; --- 120 μ M; - - - 500 μ M; — 1 mM) in 0.02 M Tris-HCl, 0.08 M KCl, pH 5, 23 °C.

et al., 1994), while at 100 μ M, the helix content of (*r*-LZ38)₂ was 94%. The midpoint of the unfolding of (*r*-LZ38)₂ induced by GuHCl, at a peptide concentration of 20 μ M, was at 2.35 M (Fig. 4). From the same experiment the free energy of dissociation/unfolding and the dissociation constant of the (*r*-LZ38)₂ dimer could be determined ($\Delta G_u = 55.0$ kJ mol⁻¹, $K_u = 1.8 \times 10^{-10}$ M). The titration curves of both *r*-LZ35 and (*r*-LZ38)₂ were sigmoidal, indicating cooperative dissociation/unfolding of the *retro*-peptide chains.

Ultracentrifugation studies of *r*-LZ35 and (*r*-LZ38)₂

The results of conventional sedimentation equilibrium analyses for *r*-LZ35 and (*r*-LZ38)₂ are shown in Tables 1 and 2. All of the listed values of $M_{w,app}$ were calculated on the assumption that the data could be represented by a single ideal species model. However, the data indicated, by their speed and concentration dependencies, that this model was inadequate. Preliminary experiments on *r*-LZ35, carried out at peptide concentrations in the 20–50 μ M range, appeared to show that the peptide was monomeric in solution (Table 2); CD measurements (Fig. 3) had already revealed that the peptide was essentially unstructured at these concentrations. Attention then switched to the disulfide-crosslinked (*r*-LZ38)₂ as CD spectroscopy had shown that this peptide remained structured into the low micromolar range. Representative data at different peptide

Fig. 4. GuHCl concentration-dependent unfolding of (*r*-LZ38)₂ calculated from the decrease of the CD signal at 222 nm. The peptide concentrations used were 20 μ M (open circles) and 50 μ M (filled squares), respectively. Inset: Change of the free energy of dissociation/unfolding of (*r*-LZ38)₂ as a function of GuHCl concentration. The intercept on the y-axis of the least-squares line obtained from the 20 μ M solutions of the peptide was at 55 kJ mol⁻¹.

concentrations and temperatures are shown in Table 1. The data clearly demonstrated that the covalently crosslinked peptide associated to a dimer that, therefore, incorporated four strands of the original sequence. At the highest concentrations, the monomeric state was only poorly populated and there was no evidence of the presence of anything larger than the dimer. The monomer contributed more significantly at 10 μ M, while it appeared that the dimer was slightly destabilized at 30 °C. Due to the stability of the system it was not possible to estimate thermodynamic parameters from the results of dilution-induced dissociation, the concentrations required being too low for reliable optical absorption measurements. Therefore, in an experiment parallel to the CD measurements described above, a range of concentrations of the denaturant GuHCl was added to (*r*-LZ38)₂ at a fixed peptide concentration of 50 μ M to promote unfolding. The apparent mass of each sample was then measured at a variety of operational speeds. The results are shown in Figure 5 and a representative data set, measured at 2.5 M GuHCl

Table 1. (*r*-LZ38)₂: Apparent whole cell masses, $M_{w,app}$, at different temperatures and peptide concentrations^a

Temperature (°C)	Concentration (μ M)	$M_{w,app}$ (Da)
30	100	15,000 $\pm$ 320
	50	15,700 $\pm$ 220
	10	12,000 $\pm$ 350
20	100	16,600 $\pm$ 150
	50	16,000 $\pm$ 250
	10	10,700 $\pm$ 400
10	100	16,300 $\pm$ 230
	50	16,300 $\pm$ 150

^a All the data were acquired from experiments at 30,000 rpm. Calculated monomer relative molecular mass of the oxidized form, 8,906 Da.

Table 2. *r*-LZ35: $M_{w,app}$ at different experimental speeds, temperatures, and peptide concentrations^a

Temperature (°C)	Speed (krpm)	Concentration (μM)	$M_{w,app}$ (Da)
20	20	250	10,610 ± 170
		125	7,280 ± 125
		50	4,410 ± 310
	30	250	10,460 ± 340
		125	6,650 ± 120
		50	3,930 ± 130
30	40	250	7,580 ± 340
		125	5,770 ± 100
		50	3,860 ± 60

^aCalculated monomer relative molecular mass, 4,238 Da.

(i.e., in the center of the transition), in Figure 6. A smooth transition from an apparent mass of $\approx 18,000 \text{ g mol}^{-1}$ in the absence of GuHCl to $\approx 9,000 \text{ g mol}^{-1}$ at 6 M GuHCl was found, which could be modeled by a simple monomer-dimer equilibrium. From the magnitudes of the equilibrium constant (i.e., K_2 of Equations 1, 2) determined for this model from multiple file fitting as a function of GuHCl concentration, the value at 0 M denaturant was obtained by linear extrapolation of points in the center of the transition in the

Fig. 5. GuHCl concentration-dependent relative molecular mass of $(r\text{-LZ38})_2$ at 23 °C. Mass data are shown as open circles and the error bars are drawn at ± 1 SD. The solid line was derived from measurements of the same transition followed by CD spectroscopy. **Fig. 6.** Representative ultracentrifugational data from the GuHCl-induced unfolding of $(r\text{-LZ38})_2$. Experiments were performed at 2.5 M GuHCl at various run speeds with a detection wavelength of 235 nm. **A:** Data at 35,000 rpm; the solid line was obtained from the simultaneous fitting of the data acquired at four different experimental speeds, assuming that the system could be modeled as a reversible monomer-dimer equilibrium (Equation 1). **B–E:** Computed residuals to the fit illustrated in **A**. **C–E:** Residuals to the fit at 20,000, 25,000, and 30,000 rpm, respectively. The values of $K_{2,a}$ obtained from the fits were used in the computation of the free energy of unfolding after suitable conversion (Equation 2), assuming an extinction coefficient of $1,420 \text{ M}^{-1} \text{ cm}^{-1}$ for the monomer.

usual manner (Pace, 1975). A value of $54.0 \pm 7.6 \text{ kJ mol}^{-1}$ for the net stability of the dimer relative to the fully unfolded state was arrived at in this way, a result in close agreement with that obtained from CD spectroscopy. The effect of GuHCl on $\bar{v}$ was ignored in these calculations. Binding of the denaturant leads to a decrease in the value of $\bar{v}$ which is normally taken to be of the order of $0.02\text{--}0.03 \text{ cm}^3 \text{ g}^{-1}$ at 6 M GuHCl, although the data used here only extended to 3 M. A fall of $\approx 2\%$ (i.e., $0.02 \text{ cm}^3 \text{ g}^{-1}$) in the value of $\bar{v}$ would lead to a corresponding fall of $\approx 7\%$ in the estimated mass.

When *r*-LZ35 was reinvestigated at higher peptide concentrations (100–250 μM) it was found that it, too, formed higher order oligomers (Table 2); by 125 μM the peptide clearly started to self-associate, a process that continued to the highest peptide concentration measured (250 μM) and that appeared to be temperature dependent. The data could only be reasonably fit to a monomer-tetramer equilibrium, there being no evidence for the presence of the dimeric or trimeric state. Both *r*-LZ35 and $(r\text{-LZ38})_2$ therefore

form four-stranded structures which, in the latter case, could be viewed as a dimer of a unit containing two covalently linked monomers.

Discussion

A large number of *retro*-peptides have already been synthesized (Chorev & Goodman, 1995, and references therein) but, apart from a 13-residue β -hairpin *retro*-D-peptide (McDonnell et al., 1997), their molecular structures are not known. Some authors believe that *retro*-proteins are topological mirror images of the native structures of their parent proteins (Guptasarma, 1992), while others predict that the topology of a *retro*-protein may be that of the corresponding native protein, as has been suggested for the *retro*-protein A three-helix bundle (Olszewski et al., 1996) on the basis of potential energy calculations. That *retro*-proteins are not only interesting structurally but also functionally was shown recently by Merrifield and coworkers (Juvvadi et al., 1996). These authors demonstrated that the two main activities of melittin, the 26-residue peptide toxin of bee venom, were resolved in isomeric forms of the peptide. Whereas natural L-melittin had strong antimicrobial activity and lysed eukaryotic cells such as ovine erythrocytes, *retro*-L-melittin and *retro*-D-melittin basically retained only the antibacterial activity.

Nature uses the coiled coil structural motif to produce homo-oligomeric as well as hetero-oligomeric proteins of diverse biological function (Cohen & Parry, 1990; Ransone & Verma, 1990; Ellenberger et al., 1992). We have previously utilized this principle to dimerize a polypeptide, R42, that bound to the HIV-1 enhancer region (Hehlhans et al., 1993; Städler et al., 1995). This peptide, which was based on the structure of the repressor-operator complex of bacteriophage 434, was dimerized using a sequentially normal coiled coil derived from the GCN4-p1 peptide (Liu et al., 1997; Fig. 2) to increase specificity and affinity of DNA binding. As discussed above, it seemed probable that R42 could also be dimerized in the desired manner by covalently linking it to a *retro*-leucine zipper.

As the first step toward the synthesis of an HIV enhancer binding-*retro*-leucine zipper fusion protein we have prepared a putative *retro*-coiled coil, (*r*-LZ35), based on the sequence of GCN4-p1 and extended by two amino acids (glutamine and leucine) to provide additional sites for interchain interactions. Interestingly, *r*-LZ35 (Fig. 2) showed 34.3% sequence identity with segment 863–897 of the yeast chromosome segregation protein SMC2 (Strunnikov et al., 1995), the highest sequence identity with a naturally occurring 35-residue sequence based on a search of the SWISSPROT data bank. This segment is part of a putative coiled-coil region of SMC2, a member of a family of prokaryotic and eukaryotic proteins that are likely to be key components for maintaining the ordered structures of chromosomes.

Initially, it was thought that, if *r*-LZ35 formed a defined conformation at all, the most probable structure it would adopt would be two-stranded, by analogy with the normal GCN4-p1 sequence. However, the CD spectrum of a 30 μ M solution (Fig. 7A) showed very little, if any, structure, and equilibrium sedimentation of the same solution indicated that *r*-LZ35 was monomeric under these conditions (data not shown). In the case of model coiled coils it is the oligomerization process that leads to mutual stabilization of structure in the contributory strands. Obviously, at lower concentrations *r*-LZ35 was not able to form oligomers and, thus, a stable structure. In an attempt to raise the local concentration of the

Fig. 7. CD spectra of (A) *r*-LZ35, (B) (*r*-LZ38)₂, and (C) (*r*-LZ38)₂ at peptide concentrations of 30, 15, and 100 μ M, respectively.

monomer chain, thereby mimicking higher solution concentration, a glycine-spaced cysteine residue was introduced, forming the peptide *r*-LZ38 and its oxidized form, (*r*-LZ38)₂. Similar maneuvers have been used in the past to establish the orientation of the helices or to increase the stability of the oligomeric structure of leucine zippers (Hodges et al., 1981; O'Shea et al., 1989; Wendt et al., 1995), to replace the leucine zipper moiety of GCN4 without affecting the binding specificity of the basic region peptide (Talanian et al., 1990), and to enhance the DNA binding affinity of an N-terminal fragment of bacteriophage λ cro protein (Gutierrez et al., 1980). It was immediately apparent that (*r*-LZ38)₂ (Fig. 2) was structurally competent; the CD spectrum of (*r*-LZ38)₂ indicated high α -helical content even at very low concentrations of the peptide (Fig. 7B). It came as something of a surprise, however, when (*r*-LZ38)₂ was found to form a very stable dimer corresponding to a tetramer of strands of the original sequence (Table 1; Fig. 5).

Tetramerization, although only at concentrations >250 μ M, was also subsequently observed for *r*-LZ35 (Table 2), which existed in an equilibrium between monomer and tetramer. It seems that covalent dimerization considerably stabilizes the tetramer, (*r*-LZ38)₂ associating even at very low concentrations, whereas appreciable oligomerization of *r*-LZ35, which could not form a disulfide bond, was only achieved at peptide concentrations >250 μ M. It is rea-

sonable to suppose that $(r\text{-LZ38})_2$, as well as $r\text{-LZ35}$ at high concentrations, forms a four-helix bundle-type structure, although more extended conformations are also possible. Sedimentation velocity experiments are currently under way to try to distinguish between these conformational possibilities.

In the *retro* form the positions of residues within the heptad sequence are effectively reversed with respect to their positions within the parent coiled coil. Perhaps the most important consequence of this is that the hydrophobic core residues, **a** and **d**, are exchanged, although their relative spacing is maintained (Fig. 2). In a careful study into the effects of amino acid substitutions in the core positions all the **a** and **d** residues of the GCN4-p1 sequence were systematically replaced with the hydrophobic amino acids commonly found in coiled coils (Harbury et al., 1993). The results of the substitutions were examined in a pairwise manner, using a nomenclature, adopted here, that indicated the nature of the residues at the **a** and **d** positions; thus p-IL had isoleucine at all **a** and leucine at all **d** positions, respectively. It was found that the arrangement of the core residues had a profound influence on the oligomerization state and that dimers, trimers, and tetramers could be produced, depending on the combination of substituents. It was concluded that the positioning of β -branched residues played a crucial role in determining configuration: β -branched residues at **d** disfavored dimers, at **a** disfavored tetramers, whereas β -branched residues at both positions favored trimers. GCN4-p1 itself is of the p-VL type with the important proviso that asparagine₁₆ is intact (see below). *retro*-GCN4-p1 would be classified as p-LV, which, were the remainder of the sequence unperturbed, would form a trimer, although p-LI, also β -branched at the **d** position, was tetrameric (Harbury et al., 1993). The role of the asparagine residue in the **a** position of the central heptad of GCN4-p1 in maintaining the dimeric configuration has been investigated in detail. Replacement of the residue with either alanine (Thomas et al., 1995) or valine (Harbury et al., 1993; Potekhin et al., 1994) produces a trimeric structure, whereas, if aminobutyric acid was used, both dimers and trimers were found in solution and could be crystallized separately (Gonzalez et al., 1996a). The higher order oligomers tend to be more stable than the dimer, underlining the importance of accommodating the asparagine to maintain the dimeric structure, even at an energetic cost. In the *retro*-peptide the asparagine residue is moved to the central **d** position, while **a** is occupied by leucine. This alone might be expected to produce a dimer to trimer shift; the formation of a tetramer therefore also seems to reflect other features created by the sequence reversal. Changes in residues at the **e** and **g** positions are also capable of altering the oligomeric state (Krylov et al., 1994; Zeng et al., 1997).

The availability of crystals, obtained under conditions differing strongly from those used for the crystallization of the GCN4-p1 leucine zipper (e.g., 0.2 mM versus 2.6 mM peptide solutions), and the high solubility at pH 5 (>1 mM) of the $(r\text{-LZ38})_2$ dimer should allow elucidation of the structure of a *retro*-peptide in the crystal as well as in solution.

Materials and methods

Peptide synthesis and purification

The *retro*-leucine zipper peptides $r\text{-LZ35}$ and $r\text{-LZ38}$ (Fig. 2) were synthesized by the solid phase method (Merrifield, 1963,

1995) on an Applied Biosystems 433A Peptide Synthesizer using Fmoc chemistry (Carpino & Han, 1970; Applied Biosystems Inc. User's Manual, 1993) and purified by high-performance liquid chromatography on an Aquapore column (Brownlee, 250 × 7 mm) using a solvent gradient (first solvent, 0.1% TFA; second solvent, 0.085% TFA in 75% acetonitrile). To obtain $(r\text{-LZ38})_2$, the disulfide dimer of $r\text{-LZ38}$, a 100 μM solution of purified reduced $r\text{-LZ38}$ was oxidized by air in 0.05 M NH_4HCO_3 , pH 9, at 4 °C for one day. The product was lyophilized and rechromatographed on the Aquapore column. The purity of the peptides was demonstrated by amino acid analysis after acid hydrolysis and by electrospray mass spectrometry on an API III triple-quadrupole Biomolecular Mass Analyzer (Sciex).

Circular dichroism

CD spectra were measured on a Jasco J-715 Spectropolarimeter using a 1-mm cuvette at 23 °C and a solvent of 0.02 M Tris-HCl, 0.08 M KCl adjusted to pH5, both in the absence and in the presence of GuHCl at various concentrations. The calculation of the helix content of the *retro*-leucine zipper peptides was based on the magnitude of the CD signals at 222 nm using the empirical formula of Chen et al. (1972). Titration of $(r\text{-LZ38})_2$ with GuHCl allowed the calculation of the free energy of unfolding (ΔG_u) using an established method (De Francesco et al., 1991).

Analytical ultracentrifugation

Conventional sedimentation equilibrium measurements were made with an XL-A analytical ultracentrifuge (Beckman, Palo Alto, California). Analytical cells were assembled from charcoal-filled epon double sector or six-channel equilibrium centerpieces and quartz windows. The fluorocarbon FC43 was used to form a false bottom, and samples were run at a variety of operational speeds, the temperatures reported being those indicated by the ultracentrifuge. The same solvent as was used for CD measurements was employed and samples were exhaustively dialysed before use. Data were fitted to

$$c_r = c_0 \cdot \exp^{[M_{w,app}(1-\bar{v}_2\rho)\omega^2(r^2-r_0^2)]/2RT} + K_n c_0^n \cdot \exp^{[n_0 \cdot M_{w,app}(1-\bar{v}_2\rho)\omega^2(r^2-r_0^2)]/2RT} \quad (1)$$

where c is the concentration (absorbance) at radial position r , $M_{w,app}$ the apparent molecular mass, $\bar{v}_2$ the partial specific volume, ρ the solvent density, ω the angular velocity, n the association stoichiometry, K_n the equilibrium constant for association step n , and R and T the gas constant and temperature, respectively. For an ideal, monomeric species $K_n = 0$. Molar equilibrium constants, $K_{n,c}$, were calculated from

$$K_{n,c} = K_{n,a} \frac{[\epsilon \ell]^{(n-1)}}{n} \quad (2)$$

where $K_{n,a}$ is the equilibrium constant in absorption units, ℓ the pathlength (= 1.2 cm), and c and ϵ the monomer concentration and molar extinction coefficient, respectively. Partial specific volumes, $\bar{v}_c$, were calculated from the amino acid composition (Laue et al., 1992) and were corrected, where necessary, for temperature variation. No adjustment was made for the presence of GuHCl in

experiments where it was employed. Values of $\bar{v}_{c,20}$ of 0.74₈ and 0.74₁ cm³ g⁻¹ were obtained for *r*-LZ35 and (*r*-LZ38)₂, respectively, at 20 °C. Solvent densities were measured at the experimental temperatures using a DSA48 density and sound analyser (Anton Paar, Graz, Austria).

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References

- Aggarwal AK, Rodgers DW, Drott M, Ptashne M, Harrison SC. 1988. Recognition of a DNA operator by the repressor of phage 434: A view at high resolution. *Science* 242:899–907.
- Anderson JE, Ptashne M, Harrison SC. 1987. Structure of the repressor-operator complex of bacteriophage 434. *Nature* 326:846–852.
- Applied Biosystems Inc. 1993. *User's manual of model 433A peptide synthesizer*, vols. 1 and 2.
- Betz S, Fairman R, O'Neil K, Lear J, DeGrado W. 1995. Design of two-stranded and three-stranded coiled-coil peptides. *Philos Trans R Soc Lond [Biol]* 348:81–88.
- Caderas G. 1997. Passive transcriptional repression by an artificial HIV-1 enhancer-binding peptide [Thesis]. University of Zürich, Switzerland.
- Carpino LA, Han GY. 1970. The 9-fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. *J Am Chem Soc* 92:5748–5749.
- Chen YH, Yang JT, Martinez HM. 1972. Determination of secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* 11:4120–4131.
- Chorev M, Goodman M. 1995. Recent developments in retro peptides and proteins—An ongoing topological exploration. *Trends Biotechnol* 13:438–445.
- Cohen C, Parry DAD. 1990. α -Helical coiled coils and bundles: How to design an α -helical protein. *Proteins Struct Funct Genet* 7:1–15.
- Crick FHC. 1953. The packing of α -helices: Simple coiled coils. *Acta Crystallogr* 6:689–697.
- De Francesco R, Pastore A, Vecchio G, Cortese R. 1991. Circular dichroism study on the conformational stability of the dimerization domain of transcription factor LFB1. *Biochemistry* 30:143–147.
- Ellenberger TE, Brandl CJ, Struhl K, Harrison SC. 1992. The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted α -helices: Crystal structure of the protein-DNA complex. *Cell* 71:1223–1237.
- Gonzalez L, Brown RA, Richardson D, Alber T. 1996a. Crystal structures of a single coiled coil peptide in two oligomeric states reveal the basis for structural polymorphism. *Nat Struct Biol* 3:1002–1010.
- Gonzalez L, Woolfson DN, Alber T. 1996b. Buried polar residues and structural specificity in the GCN4 leucine zipper. *Nat Struct Biol* 3:1011–1018.
- Guptasarma P. 1992. Reversal of peptide backbone direction may result in the mirroring of protein structure. *FEBS Lett* 310:205–210.
- Gutte B, Schindler S, Standar F, Wittschieber E. 1980. Interaction of synthetic NH₂-terminal fragments of bacteriophage λ cro protein with nucleic acids. *Biochem Biophys Res Commun* 95:1071–1079.
- Harbury PB, Kim PS, Alber T. 1994. Crystal structure of an isoleucine-zipper trimer. *Nature* 371:80–83.
- Harbury PB, Zhang T, Kim PS, Alber T. 1993. A switch between two, three and four stranded coiled coils in GCN4 leucine zipper mutants. *Science* 262:1401–1407.
- Hehlgans T, Stolz M, Klauser S, Cui T, Salgam P, Brenz Verca S, Widmann M, Leiser A, Städler K, Gutte B. 1993. The DNA binding properties of an artificial 42-residue polypeptide derived from a natural repressor. *FEBS Lett* 315:51–55.
- Hodges RS, Saund AK, Chong PCS, St-Pierre SA, Reid RE. 1981. Synthetic model for two-stranded α -helical coiled coils. *J Biol Chem* 256:1214–1221.
- Juvvadi P, Vunnam S, Merrifield RB. 1996. Synthetic melittin, its enantio, retro and retroenantio isomers, and selected chimeric analogs: Their antibacterial, hemolytic and lipid bilayer action. *J Am Chem Soc* 118:8989–8997.
- Krylov D, Mikhailenko I, Vinson C. 1994. A thermodynamic scale for leucine zipper stability and dimerization specificity: e and g interhelical interactions. *EMBO J* 13:2849–2861.
- Landschulz WH, Johnson PF, McKnight SL. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* 240:1759–1764.
- Laue TM, Shah B, Ridgeway TM, Pelletier SL. 1992. Computer-aided interpretation of analytical sedimentation data for proteins. In: Harding SE, Rowe AJ, Horton JC, eds. *Analytical ultracentrifugation in biochemistry and polymer science*. Cambridge, UK: Royal Society of Chemistry. pp 90–125.
- Liu N, Caderas G, Gutte B, Thomas RM. 1997. An artificial HIV enhancer-binding peptide is dimerized by the addition of a leucine zipper. *Eur Biophys J* 25:399–403.
- Lovejoy B, Choe S, Cascio D, McRorie DK, DeGrado WF, Eisenberg D. 1993. Crystal structure of a synthetic triple stranded leucine zipper. *Science* 259:1288–1293.
- Lumb KJ, Carr CM, Kim PS. 1994. Sub-domain folding of the coiled coil leucine zipper from the transcriptional activator GCN4. *Biochemistry* 33:7361–7367.
- McDonnell JM, Fushman D, Cahill SM, Sutton BJ, Cowburn D. 1997. Solution structures of FceRI α -chain mimics: A β -hairpin peptide and its retroenantiomer. *J Am Chem Soc* 119:5321–5328.
- Merrifield RB. 1963. Solid-phase peptide synthesis. I. The synthesis of a tetrapeptide. *J Am Chem Soc* 85:2149–2154.
- Merrifield RB. 1995. Solid-phase peptide synthesis. In: Gutte B, ed. *Peptides—Synthesis, structures and applications*. San Diego, CA: Academic Press. pp 93–169.
- Olszewski KA, Kolinski A, Skolnick J. 1996. Does a backwardly read protein sequence have a unique native state? *Protein Eng* 9:5–14.
- O'Shea EK, Klemm JD, Kim PS, Alber T. 1991. X-ray structure of the GCN4 leucine zipper: A two-stranded parallel coiled coil. *Science* 254:539–544.
- O'Shea EK, Rutkowski R, Kim PS. 1989. Evidence that the leucine zipper is a coiled coil. *Science* 243:538–542.
- Pace CN. 1975. The stability of globular proteins. *CRC Rev Biochem* 3:1–42.
- Potekhin SA, Medvedkin VN, Kashparov IA, Venyaminov SY. 1994. Synthesis and properties of the peptide corresponding to the mutant form of the leucine zipper of the transcriptional activator GCN4 from yeast. *Protein Eng* 7:1097–1101.
- Ransone LJ, Verma IM. 1990. Nuclear proto-oncogenes *fos* and *jun*. *Annu Rev Cell Biol* 6:539–557.
- Rasmussen R, Benvegna D, O'Shea EK, Kim PS, Alber T. 1991. X-ray scattering indicates that the leucine zipper is a coiled coil. *Proc Natl Acad Sci USA* 88:561–564.
- Riley LG, Ralston GB, Weiss AS. 1996. Multimer formation as a consequence of separate homodimerization domains: The human c-Jun leucine zipper is a transplantable dimerization module. *Protein Eng* 9:223–230.
- Städler K, Liu N, Trotman L, Hiltpold A, Caderas G, Klauser S, Hehlhans T, Gutte B. 1995. Design synthesis and characterization of HIV-1 enhancer binding polypeptides derived from bacteriophage 434 repressor. *Int J Pept Protein Res* 46:333–340.
- Strunnikov AV, Hogan E, Koshland D. 1995. SMC2, a *Saccharomyces cerevisiae* gene essential for chromosome segregation and condensation defines a subgroup within the SMC family. *Gene Dev* 9:587–599.
- Talanian RV, Knight CJ, Kim PS. 1990. Sequence-specific DNA binding by a short peptide dimer. *Science* 249:769–771.
- Thomas RM, Wendt H, Zampieri A, Bosshard HR. 1995. α -Helical coiled coils: Simple models for self-associating peptide and protein systems. *Prog Colloid Polym Sci* 99:24–30.
- Thomas RM, Zampieri A, Jumel K, Harding SE. 1997. A trimeric α -helical coiled coil peptide: Association stoichiometry and interaction strength by analytical ultracentrifugation. *Eur Biophys J* 25:405–410.
- Wendt H, Dürr E, Thomas RM, Przybylski M, Bosshard HR. 1995. Characterization of leucine zipper complexes by electrospray ionization mass spectrometry. *Protein Sci* 4:1563–1570.
- Zeng X, Zhu H, Lashuel HA, Hu JC. 1997. Oligomerization properties of GCN4 leucine zipper e and g position mutants. *Protein Sci* 6:2218–2226.