Solution Structure of Porcine Delta Sleep-inducing Peptide Immunoreactive Peptide A Homolog of the Shortsighted Gene Product

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Received for publication, April 17, 1997, and in revised form, July 23, 1997

The 77-residue delta sleep-inducing peptide immunoreactive peptide (DIP) is a close homolog of the Drosophila melanogaster shortsighted gene product. Porcine DIP (pDIP) and a peptide containing a leucine zipper-related partial sequence of pDIP, pDIP(9–46), was synthesized and studied by circular dichroism and nuclear magnetic resonance spectroscopy in combination with molecular dynamics calculations. Ultra-centrifugation, size exclusion chromatography, and model calculations indicated that pDIP forms a dimer. This was confirmed by the observation of concentration-dependent thermal folding-unfolding transitions. From CD spectroscopy and thermal folding-unfolding transitions of pDIP(9–46), it was concluded that the dimerization of pDIP is a result of interaction between helical structures localized in the leucine zipper motif. The three-dimensional structure of the protein was determined with a modified simulated annealing protocol using experimental data derived from nuclear magnetic resonance spectra and a modeling approach based on an established strategy for coiled-coil structures. The left-handed super helical structure of the leucine zipper type sequence resulting from the modeling approach is in agreement with known leucine zipper structures. In addition to the hydrophobic interactions between the amino acids at the heptad positions a and d, the structure of pDIP is stabilized by the formation of interhelical i to i’ + 5 salt bridges. This result was confirmed by the pH dependence of the thermal-folding transitions. In addition to the amphipatic helix of the leucine zipper, a second helix is formed in the NH2-terminal part of pDIP. This helix exhibits more 3_12-helix character and is less stable than the leucine zipper helix. For the COOH-terminal region of pDIP no elements of regular secondary structure were observed.

Delta sleep-inducing peptide immunoreactive peptide (DIP)

* This work was supported by grants from the Fonds der Chemischen Industrie (FCI) (to P. R.) and a FCI Kekulé fellowship (to G. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

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‡ The abbreviations used are: DIP, delta sleep-inducing peptide; pDIP, porcine DIP; hDIP, human DIP; bZIP, basic region/leucine zipper; MD, molecular dynamics; RMSD, root mean square deviation; Fmoc, N-(9-fluorenly)methoxycarbonyl; TBTU, O-benzotriazol-1-y1-N,N,N’,N’-tetramethyluronium tetrafluoroborate; HPLC, high pressure liquid chromatography; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy.

This paper is available on line at http://www.jbc.org

Vol. 272, No. 49, Issue of December 5, pp. 30918–30927, 1997
Printed in U.S.A.
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coil domains (21–24) or model α-helical peptides (20, 25, 26). bZIP domains were widely studied with a variety of experimental methods (27, 28). Here, however, we present experimental and computational data on an acetylated full-length leucine zipper protein not containing a DNA binding basic domain to more completely understand structural features of leucine zipper domains in the context of full-length proteins.

MATERIALS AND METHODS

Solid-phase Peptide Synthesis—Peptides were assembled using Fmoc chemistry on an automated peptide synthesizer (model 9050, PerSeptive Biosystems, Wiesbaden, Germany). Fmoc and acids were purchased from Orpegen (Heidelberg, Germany) and PerSeptive Biosystems. Fmoc-Arg(Pbf) was from Sygena (Liestal, Switzerland). N,N-Dimethylformamide (peptide synthesis grade) and polyethylene glycol-polystyrine resins were purchased from PerSeptive Biosystems. TBTU was from Pecher (Langeveli, France). Acetonitrile (HPLC grade), acetic anhydride, dichloromethane, tert-butylmethyl ether, pyridine, piperidine, 1,2-ethanedithiol, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). 1-Hydroxybenzotriazole and diisopropylethylamine were obtained from Fluka (Neu-Ulm, Germany). Solid phase synthesis of pDIP was carried out on a preloaded Fmoc-Val-polystyrine resin (loading 0.19 mmol/g, 0.78 g), phase synthesis of pDIP was carried out on a preloaded Fmoc-Val-Merck column (Darmstadt, Germany). 1-Hydroxybenzotriazole and diisopropylethylamine were added to the Fmoc-Val-polystyrine resin (loading 0.19 mmol/g, 0.78 g). Acylations were performed in 30 min with a 4-fold excess of Fmoc-L-amino acid in the presence of TBTU/diisopropylethylamine and 1-hydroxybenzotriazole (4 eq). Fmoc groups were cleaved by treatment with 20% piperidine in N,N-dimethylformamide for 10 min. After deprotection of the terminal amino groups, the peptide resins were acetylated with a mixture of dichloromethane/N,N-dimethylformamide/acetic anhydride/pyridine (40:40:19.1, volume) in 20 min at 0 °C. Subsequently, the resins were washed with N,N-dimethylformamide, 2-propanol, and dichloromethane (3×) and then dried to a constant weight. Resin cleavage and deprotection were carried out with a freshly prepared mixture of trifluoroacetic acid/ethanedithiol/water (94/5:3, volume, 10 ml/g resin) for 2 h. After filtration, the resin was washed with trifluoroacetic acid, and the crude peptide was precipitated by addition of chilled tert-butylmethyl ether, washed with tert-butylmethyl ether, and lyophilized from 5% acetic acid (crude yields: pDIP, 516 mg, 39.7%; pDIP(9–46), 225 mg, 38.7%). For purification, the dried crude products were dissolved in water (50 ml), loaded onto a Vydac C18 column (20 × 250 mm, 10 μm, 300 Å, The Separations Group, Hesperia, CA) and separated with Buffer A: 0.06% trifluoroacetic acid in water; Buffer B: 0.05% trifluoroacetic acid in acetonitrile/water, 41:59, linear gradient 20–100% buffer B in 80 min, flow rate 9 ml/min, detection at 230 nm). Pure fractions, detected by analytical HPLC (Vydac C18, 5 μm, 300 Å, 4.6 × 250 mm, flow, 0.8 ml/min, detection at 215 nm) were pooled and lyophilized. pDIP yield, 66 mg (5.1%, calculated from initial resin loading). Molecular weight (Mw) by electrospray mass spectroscopy [M + H]+: 2271.5 (Mf, calculated 2271.5). Amino acid composition (after hydrolysis with 6 N HCl at 150 °C for 90 min, 1900 Aminoquant, Hewlett-Packard): Ala 6.15 (6), Arg 3.80 (4), Asp 20.40 (19), Gly 19.50 (19), Glx 19.50 (19), Leu 9.92 (10), Lys 5.09 (5), Met 1.99 (2), Phe 1.01 (1), Pro 7.10 (7), Ser 4.69 (5), Thr 2.89 (3), Tyr 1.05 (1), Glx 19.50 (19), Leu 19.50 (20), Thr 2.89 (3), Tyr 1.05 (1), Val 5.09 (5), Phe 1.01 (1), Pro 3.79 (4), Tryptophan 0.06% (4). 3-Dimensional NMR spectra were employed for the sequence specific assignment of spin systems and the evaluation of nuclear Overhauser enhancement spectroscopy (NOESY) distance constraints: double quantum filtered correlation spectroscopy (DQF-COSY) spectra in 1H, 12C, and 13C were recorded between 300 and 120 ms of mixing time, respectively, and a spin lock field of approximately 12 kHz and NOESY with mixing times of 100 and 200 ms, respectively. Solvent suppression was performed by continuous coherent irradiation prior to the first excitation pulse and during the mixing time in the NOESY experiment. The sweep in 6o and 6q was 7246 Hz for all spectra. Quadrature detection was used in both dimensions with the proportionality factor of the increment technique in δo (37). 4 K data points were collected in 6o and 512 data points in 6q. Zero filling of the time domain data resulted in a frequency-domain matrix with 1 and 2 K data points in 6o and 6q, respectively. All two-dimensional spectra were multiplied by a squared sinebell function phase shifted by π/4 for NOESY and for total correlation spectroscopy spectra and by π/8 for COSY spectra. Base-line correction to the seventh order was used. Data were evaluated on X-window workstations with the NDee program package (Software Symbiose, Inc., Bayreuth, Germany). Shift values are reported relative to external 2,2-dimethyl-2-silapentane sulfonate.

Structure Calculations—Distance information was obtained from two-dimensional NOESY spectra in H2O/D2O (9:1) and in 99.999% H2O. NOESY cross peaks were classified into three categories according to their volume intensity as estimated from the number of contours in NOEY spectra: strong 0.18–0.27 nm; medium, 0.18–0.4 nm; weak, 1.8–5.5 nm. Pseudorotameric constraints were used to adjust distances that involved nonstereospecifically assigned protons such as methyl groups or aromatic ring protons (38). Three-dimensional structures were calculated with the X-PLOR 3.1 package (39). The standard protocols for ab initio calculations of backbone conformational preferences were applied with some modifications. The initial structure calculations started from an extended template with satisfactory local geometry. For the leucine zipper domain a modeling approach for coiled coil proteins (40) was used. The method draws upon knowledge of the oligomerization state, the helix directionality, and the properties of heptade repeat sequences. Unknown structural parameters are heavily sampled. The
coiled coil twist angle, for example, is sampled with an initial range from −35° to 35° in one degree increments. The initial Cα positions were those of a regular α-helix, and the initial separation between the helices was set to 10 Å. For each initial structure, side chain and backbone atoms were grown from the Cα position by applying a protocol similar to those used for the generation of initial coordinates in NMR structure determination (41). Each structure was relaxed with the following simulated annealing protocol: (i) a 5 ps molecular dynamics slow-cooling stage from 500 to 300 K, (ii) a 20 ps constant temperature molecular dynamics (MD) simulation at 300 K, and (iii) 1000 steps of conjugate gradient minimization. During the slow-cooling stage, Cα atoms were held in place with harmonic point restraints that were slowly reduced. The helical hydrogen bond restraints were active during all stages, but no other restraints were applied during the constant temperature MD and energy minimization stages. A time step of 0.5 fs was used for temperatures above 350 K during the slow-cooling stage, otherwise a time step of 1 fs was applied. The coordinates for the residues in the leucine zipper domain thus obtained were used as a reference set in the ab initio simulated annealing and the simulated annealing refinement to restrain the main coordinate set. The restraints were incorporated as point restraints in the form of a harmonic potential.

Unrestrained MD—Unrestrained MD calculations were carried out using the parameters for a representative leucine zipper structure and the TIP3P water model (42) that was supplied with the standard X-PLOR force field (39). The overlay was achieved by placing the protein in the center of a cubic water box (6.33 nm) and deleting all solvent molecules closer than 0.26 nm to any heavy atom of the protein. Close nonbonded solute-solvent interactions were removed in two steps. First, 100 cycles of conjugate gradient energy minimization (43) were carried out, keeping the positions of all protein atoms fixed. Second, in 300 cycles of energy minimization, a harmonic potential was used to restrain the protein to its original conformation. During the first 15 ps of the MD calculations, the system was gradually heated to 300 K while coupled to an external water bath (44). The MD calculations were carried out using the Verlet algorithm (45) with a time step of 1 fs. The SHAKE algorithm (46) was used to constrain covalent bond lengths. A dielectric constant of 1.0 was applied with a scaling factor of 0.4 for 1–4 electrostatic interactions. All nonbonded solute-solvent interactions were cut off at a distance of 0.85 nm. During the whole simulation of 200 ps, minimum image periodic boundary conditions were used. Coordinates, energies, and velocities were saved every 0.5 ps for further analysis. Simulations and analyses were performed on Hewlett Packard HP 735 computers. A 1-ps simulation required about 3 h of CPU time.

A second unrestrained MD calculation was carried out with the leucine zipper of GCN4 using the crystal structure (47) (PDB coordinates 2ZTA) as a starting structure. Protons were added to this structure, and an energy minimization was carried out.

RESULTS AND DISCUSSION

Solid-phase Synthesis—In contrast to many other peptides with comparable lengths prepared by solid-phase peptide synthesis, application of well established Fmoc chemistry to the synthesis of pDIP resulted in a crude product containing the desired product as the major component. The analytical reversed-phase HPLC of the crude product shows pDIP as the last eluting compound of the highest absorbance (Fig. 1). Because most other components in the crude product are characterized by significantly shorter retention times, it is most probable that these are by-products were derived from truncations during solid-phase assembly of the peptide. The COOH-terminal Pro/Glu-rich domain, however, caused no difficulties. HPLC from the crude pDIP(9–46) indicated a similar quality of the crude product. Both peptides were obtained in a purity of about 93% after only one preparative HPLC separation according to mass spectrometry and HPLC analysis. pDIP and the leucine zipper-containing fragment are eluted from the analytical HPLC at about 60% buffer B (Fig. 1), demonstrating that the peptides are retarded by significant interactions with the hydrophobic column.

Sequence Analysis—Residues 19 to 40 of pDIP have been proposed to form a leucine zipper (2). The arrangement of the sequence in an α-helical structure may be modeled schematically (Fig. 2). For clarity, the helical wheel representation for the maximal five heptades (Met9-Leu43) is shown as a helix with a pitch of 3.5 residues/turn as found in coiled coil structures. The interface between the two helices consists of hydrophobic residues with the exception of Asn31 and Asn38. Asparagine pairs are proposed to form an interhelical H-bond at the GCN4 homodimer interface (47). Recently, Lamb and Kim (48) showed that these Asn residues impart specificity for the formation of a two-stranded parallel coiled coil at the expense of stability. Electrostatic interactions between charged residues at positions e and g are sterically allowed in coiled coil structures (47) (Fig. 2, curved arrows), and formation of salt bridges between the two helices could further enhance dimer stability (49, 50).

Protein secondary structure prediction suggests a high amount of helical secondary structure for pDIP, and a long helix starting from the NH2 terminus and spanning the leucine zipper domain (with the exception of the residues around Asn30) is predicted by all of the methods used (Fig. 2). The algorithm by Wolfson and Alber (51) distinguishes between amino acid sequences of dimeric and trimeric coiled coils. This method predicts a preference for dimer formation of the pDIP leucine zipper domain. The Δ-score measuring the difference of
dimer and trimer formation propensities of the test sequence was found to be 2.4, characteristic for dimer formation. Sequence analysis thus suggests that pDIP forms a two-stranded coiled coil. Dimerization may be achieved through a leucine zipper in which the hydrophobic interaction is complemented by the interaction of oppositely charged residues. Our subsequent spectroscopic studies were aimed at further defining structural characteristics of pDIP in solution.

Oligomerization State—pDIP fragment-(9–46) eluted from a Superdex 75 gel-filtration column as a symmetric single peak with the molecular weight expected for a dimer (Fig. 3). In contrast, full-length pDIP eluted as a symmetric peak with an apparent molecular mass of 41.2 kDa, closer to a pentameric (43.8 kDa) than to the expected dimeric (17.5 kDa) form, indicating the presence of higher oligomers in solution (Fig. 3).

The state of association of pDIP was further analyzed by sedimentation equilibrium ultracentrifugation, because this technique does not suffer as much from peak shape dependence as does gel filtration. On the contrary, boundary analysis and curve fitting allow the minimal molecular mass and the association constant of assemblies to be quantified. Making use of an ultrathin cell (2 mm pathlength), combined with schlierenoptics, the sedimentation coefficient and diffusion constant were determined under the conditions of the NMR experiments. At $c_{pDIP} = 2$ mM and 68,000 rpm, no significant trailing of the schlieren peak was detectable; with $s_{20,w} = 1.09 \pm 0.02$ S and $D_{20,w} = (6.59 \pm 0.10) \times 10^{-7}$ cm$^2$ s$^{-1}$, the maximum molecular mass is found to be $M_{s,D} = 15,720 \pm 1300$ Da.

Sedimentation equilibrium experiments made use of the meniscus depletion technique (52) applying A. Minton’s “multeq 3b” program, the $A_{280 \text{ nm}}$ versus $t$ profiles were fitted assuming a monomer-dimer equilibrium. Fig. 4 shows the result; the fit is perfect for both potassium phosphate buffer in the absence and presence of 100 mM Na$_2$SO$_4$. The residuals do not exceed ±0.01.

The association constant is $K_a = 8.0 \times 10^6$ M$^{-1}$. The minimum molecular mass, extrapolating the ln(c) versus $r_0^2$ profile to zero concentration (meniscus depletion), yields weight average $M_w = 8730 \pm 1295$ in accordance with the calculated monomer.

CD Spectroscopy—To examine helical content and thermal stability of pDIP, CD studies were performed at different temperatures (Fig. 5B). The well defined isodichroic point around 202 nm suggests that the denaturation equilibrium can be described on the basis of a two-state structural transition. Minima at 207 and 221 nm and a maximum at 190 nm dem-
onstrate that pDIP is largely α-helical as predicted. Spectra for pDIP(9–46) also indicate predominantly α-helical structure (Fig. 5A). The helix content for pDIP and pDIP(9–46) was determined according to Price (53) to be 57 and 61%, respectively. The lower relative helical content for pDIP(9–46) (74% expected for the four heptades) could reflect the occurrence of two pairs of Asn at α positions. These polar residues are known to be less stabilizing compared with hydrophobic residues at this position in the heptade repeat (48). A similar result was obtained for CD studies of the Max homodimer, a member of the b-HLH-ZIP family (54).

Thermal Denaturation—The peak of negative ellipticity at 222 nm differs significantly in the spectrum of native and denatured pDIP providing a probe for the thermal unfolding reaction that was studied at acidic and neutral pH (Fig. 6A). The sigmoidal shape of the curves suggests a two-state transition in both pH regions, implying a cooperative transition. The melting temperature, defined as the temperature where 50% of the peptide is in the unfolded state, increases from 326 to 337 K when the pH is increased from 2.5 to 6.

The melting temperature is concentration dependent, since it decreases with decreasing protein concentration (Fig. 6B). This observation is consistent with the idea of unfolding connected to dissociation of the two pDIP subunits. If the folded

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**FIG. 4.** Sedimentation equilibrium analysis of pDIP. A sample was run at 23 °C in 50 mM potassium phosphate, 100 mM Na₂SO₄, pH 7. Rotor speed used was 30,000 rpm. Scans at two different concentrations (○ and ●) were fitted assuming a 2M = M₂ equilibrium with M = 8755 Da. The curve depicts the fit; the upper frames depict the residuals.

**FIG. 5.** Far UV circular dichroism spectra. A, CD spectra of pDIP(9–46) recorded at pH 6 and a peptide concentration of 80 μM with varying temperature. Measured rotations are converted to mean residue ellipticities. B, CD spectra of pDIP recorded at pH 2.5 and a peptide concentration of 80 μM with varying temperature. Measured rotations are converted to mean residue ellipticities.

**FIG. 6.** A, thermal unfolding curves for pDIP as a function of pH. The data were recorded at pH 2.5 (●) and pH 6 (○) at a concentration of 80 μM. Thermal unfolding curves as a function of concentration are shown as follows: B, data for pDIP at concentrations of 8 μM (■) and 80 μM (●) at pH 6; C, data for pDIP(9–46) at concentrations of 8 μM (■) and 80 μM (○) at pH 6.
monomer state is essentially unpopulated, unfolding can be described with the two-state model and the energetics can be readily determined in a manner similar to that for monomer denaturation:

\[ N_0 = 2D \]  
\[ K_U = \frac{[N_0]_2 = 2P_f f_m (1 - f_m)}{[D]} \]

with \( P_t \) being the total protein concentration and \( f_m \), the fraction of the unfolded protein. The two states are the native dimer, \( N_0 \), and the denaturated monomer, \( D \). If this two-state model for the unfolding reaction of a dimer provides a reasonable description, then identical values of \( \Delta G^\circ \) (value for a protein concentration of 1 m) should follow from experiments performed at different protein concentration. Fig. 7 shows unfolding free energies calculated for two different protein concentrations of pDIP as a function of temperature. The denaturation: 

\[ \Delta G^\circ = -RT \ln K_U \]

with \( K_U \) being the equilibrium constant for the unfolding reaction. 

**Table I**

| Residue | HN | C,H | C,H | Others |
|---------|----|-----|-----|--------|
| Met\(^1\) | 8.40 | 4.31 | 1.97 | 2.58; 2.50 |
| Asp\(^2\) | 8.56 | 4.57 | 2.82 | 2.71 |
| Leu\(^3\) | 8.02 | 4.21 | 1.67 | 1.55 |
| Val\(^4\) | 7.83 | 3.88 | 2.07 | 0.95; 0.87 |
| Lys\(^5\) | 8.18 | 4.12 | 1.75 | 1.42; 1.32 |
| Asn\(^6\) | 8.13 | 4.60 | 2.78 | |
| His\(^7\) | 8.41 | 4.53 | 3.30 | |
| Leu\(^8\) | 8.19 | 4.21 | 1.70 | 1.57 |
| Met\(^9\) | 8.15 | 4.31 | 2.03 | 2.48; 2.55 |
| Tyr\(^10\) | 7.99 | 4.21 | 3.04 | |
| Ala\(^11\) | 8.07 | 4.12 | 1.45 | |
| Val\(^12\) | 7.93 | 3.88 | 2.10 | 1.02 |
| Arg\(^13\) | 8.04 | 3.90 | 1.85 | 1.75; 1.58 |
| Glu\(^14\) | 8.45 | 4.23 | 1.95 | 2.44 |
| Glu\(^15\) | 8.29 | 4.24 | 1.99; 1.88 | 2.41 |
| Val\(^16\) | 8.10 | 3.39 | 2.17 | 0.94; 0.85 |
| Glu\(^17\) | 7.59 | 3.98 | 2.16 | 2.48 |
| Ile\(^18\) | 8.04 | 3.75 | 1.95 | 1.66; 1.17; 0.89 | 0.78 (6) |
| Leu\(^19\) | 8.59 | 3.90 | 1.81 | ND | 0.92; 0.78 (5) |
| Lys\(^20\) | 8.27 | 4.07 | ND | 1.48 |
| Glu\(^21\) | 7.94 | ND | 2.11 | ND |
| Glu\(^22\) | 8.62 | 4.09 | ND | ND |
| Ile\(^23\) | 7.65 | 3.76 | 1.97 | 1.63; 1.20 |
| Arg\(^24\) | 7.96 | 3.91 | 1.94 | 1.80; 1.58 |
| Glu\(^25\) | ND | |
| Leu\(^26\) | 8.35 | 3.47 | 2.11 | ND |
| Val\(^27\) | 8.99 | 3.63 | 2.15 | 1.07; 0.93 |
| Glu\(^28\) | 7.84 | 4.13 | 1.97 | 2.20 |
| Lys\(^29\) | 8.26 | 4.04 | 1.49 | 1.31 |
| Asp\(^30\) | 8.86 | 4.08 | 3.26; 2.65 | |
| Ser\(^31\) | 8.42 | 4.21 | 4.06; 4.00 | |
| Glu\(^32\) | ND | |
| Leu\(^33\) | ND | |
| Glu\(^34\) | ND | 3.94 | 1.95 | 2.07 |
| Arg\(^35\) | 7.81 | 4.07 | 1.94 | 1.79; 1.58 |
| Glu\(^36\) | 7.96 | 4.21 | 2.01 | 2.46 |
| Asn\(^37\) | 7.83 | 4.06 | 3.16; 2.66 | |
| Thr\(^38\) | 7.71 | 4.34 | 4.14 | 1.31 |
| Leu\(^39\) | 7.50 | 4.21 | 1.66 | 0.79 (6) |
| Leu\(^40\) | ND | |
| Lys\(^41\) | 8.60 | 4.11 | 1.45 | 1.34 |
| Thr\(^42\) | 7.94 | 4.41 | 4.02 | 1.12 |
| Leu\(^43\) | ND | |
| Ala\(^44\) | 8.10 | 4.35 | 1.27 | |
| Ser\(^45\) | 8.34 | 4.26 | 3.94; 3.90 | |
| Pro\(^46\) | 4.38 | 1.88 | 3.75; 3.63 (5) |
| Glu\(^47\) | 8.40 | 4.26 | 1.93 | 2.42 |
| Glu\(^48\) | 7.65 | 4.08 | 1.98 | 2.23 |
| Leu\(^49\) | 6.83 | 3.76 | 1.82 | 1.56 |
| Glu\(^50\) | 8.04 | 4.11 | 2.10 | 2.47 |
| Lys\(^51\) | 8.10 | 4.16 | 1.59 | 1.46 |
| Phe\(^52\) | 8.15 | 4.30 | 3.30; 3.00 | |
| Glu\(^53\) | 8.07 | 4.02 | 2.31 | 2.58 |
| Val\(^54\) | 7.96 | 4.25 | 3.77 | 7.25 (H4); 7.16 (H3/5); 7.13 |
| Glu\(^55\) | 7.78 | 4.27 | 1.90 | 1.75; 1.66 |
| Ser\(^56\) | 7.63 | 4.18 | 1.44 | ND |
| Ser\(^57\) | 7.89 | 4.66 | 3.84; 3.76 | 0.67 (5) |
| Pro\(^58\) | 4.36 | 2.25; 1.86 | 1.97 | 3.66 (6) |
| Glu\(^59\) | 8.34 | 4.27 | 2.04; 1.91 | 2.44 |
| Glu\(^60\) | 8.22 | 4.65 | 2.06; 1.85 | 2.43 |
| Pro\(^61\) | 4.27 | 2.03; 1.91 | 1.97 |
| Ala\(^62\) | 8.27 | 4.54 | 1.31 |
| Pro\(^63\) | 4.39 | 2.25; 1.85 | 1.96 |
| Glu\(^64\) | 8.41 | 4.32 | 2.06; 1.93 | 2.43 |
| Thr\(^65\) | 8.17 | 4.54 | 4.11 | 1.19 |
| Pro\(^66\) | 8.36 | 4.22; 1.85 | 1.98 | 3.80; 3.67 (5) |
| Glu\(^67\) | 8.34 | 4.27 | 2.03; 1.91 | 2.44 |
| Ala\(^68\) | 8.31 | 4.55 | 1.30 |
| Pro\(^69\) | 4.41 | 2.24; 1.85 | 1.95 |
| Glu\(^70\) | 8.30 | 4.27 | 2.04; 1.91 | 2.41 |
| Ala\(^71\) | 8.34 | 4.52 | 1.31 |
| Pro\(^72\) | 4.38 | 2.24; 1.87 | 1.97 |
| Gly\(^73\) | 8.48 | 3.93 |
| Gly\(^74\) | 8.25 | 3.96 |
| Ser\(^75\) | 8.19 | 4.42 | 3.80 |
| Ala\(^76\) | 8.31 | 4.34 | 1.35 |
| Val\(^77\) | 7.91 | 4.11 | 2.09 | 0.87 |

* ND, not determined.*
NMR—The NH–NH region of the NOESY spectrum often can be used to define elements of secondary structure. From the large α-helix content observed in the CD spectra, an extensive array of interresidue connectivities is expected in the NH–NH region of the NOESY spectrum of pDIP. The sharp cross peaks observed in this region were found, however, to correspond only to the residues NH₂-terminal of the leucine zipper domain. In addition to the absence of narrow interresidue NH–NH cross peaks between amino acids of the leucine zipper domain, the fingerprint region in the double quantum filtered COSY and the total coherence spectroscopy experiments show only 60% of the total number of cross peaks expected for the amino acid sequence of pDIP. Following standard methodology (38) it was possible to assign these resonances to the residues NH₂- and COOH-terminal of the leucine zipper domain.

In addition to the narrow cross peaks, broad resonances of generally small intensity were found in the NOESY spectrum. These resonances correspond to residues from the leucine zipper domain. The lineshape of these cross peaks could result from an exchange between monomeric and dimeric or oligomeric states of pDIP, the life time of these states being intermediate on the NMR time scale. Additionally, the leucine zipper domain may indeed be a rigid region, and thus protons therein would be subject to strong cross-relaxation and poor J-coupling (58). A similar behavior of the two subdomains in BR-LZ containing the DNA-binding domain and the leucine zipper domain of GCN4 was observed earlier (27).

Despite these difficulties, it was possible to assign a large number of backbone resonances starting from some well separated NH resonances in the downfield region. For this assignment, NH–NH NOEs and both sequential CaH–NH(i + 1) NOEs and medium range CaH–NH(i + 3) NOEs from the better resolved CaH resonances were used (59). The strong sequential overlap in the side chain region does not allow tracing of the complete spin system for each backbone assignment. Using the two different assignment procedures for the residues from the leucine zipper domain and the remaining residues, the unambiguous sequence-specific assignment of 72 out of 77 amino acids was possible.

Comparing the chemical shifts of the Asn side chain protons, the differences between Asn⁶ in the NH₂-terminal region, and Asn³⁰ and Asn³⁷ in the leucine zipper (Table I) are obvious. Whereas the side chain chemical shifts of Asn⁶ show random coil values (38), Asn³⁰ and Asn³⁷ show an extreme chemical shift separation (0.5–0.6 ppm) of the Cβ protons and (1.1–1.3 ppm) of the γNH₂. This observation may be explained by the formation of one or more hydrogen bonds (22, 47).

**Fig. 8.** A, chemical shift index of the Ca proton frequencies. Amino acids for which the chemical shift of the Ca proton was not determined are marked by diamonds (♦). Stretches of negative chemical shift indices (−1), which are characteristic for α-helical structure, are boxed. B, chemical shift differences Δδ in ppm of the Ca proton frequencies. Dashed lines indicate chemical shift differences of +0.1 ppm and −0.1 ppm, respectively, the values characteristic for the formation of secondary structure.

**Fig. 9.** Survey of the short and intermediate range NOEs observed for pDIP. The height of the bars indicate the intensities of the NOEs. Gray bars indicate that the NOE could not be observed because of frequency degeneration.
The deviation of C\textsubscript{a} proton chemical shifts from their random coil positions are sensitive indicators of secondary structure. Therefore, a secondary structure estimate according to the chemical shift index strategy (60) was used (Fig. 8A). C\textsubscript{a} proton resonances shifted to high field, relative to the corresponding random coil C\textsubscript{a} proton resonances, indicate local \(\alpha\)-helical structure. High field shifts more than 0.1 ppm are marked by \(-1\). For interpretation of the chemical shift index only resonances should be taken into account with the same sense chemical shift deviation for a stretch of more than three sequential residues (60). This procedure indicates for pDIP two chemical shift deviation for a stretch of more than three resonances should be taken into account with the same sense structure. High field shifts more than 0.1 ppm are marked by \(\beta\). Hydrophobic and electrostatic interactions in the modeled leucine zipper. Positive twist angles correspond to a left-handed conformation of the super helix, which is in agreement with proposals by Crick (8) and with known leucine zipper structures (47). Further structural analyses show that the hydrophobic and electrostatic interactions are stronger in the case of left-handed conformations (Fig. 11). Most of the leucine-leucine distances in these structures are in the range of 5–6 Å when measured as average distances of the C\textsubscript{a} Identical values are found in the x-ray structure of GCN4 (47). Formation of salt bridges as defined by the distance between ionizable side chains being less than 4 Å (62) is indicated between Glu\textsuperscript{36} and Lys\textsuperscript{29} and between Glu\textsuperscript{36} and Lys\textsuperscript{41} (Fig. 2). In the modeled coiled coil structures, the leucine zippers corresponding to a left-handed conformation are stabilized by such intermonomer electrostatic interac-

**Fig. 10.** Correlation plot for the structures with different initial twist angles. Negative twist angles correspond to a right-handed super helix; positive twist angles correspond to a left-handed super helix. Dots at position \((i, j)\) indicate an RMSD value of less than 1.6 Å between structures \(i\) and \(j\).

**Fig. 11.** Hydrophobic and electrostatic interactions in the modeled leucine zipper. A, distances between the C\textsubscript{a} atoms of the leucines at position \(d\) and \(d^{\prime}\) \((\bullet, \text{Leu}^{17-}\text{Leu}^{19}; \blacksquare, \text{Leu}^{26-}\text{Leu}^{28}; \blacktriangle, \text{Leu}^{33-}\text{Leu}^{35}; \blacktriangleleft, \text{Leu}^{40-}\text{Leu}^{42})\) versus the helix twist angle. Stronger hydrophobic interactions are formed in structures with a positive starting twist angle. B, distances between the ionizable side chains of charged residues at positions \(g\) and \(e\) \((\bullet, \text{Glu}^{34-}\text{Lys}^{29}; \blacksquare, \text{Lys}^{32-}\text{Glu}^{34}; \blacktriangle, \text{Glu}^{36-}\text{Lys}^{41}; \blacktriangleleft, \text{Lys}^{41-}\text{Glu}^{36})\). The solid line indicates distances between ionizable side chains of less than 4 Å (61). The formation of intermonomeric \(i\) to \(i + 5\) salt bridges is only possible for left-handed conformations of the super helix.
already indicated from analysis of the NOE pattern (Fig. 9) and shows this part to form a helical secondary structure, as was tide. Structural analysis with the PROMOTIF program (63) leucine zipper domain. For the residues NH2- and COOH-terminal to the leucine zipper domain, only the NOEs determined from the 100 ms NOESY spectrum were used as exper-imential restraints, and no symmetry was imposed on the structure.

A schematic picture of the pDIP structure is shown in Fig. 12, and the energy and root mean square deviation (RMSD) values for 10 structures that resulted from this molecular dynamics calculation procedure are listed in Table II. The positive term for the NOE effective energy is caused by no more than two NOE violations larger than 0.05 nm per structure.

The leucine zipper region, Val16–Ala44, shows the lowest RMSD values due to the harmonic fitting. A second region with low flexibility was found in the NH2-terminal part of the peptide. Structural analysis with the PROMOTIF program (63) shows this part to form a helical secondary structure, as was already indicated from analysis of the NOE pattern (Fig. 9) and the chemical shift index (Fig. 8). The helix is extending from Val⁴ to Tyr¹⁰ and shows 3₁₁-helix character in the region from Leu⁸ to Tyr¹⁰ to some extent. The existence of a second helix, aminoo-terminal to the leucine zipper, is somewhat reminiscent of the arrangement of helices in bZIP proteins. In pDIP, however, this first helix is not basic and does not contain the conserved sequence found in most bZIP proteins, that is NXX(A)/XXXXC/ S/R (64). Within the bZIP protein family members with a dimerization domain but no intact basic region responsible for the DNA-binding such as CHOP (65) act by dimerization with proteins containing the basic region and inhibiting their DNA-binding activity. The formation of such heterodimers would be a possible mode of action of pDIP.

The COOH-terminal region of the peptide is very flexible and does not show stable elements of regular secondary structure (Fig. 12). Structured parts of pDIP show higher homology within the sequences of DIP, TSC-22 proteins from different mammals, and two shs variants (Fig. 13): The NH2-terminus is highly conserved (80% identity). Residues responsible for the hydrophobic and electrostatic interactions between the two α-helices in the leucine zipper domain are 88% identical. Lys²⁹, Glu³⁴, Glu³⁶, and Lys⁴¹ (sequence position in pDIP), all involved in salt bridge formation, are conserved in all sequences and the hydrophobic amino acids at positions a and d show high homology. The evolutionary conservation of these residues underscores their functionality. It is known that the residues at positions a and d influence the oligomerization state of the

![Fig. 12. MOLSCRIPT representation (69) of the three-dimen-sional structure of pDIP.](image)

![Fig. 13. Amino acid sequence alignment according to the ClustalW program using the standard set of parameters (70). Asterisks below the sequences denote amino acid identity among all proteins. Dots mark conservative amino acid replacements. The heptade positions of the leucine-zipper are marked (abcdefg). The sequences shown are as follows: pDIP (DIP_PIG), hDIP (DIP_HUMAN), TSC-22 protein from chicken (CHKTSC), mouse (TSC2_MOUSE) and rat (TSC2_RAT) and two shs gene product sequences (DROSHS and DROSHS_II).](image)
coiled coil protein and the distribution of charged residues at positions e and g control the topology of the mutual helix arrangement (18, 66). Therefore, the leucine zipper domains of the other proteins in the sequence alignment (Fig. 13) also have an intrinsic disposition to form parallel two-stranded coiled coils.

The unstructured COOH-terminal region of pDIP, in contrast, shows only 20% identity within the aligned sequences. Although the proline-rich sequence of pDIP does not show homology to SH1-binding domains (67) it is possible that this region is responsible to protein-protein interaction. Proline-rich polypeptides have highly restricted mobility even before binding. Thus, binding leads to a smaller drop in entropy than for a normal more flexible peptide, and hence a larger overall binding energy is achieved (68).

Unrestrained Molecular Dynamics—Unrestrained MD calculations of the leucine zipper of pDIP were performed to compare its structural stability to that of the well known GCN4 leucine zipper structure from the bZIP family. The RMSD values of the backbone and side chain heavy atoms of the structures in the unrestrained molecular dynamics calculation as compared with the starting structure were determined for both structures (Fig. 14). For both proteins, the RMSD values increase early in the calculation as expected due to an equilibration with the solvent. For the remaining time the RMSD values of the backbone atoms remain relatively stable, whereas the RMSD values of the side chain heavy atoms still increase. The RMSD values for the backbone atoms of pDIP and GCN4 leucine zippers are of the same order after the simulation time of 200 ps (0.96 and 0.98 Å, respectively), indicating virtually identical conformational stability of these leucine zippers. This indicates the modeled leucine zipper of pDIP to exhibit the same degree of flexibility as the leucine zippers of the bZIP family.

Acknowledgment—We are grateful to A. T. Brunger for providing protocols for a modeling approach of coiled coil proteins.

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