Unequivocal Determination by Fluorescence Spectroscopy of the Formation of a Parallel Leucine-Zipper Homodimer

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Leucine-zipper polypeptides are characterized by a domain of around 30 amino acids containing a periodic repeat of leucine and, in some cases, isoleucine residues in every seventh position.[1] Peptide sequences of this class, which have been found in many DNA-binding transcriptional activators,[2] mediate the formation of homo- and/or heterodimers by specific interactions of the polypeptide chains. This is the case for the Max and *c*-Myc gene products, which contain a basic-region helix–loop–helix leucine-zipper motif (BHLH-Zip) responsible for the specific formation of Max homodimers and Max–*c*-Myc heterodimers.[3] The biological importance of the dimerization of the above gene products for DNA[4] binding prompted us to investigate by circular dichroism (CD) whether the leucine-zipper domains of the Max and *c*-Myc gene products are autonomous dimerization sites to mediate specific homo- and heterodimeric formation. As part of this ongoing study, a method has been developed to monitor the formation of noncovalent leucine-zipper dimers and to identify the relative orientation of the two peptide chains. The dimer/monomer transition of a leucine-zipper polypeptide can be followed by fluorescence spectroscopy. A pyrene-labeled leucine-zipper peptide shows excimer fluorescence (maximum at 480 nm) when the polypeptide self-associates to form a parallel homodimer and monomer fluorescence (maxima at 380, 400 nm) when the peptide adopts a single-stranded conformation.

Peptides **1** and **2** were assembled on an automated continuous-flow synthesizer, employing the fluorenylmethoxycarbonyl (Fmoc) strategy. Incorporation of glycine and 1-pyrenebutyric acid

Ac-Arg-Arg-Lys-Val-Asp-Thr-Leu-Gln-Gln-Asp-Ile-Asp-Asp-Leu-Lys-Arg-Gln-Val-Ala-Leu-Leu-Glu-Gln-Gln-Val-Arg-Ala-Leu-Glu-NH_2 (peptide **1**)

N-1-pyrenebutyrolyl-Gly-Gly-Arg-Arg-Lys-Val-Asp-Thr-Leu-Gln-Gln-Asp-Ile-Asp-Asp-Leu-Lys-Arg-Gln-Val-Ala-Leu-Leu-Glu-Gln-Gln-Val-Arg-Ala-Leu-Glu-NH_2 (peptide **2**)

(double coupling) was mediated by 2-(2-pyridon-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate/1-hydroxybenzotriazole coupling, and the other amino acids were coupled using their trichlorophenyl active esters. Once chain assembly was completed, the peptide resins were simultaneously cleaved/deprotected with trifluoroacetic acid/water/ethanedithiol (76:4:20 v/v/v), and the crude compounds were purified by reverse-phase medium-pressure liquid chromatography. The purity of the peptides was verified by reverse-phase analytical HPLC, and the identity of the final products was assessed by correct amino acid and mass spectral (matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MALDI-TOF) analyses.

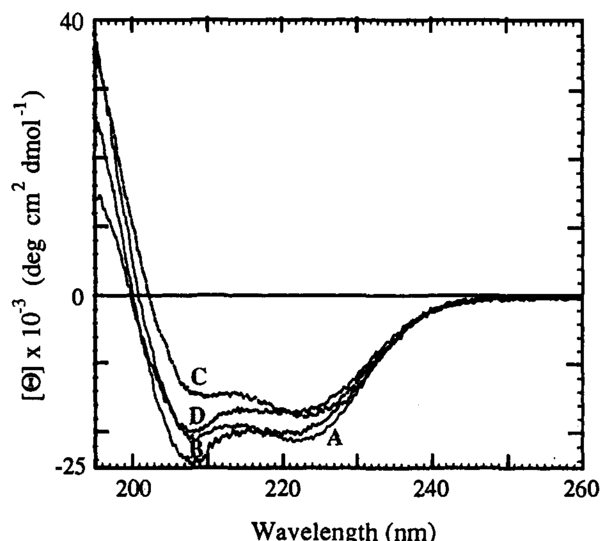

Figure 1. Circular dichroism of peptide **1** ($c = 40\ \mu M$; $T = 22$ °C). (A) 100 mM NaCl, 10 mM phosphate buffer, pH = 7.0. (B) 2,2,2-Trifluoroethanol. The CD of peptide **2** is also shown ($c = 40\ \mu M$; $T = 22$ °C). (C) Water. (D) 2,2,2-Trifluoroethanol.

The leucine-zipper peptide **1**, which was modeled on the basis of the leucine-zipper sequence of the Max gene product,[5] showed in a phosphate buffer a characteristic coiled coil CD spectrum with minima at 208 and 222 nm, and a $\Theta_{222}/\Theta_{208}$ ratio of 1.04 (Figure 1).[6] This polypeptide was labeled at the *N*-terminus with 1-pyrenebutyric acid, using a glycine–glycine dipeptide as a spacer (peptide **2**). It is known from the literature[7] that an excited pyrene can form an intramolecular excimer with a ground-state molecule if the latter is near during the excited pyrene lifetime. The formation of an intramolecular excimer is followed by a broad fluorescence emission spectrum with a maximum at 480 nm, which is absent in the monomer fluorescence band. In accordance with the above properties, excimer fluorescence due to intramolecular label/label interactions would be observed in a parallel dimer with the *N*-terminus of the two polypeptide monomers in close proximity, whereas in an antiparallel orientation or without dimer formation, intramolecular label/label interactions would be absent, and the monomer fluorescence would be detected. As expected from the solution conformation of **1**, an excimer fluorescence band for peptide **2** was observed in aqueous media (Figure 2), indicating that the peptide self-associates to form a parallel dimer. The possibility of intramolecular excimer formation due to aggregation of the molecular

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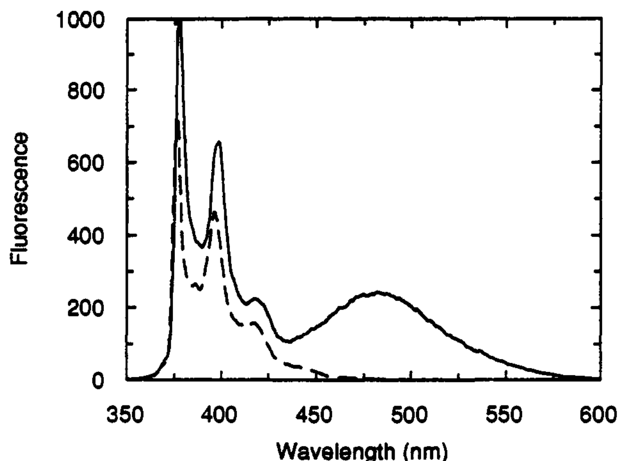

Figure 2. Fluorescence emission spectra (λ_{ex} = 350 nm) of peptide **2** at 22 °C in 10 mM phosphate buffer/dimethyl sulfoxide (4:1 v/v; c = 36 μM; solid line)[9] and 2,2,2-trifluoroethanol (c = 32 μM; dotted line).

label was ruled out by obtaining a monomer fluorescence band for 1-pyrenebutyric acid under identical experimental conditions as for peptide **2** (data not shown).[8] The dimeric structure of peptide **2** in water was also proved by circular dichroism (Figure 1). The $\Theta_{222}/\Theta_{208}$ ratio and the CD pattern clearly indicate that peptide **2** adopts a double-stranded α-helix structure in water.[6] The CD data (Figure 1) were also consistent with the observed monomer fluorescence emission spectrum of the labeled peptide in 2,2,2-trifluoroethanol (Figure 2), a disrupter of the coiled coil structure.[10] In this solvent, peptide **2** shows the CD profile of a single-stranded α-helix ($\Theta_{222}/\Theta_{208}$ = 0.81; estimated fraction of α-helix = 56%).[11]

This communication shows that fluorescence-labeled polypeptides can be a valuable tool for use in the study of macromolecular interactions and in the development of novel spectroscopic assays for relevant biological processes. The data obtained from fluorescence and circular dichroism studies were well consistent to prove the dimer/monomer transition of a leucine-zipper polypeptide. It remains for future work to evaluate the application of the technique described in this paper for examining other protein–protein interactions.

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(8) Concentration range: 32–97 μM in 10 mM phosphate buffer/dimethyl sulfoxide (4:1 v/v); T = 22 °C.

(9) An identical fluorescence spectra was obtained in water (data not shown).

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