Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 13, pp. 1695-1698, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00290-7

PROBING THE FORMATION OF A PARALLEL HETERODIMERIC COILED COIL BY FLUORESCENCE QUENCHING

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Abstract. *Engineered leucine zipper peptides that are unfolded in isolation were appended at the N-terminus with the 2-aminobenzoyl probe or the 2,4-dinitrophenyl-β-alanine amino acid. The intrinsic fluorescence of the 2-aminobenzoyl group is quenched when the peptides associate in benign medium to form a parallel heterodimeric coiled coil.* © 1997 Elsevier Science Ltd.

The leucine-zipper polypeptide[1] is a protein-protein interaction motif that promotes the formation of homo- and/or heterodimers by specific interactions of the two peptide chains. Leucine zippers are frequent domains in DNA-binding transcription factors[2] and have been extensively used as scaffolds in the *de novo* protein design[3]. Recently, we have shown that the dimer/monomer transition of a leucine zipper homodimer can be followed by fluorescence spectroscopy[4]. This work has been extended with a method to monitor heterodimer formation. The assay reported here is based on fluorescence quenching by resonance energy transfer[5] and uses the 2-aminobenzoyl group as fluorescent energy donor and the 2,4-dinitrophenyl group as energy acceptor.

Ac-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-NH_2 (Peptide **1**)

Ac-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-NH_2 (Peptide **2**)

Abz-Gly-Gly-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-Glu-Leu-Gly-Ala-Leu-Glu-Lys-NH_2 (Peptide **3**)

2,4-DNP-βAla-Gly-Gly-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-Lys-Leu-Gly-Ala-Leu-Lys-Glu-NH_2 (Peptide **4**)

Figure 1. Sequences of the leucine zipper peptides.

Peptides **1-4** (Figure 1) were synthesised on a Milligen 9050 automated peptide synthesiser (continuous flow), employing the fluorenylmethoxycarbonyl strategy[6]. The required Fmoc amino acids (3 equiv.) were incorporated using their 2,4,5-trichlorophenyl esters with minimum reaction times of 30 min. 2-*tert*-Butyloxycarbonyl-aminobenzoic acid and 2,4-dinitrophenyl-β-alanine (3 equiv. each) were coupled with benzotriazolyloxy-tris-(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole[7] (1:1, v/v; 3

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equiv.; double coupling) in the presence of diisopropylethylamine (6 equiv.). The completed peptide resins were simultaneously deprotected and cleaved by treatment with trifluoroacetic acid/water (95:5, v/v) for 3 h at room temperature. The filtrate from each cleavage reaction was precipitated in diisopropyl ether-petroleum ether (1:1; v/v) at 0 °C, and the precipitate was collected by filtration. The crude peptides were purified by medium-pressure liquid chromatography on a C_{18}-column using an acetonitrile-water gradient. The purity of the final compounds was verified by reversed-phase analytical HPLC[8], and the identity was assessed by correct mass spectral (matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry, MALDI-TOF) and amino acid analyses[9].

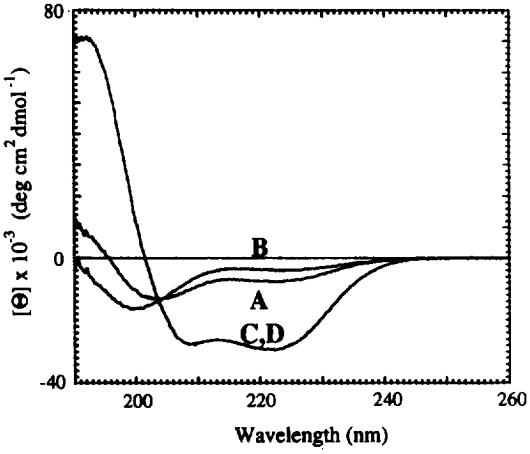

Figure 2. Circular dichroism spectra of: **A**, peptide **1** (c = 300 μM); **B**, peptide **2** (c = 300 μM); **C**, peptide **1**:peptide **2** (1:1; c_t= 300 μM); **D**, and peptide **3**:peptide **4** (1:1; c_t= 300 μM) in 100 mM NaCl, 10 mM phosphate buffer, pH= 7.0, T = 20 °C.

The design of the above leucine zipper peptides was based on the sequences described by Hodges *et al.*[10]. The peptides contain leucine residues at the **a** and **d** positions[11], which are included to achieve a hydrophobic stabilisation of the structure, and also demonstrate interhelical electrostatic interactions between glutamic acid and lysine residues on neighbouring helices. As expected from the original report[10], an equimolar mixture of peptides **1** and **2** displays in a phosphate buffer a characteristic coiled coil circular dichroism spectrum with double minima at 208 and 222 nm, and a $\Theta_{222}/\Theta_{208}$ ratio of 1.08 (Figure 1)[12]. By contrast, the peptides are unfolded in isolation under the same experimental conditions (Figure 1). Peptides **1** and **2** were labelled at the *N*-terminus with 2-aminobenzoic acid (peptide **3**) and 2,4-dinitrophenyl-β-alanine (peptide **4**), respectively, using a glycine-glycine dipeptide as a spacer. The derivatised leucine zipper peptides showed folding preferences (Figure 1) and an oligomerisation state (data not shown) identical to those of the parent peptides[13].

The 2-aminobenzoyl and 2,4-dinitrophenyl groups have been extensively used as donor-acceptor pairs in intramolecularly quenched fluorogenic protease substrates[14]. Quenching occurs by resonance energy transfer[5], a phenomenon in which excitation energy is transferred from the excited fluorescent donor chromophore to the quenching chromophore acceptor. We posited that intermolecular fluorescence quenching would be observed when conveniently tagged leucine zipper peptides adopt a parallel heterodimeric coiled-coil conformation that

bring the fluorophore and the quencher into close proximity with one another. This is in fact what happened when peptide **3** was titrated with peptide **4** (Figure 3). In accordance with the solution conformation of the peptides and the energy transfer principle, the intrinsic fluorescence of the 2-aminobenzoyl group was quenched when peptide **4** was added to a solution containing peptide **3**. The possibility that this quenching may have been due to aggregation of the fluorophore and the quencher was ruled out by titrating peptide **3** with 2,4-dinitrophenyl-β-alanine under the same experimental conditions as above[15]. In this case, no change in the fluorescence was observed (Figure 3B).

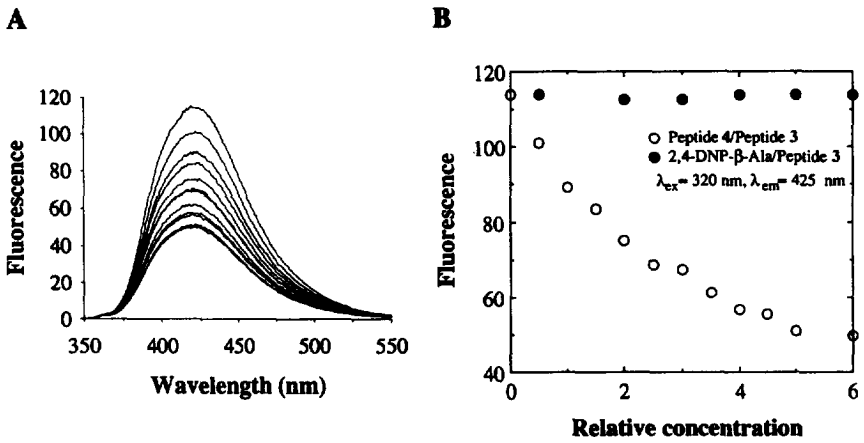

Figure 3. (A) Fluorescence emission spectra of peptide **3** (c = 5 μM) in the presence of different amounts of peptide **4** (c = 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 μM) in 100 mM NaCl, 10 mM phosphate buffer, pH= 7.0, T = 20 °C. The excitation wavelength was 320 nm with an excitation slit of 3.0 nm and an emission slit of 5.0 nm. The spectra were recorded using a Shimadzu spectrofluorophotometer model RF-5001 PC. (B) Variation of the emission fluorescence maxima of peptide **3** following addition of peptide **4** (o) or 2,4-dinitrophenyl-β-alanine (•) in 100 mM NaCl, 10 mM phosphate buffer, pH= 7.0, T = 20 °C.

In summary, we have shown that fluorescence quenching by resonance energy transfer[16] can be applied to the study of macromolecular self-assembling structures. The data obtained from fluorescence, in combination with the circular dichroism studies, were sufficiently consistent to confirm the formation of a parallel heterodimeric coiled coil.

Acknowledgements

I thank R. Wille and D. Arz for their technical assistance, Dr. E. John for the use of the CD instrument and Dr. P. Senn for allowing me to use the fluorescence spectrometer.

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8. The purity of the peptides was verified by reversed-phase analytical HPLC on a Nucleosil column (250 x 4.0 mm; 5 μm, 100 Å): linear gradient over 10 min of MeCN/0.09% TFA and H_2O/0.1% TFA from 1:49 to 3:2; flow rate 2.0 mL/min, detection at 215 nm; single peak at t_R= 8.22 (peptide **1**); t_R= 8.32 (peptide **2**); t_R= 8.14 (peptide **3**); t_R= 8.74 (peptide **4**).
9. Mass spectral analyses (matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry, MALDI-TOF) revealed molecular masses within 0.1% of the expected values (negative-ion mode): 3762.8 (calc. 3762.4, $C_{167}H_{284}N_{41}O_{56}$; peptide **1**); 3757.1 (calc. 3757.7, $C_{172}H_{309}N_{46}O_{46}$; peptide **2**); 3953.6 (calc. 3953.6, $C_{176}H_{293}N_{44}O_{58}$; peptide **3**); 4066.0 (calc. 4066.9, $C_{183}H_{320}N_{51}O_{52}$; peptide **4**). Quantitative amino acid analyses of the purified peptides after acid hydrolysis gave the following composition, with expected values in parentheses: 4.9 Lys (5); 10.1 Glu (10); 5.0 Gly (5); 5.0 Ala (5); 9.5 Leu (10) for peptide **1**; 9.5 Lys (10); 5.2 Glu (5); 5.3 Gly (5); 5.1 Ala (5); 9.5 Leu (10) for peptide **2**; 4.9 Lys (5); 9.8 Glu (10); 7.1 Gly (7); 5.3 Ala (5); 9.8 Leu (10) for peptide **3**; 10.0 Lys (10); 5.2 Glu (5); 6.7 Gly (7); 5.1 Ala (5); 9.9 Leu (10) for peptide **4**.
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(*Received in Belgium* 30 *September* 1996; *accepted* 28 *May* 1997)