Synthesis of a Disulphide Linked Heterotrimeric Active Site Peptide Segment of Laminin

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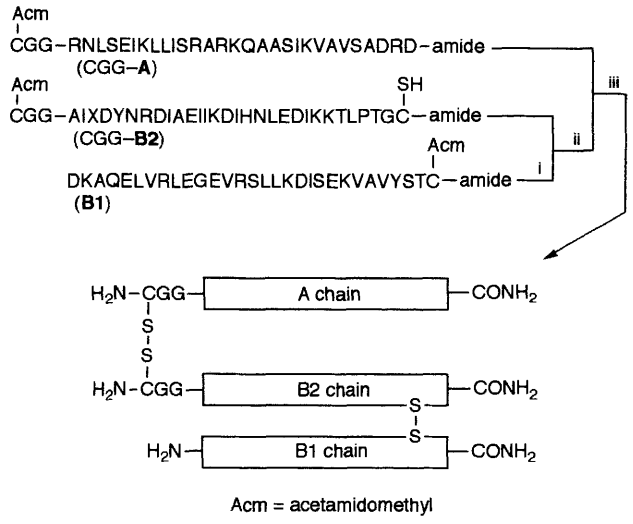
A disulphide linked 95-mer heterotrimeric active site segment of laminin (B2 chain Met 1538 replaced with Nle) was synthesized by the solid phase peptide synthesis method utilizing the two-step trimethylsilyl bromide–HF deprotection procedure and the interlinking of the three subunits by the stepwise selective formation of two disulphide bridges using air-oxidation and thallium(III) trifluoroacetate oxidation.

Laminin is a major glycoprotein (M_r = 900000) in basement membranes with diverse biological activities.[1] A prominent form of laminin consists of the A, B1 and B2 chains which are held by disulphide bonds. Several active sites of laminin have been localized previously. In particular, an IKVAV (Ile-Lys-Val-Ala-Val) sequence of the A chain of laminin was found to be one of the most potent sites for promoting cell attachment, migration and neurite outgrowth.[2] However, the activity of the single chain of the IKVAV-containing synthetic peptide was not as high as that of intact laminin when compared at molar ratios. It has been proposed that the region surrounding the active site is composed of the three chains, A, B1 and B2 which form a heterotrimer with coiled-coil triple helical conformation.[3]

As a model for the coiled-coil segment of laminin, we designed and synthesized the 95-mer peptide which consists of three chains (CGG-**A**, **B**1 and CGG-**B**2) and is stabilized by covalent linking through two disulphide bridges (Scheme 1). The native sequence subunits, **A** and **B**2, were *N*-terminally extended with CGG tripeptide sequences to serve as linker arms.[4] Met 1538 in B2 chain was replaced with norleucine in order to assure better stability of the resulting peptides.

The preparation of the three protected peptide resins was carried out by using the Boc based solid phase peptide synthesis procedure.[5] Cysteines were protected as the acetamidomethyl (Acm)[6] derivatives during the synthesis of subunits CGG-**A** and **B**1. The *N*- and *C*-terminal cysteines of subunit CGG-**B**2 were strategically protected with Acm and the HF susceptible 4-methylbenzyl[7] group, respectively. Using the 4-methylbenzhydrylamine resin,[8] the respective amino acids corresponding to the three peptides were condensed in a stepwise manner using an automated peptide synthesizer. The protected peptide resins were deprotected using the two-step deprotection–cleavage method, which consisted of the consecutive trimethylsilyl bromide–thioanisole and HF procedure.[9] The crude peptides were purified by gel filtration and HPLC. Removal of the Acm protective group from **B1**[Cys(Acm)] was accomplished with silver trifluoromethanesulphonate (AgOTf) in trifluoroacetic acid (TFA) in the presence of anisole at 4 °C for 2 h.[10] After treatment with dithiothreitol (DTT), the reduced peptide was purified by gel filtration.

B1(Cys-SH) and C(Acm)GG-**B2**(Cys-SH) (molar ratio, 1 : 1) were mixed in 50 mmol dm^{-3} Tris-HCl (pH 7.6) and kept at room temperature for 3 days. After air-oxidation, the resulting heterodimer was purified by HPLC, on a Vydac C4 column (10 × 250 mm) using a water–90% MeCN gradient containing 0.05% TFA (30–70% in 30 min), flow rate, 2.5 ml per min; retention time, 17.2 min; yield of **B1**-disulphide-**B2**-GGC(Acm): 36%, based on **B1**(Cys-SH).



Scheme 1 Synthetic scheme for **B1**-disulphide-(**B2**-GGC)-disulphide-(CCG-**A**). **A**: mouse laminin A chain position 2081–2109; **B2**: mouse laminin B2 chain position 1536–1565, Met(position 1538) replaced with norleucine (X = Nle); **B1**: mouse laminin B1 chain position 1735–1764. *Reagents and conditions*: i, AgOTf–TFA; ii, air-oxidation; iii, $(CF_3CO_2)_3$ Tl–TFA.

The above dimer and C(Acm)GG-**A** (2 equiv.) were oxidatively coupled using thallium(III) trifluoroacetate (10 equiv.) in TFA in the presence of anisole at 4 °C for 2 h.[11] The resulting trimeric product was purified by gel filtration followed by HPLC using the above conditions. Retention time, 16.0 min; yield 23%, based on the **B1**-disulphide-**B2**-GGC(Acm). The trimer and the dimer were identified by amino acid analysis, and by analytical HPLC after reduction of disulphide bridges by DTT to the respective monomers. The successful synthesis of our trimeric peptide also serves to demonstrate for the first time that thallium trifluoroacetate is an effective reagent for the intermolecular oxidative coupling of Acm protected Cys-peptides.

The conformations of the dimer and the trimer were compared by circular dichroism spectroscopy. The spectra of both peptides exhibited a similar α-helical pattern at room temperature. However, at higher temperature (42 °C), only the spectrum of the dimer indicated substantial denaturation, suggesting that the covalent A chain in the trimer, while not increasing the gross α-helical content, nevertheless imparts additional conformational stability to it, when compared to the dimer.

When tested in cell adhesion assays of rat pheochromocytoma cells (PC-12), the trimer was found to be as effective as the A subunit peptide [C(Acm)GG-**A**].[12]

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