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β-CELL TROPIN: SYNTHESIS AND BIOLOGICAL ACTIVITY

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SUMMARY The structure of β-cell tropin, an insulin secretagogue released by the neurointermediate lobe of the obese (ob/ob) mouse, has recently been determined as the 22–39 moiety of ACTH. A method for the preparation of this octadecapeptide using mild solid-phase procedures followed by preparative high pressure liquid chromatography is described. The molecular weight of the synthetic peptide has been confirmed by Fast Atom Bombardment mass spectrometry. Synthetic β-cell tropin is indistinguishable in its chromatographic, antigenic and biological properties from natural β-cell tropin.

β-Cell tropin (βCT) is a newly discovered peptide which is released from the neurointermediate lobe of the pituitary of the genetically obese (ob/ob) mouse (1). The hormonal nature of βCT has been suggested by the finding that it is present in the plasma of this animal (2). βCT stimulates insulin release in a monophasic manner at sub-stimulatory glucose concentrations (1) and potentiates glucose-induced secretion from the perfused rat pancreas (3, 4). The peptide has an insulin-like effect on lipogenesis (5). βCT is also released from the pituitaries of lean mice, although in smaller quantities (3).

We have recently determined the structure of βCT as the octadecapeptide, H-Val^{1}-Tyr-Pro-Asn-Gly^{5}-Ala-Glu-Asp-Glu-Ser^{10}-Ala-Glu-Ala-Phe-Pro^{15}-Leu-Glu-Phe^{18}-OH, which is ACTH-(22–39) (6). Although βCT can be prepared by trypsinisation of synthetic CLIP, it was considered that a direct synthetic route to βCT would be essential for the generation of sufficient peptide for biological investigations and the development of a specific immunoassay.

This communication describes the preparation of synthetic βCT and a comparison of its biological and chromatographic properties with those of natural mouse-derived βCT.

MATERIALS AND METHODS

Materials Dichloromethane and 2-propanol were Analar grade. Trifluoroacetic acid (TFA) was of 99% purity (Aldrich Chem. Co.). The remaining solvents and reagents were redistilled. Alkoxybenzyl resin (Merseyside Laboratories, Warrington, U.K.) was washed with dichloromethane, DMF, 10% diisopropylethylamine in dichloromethane (v/v), DMF, and dichloromethane (5-times each), prior to attachment of the first amino acid. 9-Fluorenylmethyloxycarbonyl (Fmoc) amino acids, having *tert.*-butyl side chain protections for Tyr, Glu, Asp and Ser, were converted to their respective symmetrical anhydrides by a 15 min treatment with 0.5 equiv of dicyclohexylcarbodiimide (DCC) in dichloromethane (for Glu, Pro, Ser, Tyr and Val) or 10% DMF in dichloromethane (for Leu, Asp, Phe, Ala and Gly) and used immediately (modification of method in ref. 7). The anhydrides of Phe, Ala and possibly Gly co-precipitated with the DCC-urea. Consequently, for these residues the anhydrides were used without being freed from DCC-

Abbreviations: βCT, β-cell tropin; ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate lobe peptide, ACTH (18–39); HPLC, high pressure liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; DMF, dimethylformamide; TFA, trifluoroacetic acid.

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urea. Valine was introduced as the *tert.*-butyloxycarbonyl (Boc) derivative in order to achieve simultaneous cleavage and deblocking during the final acidolysis step.

Coupling Cycles The peptide was synthesised with a Beckman model 990B peptide synthesiser. The programmes were: *1.* Fmoc-deprotection – DMF wash, 5x1 min; 50% piperidine in DMF (v/v), two consecutive treatments for 5 min and 20 min; DMF wash, 5x1 min: *2.* Coupling – DMF wash, 5x1 min; symmetrical anhydride (4 equiv), or for Asn a mixture of the Fmoc-*p*-nitrophenyl active ester (6 equiv) and 1-hydroxybenzotriazole (1 equiv) (7) in DMF, coupling time; DMF wash, 5x1 min: *3.* Washing – DMF, 5x1 min; dichloromethane, 3x1 min; 2-propanol, 3x1 min; dichloromethane; 4x1 min. The total coupling sequence comprised programmes 3, 1, 2 and then 3. This was followed, for double couplings, by programme 2 and then 3. The coupling reactions were monitored by the Kaiser ninhydrin test (8). At various stages the peptide resins were submitted for amino acid analysis on a Rank-Hilger Chromaspek after being hydrolysed for 24 hr at 110°C with 12 *N* HCl-propionic acid (1:1 v/v) or 6 *N* HCl containing two crystals of phenol.

High Pressure Liquid Chromatography Separations were carried out on an analytical scale in a Kontron (St. Albans, Herts., U.K.) system with detection at 280 and 254 nm and by radioimmunoassay at Imperial College and on a preparative scale on an LDC (Stone, Staffs., U.K.) system at 274 nm at Beecham. The elution conditions were: *A,* Reverse Phase, RP8 (25 cm x 0.45 cm, Perkin-Elmer, Beaconsfield, Bucks., U.K.) eluted at 1 ml/min with 35% acetonitrile-0.1 *M* phosphate buffer, pH 7: *B,* μBondapak-C_{18} (30 x 0.7 cm, Jones Chromatography, Llanbradach, Glam., U.K.) eluted isocratically at 3.5 ml/min with 2-propanol, 5% aqueous acetic acid (20:80 v/v) for 2 min followed by a 33 min linear gradient to 2-propanol, 5% aqueous acetic acid (40:60 v/v): *C,* Zorbax-C_8 (30 x 0.8 cm, packed at IC) eluted isocratically at 2 ml/min with acetonitrile, H_2O, TFA (22:78:0.1 v/v/v) for 5 min followed by a 20 min linear gradient to acetonitrile, H_2O, TFA (50:50:0.1 v/v/v): *D,* Lichroprep RP8 (25 x 1.6 cm, Roth Scientific, Farnborough, Hants., U.K.) eluted isocratically at 8 ml/min with 83% of acetonitrile, H_2O, TFA (22:78:0.1 v/v/v) and 17% of acetonitrile, H_2O, TFA (80:20:0.1 v/v/v): *E,* μBondapak-C_{18} (25 x 0.8 cm), Bennett Co., Berks., U.K.) eluted at 1 ml/min with a linear 30 min gradient from 1-propanol, 5% aqueous acetic acid (10:90 v/v) to 1-propanol, 5% aqueous acetic acid (40:60 v/v) (6).

Fast Atom Bombardment Mass Spectrometry Spectra were obtained on a ZAB-HF mass spectrometer at 8 kV accelerating voltage. The sample was loaded in 1–2 μl of 1-propanol, 5% aqueous acetic acid (20:80 v/v) into the glycerol matrix. The primary ionising beam was generated at 8–10 kV from xenon.

Biological Methods Insulin release from the perfused rat pancreas was measured by a conventional double-antibody radioimmunoassay as described (3). Measurement of CLIP (ACTH C-terminal) immuno-reactivity has also been described (1). The peptide was dissolved initially in 5% aqueous acetic acid and rapidly diluted into aliquots in Krebs-Ringer bicarbonate buffer, then stored at 4° until required.

RESULTS AND DISCUSSION

Synthesis of βCT Alkoxybenzyl resin (3g) was acylated for 5 hr with the symmetrical anhydride of Fmoc-phenylalanine (7.5 mM) in DMF (48 ml) containing N-methylmorpholine (7.5 mM) and *p*-dimethylaminopyridine (0.75 mM), washed (2 x programme 3) and dried. A test portion (62 mg), cleaved by reaction with 55% TFA in methylene chloride (v/v, 2.5 ml) for 3 hr gave essentially one peak at 268 nm due to Fmoc-phenylalanine on analytical HPLC with system *A*. This indicated that the Fmoc protecting group was stable to the basic catalysts used in the attachment reaction (9). The remaining Fmoc-Phe-resin (3.77 g) was benzoylated with benzoyl chloride (1.56 ml) in pyridine (1.08 ml) for 30 min at 0°C then 1 hr at room temperature, washed (programme 3) and dried to give 3.81 g. The loading of phenylalanine was 0.64 mM/g by amino acid analysis and 0.69 mM/g by total nitrogen analysis.

The Fmoc-Phe-resin (1.33 g, 0.851 mM Phe) was taken through eight coupling cycles to give the nonapeptide resin (1.91 g). Part (0.94 g) of this resin was further elongated to the tetradecapeptide resin (1.06 g). Four more coupling cycles with 0.54 g of the latter gave 0.59 g of the protected βCT resin. Amino acid analysis (uncorrected) gave Val 0.78, Tyr 0.95, Pro 2.17, Asx 1.94, Gly 0.99, Ala 2.93, Glu 4.09, Ser 0.85, Phe 2.24 and Leu 1.06. Most of the coupling reactions were complete within 90 min, but a double coupling was performed for the incorporation of tyrosine and proline in positions 2 and 3, respectively. Direct hydrolysis of the Fmoc-peptide resin with HCl-propionic acid gave satisfactory amino acid ratios at the tri- and hexapeptide stage. However, at the nona-peptide stage, when serine was the amino terminal residue, these conditions gave the abnormally low ratio of 0.32 for serine. This ratio was increased to 0.86 when the Fmoc-protection was removed (piperidine-DMF) prior to hydrolysis with 6 *N*

HCl in the presence of phenol. The latter procedure gave a value of 0.94 for serine at the dodecapeptide stage.

Cleavage of the βCT-resin (330 mg) with TFA (50 ml) containing anisole (0.5 ml) for 1 hr gave on evaporation a brown oil which produced a white solid on washing with ether. Lyophilisation from 10% aqueous acetic acid gave 75 mg of crude βCT.

Purification The crude βCT (39.58 mg, load volume 2 ml) was purified by HPLC on μBondapak-C_{18} with system *B* and eluted at 7.3 min as the only major peak. On examination of the purified βCT by FAB-MS, an intense quasimolecular $[M+H]^+$ ion was observed at m/z 1984, defining the nominal mass of the peptide as 1983 (Fig. 1: note the more intense ^{13}C isotope peak at this mass). Interestingly, a less intense ion at m/z 1966 was observed, corresponding to a dehydrated form of βCT. Chromatography of the above purified βCT on Zorbax-C_8 with system *C* produced two uv absorbing peaks. The major peak (80% of total at 280 nm) eluted first at 6.8 min and was shown to be βCT by FAB-MS (m/z 1984) and this was confirmed by amino acid analysis. The later eluting minor peak at 8.4 min gave on FAB-MS the quasimolecular ion at m/z 1966. The structure of the dehydration product has not been established but a possibility is ΔAla^{10}-βCT.

A convenient one-column preparative separation of crude βCT (32.3 mg, load volume 2 ml) was later achieved on Lichroprep RP8 with system *D*. βCT and the dehydrated form emerged at 11.8 min and 15.6 min, respectively.

Comparison of natural and synthetic βCT The purified synthetic βCT co-eluted on μBondapak-C_{18} with system *E* with both natural and tryptically derived βCT from CLIP. Following HPLC the synthetic and natural peptides possessed indistinguishable cross-reactivity with CLIP antisera.

In the rat pancreas perfusion system, synthetic βCT produced a monophasic release of insulin at 5.6 mM glucose (Fig. 2) which is consistent with that found for natural βCT (1). The total insulin release in this peak, expressed as a mean of seven perfusions, was not significantly different from those obtained using natural or tryptic βCT (Fig. 3). The dehydrated βCT product was also fully active.

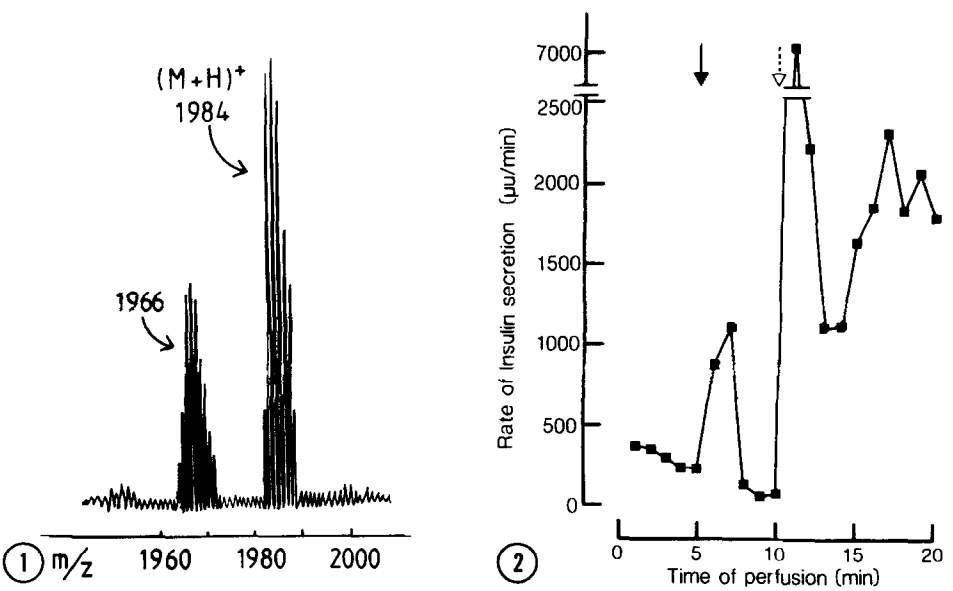

Figure 1: Molecular ion region of the Fast Atom Bombardment mass spectrum of synthetic βCT. An intense quasimolecular $[M+H]^+$ ion was observed at m/z 1984, defining the nominal molecular weight of the peptide as 1983. The ion at m/z 1966 arises from a dehydrated form of βCT, possibly [ΔAla^{10}] βCT, which was separated by high pressure liquid chromatography. Note the more intense ^{13}C isotope peaks at this mass.

Figure 2: Effect of synthetic βCT on insulin secretion: a typical stimulation. Solid arrow indicates point of introduction of buffer containing βCT (1.9 ng/ml). Broken arrow shows switch-over to high glucose (16.7 mM) from the normal (5.6 mM) glucose concentration.

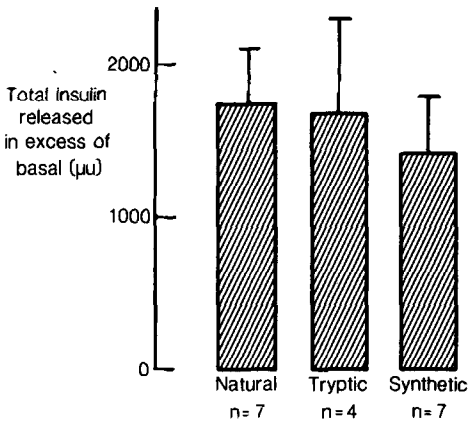

Figure 3: Effect of natural, tryptic (from CLIP) and synthetic βCT, at concentrations in the range 0.2 to 2.0 ng/ml, on insulin release from the perfused rat pancreas. Columns indicate mean total insulin release, in the monophasic stimulation, in excess of the basal secretion; SEM values are indicated by the bars.

These data show that the synthetic material is indistinguishable in its chromatographic, antigenic and biological properties from natural βCT.

With the availability of large quantities of synthetic βCT, work is now in progress to develop a more specific radioimmunoassay, by raising an N-terminal antiserum and to further define structure-function relationships.

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