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Research Article

Unordered structure of proinsulin C-peptide in aqueous solution and in the presence of lipid vesicles

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Abstract. Proinsulin C-peptide ameliorates renal and autonomic nerve function and increases skeletal muscle blood flow, oxygen uptake and glucose transport in patients with insulin-dependent diabetes mellitus. These effects have in part been ascribed to the stimulatory influence of C-peptide on Na^+,K^+-ATPase and endothelial nitric oxide synthase. To evaluate the capacity of C-peptide to insert into lipid bilayers and form ion channels, C-peptide secondary structure and membrane interactions were studied with circular dichroism spectroscopy and size exclusion chromatography. C-peptide is shown to lack a stable secondary structure, both when part of proinsulin and when free in aqueous solution, although the N-terminal third of the peptide exhibits an α-helical conformation in trifluoroethanol. Moreover, C-peptide remains disordered in the aqueous solvent in the presence of lipid vesicles, regardless of vesicle composition. In conclusion, C-peptide is unlikely to elicit physiological effects through stable conformation-dependent interactions with lipid membranes.

Key words. Diabetes mellitus; protein secondary structure; circular dichroism; peptide-lipid interactions; insulin.

Introduction

Proinsulin is cleaved by membrane-bound proteases into equimolar amounts of insulin and connecting peptide (C-peptide), which are eventually co-released into the circulation. The 31-residue C-peptide has long been considered to lack biological effects beyond the generation of correctly folded proinsulin. Renewed interest in C-peptide was prompted by the finding that C-peptide may prevent or retard the development of microvascular complications in insulin-dependent diabetes mellitus (IDDM) patients [1]. Thus, C-peptide has been found to improve renal function [2, 3], stimulate glucose transport in skeletal muscle [4, 5], increase skeletal muscle blood flow and oxygen uptake [4, 6], and improve autonomic and motor nerve function [7, 8] in IDDM patients and in animals with experimental diabetes.

The mechanism(s) behind these physiological effects are not fully known. Rat C-peptide 1 produces a dose-dependent stimulation of Na^+,K^+-ATPase activity in rat renal tubular cells, which can be inhibited by pertussis toxin [9]. Human C-peptide prevents decreased Na^+,K^+-ATPase activity in the sciatic nerve of rats with streptozotosin-induced diabetes [7]. C-peptide also increases release of nitric oxide (NO) from bovine aortic endothelial cells by stimulating endothelial NO synthase (eNOS) through a Ca^{2+}-dependent mechanism [10]. Finally, C-peptide stimulates Ca^{2+} influx into rat renal

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tubular cells and bovine aortic endothelial cells [9, 10]. From these data and results from studies of C-peptide fragment activities [11], it has been suggested that C-peptide activates a G-protein-coupled receptor with subsequent activation of Ca^{2+}-dependent intracellular pathways, resulting in stimulation of Na^+,K^+-ATPase and eNOS activities [12].

Binding of ^{125}I-labelled rat C-peptide 1 to rat islet tumour cells composed of insulin-secreting β-cells has been reported [13]. Specific binding of human C-peptide to various human cell types has recently been detected [14]. In another study, human C-peptide was found to compete for ^{125}I-proinsulin, but not for ^{125}I-insulin binding to IM-9 lymphoblasts [15]. However, an alternative mechanism of C-peptide action has been proposed [7]. A reverse sequence (retro) C-peptide and a C-peptide with D-enantiomer amino acids (all-D C-peptide) instead of the naturally occurring L-forms were observed to be almost as effective as native human C-peptide with regard to vascular effects in diabetic rats, whereas a peptide with the same amino acid composition as C-peptide but in random order (scrambled C-peptide) was inactive. Similar effects on Na^+,K^+-ATPase activity were observed with D-amino acid fragments of C-peptide [11]. These observations prompted the suggestion that C-peptide effects could be mediated by non-chiral mechanisms rather than by stereospecific receptors or binding sites [7, 11]. It was recently found that cation (K^+)-selective channels were formed in lipid bilayers after addition of C-peptide or retro-C-peptide, but not in the presence of scrambled C-peptide [16]. C-peptide may thus be able to insert into lipid bilayers and form cation-selective channels, thereby providing a mechanism by which C-peptide alters ion homeostasis in cells independently of binding to a specific receptor. To further examine possible interactions between C-peptide and lipid membranes, we have investigated the secondary structure and membrane-binding capacity of C-peptide.

Materials and methods

Preparation of lipid vesicles and micelles. Phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylserine (LPS) and cholesterol (Sigma) were dissolved in chloroform/methanol, 1:1 (v/v), and mixed in the mass proportions given in table 1. After solvent evaporation under nitrogen and reduced pressure, the mixtures were resuspended in 50 mM sodium phosphate (NaPi) buffer, pH 5 or pH 7, at 55 °C, under vigorous vortexing. Small unilamellar vesicles (approximately 60–100 nm diameter) were prepared from the vesicle mixtures by sonication at 55 °C for 5×20 s at 30 W, using an MSE 150 W ultrasonic disintegrator Mk2 sonifier (MSE Scientific Instruments) [17]. Micelles were formed from the lysophospholipid-containing mixtures by vortexing. Both vesicles and micelles were made at a total lipid concentration of 1 mg/ml.

Circular dichroism spectroscopy. Human C-peptide, rat C-peptide 2, human insulin and human proinsulin (Ferring and Sigma) were analysed. For evaluation of peptide secondary structure in different solvents, 10 µl peptide stock solution in water was mixed with 190 µl solvent. Solvents used were distilled water, plasma-like ion solution (113 mM NaCl, 24 mM $NaHCO_3$, 0.6 mM $MgCl_2$, 1.3 mM $CaCl_2$, 3.9 mM KCl, adjusted to pH 7.3 with 2 M HCl), 50 mM NaPi at pH 5 or 7, and 50–100% trifluoroethanol (TFE). For analysis of pep-

Table 1. Compositions of lipid vesicles and micelles used to study C-peptide/lipid interactions.

Mixture	Composition					
	PC	PG	PS	LPC	LPS	cholesterol
PC	100					
PC + 10% cholesterol	90					10
PG		100				
PG + 10% cholesterol		90				10
PS			100			
PS + 10% cholesterol			90			10
PC:PG 4:1	80	20				
PC:PG 4:1 + 10% cholesterol	72	18				10
PC:PS 4:1	80		20			
PC:PS 4:1 + 10% cholesterol	72		18			10
LPC:LPS 4:1				80	20	
LPC:LPS 4:1 + 10% cholesterol				72	18	10

Relative lipid concentration in mass% is given for each mixture. Mixtures containing lysophospholipids form micelles, the other mixtures form vesicles. All lipid mixtures with C-peptide added were analysed by circular dichroism spectroscopy at pH 5 and pH 7. PC:PS 4:1 + 10% cholesterol plus C-peptide, pH 5, was studied by gel filtration.

tide secondary structures in the presence of phospholipid vesicles and lysophospholipid micelles, 10 µl peptide stock solution in water was dissolved in 95 µl lipid solution and 95 µl distilled water. The final solvent composition was 0.5 mg/ml lipid and 25 mM NaPi.

Circular dichroism (CD) spectra between 184 and 260 nm were recorded at room temperature with a Jasco-720 Spectropolarimeter and with an AVIV CD Spectrometer 62 DS at a resolution of 1 data point/nm. Polypeptide concentrations were determined by amino acid analysis. The residual molar ellipticity ($[\theta]$) was expressed in kdeg × cm^2 × dmol^{-1}.

Nuclear magnetic resonance spectroscopy. Human C-peptide was dissolved in 95% TFE, 5% ^2H_2O at a peptide concentration of 1.83 mM, pH 3.6. Sequential assignments were obtained from 2D [^{1}H, ^{1}H]-TOCSY (τ_{mix} = 100 ms, data size 2048 × 1024, t_{1max} 85 ms, t_{2max} 170 ms) and [^{1}H, ^{1}H]-ROESY (τ_{mix} = 200 ms, data size 2048 × 1024, t_{1max} 85 ms, t_{2max} 170 ms) spectra recorded at 40 °C with a Bruker DMX 600 NMR spectrometer (600 MHz ^{1}H frequency). The proton chemical shifts are relative to tetramethylsilane. Secondary structure elements were assigned from the deviation of α-proton chemical shifts from random coil values [18]. This is based on the observation that the ^{1}H-nuclear magnetic resonance (NMR) chemical shifts of the α-protons of all amino acids in α-helical conformation experience upfield shifts of > 0.1 ppm with respect to random-coil values. Comparable downfield shifts are seen when the peptide is in β-strand conformation.

Size exclusion chromatography. Two hundred microlitres 40 µM human C-peptide and 0.4 mg/ml PC:PS 4:1 + 10% cholesterol, in 20 mM NaPi, pH 5, was incubated for 30 min at room temperature, and then separated according to size on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech). The sample was eluted with 20 mM NaPi, 100 mM NaCl, pH 7.0, at a flow of 0.5 ml/min, and 2-ml fractions were collected. As control experiments, 200 µl 40 µM human C-peptide, and 200 µl 0.4 mg/ml PC:PS 4:1 + 10% cholesterol, in 20 mM NaPi, pH 5, were analysed on the same column. Absorbance at 214 nm was measured for the fractions collected. Protein concentrations in relevant fractions were determined by amino acid analysis.

Amino acid analysis. Samples were hydrolysed for 24 h with 6 M HCl, 0.5% phenol in evacuated tubes at 110 °C. Amino acids were separated by ion-exchange chromatography and detected by ninhydrin.

Results

Unordered structure of the C-peptide part of proinsulin and of free C-peptide in aqueous solution. The conformation of the insulin part of proinsulin is similar to that of free insulin [19]. Consequently, the CD spectrum obtained by subtraction of the insulin spectrum from that of proinsulin reflects the conformation of the C-peptide part of proinsulin. The resulting CD spectrum shows a single minimum at about 197 nm and a broad maximum centred around 220 nm (fig. 1A). These features are typical of CD spectra of unstructured peptides [20]. The C-peptide part of proinsulin is therefore concluded to be largely unstructured, in agreement with an earlier study [21]. The CD spectra of free human C-peptide (fig. 1) and rat C-peptide 2 (data not shown) in water and plasma-like ion solution are similar to the subtraction spectrum, with a minimum below 200 nm as an outstanding feature. This shows that C-peptide is unstructured, both in aqueous solution and when part of proinsulin.

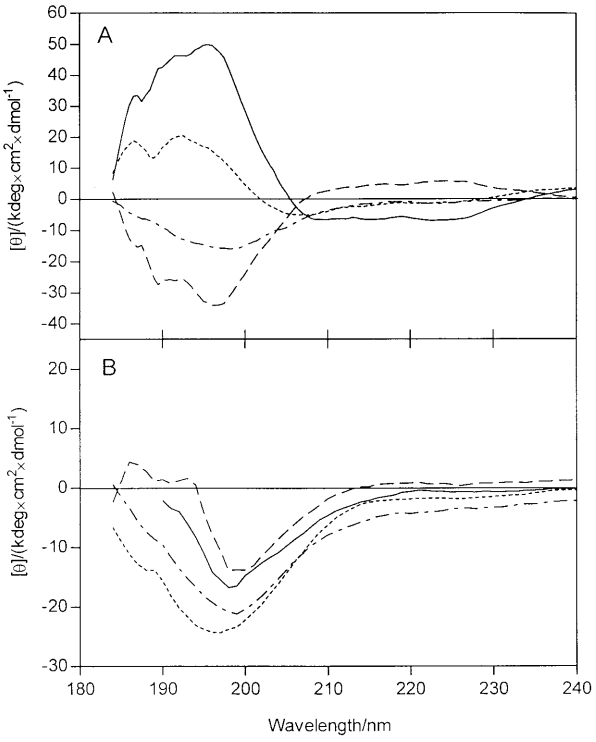

Figure 1. C-peptide secondary structure determination with CD spectroscopy. (*A*) C-peptide is unstructured in proinsulin and in aqueous solution. CD spectra were recorded for proinsulin (----), insulin (—), and free C-peptide (–·–·-) in 50 mM sodium phosphate buffer, pH 7.0. The difference spectrum (– – –) was obtained by subtraction of the insulin spectrum from the proinsulin spectrum. Polypeptide concentrations were 5 µM for proinsulin, 6 µM for insulin, and 34 µM for C-peptide. (*B*) C-peptide is unstructured in the presence of lipid vesicles. CD spectra of C-peptide in water (–·–·-), plasma-like ion solution (– – –), in the presence of lipid vesicles composed of PC:PS 4:1 and 10% cholesterol at pH 5 (—), or lipid micelles composed of LPC:LPS 4:1 and 10% cholesterol at pH 5 (----). Spectra nearly identical to those shown were obtained with all lipid mixtures in table 1 irrespective of pH (5 or 7). C-peptide concentration, 40 µM.

C-peptide secondary structure does not change in the presence of artificial membranes. The CD spectrum for human C-peptide is not significantly altered in the presence of any type of lipid vesicle or micelle (fig. 1B). Neither the shape of the curve nor the molar ellipticity at 222 nm ($[\theta]_{222}$), both of which are indicative for helical structure, are altered when compared to spectra in water and in plasma-like ion solution. Neutral and anionic vesicles and micelles, with or without cholesterol at pH 5 and pH 7, were tested (table 1).

C-peptide migrates separately from lipid vesicles upon gel filtration. C-peptide and lipid vesicles composed of PC:PS 4:1 and 10% cholesterol at pH 5 do not co-migrate upon size exclusion chromatography. From the chromatogram shown in figure 2, fractions 4–5 and 8–10 were pooled. Amino acid analysis of these samples revealed no detectable C-peptide in fractions 4–5. In contrast, all C-peptide loaded on the column was present in fractions 8–10. Control experiments with only peptide or vesicle solutions confirmed that C-peptide elutes in fractions 8–10 and vesicles in fractions 4–5.

C-peptide forms a partial α-helix in TFE. The only significant change in C-peptide secondary structure that could be observed by CD spectroscopy occurs at high concentrations of TFE (50–95% v/v). When the TFE content is increased, the CD spectra (fig. 3A) display a transition towards a maximum at about 192 nm and a double minimum at 208 and 222 nm. This change indicates a coil to helix transition. The secondary structure of C-peptide in TFE was also investigated with NMR spectroscopy. Analysis of C-peptide α-proton chemical shifts in 95% TFE showed that the first eleven residues form an α-helix, while residues 12–31 are in a flexibly disordered random-coil conformation (fig. 3B).

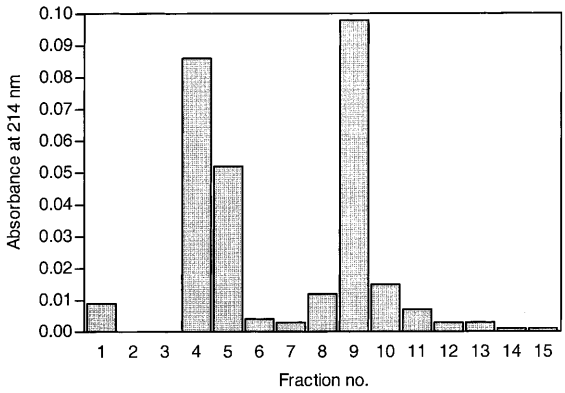

Figure 2. C-peptide and lipid vesicles do not co-migrate. Gel filtration of a mixture of C-peptide and lipid vesicles (PC:PS 4:1 + 10% cholesterol). C-peptide elutes in fractions 8–10, lipid vesicles in fractions 4 and 5.

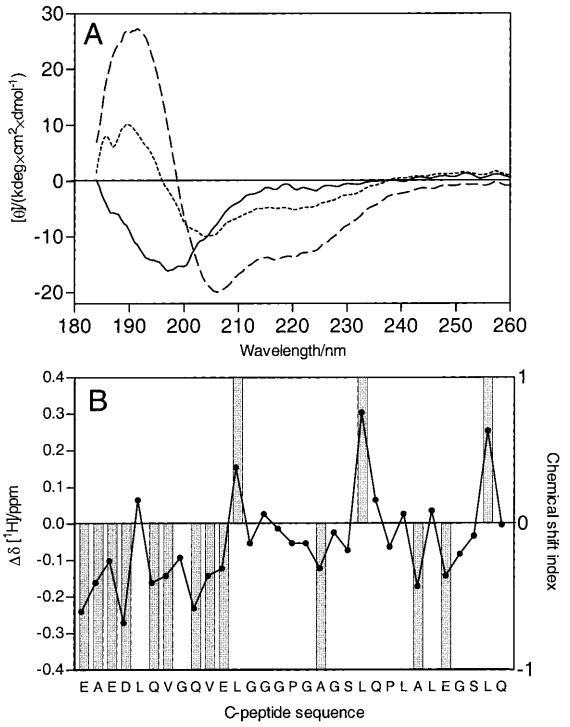

Figure 3. N-terminal α-helical secondary structure of C-peptide in TFE. (*A*) CD spectra of human C-peptide in water (—), in 50% TFE (----) and 95% TFE (– – –). The peptide concentration is 35 μM in all cases. (*B*) NMR analysis of C-peptide in TFE. Deviation of C-peptide α-proton chemical shifts in 95% TFE from random-coil values (solid line) and the corresponding indices (bars) [18]. At least four consecutive −1s not interrupted by a +1 correspond to helix formation, and C-peptide residues 1–11 thus form a helix in TFE.

Discussion

C-peptide/membrane interactions. C-peptide effects have been suggested to be mediated by non-chiral interactions [7, 11]. Moreover, in lipid bilayers composed of PC:PS 4:1 and 10% cholesterol at pH 5 and 7, cation (K^+)-selective channels were found after addition of C-peptide or retro C-peptide, but not in the presence of scrambled C-peptide [16]. These data suggest that C-peptide may be able to insert into lipid bilayers and form ion channels. Such interactions should be detectable by structural studies, as peptide secondary structure is expected to change upon transfer from a polar aqueous solution into a non-polar membrane [22].

The CD data obtained in this study show clearly that C-peptide exhibits a random coil structure in water and plasma-like ion solutions, and that the structure is not altered in the presence of lipid vesicles or micelles of different compositions (fig. 1). The lipid compositions of the vesicles and micelles used are similar to that of plasma membranes [23]. Furthermore, vesicles and micelles containing different ratios of acidic/neutral phospholipids and cholesterol were evaluated at different pHs. Micelles have been used extensively as in vitro models of biological membranes in studies of interactions between membranes and peptides, including peptide hormones [24]. Our observations thus contradict the suggestion that C-peptide can interact with membranes to form pores [7, 16]. The absence of C-peptide/membrane interactions obtains further support from the observation that C-peptide upon gel filtration does not co-elute with lipid vesicles (fig. 2). The lipids used for gel filtration were the same as those in the bilayers in which C-peptide was previously concluded to form cation channels [16].

Our data are in line with remarks that C-peptide does not possess the hallmarks of pore-forming peptides: it shows no tendency to self-associate and it is polar [25]. The possibility remains that C-peptide may interact with membranes or membrane proteins in a transient manner and modify their functional properties, but a stable physical association between C-peptide at physiological concentrations and membranes, mediating biological effects, is not favoured by these data.

C-peptide secondary structure. The mid-portion of human C-peptide was predicted to mediate biological activity [7, 11]. This part of C-peptide is highly similar in all mammalian species studied and contains a high proportion of non-polar amino acids flanking Pro16. From secondary structure predictions, molecular modelling, and CD spectroscopy, a conformation of C-peptide was previously suggested with N- and C-terminal helical segments and a non-polar turn-like structure in the middle of the peptide [7]. The present CD data (fig. 1) provide qualitative evidence against a stable C-peptide structure in aqueous solution. The appearances of the C-peptide spectra are typical for unstructured peptides [26] with a minimum below 200 nm, and show no features typical for helical structures, such as a maximum at 190 nm and minima at 208 and 222 nm. The reasons for the discrepancy between our data on C-peptide structure in solution and those previously presented [7] are not obvious. It should be pointed out, however, that the CD signal intensities shown earlier [7] are unexpectedly low, about 10% of the intensities usually detected [26] (figs 1, 3A).

The only significant change in C-peptide secondary structure compared to that in water which we could detect by CD spectroscopy occurred at high concentrations of TFE (50–95%) (fig. 3A). From NMR chemical shifts of human C-peptide α-protons in 95% TFE/5% ^2H_2O compared to random-coil values, the N-terminal 11 residues are concluded to adopt an α-helical conformation in this medium (fig. 3B). Leu5 does not give a negative chemical shift index and might represent a kink in the helix. In other species, Leu5 is replaced by Pro, which generally produces a kink when situated in a helix. The helical content derived by NMR correlates well with the CD data obtained, suggesting 35–40% helicity for C-peptide in 95% TFE. An α-helix for residues 1–11 is also predicted from the criteria of Chou and Fasman [27]. Peptide fragment α-helicity in TFE correlates with the secondary structure of the corresponding part of the parent protein [28–30]. This is, however, probably not the case with the C-peptide part of proinsulin, considering the CD data obtained (fig. 1).

The homogeneous environment of 95% TFE is not favourable for intermolecular peptide/solvent hydrogen bonds, and a partly helical C-peptide secondary structure with intra-peptide hydrogen bonds is induced. In contrast, in the anisotropic environment of phospholipid vesicles in aqueous solution, C-peptide does not form a stable secondary structure. In the latter case, an unordered peptide conformation due to hydrogen bonding with the aqueous solvent is favoured over insertion of C-peptide into lipid vesicles with concomitant intra-peptide hydrogen bonding. Physiological conditions that promote a partly helical state of C-peptide have not been identified.

C-peptide mechanism of action. The above findings indicate that physiological effects of C-peptide are unlikely to be mediated via non-chiral membrane interactions, although it is possible that C-peptide at supraphysiological concentrations might interact with membranes. Specific binding of C-peptide to cell membranes has recently been demonstrated with fluorescence correlation spectroscopy [14]; addition of excess concentrations of both all-D C-peptide or scrambled C-peptide fail to elicit competitive displacement. The latter data thus support previous observations [13, 31] which indicate that C-peptide effects are mediated through stereospecific membrane-bound receptors, rather than via non-chiral interactions between C-peptide and the cell membrane [7, 16].

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