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Research Article

Proinsulin C-peptide and its C-terminal pentapeptide: degradation in human serum and Schiff base formation with subsequent CO_2 incorporation

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Abstract. Processing of human proinsulin C-peptide and its C-terminal pentapeptide in blood serum was studied using reverse-phase HPLC and electrospray mass spectrometry. The results reveal degradation of both peptides, with a longer half-life for intact C-peptide than for the C-terminal pentapeptide. Products from C-peptide degradation were not distinguishable from the peptide background, suggesting endopeptidase degradation of C-peptide. In contrast, a set of products from the C-terminal pentapeptide were identifiable and corresponded to successive losses from the N terminus, showing that the pentapeptide is degraded by aminopeptidase in serum. Consistent with this finding, a slower degradation was found for the N-acetyl-protected pentapeptide. Removal of serum proteins by acetone precipitation produced N-terminally carbamate-modified C-peptide via a Schiff base intermediate (a ketimine with acetone), to which CO_2 was added and acetone removed, generating a cyclic side chain via anhydride formation. The modification was not seen with the pyroglutamate form of C-peptide, with the N-terminally acetylated C-peptide, or with a control peptide having N-terminal Phe, but was found with human C-peptide, its N-terminal tetrapeptide, and a rat C-peptide fragment (all with N-terminal Glu). Hence, the modification appears to require N-terminal Glu, but this is not the only prerequisite since the C-terminal pentapeptide and another control peptide (also starting with Glu) were not modified. A peptide aldimine Schiff base leading to CO_2 incorporation was detected with formaldehyde in $NaHCO_3$. The observation that C-peptide forms Schiff bases with ketones/aldehydes, enhancing covalent attachment of CO_2, may have biological implications.

Key words. Proinsulin; C-peptide; aminopeptidase; proteolytic processing; Schiff base; carbamate modification; CO_2 polypeptide incorporation; electrospray mass spectrometry.

The C-peptide of proinsulin is secreted as a 31-residue entity in amounts equimolar with those of insulin from the pancreatic β-cells of the islets of Langerhans [1]. It is now considered to be biologically active by binding to target cells, activating a G protein-coupled Ca^{2+}-signalling response [2, 3] and giving end effects on Na^+, K^+-ATPase [2, 4] and endothelial nitric oxide (NO) synthase (eNOS) [5–7] and on clinically observable parameters [8, 9]. The latter include improvements in renal and nerve functional variables and increased blood flow in type I diabetic patients [8]. C-peptide binding is displaceable with the C-terminal pentapeptide of C-peptide [10], and this pentapeptide also has the effect on the enzyme Na^+,K^+-ATPase [4] and on the Ca^{2+} rise [3].

With these recent findings regarding C-peptide, its processing and mode of degradation in serum are of interest,

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although major routes of elimination are via the kidneys and other organs. The in vivo half-life of C-peptide circulating in blood is about 30 min [cf. 11], compared to 4–5 min for insulin [12]. The relative stability of C-peptide versus its C-terminal pentapeptide is not known, nor the pattern of degradation of the two peptides. We therefore investigated C-peptide and pentapeptide degradations in the presence of serum. The proteolytic enzyme in the pentapeptide degradation is identified, as is a carbamate derivative at the C-peptide α-amino group. The modification appears specific since control peptides were not modified under the same conditions. Schiff bases are frequently encountered in metabolic turnover of molecules [13], and carbamate attachment to N-terminal α-amino groups is well known in haemoglobin [14], but now also shown in a stabilized form in C-peptide.

Materials and methods

Materials

Human blood was left to coagulate for 30 min at room temperature and centrifuged at 1800 g, after which the serum supernatant was recovered, aliquoted (2 ml) and stored frozen at −20 °C. Human C-peptide, pyroglutamate C-peptide, C-terminal pentapeptide and a 17-residue segment of the rat C-peptide sequence (from Sigma Genosys and PolyPeptides), the N-terminal tetrapeptide, and two unrelated control peptides, 23 and 10 residues in length, were synthesized by solid-phase technology [15] and purified to better than 95% with reverse-phase HPLC.

Acetylation

N-terminal acetylation (50 nmol peptide dissolved in 100 µl water) was carried out by addition of acetic anhydride (Sigma) in methanol (500 µl acetic anhydride/methanol, 1:3, by vol) and subsequent incubation at room temperature for 30 min. N-terminally acetylated peptides were purified using reverse-phase HPLC.

Desodiation

In efforts to remove sodium and potassium ions bound to the acidic groups of the peptides, preparations were agitated for approximately 30 s with 3 mg mixed-bed ion exchange resin beads (AG 50W-X8; Bio-Rad) that had been previously washed twice with water.

Analytical protocol

Human serum was diluted fivefold in water and placed in an Eppendorf tube (1.5 ml). The synthetic peptide (8 nmol) was added and incubation at 37 °C was started. Aliquots were withdrawn directly and after specific times up to 48 h. For samples from C-peptide incubations, acetone precipitation was carried through by addition of acetone to a final concentration of 70% (v/v), incubation at room temperature for 30 min and subsequent centrifugation at 10,000 rpm for 10 min. The supernatant was recovered, acetone was evaporated under a stream of nitrogen, and 0.1% TFA was added to yield a pH of about 2. After filtration (0.2-µm membrane filter; NanoSep, Pall Gelman Laboratory), the sample was submitted to reverse-phase HPLC on Vydac C_4 (4.6 × 250 mm, 5-µm particles, flow 1 ml/min) with a linear gradient of acetonitrile from 0 to 60% in 0.1% TFA during 60 min. Fractions were collected manually in Eppendorf tubes (1.5 ml), dried completely under a stream of nitrogen and dissolved in acetonitrile/water (1:1, by vol) for direct mass spectrometry. When necessary, HPLC fractions were cleaned using ZipTips (C_{18}; Millipore). The fractions were applied in 0.1% TFA, washed with the same solvent and eluted in 60% acetonitrile containing 1% acetic acid for electrospray mass spectrometry. For the pentapeptide time aliquots, the sample workup excluded the acetone precipitation step.

Nano-electrospray mass spectra were recorded using a quadrupole time-of-flight tandem mass spectrometer, Q-TOF (Micromass), equipped with an orthogonal sampling electrospray ionization (ESI)-interface (Z-spray; Micromass), and metal-coated nano-ESI needles (Protana) which gave a spraying orifice of about 5 µm and a flow of approximately 20–50 nL/min at a capillary voltage of 0.8–1.2 kV. For the acquisition of collision-induced dissociation (CID) spectra, the collision energy was optimized in the range 30–80 eV with argon as collision gas.

Results and discussion

Protocol

Using the protocol described above, human C-peptide and its C-terminal pentapeptide were tested for stability upon incubation with human serum. In general, direct HPLC to detect peptides at low concentrations (like those physiological for C-peptide) and in particular small peptides like the C-terminal pentapeptide is complicated by a background of peptides and other components in serum. We therefore tested different protocols and found that detecting the pentapeptide directly is feasible, provided the samples are pretreated with acid (TFA) and filtered at 0.2 µm. Even then, some artefacts remain in the subsequent HPLC step, but the pentapeptide is directly visible at low nanomole amounts. For the C-peptide, an acetone precipitation step was necessary to spot the peptide peak in the chromatogram. At 70% acetone, a substantial protein precipitate was removed, including serum polypeptides interfering with the subsequent reverse-phase HPLC step. Tests showed that the C-peptide did not co-precipitate using 70% acetone.

To further decrease artefacts, diluting the serum 1 to 5 before mixing with the test peptides was suitable. However, for determination of the half-life of C-peptide only, a 1:1 dilution was employed because of the slow degradation rate of C-peptide at a 1:5 dilution. Using these precautions, even minor degradation products of the pentapeptide were possible to analyse as shown. We also tested further extensions to our protocol, including the use of capillary electrophoresis [16], but did not achieve routine results better than those with the protocol now chosen. However, with maximal sample volumes and pretreatments, we are at a detection level in serum less than two orders of magnitude above the physiological concentration (C-peptide at 64 nM detectable using capillary electrophoresis and absorbance at 200 nm). The present protocol with reverse-phase HPLC is suitable for estimation of peptide stabilities and degradation patterns. Intact C-peptide then elutes at 37 min and the C-terminal pentapeptide at 16 min, both free from interference with serum components.

Degradation in serum

Degradations of C-peptide and the pentapeptide at 37 °C in serum (diluted 1:1 and 1:5 with water, respectively) were monitored via HPLC analysis of aliquots (fig. 1) from a 16 µM starting concentration. The parent peaks decreased with time, but with a much lower rate for C-peptide than for the pentapeptide. To achieve measurable data with C-peptide, it was studied at a higher serum concentration (1:1 dilution) than was the pentapeptide (1:5 dilution). In addition, the C-peptide, when exposed to serum followed by acetone precipitation of proteins, appeared as a double peak in the chromatogram at about a 1:1 peak height relationship (fig. 2) indicating a modification of the peptide structure (identified below). Monitoring of the degradations by plotting the peak heights versus time (for C-peptide, the sum of the two peak heights) revealed straight-line relationships, with a half-life of 174 h for the C-peptide and 5.8 h for the pentapeptide under these conditions. Hence, C-peptide is significantly more stable in serum incubations than the pentapeptide. Similar incubations with N-terminally acetylated pentapeptide resulted in a half-life of 24.4 h (20% serum), showing that protection of the N terminus slows down the degradation, prolonging the half-life by close to 80%.

Upon long-time serum incubation of C-peptide, no further peptides other than the intact C-peptide and its modified product identified below were detectable in the supernatant after acetone precipitation of the serum proteins. With time, the two C-peptide peaks only decreased in size, reflecting elimination of C-peptide. The lack of additional products in the C-peptide size range suggests that C-peptide elimination upon incubation with serum generates products of a different size (hence not recovered in the C-peptide fraction), and therefore largely derived from endopeptidase rather than exopeptidase cleavages. In contrast, the C-terminal pentapeptide revealed a clear degradation pattern, with successive formation of peaks eluting earlier in the HPLC separation (fig. 1). The products were identified by mass spectrometry which

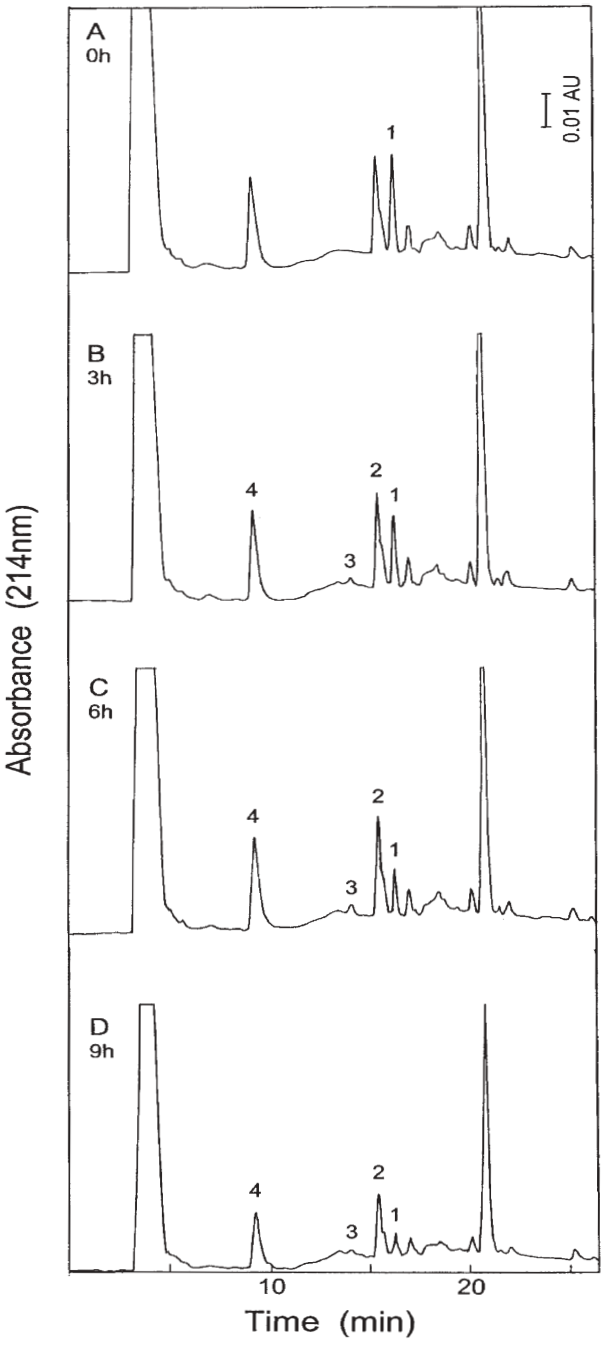


Figure 1. Reverse-phase HPLC of aliquots from incubation of the C-terminal pentapeptide in serum at 37 °C. Detection at 214 nm. Mass spectrometry revealed the following identities: peak 1 (EGSLQ), peak 2 (GSLQ), peak 3 (SLQ), and peak 4 (LQ together with free Q, plus serum components). (*A*) Directly after mixing serum and pentapeptide; (*B*) After 3 h; (*C*) After 6 h; (*D*) After 9 h.

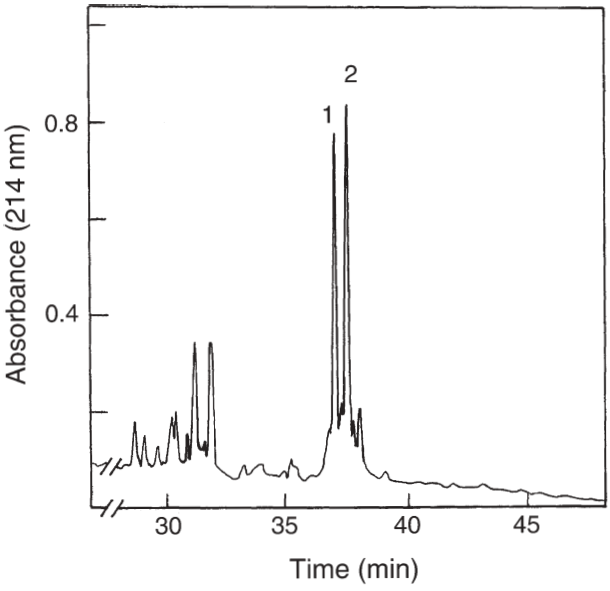


Figure 2. Reverse-phase HPLC directly after mixing serum and C-peptide followed by acetone precipitation of proteins. Peak 1 corresponds to intact C-peptide while peak 2 corresponds to C-peptide N-terminally modified by a carbamate derivative at the α-amino group (see text).

showed that each of the successively earlier peak fractions corresponded to degradation by removal of a residue from the N terminus of the subsequent fraction (fig. 3). Hence, one may conclude that the C-terminal pentapeptide is degraded by aminopeptidases in serum. This is visible both in the fragment pattern upon degradation and in the partial protection against degradation by N-terminal acetylation before the serum treatment.

C-peptide chemical modification

As mentioned above, C-peptide upon incubation with serum, followed by protein precipitation in 70% acetone, gives rise to a second peak (in about equimolar amount to the remaining intact C-peptide). This new component elutes slightly after the intact peptide (fig. 2, peak 2). Electrospray mass spectrometry showed the new component to contain the original C-peptide mass (M, 3018 Da) plus 26 and 44 Da, while the M+26 compound could not be detected in the fraction corresponding to peak 1 (fig. 4 A, B). The mass increase M+26 is not obtained from intact C-peptide (fig. 4 A) and further analyses (below) suggested the incorporation of a carbamate group at the N terminus of the C-peptide when exposed to serum and acetone, followed by dehydration and anhydride formation between the carbamate group and the side chain carboxyl group of the N-terminal Glu residue, and finally rearrangement of this structure to a more stable five-membered cyclic derivative. The other mass increase, at M+44, was interpreted to correspond partly to a sodium adduct (two Na^+ bound to the highly acidic peptide structure) and partly to a fraction of the non-cyclic carbamate derivative, not dehydrated. The strategy to find the evidence supporting these conclusions was as follows below.

The early peak from C-peptide incubation, eluting at 37 min (fig. 2, peak 1), corresponds to the intact C-peptide (M and Na^+/K^+ adducts to M detected). The second peak (fig. 2, peak 2) corresponds to a structurally modified peptide (M+26 and Na^+/K^+ adducts to M detected). A mass increase of 26 Da corresponds theoretically to a carboxylation/carbamate incorporation (M+44) followed

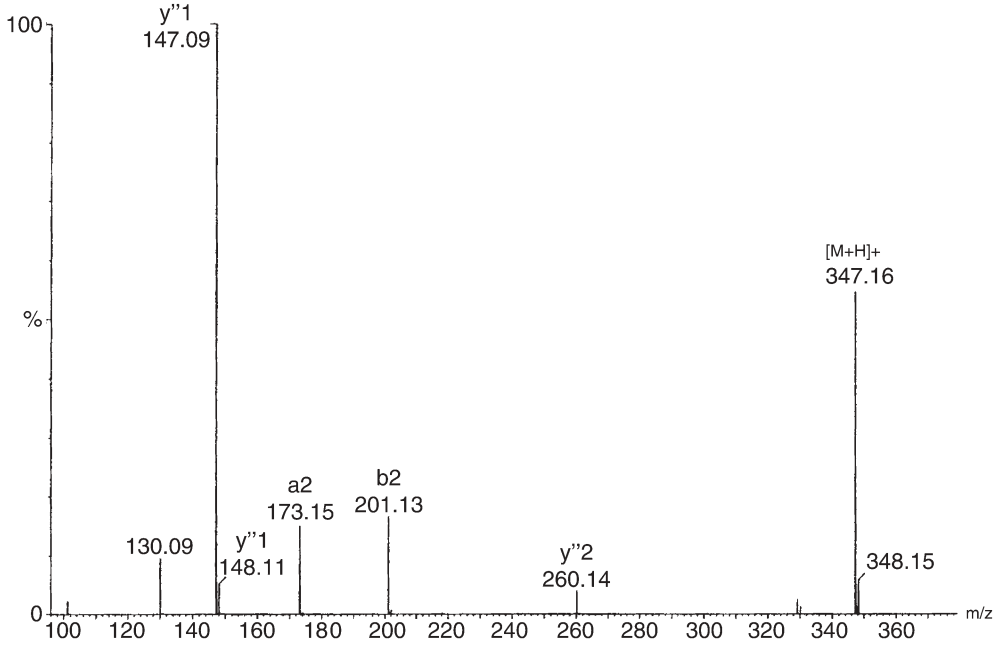


Figure 3. Product ion spectrum of the protonated SLQ tripeptide (peak 3, fig. 1).

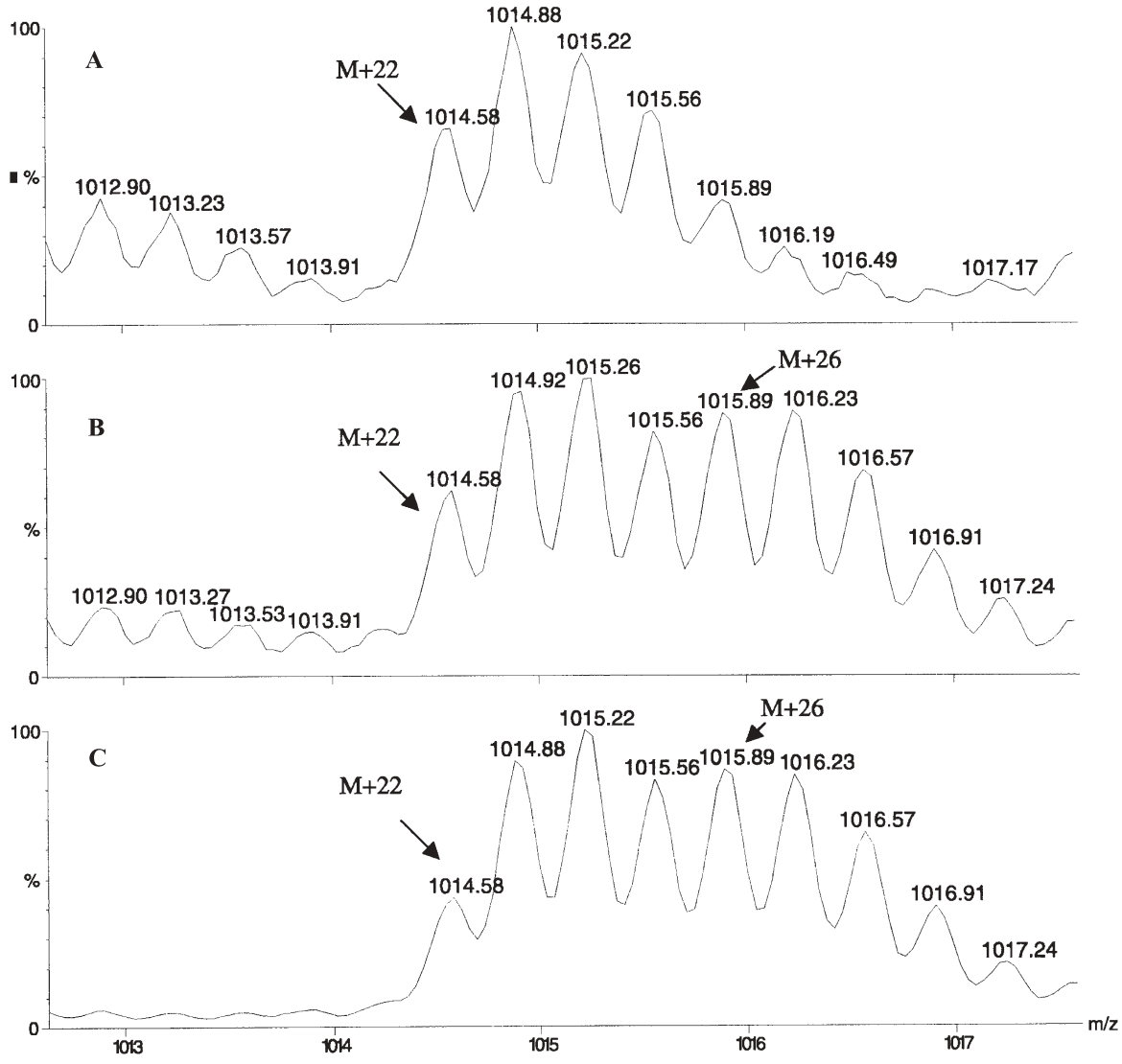
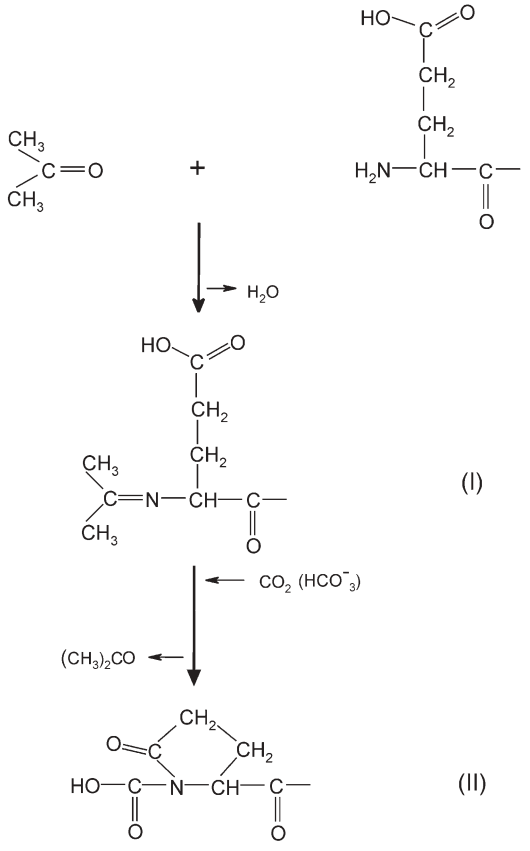


Figure 4. Sections of mass spectra after incubation of C-peptide in serum showing the component corresponding to peak 1 in the HPLC separation (fig. 2) with a triply charged ion corresponding to the C-peptide plus one sodium ion (M+22) only (*A*), the component corresponding to peak 2 in the HPLC separation (fig. 2) with triply charged ions corresponding to the C-peptide plus one sodium ion (M+22) and the C-peptide plus 26 Da (M+26) (*B*) and C-peptide incubated in 0.1 M $NaHCO_3$ instead of serum with both M+22 and M+26 signals visible as in *B* (*C*).

by a loss of water (44 – 18 = 26), the latter either naturally or an artefact produced in the electrospray ionization process. The dehydrated derivative is stabilized via a rearrangement reaction generating a five-membered ring structure (scheme 1). The carbon source in serum for such a modification is likely to be HCO_3^- ions or dissolved CO_2. To test this hypothesis, the C-peptide was incubated in 0.1 M $NaHCO_3$/70% acetone. The HPLC chromatogram again showed two peaks that upon mass spectrometry revealed the same result as for C-peptide incubated in serum (fig. 4C). In the mass spectra, sodium and potassium adducts were also visible.

To exclude the possibility that adduct formation with alkali metal salts was responsible for the dual-peak pattern in reverse-phase chromatography, the C-peptide was incubated in acetone (70%) with 28 mM NaCl or with 1 mM KCl (corresponding to physiological levels diluted fivefold), but no serum. In both these cases, only one peak was detected in the resulting chromatogram. Nevertheless, the many salt adducts present in the mass spectra complicated interpretations and the samples were therefore treated with a mixed-bed ion-exchange resin before analysis, which removed or diminished most of the adducts. Detection of the M+26 signal was thereby much enhanced compared to the situation without ion-exchanger when M+26 suffered from severe interference with the signal from the M+22 sodium adduct. To localize the M+26 modification in the C-peptide structure,



Scheme 1. Proposed reaction outline for the formation of the cyclic carbamate derivative at the N terminus of the C-peptide. Components are: (I) a Schiff base with acetone, (II) the final product, a cyclic derivative resulting from incorporation of CO_2, removal of acetone, and dehydration, followed by rearrangement to form a stable five-membered ring structure. The structures are based on theoretical considerations and on the masses detected in electrospray mass spectrometry.

tandem mass spectrometry was carried out. The CID spectrum revealed a series of b-ions that account for the intact amino acid sequence without modifications up to the third residue from the N terminus. The N-terminal dipeptide sequence Glu-Ala- is detected as one fragment in the CID spectrum having an extra mass of 26 Da added (200+26). This indicates that the modification is located at the N-terminal Glu (since the penultimate residue Ala is inert, not likely to be modified). To further dissect the location, the C-peptide was N-terminally acetylated and then incubated in both serum and 0.1 M $NaHCO_3$ with 70% acetone, followed by application to reverse-phase HPLC. The chromatogram revealed in both these cases only one peak, indicating that the modification was not introduced when the N terminus was acetyl-blocked, and hence resides at the α-amino group, not at the side chain carboxyl group of Glu-1. This result assigns the modification as a carbamate derivative at the α-amino group secondarily forming a cyclic derivative with the Glu side chain carboxyl group at the expense of a water molecule (scheme 1).

We also studied synthetic peptides in 0.1 M $NaHCO_3$/70% acetone to further evaluate the introduction of the carbamate group (table 1). The N-terminal tetrapeptide (EAED), like the whole C-peptide, revealed two peaks in the chromatography after incubation in $NaHCO_3$ and acetone (table 1), showing that the carbamate modification still occurs in this shorter peptide. Electrospray mass spectrometry showed that the first peak contained only M (462 Da) while the later eluting peak contained both M+26 and M+40. The latter mass could be clearly detected since the degree of sodiation for the tetrapeptide is much lower than for the intact 31-residue parent molecule. M+26 is again interpreted as the carbamate cyclic derivative resulting from incorporation of CO_2, and M+40 as the ketimine (Schiff base) structure resulting from nucleophilic attack on the carbonyl carbon of acetone by the α-amino group nitrogen of the tetrapeptide (scheme 1), a reaction that is well known [17]. Since both these products are present, the modification is thought to proceed over the incorporation of CO_2 by nucleophilic addition to the ketimine nitrogen (scheme 1). Dehydration follows and a cyclic carbamate derivative is formed via reaction with the glutamic acid side chain. This structure is rearranged to yield a stable final product (II, scheme 1). When the tetrapeptide was tested with formaldehyde instead of acetone, two chromatographic

Table 1. Synthetic peptides incubated in 0.1 M $NaHCO_3$/70% acetone followed by reverse-phase HPLC.

Peptide	Susceptible to carbamate modification
EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ	yes
EAED	yes
EGSLQ	no
Pyr-EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ	no
Ac-EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ	no
ELGGGPGAGDLQTLALE	yes
EPSHWKHVEL	no
FTPQAGKARVAKHPEGNFALKND	no

The five top peptides correspond to or are derived from the human C-peptide structure, the sixth corresponds to a rat C-peptide part, and the bottom two peptides are unrelated control peptides.

peaks were again detected and electrospray mass spectrometry of the material corresponding to the later peak contained the aldimine (M+12), in addition to M+26, the cyclic carbamate derivative. Tests of additional peptides showed that a segment of the rat C-peptide starting ELGGG (table 1) revealed two chromatographic peaks in reverse-phase HPLC. However, the C-terminal pentapeptide, which also contains an N-terminal Glu residue (EGSLQ), resulted in only one peak and is therefore not sensitive to the modification. Similarly, in tests of two control peptides, a 10-residue peptide with Glu at the N terminus and a 23-residue peptide with Phe at the N terminus (table 1), only one chromatographic peak and no carbamate formation was detected. This was also the case for the pyroglutamate form of the C-peptide (table 1) when it was tested in 0.1 M $NaHCO_3$/70% acetone. Combined, these observations show that the modification is not random but provides some specificity for the N-terminal carbamate incorporation. Apparently, a Glu residue is favourable but not sufficient for modification. Potentially, primary as well as higher-level structural features can be involved in the specificity [18], and the involvement of a Schiff base intermediate is well known in the metabolism of ketone/aldehyde substrates [13].

In conclusion, the results give a protocol for direct analysis of small peptides in serum, establish the major pentapeptide-degrading enzyme in serum to be an aminopeptidase, show a substantially larger stability in serum incubations of C-peptide than of its bioactive C-terminal pentapeptide fragment, and identify an incorporation of carbamate into the C-peptide via Schiff base formation at the N-terminal Glu residue.

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