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Insulin proteinase liberates from glucagon a fragment known to have enhanced activity against $Ca^{2+} + Mg^{2+}$-dependent ATPase

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We find, contrary to previous reports, that substantial cleavage of glucagon by insulin proteinase occurs at only one region, namely the double-basic sequence -Arg^{17}-Arg^{18}-. Cleavage takes place almost exclusively between these two residues, liberating fragments glucagon-(1–17) and glucagon-(18–29). Others have shown that the fragment glucagon-(19–29) is 1000-fold more efficient compared with intact glucagon, at inhibiting the Ca^{2+}-activated and Mg^{2+}-dependent ATPase activity and the Ca^{2+} pump of liver plasma membranes. We show that this fragment is not liberated in detectable quantities by our insulin proteinase preparation. On the other hand, others have shown that glucagon-(18–29), though less active than glucagon-(19–29), was still 100-fold more active than glucagon itself in the above-mentioned system. Our observations represent the first demonstration of the release by insulin proteinase of a hormone fragment having enhanced activity, although it has yet to be shown that the activity of this fragment is important *in vivo*. Since the formation of glucagon-(19–29) from glucagon-(18–29) would involve merely removal of Arg^{18}, a second enzyme might exist to provide the more active fragment.

INTRODUCTION

The term 'insulin proteinase' describes an enzymic activity that cleaves certain biologically important polypeptides (e.g. insulin, glucagon) but not others (e.g. vasoactive intestinal polypeptide) [1]. Apart from an apparent difference in M_r, insulin proteinase appears to be functionally the same enzyme as neutral thiopeptidase [2]. Support for a role of insulin proteinase in the cellular processing of insulin has been obtained ([3], and references cited therein).

There is much interest in identifying fragments of insulin and glucagon that may have physiological roles, and testing them for possible biological activity (see, e.g., [2,4–6]). The degradation of insulin *in vitro* by insulin proteinase produces many fragments, most of which have been identified by the application of h.p.l.c. and f.a.b.-m.s. [5–7]. Identification of the degradation products of insulin is complicated to some extent by the two-chain nature of the substrate, and insulins labelled with stable isotopes were used to assist peptide identification. The single-chain nature of glucagon simplifies fragment analysis by f.a.b.-m.s., and we present below the identification of the major fragments produced by its degradation by insulin proteinase. The possible significance of these results is discussed.

MATERIALS AND METHODS

All reagents and solvents were of analytical grade or better unless otherwise stated.

The preparation of insulin proteinase, separation of fragments by h.p.l.c. and analysis by f.a.b.-m.s. were as previously described [7]. Glucagon (crystallized; Eli Lilly and Co., Indianapolis, IN, U.S.A.) dissolved at 82 nM in 50 mM-Tris/HCl buffer, pH 7.5, was incubated with a preparation of insulin proteinase at 37 °C for 10 min. The quantity of the preparation of insulin proteinase added (0.066 ml/ml of glucagon solution) was determined from preliminary experiments to be sufficient to give about 50% digestion of glucagon as determined by reversed-phase h.p.l.c. The reaction was stopped by acidification to about pH 4 with pure acetic acid and the products were isolated by concentration on a C_{18} Sep-Pak (Waters Associates) followed by h.p.l.c., as previously described for insulin [7] except that a 4 mm-internal-diam. × 250 mm Nucleosil 30 nm-pore-size 5 μm-particle-size C_8 column (Machery–Nagel, Düren, W. Germany) was used for the separation and a shallower gradient [generally 0.5% (v/v) acetonitrile/min] was used. Appropriate controls (glucagon without enzyme, and enzyme without glucagon) were treated similarly. A very shallow gradient [3% (v/v) acetonitrile/h] was used in one experiment in order to separate completely three closely eluted components.

Reduction of methionine sulphoxide to the thioether with *N*-methylmercaptoacetamide (purum grade; Fluka, Buchs, Switzerland) was performed according to the literature procedure [8]. The reduced samples were analysed directly by reversed-phase h.p.l.c., with monitoring at 280 nm.

Hydrolysis was performed with 6 M-HCl at 112 °C for 24 h and hydrolysates were analysed on a Beckman 6300 automatic amino acid analyser.

A simple computer program was written to calculate the exact masses of all possible fragments of glucagon of mass up to that of the intact chain. The resulting table was interrogated by computer to provide all possible sequences corresponding to a given mass.

Abbreviation used: f.a.b.-m.s., fast-atom-bombardment mass spectrometry.

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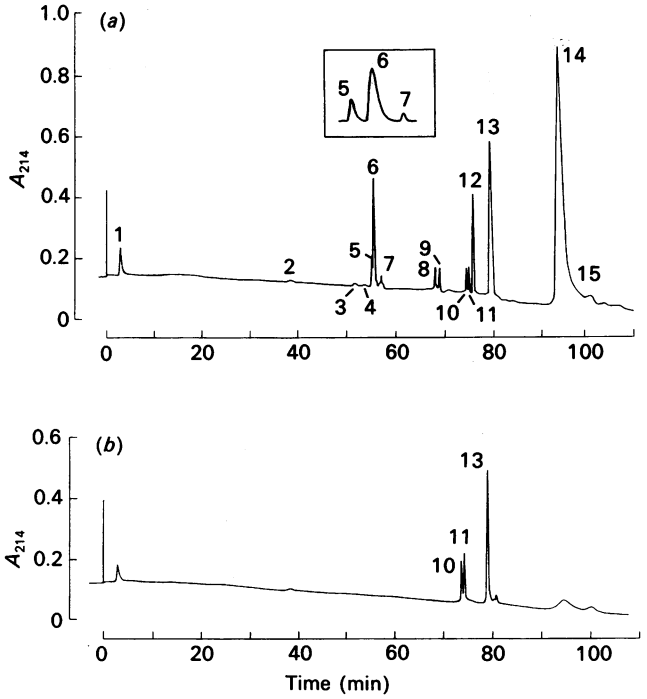

Fig. 1. H.p.l.c. profile of the degradation of glucagon by insulin proteinase

(*a*) H.p.l.c. profile of the degradation of glucagon by insulin proteinase. Conditions of the incubation, work-up and chromatography were as described in the Materials and methods section. The inset shows the complete resolution of peaks 5, 6 and 7 obtained under shallow gradient conditions. (*b*) H.p.l.c. profile of glucagon after incubation in digestion medium without enzyme. Chromatography conditions were the same as for (*a*).

RESULTS AND DISCUSSION

Separation of the degradation products by h.p.l.c.

Fig. 1(*a*) shows the elution profile of the degradation of glucagon by insulin proteinase. For the purposes of amino acid analysis, the products of a similar degradation experiment were separated by using a gradient of 3% acetonitrile/h in order to obtain complete separation of peaks 5, 6 and 7 (Fig. 1*a* inset). Control experiments showed that only peaks 3–13 are derived from glucagon, and of these peaks 3 and 4 were not considered large enough to merit further analysis. The broad peaks (14 and 15) are always associated with the enzyme preparation whether or not substrate is present and give no interpretable mass spectrum ([7]; note that a different column was used in this earlier work). Peak 14 corresponds in shape and retention time to bovine serum albumin, which was used to rinse the sample containers [7]. Fig. 1(*b*) shows the elution profile of a glucagon blank incubation (no enzyme present). In addition to residual traces of peaks 14 and 15, we note the presence of three sharp peaks, 10, 11 and 13, which are discussed below.

Identification of components of Fig. 1(*a*)

Peaks 5 and 6 were not fully separated under the steeper gradient conditions of Fig. 1(*a*) used to collect fragments for m.s. analysis, but this does not affect the conclusions. Fig. 2(*a*) shows the mass spectrum obtained with material containing both peaks 5 and 6. In all mass spectra shown, measured mass values (given in Table 1) have been rounded down, for plotting purposes, to the nearest integer, which corresponds to the nominal mass value for fragments of less than 2000 atomic mass units. At masses approaching 2000 atomic mass units the ^{13}C isotope peak is of similar intensity to that of the monoisotopic species and may, for ion-statistical reasons, exceed the latter in intensity (see spectra and discussion in ref. [7]). In Fig. 2(*a*) signals are found around m/z 1989–1994. Computer-assisted interrogation of the table of masses of all possible protonated glucagon fragments showed (Table 1) only two fragments close in mass, residues 9–24 (1990.96) and residues 1–17 (1991.907). In view of the relative intensities found in the range m/z 1989–1994, we interpret the signals as being due to residues 1–17 (1991.84 found, plotted at m/z 1991, with a slightly more intense isotope peak plotted at 1992). This interpretation is supported by results of amino acid analysis of peak 6 (Table 2) and our finding evidence for the existence of the complementary fragment containing residues 18–29 (see below). Fig. 2(*a*) also shows signals in the range 1834–1837. Reference to Table 1 again shows two possibilities, this time residues 1–16 and 6–20. In view of the relative intensities found in the range m/z 1834–1837, we interpret the signals as being due to residues 1–16 (1835.78 found, plotted at m/z 1835, with a slightly more intense isotope peak plotted at 1836). This interpretation is supported by the results of amino acid analysis (Table 2, peak 5) and by our finding evidence for the existence of the complementary fragment containing residues 17–29 (see below).

Peak 7, a minor product of the degradation (Fig. 1*a*), was identified as glucagon-(4–17) by f.a.b.-m.s. In the positive-ion mass spectrum shown in Fig. 2(*b*) the signal at m/z 1639 is interpreted as being due to the protonated molecular ion of the peptide containing residues 4–17 of glucagon (1639.76 in Table 1): the fragment next closest in mass would appear at m/z 1637.79, and so can be excluded. This finding was confirmed amino acid analysis (Table 2).

Peaks 8 and 9, also minor products of the degradation (Fig. 1*a*), gave the positive-ion mass spectra shown in Figs. 2(*c*) and 2(*d*) respectively. Both peaks give rise to a signal at m/z 1524. From Table 1 it might be supposed that residues 3–15 were responsible for the signal, but this is not so. Peaks 8 and 9 correspond to oxidized forms of peak 12 (fragment 18–29); just in the same way, as is shown below, peaks 10 and 11 (Figs. 1*a* and 1*b*) correspond to oxidized forms of glucagon (native glucagon is peak 13). The signal at m/z 1524 corresponds to m/z 1508 (peak 12, residues 18–29; see below) plus one oxygen atom. Indeed, a signal at m/z 1508 is evident in Figs. 2(*c*) and 2(*d*), and most probably arose by partial reduction of the oxidized fragment by the thioglycerol matrix. The most likely explanation is that the extra oxygen atom is attached to the thioether side chain of the Met^{27} residue. Indeed, treatment of peaks 8 and 9 under conditions (see the Materials and methods section) known to reduce methionine sulphoxide residues back to methionine, followed by analysis by h.p.l.c., led to the complete disappearance of the respective peaks and the appearance of a peak with the retention time of peak 12. In view of the fact that peaks 8 and 9 are formed in approximately equal amounts, are reducible with *N*-methylmercaptoacetamide and contain a single extra

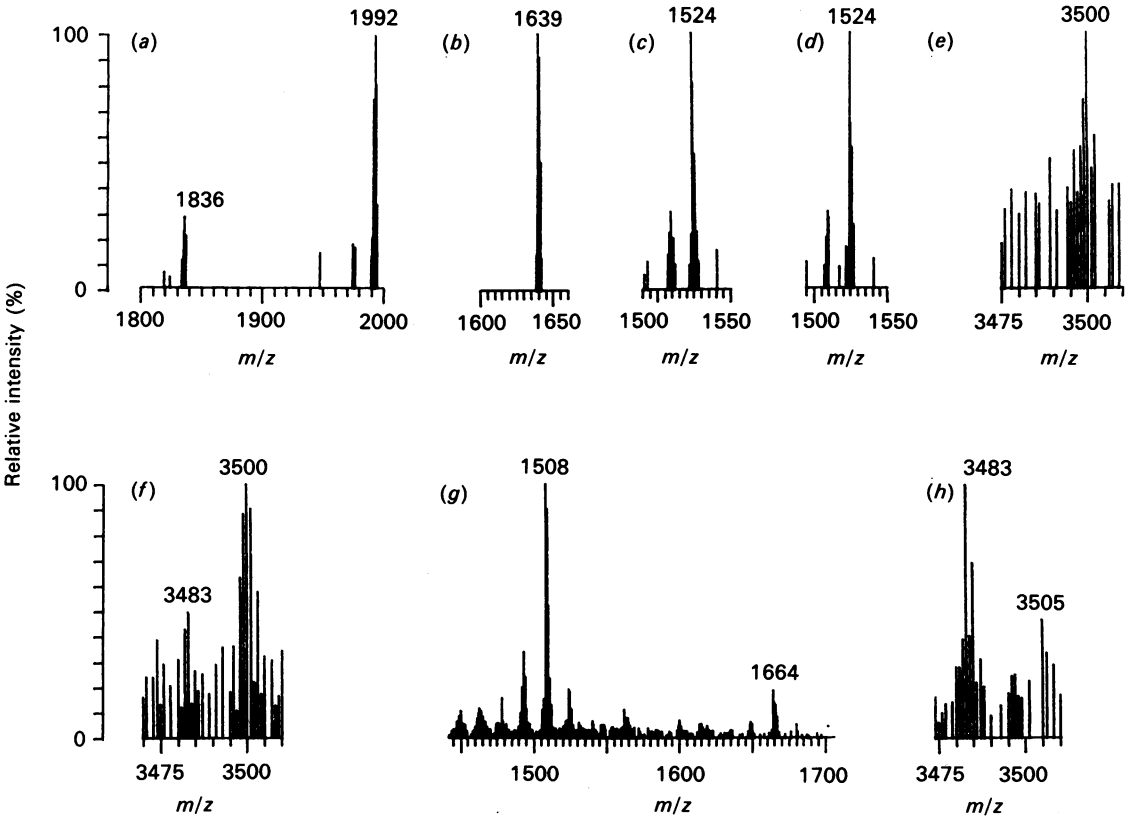

Fig. 2. Positive-ion mass spectra of fractions isolated by h.p.l.c. (Fig. 1)

(*a*) Mixture containing fractions 5 and 6; (*b*) fraction 7; (*c*) fraction 8; (*d*) fraction 9; (*e*) fraction 10 isolated from an incubation of glucagon without enzyme (Fig. 1*b*); (*f*) fraction 11 isolated from an incubation of glucagon without enzyme (Fig. 1*b*); (*g*) fraction 12; (*h*) fraction 13.

oxygen atom, we deduce that these earlier-eluted forms are diastereoisomeric pairs (the sulphur atom of methionine sulphoxide is a chiral centre). This interpretation is supported by the finding, in the f.a.b. mass spectra of peaks 8 and 9, of signals corresponding to small amounts of reduced species (reduction being due to thioglycerol in the f.a.b. matrix).

Peaks 10 and 11 (Fig. 1*a*) were also found in the glucagon blank (Fig. 1*b*). They were reduced quantitatively with *N*-methylmercaptoacetamide to a compound with the same retention time as unmodified glucagon. M.s. of peaks 10 and 11 isolated from a blank incubation of glucagon showed incorporation of a single oxygen atom (Figs. 2*e* and 2*f* respectively). These peaks are due to the formation of methionine sulphoxide analogues of glucagon and separation of the resulting diastereoisomeric pair. A glucagon standard made up in 0.1% trifluoroacetic acid at 1 mg/ml and analysed at once by reversed-phase h.p.l.c. showed only trace amounts of peaks 10 and 11 (results not shown). The resolution of diastereoisomeric sulphoxides is known for the cases of small molecules (amino acids), and the resolution of polypeptide pairs containing a single amino acid residue partially racemized at the α-carbon atom is also known. To our knowledge, the present report is the first published example of the resolution by reversed-phase h.p.l.c. of a diastereoisomeric pair of methionine sulphoxides of a large polypeptide (29 residues). Workers at Novo Industri, Bagsvaerd, Denmark, have previously achieved the separation of oxidized glucagon diastereoisomers but have not published their observations (J. Markussen, personal communication).

Peak 12 (Fig. 1*a*) gave the positive-ion mass spectrum shown in Fig. 2(*g*). The major signal appears at m/z 1508, which can only correspond (Table 1) to the protonated molecular ion of a fragment containing residues 18–29 (1508.66 found, 1508.73 theoretical). This assignment is confirmed by results of amino acid analysis (Table 2), although there appears to be some contamination with glycine and serine. The signal at m/z 1508 is associated with less intense signals 15 and 16 mass units lower in mass, a normal feature of f.a.b. mass spectra (cf. Fig. 2*a*). In addition, we note the presence of a signal at m/z 1524. This signal most probably is due to a small degree of oxidation of methionine to the sulphoxide during work-up of this fragment (see the discussion of peaks 8 and 9 above). Another signal present in Fig. 2(*g*) is at m/z 1664. Table 1 shows that this can only be due to the protonated molecular ion of a fragment containing residues 17–29 (1664.831 theoretical; 1664.69 found). We would expect to find glucagon-(17–29) in the digest as it is the fragment complementary to glucagon-(1–16) discussed above. The signal at m/z 1664 is much less intense than that at m/z 1508, and the presence in peak 12 of glucagon-(17–29) has not increased the arginine value obtained on amino acid analysis of this peak (Table 2). For these reasons, and from the relative areas of the h.p.l.c. peaks corresponding to the com-

Table 1. Assignments of m/z values for fragments of glucagon

The Table shows consecutive entries in a table of calculated masses of protonated molecular ions of all possible fragments of glucagon together with the corresponding residue numbers. Experimental m/z values, and corresponding fraction number, have been aligned. When isolated for the purpose of mass spectrometry, h.p.l.c. fraction 6 contained peaks 5 and 6 (see steeper gradient run in Fig. 1*a*, and the text). The calculated m/z values are for monoisotopic (all-^{12}C) species; for intact glucagon, the most abundant masses are calculated to appear at about m/z 3482.6 and 3483.6, with a calculated average mass of 3483.81 [9].

Calculated m/z	Residues	m/z found	H.p.l.c. fraction
1501.739	9–20, 10–21		
1508.730	18–29	1508.66	12
1520.663	1–13		
1520.760	15–26		
1524.683	3–15	1524.53	8,*9*
1536.773	16–27		
1637.790	6–18		
1639.758	4–17	1639.46	7
1648.807	10–22		
1651.800	15–27		
1664.831	17–29	1664.69	12
1683.822	13–25		
1811.917	12–25		
1835.806	1–16	1835.78	6
1836.886	6–20		
1850.866	8–22		
1979.974	14–29		
1990.960	9–24		
1991.907	1–17	1991.84	6
1994.955	3–19		
3380.572	1–28		
3481.619	1–29	3483	13

* These fractions were shown to derive from methionine sulphoxide formation of fragment 18–29 (see the text).

Table 2. Results of amino acid analysis of fragments of glucagon

The values given below have been corrected neither for destruction of serine, threonine, tyrosine or methionine, nor for background (serine, glycine and glutamic acid/glutamine being most prominent). The quantities analysed ranged from about 172 pmol (peak 7; manual integration of Phe was necessary at this low level) to about 2040 pmol (peak 12). For the purpose of amino acid analysis, peaks 5, 6 and 7 were separated completely (see Fig. 1*a* inset and the text). In parentheses are the theoretical ratios for glucagon-(1–16) (peak 5), -(1–17) (peak 6), -(4–17) (peak 7) and -(18–29) (peak 12). Abbreviation: N.D., not determined by amino acid analysis. The presence of tryptophan was confirmed by f.a.b.-m.s. of unhydrolysed peptide.

	Amino acid composition (mol of residue/mol)			
Residue	Peak 5	Peak 6	Peak 7	Peak 12
Asx	1.89 (2)	1.92 (2)	2.06 (2)	2.21 (2)
Thr	1.67 (2)	1.75 (2)	1.86 (2)	1.21 (1)
Ser	3.04 (4)	3.15 (4)	2.58 (3)	0.69 (0)
Glx	1.11 (1)	1.06 (1)	0.44 (0)	1.89 (2)
Gly	1.41 (1)	1.05 (1)	1.80 (1)	0.32 (0)
Ala				0.89 (1)
Val				0.91 (1)
Met				0.84 (1)
Leu	1.04 (1)	0.99 (1)	1.02 (1)	1.06 (1)
Tyr	1.78 (2)	1.87 (2)	1.58 (2)	
Phe	1.00 (1)	1.06 (1)	1 (1)	1.13 (1)
His	0.89 (1)	0.89 (1)		
Lys	0.91 (1)	0.93 (1)	0.95 (1)	
Arg		0.84 (1)	0.88 (1)	0.98 (1)
Trp				N.D. (1)

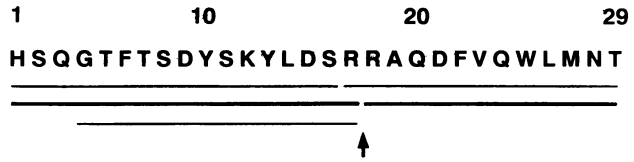

Fig. 3. Fragments of glucagon identified by f.a.b.-m.s.

The sequence of glucagon is shown. Fragments identified by f.a.b.-m.s. are represented by lines spanning the corresponding parts of the sequence, and the major cleavage site is marked with an arrow. Cleavage between Gln^3 and Gly^4 is quantitatively much less important than cleavage between Ser^{16} and Arg^{17} and between Arg^{17} and Arg^{18}.

plementary fragments (peaks 5 and 6), we conclude that cleavage of the Ser^{16}–Arg^{17} bond is much less prominent than that of the Arg^{17}–Arg^{18} bond.

Peak 13 (Fig. 1*a*) had the retention time of undigested glucagon, and produced a mass spectrum (Fig. 2*h*) consistent with this interpretation (Table 1).

Fragments found

Fig. 3 shows the cleavage identified. The major site of cleavage is that between residues Arg^{17} and Arg^{18}, and a minor site between residues Ser^{16} and Arg^{17}. In addition, cleavage between Gln^3 and Gly^4 must occur to some small extent, since we identified a very small quantity of fragment 4–17. The property of insulin proteinase to cleave both sides of particular residues has been previously noticed with insulin as substrate [6,7], but the physiological consequences (if any) of this feature have yet to be elucidated.. We have demonstrated [6,7] cleavage sites of insulin by insulin proteinase between Ser^{B9} and His^{B10}, His^{B10} and Leu^{B11}, Leu^{A13} and Tyr^{A14}, Tyr^{A14} and Gln^{A15}, Phe^{B24} and Phe^{B25}, Phe^{B25} and Tyr^{B26}, Glu^{B13} and Ala^{B14}, and Asn^{B3} and Gln^{B4}. The primary structures of insulin and glucagon offer no obvious reason for the observed specificity of insulin proteinase.

Duckworth *et al.* [10], using a fluorescence assay, deduced that about five peptide bonds were cleaved during the digestion of glucagon with highly purified insulin proteinase. The digestion was carried out to completion in 50 mM-phosphate buffer, pH 7.5 at 37 °C, and the glucagon concentration was 20 μM. Baskin *et al.* [11] went on to characterize partially the degradation products, suggesting cleavages between Ser^{11} and Lys^{12}, Tyr^{13} and Leu^{14}, Arg^{18} and Ala^{19}, Trp^{25} and Leu^{26}, either Thr^5 and Phe^6 or Asp^{21} and Phe^{22}, and either Asp^9 and

Tyr^{10} or Lys^{12} and Tyr^{13}. Since we do not find evidence to support any of these cleavages, it is a little difficult to compare them with those that we find. The digestion conditions used by Baskin *et al.* [11] were 10 mM-phosphate buffer, pH 7.5, a glucagon concentration of 41.4 μM, at 37 °C for 60 min. As stated above, our digestions took place in 50 mM-Tris buffer, pH 7.5, at 37 °C for 10 min and the glucagon concentration was 82 nM. Possible differences in purity of glucagon substrate and of enzyme preparation do not seem likely to provide an explanation of the differences between the degradation patterns that we observe and those that they reported. Insofar as glucagon purity is concerned, Baskin *et al.* [11] used a preparation from the same supplier as our own (Eli Lilly and Co.). They do not specify whether it was the crystallized grade which we used, but it would be expected to have been substantially pure. It is unlikely that differences in purity between our enzyme preparation and that of Baskin *et al.* [11] can explain the differences in degradation patterns. It has been shown (W. C. Duckworth, personal communication) that the pattern of degradation of four specifically labelled monoiodo-insulin isomers (iodinated at A^{14}, A^{19}, B^{16} and B^{26}) is unchanged for a wide range of enzyme purities, a range that includes both our preparation and that of Baskin *et al.* [11].

The most likely explanation for the differences in degradation patterns would seem to lie in the difference in substrate concentration (a factor of about 250–500-fold). The very high concentrations of glucagon used in previous studies [10,11], which are close to the limit of solubility of the hormone at pH 7.5, may have caused aggregation of the substrate and so affected the results.

Dog and rat hepatocytes have been reported ([12], and references cited therein) to convert [[^{125}I]iodoTyr^{10}]-glucagon into a peptide lacking the first three *N*-terminal residues of the hormone. Those authors attribute the formation of this fragment to the action of amino-peptidases. We have shown that insulin proteinase cleaves the Gln^3–Gly^4 bond of glucagon, but the fragment that we isolate is also cleaved at Arg^{17}.

Mallat *et al.* [4] obtained fragments of glucagon containing residues 18–29 and 19–29 by digestion of glucagon with trypsin. They report that these fragments inhibit both the Ca^{2+}-activated and Mg^{2+}-dependent ATPase activity and Ca^{2+} transport in liver plasma membranes with an efficiency 100- and 1000-fold higher respectively relative to intact glucagon. A standard of fragment 19–29 (prepared by digestion of glucagon with trypsin and characterized by f.a.b.-m.s. [13]) was found by co-injection to be eluted 2 min after peak 12 and well before peak 13, so there is no possibility that significant amounts of fragment 19–29 were present in the degradation mixture (Fig. 1*a*).

CONCLUSION

Our observations represent the first demonstration of the release by insulin proteinase of a hormone fragment having enhanced activity, although it has yet to be shown that the activity of this fragment is important *in vivo*. Nor can it be said if the fragment lies on the physiological pathway of activation proposed by Mallat *et al.* [4]. If, in the future, these two questions were to be answered in the affirmative, a second enzyme would be required, in addition to insulin proteinase, for the complete activation. The formation of glucagon-(19–29) from glucagon-(18–29) would involve merely removal of Arg^{18}.

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