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Identification of radioactive insulin fragments liberated by insulin proteinase during the degradation of semisynthetic [[^{3}H]Gly^{A1}]insulin and [[^{3}H]Phe^{B1}]insulin

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(1) We [Muir, Offord & Davies (1986) Biochem. J. 237, 631–637 and Davies, Muir & Offord (1986) Biochem. J. 240, 609–612] have previously identified a major product in the degradation of insulin by insulin proteinase (the *N*-terminal fragment produced by cleavage between residues Leu^{A13} and Tyr^{A14}, Ser^{B9} and His^{B10}) together with evidence for a minor cleavage site between His^{B10} and Leu^{B11} or between Leu^{B11} and Val^{B12}. (2) We now present evidence for minor sites of cleavage between Tyr^{A14} and Gln^{A15}, Glu^{B13} and Ala^{B14} as well as His^{B10} and Leu^{B11}.

INTRODUCTION

The mechanism whereby insulin is degraded by its target tissues and the relationship of this process to the physiological actions of insulin remain undetermined. Several studies have suggested that proteolytic cleavage of the insulin molecule occurs in isolated hepatocytes, and some of the degradation products have been partially characterized (Assoian & Tager, 1982; Misbin & Almira, 1984; Juul *et al.*, 1986). One of these products resembles the major fragment found after degradation of insulin *in vitro* by the enzyme insulin proteinase (Muir *et al.*, 1986; Davies *et al.*, 1986; Varandani & Schroyer, 1987).

The purpose of the present study was to identify other products of this reaction, and in order to do so we have employed h.p.l.c. as the initial separation method. Characterization of the products thus detected was then carried out by paper electrophoresis and h.p.l.c. Further information was obtained by following the behaviour of the products in these separating systems after chemical modification. The methodology was developed in such a way as to be applicable in future not only to the identification of fragments liberated by purified insulin proteinase, but also to the fragments forming part of the complex biochemical mixtures to be expected from experiments *in vivo*.

MATERIALS AND METHODS

All reagents were analytical grade or better unless otherwise stated.

^{3}H-labelled insulins were prepared as described previously (Halban & Offord, 1975; Halban *et al.*, 1976; Davies & Offord, 1985). Insulin proteinase was prepared from rat skeletal muscle (Duckworth *et al.*, 1972) up to the second $(NH_4)_2SO_4$ precipitation. Degradation of ^{3}H-labelled insulins by insulin proteinase was carried out as described by Muir *et al.* (1986) except that the insulin concentration was 40 nM and the bovine serum albumin concentration was reduced to 0.01%. Degradation was carried out in plastic tubes which had previously been equilibrated with 0.35% bovine serum albumin/0.05 M-Tris/HCl, pH 7.5.

H.p.l.c. and paper electrophoresis were as described by Muir *et al.* (1986) and Davies *et al.* (1986), except that in some cases radioactivity was detected in the h.p.l.c. effluent by a B-cord flow detector (LKB). Gradients for individual separations are given in the figure legends.

Authentic standards of A and B chain peptides were prepared as described by Muir *et al.* (1986) and Davies *et al.* (1986) or by the following methods. (Gly^{A1}–Glu^{A17})-peptide was prepared by the limited cleavage of the oxidized A chain by *Staphylococcus aureus* V8 protease. (Phe^{B1}–Leu^{B11})-peptide was prepared by pepsin treatment of the oxidized B chain. (Phe^{B1}–His^{B10})-peptide was prepared by carboxypeptidase A cleavage of (Phe^{B1}–Leu^{B11})-peptide. (Phe^{B1}–Glu^{B13})-peptide was prepared by limited cleavage of the oxidized B chain with *Staphylococcus aureus* protease. (Phe^{B1}–Leu^{B15})-peptide was prepared by cleavage of the oxidized B chain with subtilisin Carlsberg and this peptide was treated with carboxypeptidase A to form (Phe^{B1}–Ala^{B14})-peptide. (Phe^{B1}–Tyr^{B16})-peptide was prepared by treatment of the oxidized B chain with chymotrypsin. Peptides were purified by reversed-phase h.p.l.c. and characterized by electrophoresis and by amino-acid analysis, except in the case of carboxypeptidase A reactions where the release of the *C*-terminal amino acid was followed by paper electrophoresis.

RESULTS AND DISCUSSION

The elution profile on h.p.l.c. of the result of the treatment of both [[^{3}H]Gly^{A1}]insulin and [[^{3}H]Phe^{B1}]-insulin with insulin proteinase is shown in Fig. 1. With the exception of one radioactive peak in each profile (labelled G1 and F1), these profiles are very similar. This suggests that the same or very similar fragments are released in each case and that these fragments contain the *N*-terminal regions of both the A- and B-chains. The observed peaks are absent in the original insulins and in control digests in which ^{3}H-labelled insulins were

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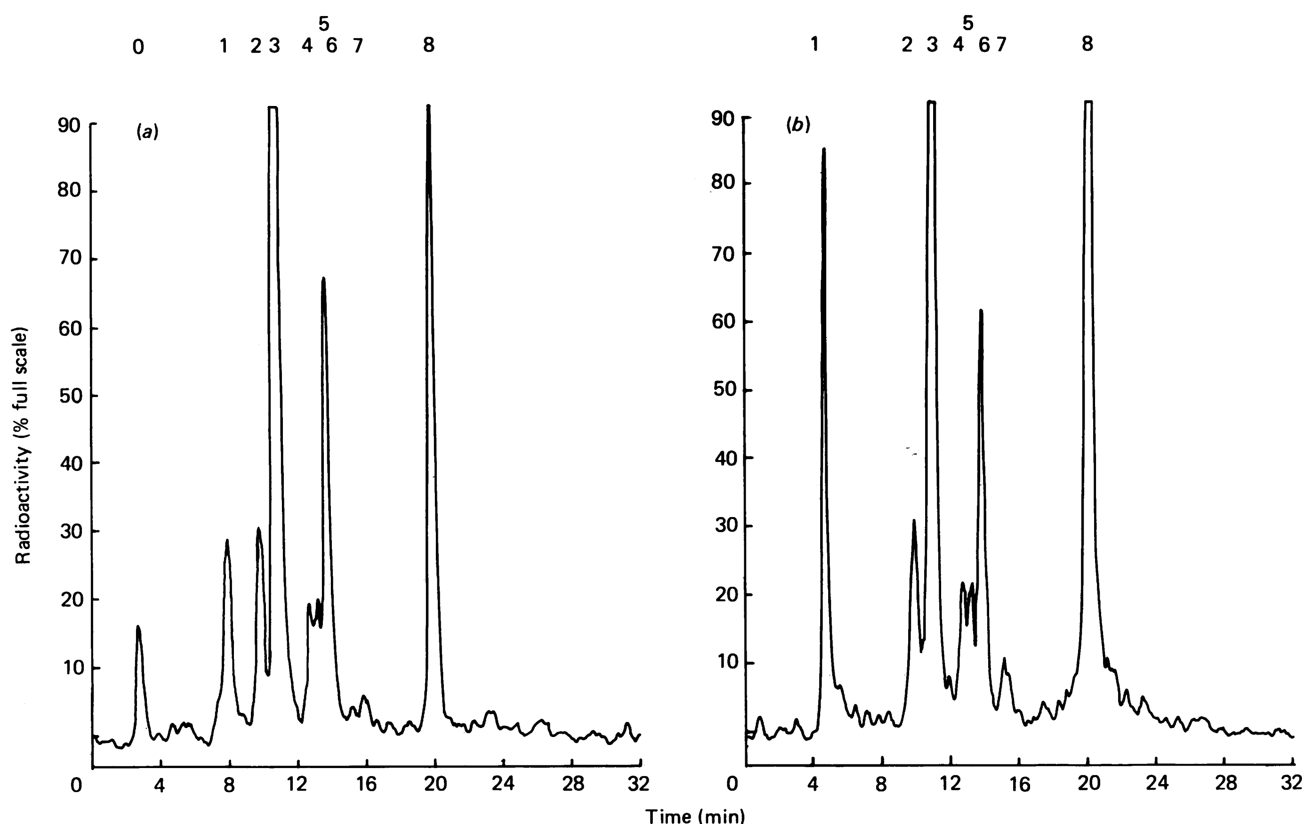

Fig. 1. Elution profile on reversed-phase h.p.l.c. of the products of the reaction between ^{3}H-labelled insulin and insulin proteinase

(*a*) ('F' fragments), [[^{3}H]Phe^{B1}]insulin; (*b*) ('G' fragments), [[^{3}H]Gly^{A1}]insulin. Peaks, numbered as shown in the Figure, were collected and analysed further (see the text). Peak 8 in each profile corresponds to the elution position of intact insulin. An acetonitrile gradient was run at 1 ml/min from 20% to 30% over 15 min and then to 35% over 5 min.

incubated in the presence of enzyme inhibited by the presence of *N*-ethylmaleimide (results not shown). Although *N*-ethylmaleimide is a known inhibitor of insulin proteinase, it might also be acting by suppressing disulphide interchange reactions. However, in none of our studies on the structures of the cystine-containing fragments has there been the slightest sign of the products of disulphide exchange. When the inhibitor was present, peaks of ^{3}H were observed eluting after the insulin peak, which were not present in the original insulin and which control incubations showed were due to reaction of *N*-ethylmaleimide with insulin under the incubation conditions. This does not affect the digests with uninhibited enzyme as *N*-ethylmaleimide was not added in this case. Fig. 1 shows typical elution profiles. Fractions, numbered as indicated in Fig. 1, were pooled, desalted on Sep-Pak cartridges, and lyophilized for electrophoresis.

Table 1 shows that, after performic-acid oxidation, the radioactive peptides corresponding to the A-chain-labelled fragments all have *m* values between -0.33 and -0.40. Reference to the graph of predicted *m* values for performate-oxidized A1-labelled fragments of insulin (Fig. 6 of Muir *et al.*, 1986) shows that these mobilities can only relate to a limited number of peptides (see Table 1). Thus, having eliminated by this means a large number of possible structures, we proceeded to choose among those remaining by comparing the h.p.l.c. retention times of each oxidized fragment with those of authentic standards (Table 2). The effect of possible day-to-day variations in retention times was eliminated by mixing the appropriate authentic standards with the radioactive fragments before the run.

An authentic sample of (Gly^{A1}–Leu^{A16})-peptide proved difficult to obtain, but its retention time would presumably equal or exceed that of (Gly^{A1}–Glu^{A17})-peptide given that the glutamic-acid residue that distinguishes these two peptides is hydrophilic. Table 2, therefore, shows that it is not reasonable to suppose that any of the radioactive fragments correspond to (Gly^{A1}–Leu^{A16})-peptide. A similar argument, based on the observed retention time of (Gly^{A1}–Ser^{A12})-peptide, can be used to dismiss (Gly^{A1}–Cys^{A11})-peptide as a possible structure for any of the A-chain fragments. Ser^{A12}, which distinguishes these two peptides, would have to balance the effect of removal of Leu^{A13} on the retention time of (Gly^{A1}–Leu^{A13})-peptide in order for any of the radioactive peptides to elute with (Gly^{A1}–Cys^{A11})-peptide. The change in retention time observed on removal of Ser^{B9} from (Phe^{B1}–Ser^{B9})-peptide suggests this is unlikely. We therefore conclude that the structure proposed for the various A-chain labelled fragments are those that appear in column 2 of Table 2. For fraction G8 the possibility that Asn^{A21} has been removed cannot be ruled out, as the resulting (Gly^{A1}–Cys^{A20})-peptide does not separate from the oxidized A chain in the systems used. Fraction G3 (cleavage at Leu^{A13}) had already been identified as the major A-chain component that retains residue Gly^{A1} (Muir *et al.*, 1986; Davies *et al.*, 1986). The less abundant

Table 1. Electrophoretic mobilities in a strong acid system of A-chain-labelled peptides

The electrophoretic mobilities (m) of the fractions isolated by reversed-phase h.p.l.c. were measured on paper in water/formic acid (7:3, v/v) Only G1 fails to correspond when unoxidized to a fragment in the digest of B1-labelled insulin. The possible structures of the oxidized labelled fragments are deduced from Fig. 6 of Muir *et al.* (1986).

	m		
Fragment	Non-oxidized	Oxidized	Possible structures of oxidized fragments
G1	0.5/0.65	−0.38	Any peptide from A1–A11 to A1–A14 inclusive, A1–A20, A1–A21
G2	0.58	−0.38	Any peptide from A1–A11 to A1–A14 inclusive, A1–A20, A1–A21
G3	0.45	−0.36	Any peptide from A1–A11 to A1–A15 inclusive, A1–10, A1–A21
G4	0.53	−0.4	Any peptide from A1–A11 to A1–A13 inclusive, A1–A20, A1–A21
G5	0.53	−0.37	Any peptide from A1–A11 to A1–A14 inclusive, A1–A20, A1–A21
G6	0.47	−0.33	Any peptide from A1–A13 to A1–A17 inclusive, A1–A20, A1–A21
G7	0.46	−0.35	Any peptide from A1–A12 to A1–A15 inclusive, A1–A20, A1–A21
G8	0.56	−0.37	Any peptide from A1–A11 to A1–A14 inclusive, A1–A20, A1–A21

fragments resulting from cleavage at Tyr^{A14} have not been previously reported. It is now possible to see these two cleavages represented in several fragments, each having a B-chain portion also susceptible to structural analysis (see below).

Tables 3 and 4 present the analytical data for the Phe^{B1}-labelled fragments. Of those fractions that give the same m value before and after performate oxidation, only F1 has no counterpart in the Gly^{A1}-labelled digest. Its position of elution on h.p.l.c. suggests that it is a small peptide, and we conclude that this fragment does not contain any portion of the A-chain, and thus lacks a disulphide bridge. Its m value at pH 1.9 relative to an authentic standard of Phe-Val (residues 1 and 2 of the B chain) in the same electrophoretic track agrees with the predicted value for (Phe^{B1}–Asn^{B3})-peptide [m (predicted relative to Phe-Val), 0.8; m (observed), 0.78].

In contrast with fraction F1, all the others (including those that do not show a significant mobility change

Table 2. H.p.l.c. analysis of oxidized A-chain-labelled fragments

The oxidized A-chain-labelled fragments were eluted from reversed-phase h.p.l.c. in the presence of authentic standards of the peptides listed in column 2. A linear gradient was applied over 15 min from 10% to 35% acetonitrile at a flow rate of 1 ml/min. Retention times relative to the intact A chain, to allow for day-to-day variations, were: (Gly^{A1}–Ser^{A12})-peptide, 0.63; (Gly^{A1}–Leu^{A13})-peptide, 0.87; (Gly^{A1}–Tyr^{A14})-peptide, 0.94; (Gly^{A1}–Gln^{A15})-peptide, 0.9; (Gly^{A1}–Glu^{A17})-peptide, 1.02; [Gly^{A1}–Cys(SO_3)^{A20}]-peptide, 1.0. [Gly^{A1}–Cys(SO_3)^{A20}]-peptide cannot be distinguished from intact A chain. All other separations were baseline apart from that between (Gly^{A1}–Glu^{A17})-peptide and intact A chain or [Gly^{A1}–Cys(SO_3)^{A20}]-peptide. Even in this case separation was sufficient to distinguish between the peptides [typical values: intact A chain, R_t = 16.0 min; (Gly^{A1}–Glu^{A17})-peptide, R_t = 16.4 min, half-height peak width was 0.2 min].

Oxidized fragment	A-chain-labelled peptide after oxidation co-elutes with:
G1	(Gly^{A1}–Leu^{A13})-peptide
G2	(Gly^{A1}–Leu^{A13})-peptide
G3	(Gly^{A1}–Leu^{A13})-peptide
G4	(Gly^{A1}–Leu^{A13})-peptide
G5	(Gly^{A1}–Tyr^{A14})-peptide
G6	(Gly^{A1}–Tyr^{A14})-peptide
G7	(Gly^{A1}–Tyr^{A14})-peptide
G8	(Gly^{A1}–Asn^{A21})-peptide

after performate oxidation) correspond in relative amount and h.p.l.c. retention time to fractions already encountered in the Gly^{A1}-labelled digest. We conclude that all these fractions contain portions of both the A and the B chains. Calculation on the possible structures (see below) of the two fractions of which m for the Phe^{B1} label is unaffected by performate oxidation show that the increase of mobility due to separation from the A chain would be almost exactly counterbalanced by the new negative charge resulting from the conversion of cystine into cysteic acid.

Fraction G1 is the only Gly^{A1}-labelled peak not to correspond in h.p.l.c. retention time and peak size to a component of the Phe^{B1}-labelled digest. Its A-chain portion is, as has been demonstrated above, (Gly^{A1}–Leu^{A13})-peptide. Its response to performate oxidation indicates that it also contains a portion of the B chain. Since the structure of F1 calls for a cleavage between residues B3 and B4, it is tempting to suggest that the B-chain section of G1 begins at residue Glu^{B4}, which would, of course, explain why the B-chain fragment is not labelled. The observed m values for the unoxidized peak (two components, m = 0.5 and 0.65) are roughly consistent with the peak being a mixture of (Gly^{A1}–Leu^{A13})-peptide joined to (Gln^{B4}–Ser^{B9})-peptide (m predicted 0.55) and (Gly^{A1}–Leu^{A13})-peptide joined to (Gln^{B4}–His^{B10})-peptide (m predicted 0.7). However the possibility cannot be ruled out of there being present a component due to (Gly^{A1}–Leu^{A13})-peptide joined to the pyrrolidone carboxylic acid form of (Gln^{B4}–His^{B10})-peptide (m predicted 0.53). Means exist whereby the structure of this component of the digest could be

Table 3. Electrophoretic mobilities in a strong acid system of B-chain-labelled fragments

The electrophoretic mobilities of the fractions isolated on reversed-phase h.p.l.c. were measured on paper in water/formic acid (7:3, v/v). Only F1 fails to correspond when unoxidized, by retention time or mobility, to a fragment in the digest of A1-labelled insulin. The possible structures of the oxidized labelled fragments were deduced from Fig. 4 of Muir *et al.* (1986).

	m		
Fragment	Non-oxidized	Oxidized	Possible structures of oxidized fragments
F1	0.57	0.57	B1–B2, B1–B3 or B1–11
F2	0.61	0.53	B1–B3, B1–B10 or B1–B11
F3	0.49	0.28	B1–B7, B1–B8, B1–B9 or any peptide from B1–B22 to B1–B28 inclusive
F4	0.52	0.47	B1–B3, B1–B4 or any peptide from B1–B10 to B1–B14 inclusive
F5	0.55	0.54	B1–B3, B1–B10 or B1–B11
F6	0.47	0.31	B1–B7, B1–B8, B1–B9 or any peptide from B1–B22 to B1–B28 inclusive
F7	0.46	0.46	B1–B3, B1–B4, or any peptide from B1–B10 to B1–B15 inclusive
F8	0.62	0.39	B1–B4, any peptide from B1–B12 to B1–B17 inclusive, B1–B29, B1–B30

verified (see, e.g., the following paper; Savoy *et al.*, 1988). Like all the other fragments except F3 (= G3) it is relatively minor in all digests observed to date.

The reasoning that permits us to attribute structures to the B-chain part of each of the fractions F2 to F8 is analogous to that used for the A-chain portions of the corresponding fractions, G2 to G8. Thus fragment F3 corresponds to G3 above, i.e. to that characterized by Muir *et al.* (1986) and Davies *et al.* (1986) for which all possibilities for the B-chain portion were eliminated other than (Phe^{B1}–Ser^{B9})-peptide fragment. In addition, Table 4 permits us to conclude that the minor fragment tentatively assigned by Davies *et al.* (1986) to the region between His^{B10} and Val^{B12} in fact results from cleavage after residue His^{B10}.

The evidence relating to fragments F4 and F7 (proposed cleavage after Glu^{B13}) requires further discussion, since, in the absence of an authentic standard for h.p.l.c., the possible structure (Phe^{B1}–Val^{B12})-peptide has yet to be eliminated. Here, m values for certain authentic standards and for the oxidized fragments were measured at pH 6.5. The B-chain labelled fragment of oxidized F4 and F7 was neutral at pH 6.5 whilst authentic (Phe^{B1}–His^{B10})-peptide and (Phe^{B1}–Leu^{B11})-peptide migrated towards the cathode, suggesting that the side chains of the histidine residues had nearly their full positive charges [(Phe^{B1}–Leu^{B11})-peptide: m predicted, 0.21; m observed, 0.17]. There are in the sequence sufficient negative charges to explain these observations. (Phe^{B1}–Val^{B12})-peptide would have migrated at about $m = 0.20$ since the valine residue that distinguishes it from (Phe^{B1}–Leu^{B11})-peptide is neutral and the ionization of the side chain of the histidine residues is unlikely to be drastically different between the peptides with and without *C*-terminal valine. Thus, for the radioactive fragments to be neutral, they must possess the acidic residue Glu^{B13}. This residue must represent the *C*-terminus of the B-chain portions of F4 and F7, since all other possibilities are excluded by the tabulated mobilities and h.p.l.c. measurements.

Table 4. H.p.l.c. analysis of oxidized B-chain-labelled fragments

The oxidized B-chain-labelled fragments were eluted from reversed-phase h.p.l.c. in the presence of authentic standards of the peptides listed in column 2. The elution conditions were chosen to suit the particular peptide being tested. Thus oxidized F2, F3, F5, F6 were analysed in isocratic 10% acetonitrile. The relative retention times were: (Phe^{B1}–Gly^{B7})-peptide, 0.93; (Phe^{B1}–Ser^{B9})-peptide, 1.0; (Phe^{B1}–His^{B10})-peptide, 0.83. Other authentic standards tested of chain length greater than (Phe^{B1}–His^{B10})-peptide were eluted on a gradient of 10% to 35% acetonitrile over 15 min. In this system retention times relative to the intact B chain were: (Phe^{B1}–Ser^{B9})-peptide, 0.43; (Phe^{B1}–Leu^{B11})-peptide, 0.65; (Phe^{B1}–Glu^{B13})-peptide, 0.7; (Phe^{B1}–Ala^{B14})-peptide, 0.72; (Phe^{B1}–Leu^{B15})-peptide, 0.87; (Phe^{B1}–Tyr^{B16})-peptide, 0.91. The oxidized radioactive fragments F4 and F7 were run in a modified gradient such that (Phe^{B1}-Glu^{B13})-peptide eluted isocratically. Oxidized F8 was run on a linear gradient of 25% to 35% acetonitrile over 15 min.

Oxidized fragment	B-chain-labelled peptide after oxidation co-elutes with
F2	(Phe^{B1}–His^{B10})-peptide
F3	(Phe^{B1}–Ser^{B9})-peptide
F4	(Phe^{B1}–Glu^{B13})-peptide
F5	(Phe^{B1}–His^{B10})-peptide
F6	(Phe^{B1}-Ser^{B9})-peptide
F7	(Phe^{B1}–Glu^{B13})-peptide
F8	(Phe^{B1}–Ala^{B30})-peptide

For fraction F6 the range of possibilities from (Phe^{B1}–Arg^{B22})-peptide to (Phe^{B1}–Pro^{B28})-peptide (Table 3) are unlikely as the retention time of the oxidized labelled fragment on h.p.l.c. differs very greatly from that of the intact B chain. Whilst standards for the range (Phe^{B1}–Arg^{B22})-peptide to (Phe^{B1}–Pro^{B28})-peptide were not

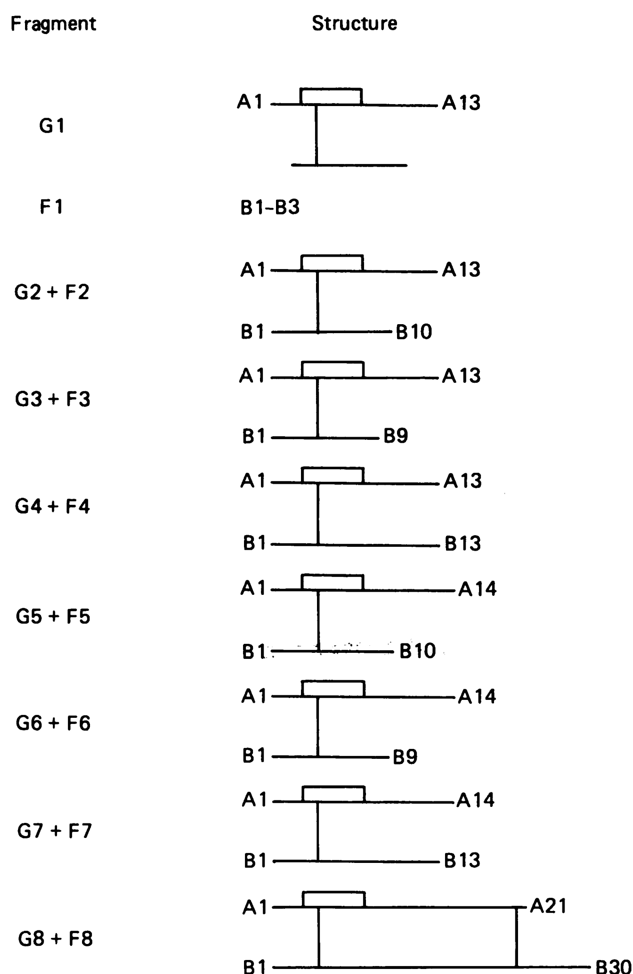

Fig. 2. Fragments of insulin identified in the degradation of insulin by insulin proteinase

The B chain portion of G1 is not labelled, as the termini are uncertain in this case.

prepared and tested, they are likely to have retention times much closer to that of the intact B chain. In addition the cleavage site at Ser^{B9} had already been identified for F3 after elimination of any possibility of an alternative site between Arg^{B22} and Lys^{B29} (Muir *et al.*, 1986).

Fraction F8 (= G8) corresponds in its electrophoretic mobility and h.p.l.c. retention time to insulin. That it is in fact substantially insulin, with no major component having truncated chains, is indicated both by h.p.l.c. measurements of the oxidized chains and by our finding, in preliminary experiments, that this peak, when obtained from digests of Ala^{B30}-labelled insulin, retains a substantial amount of label. Although the possibility of removal of Ala^{B30} (or Asn^{21}; see above) cannot be completely eliminated with the present data, we assume the most likely structure for F8 and G8 is that of intact insulin.

We therefore conclude that the structures of the various fragments are as shown in Fig. 2. Fragment G3 (= F3) is the major component that retains *N*-terminal labelling and is in agreement with studies by other workers using alternative approaches (Hamel *et al.*, 1986; Varandani & Schroyer, 1987). The other components observed in our study are minor. Does this mean that the cleavages that gave rise to these other components are also relatively minor? Any attempt to answer such a question on the basis of the data presented here is made difficult by two factors. First, as soon as a fragment loses the *N*-termini of both the A-chain and the B-chain, it disappears from our analysis (e.g. the peptide B10–B13 which is undoubtedly liberated during the digestion). Secondly, for each case in which one of the original *N*- or *C*-termini of insulin is retained by a fragment, that fragment will be liberated by one less cleavage than the number needed to liberate a molecule lacking the terminal region in question. Assuming for simplicity that all cleavages are independent events, fragments that retain the original termini will thus be liberated more rapidly than those that do not, even if the cleavages that produce the latter are as fast as those that produce the former.

In each case in this study, the *C*-terminal region of the molecule is lost to the analysis, since the labels are on the *N*-termini of the chains. We have recently prepared [[^{3}H]Ala^{B30}]insulin for this purpose (Davies *et al.*, 1987). An accompanying paper (Savoy *et al.*, 1988) using an alternative approach gives further information about the *C*-terminal region; further investigation at the radiochemical level will have to await the results of studies with insulin labelled in this region.

Specificity of the enzyme

Our preparation of insulin proteinase cleaves insulin after residues B9 and B10; A13 and A14; B13 and B3. It is not possible to deduce any simple general rules for the enzyme's specificity from these data, since the sequences around these sites have little in common. As pointed out by Muir *et al.* (1986), both residues B9 and A13 have cystine in the P3 site (notation of Schechter & Berger, 1967). Savoy *et al.* (1987) find another cleavage site, B25, that has, like B13 mentioned above, cystine in the P7 position. Such coincidences should be mentioned, but it would be unwise to draw conclusions from them.

The existence of paired cleavages (of which a further example, B24, B25, is given in the accompanying paper) might indicate a carboxypeptidase-like activity for our preparation. However, preliminary studies with [[^{3}H]Ala^{B30}]insulin do not indicate a rapid release of the *C*-terminal Ala^{B30}, as would be expected if our preparation had any simple carboxypeptidase activity. Further purification of insulin proteinase on DEAE-Sephadex and phenyl-Sepharose leaves the h.p.l.c. profile of the degradation fragments essentially unchanged (A. V. Muir & J. G. Davies, unpublished work).

The cleavage sites reported here are all distant from the proposed receptor-binding region (Pullen *et al.*, 1976). Thus insulin bound to the receptor could still be a substrate for insulin proteinase as far as these cleavages are concerned. However, the cleavages reported in the accompanying paper after B24 and B25 occur in the receptor-binding site.

As previously noted for the major fragment, the cleavage sites, with the exception of A14–A15, are all consistent with analysis carried out on fragments of iodinated insulins observed in isolated hepatocytes. This lends further support to the proposition that insulin proteinase may play a role in physiological insulin degradation; in the liver alone, the quantities of this enzymic activity reported to be present (Burghen *et al.*,

1972) are comparable with the amounts needed to degrade physiological levels of circulating insulin (cf. Karakash & Jeanrenaud, 1983).

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