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The identification of a major product of the degradation of insulin by 'insulin proteinase' (EC 3.4.22.11)

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1. We have studied a major product in the degradation of insulin by insulin proteinase (EC 3.4.22.11). Semisynthetic [[^{3}H]Phe^{B1}]insulin and [[^{3}H]Gly^{A1}]insulin were used in the experiments. 2. The structure of the fragment was deduced by observing the chromatographic and electrophoretic migration of the label both before and after further digestion of the fragment with proteinases of known specificity, with and without additional treatment by performic acid. Ambiguities were resolved by studying the behaviour of authentic fragments of known structure, isolated and characterized after digestion of intact insulin by proteinases of known specificity. 3. We conclude that a major product in the degradation of insulin by insulin proteinase consists of a truncated section of the A chain, joined by the disulphide bridge B7–A7 to a truncated section of the B chain. The A-chain fragment consists most probably of residues A1–A13, and the B-chain fragment consists most probably of residues B1–B9. 4. The similarity between this fragment and that found by other workers when insulin is degraded by intact hepatocytes is significant in the light of proposals that insulin proteinase is a possible participant in the physiological degradation of insulin by target cells.

INTRODUCTION

Insulin is degraded after binding to cell-surface receptors on its target tissues (Terris & Steiner, 1975; Gliemann & Sonne, 1978). The cellular processes that are responsible for this degradation remain undetermined. In order to define these processes it is necessary to isolate and characterize the intermediates and products of the degradation pathway. The intracellular concentration of such products is extremely low, and a radioactive label is normally necessary for their detection. Fragments of labelled insulins have been isolated from intact hepatocytes, giving a partial description of this pathway (Assoian & Tager, 1982; Misbin & Almira, 1984).

An alternative approach is to study insulin-degrading enzymes that have been isolated from target tissues. One such enzyme is insulin proteinase (EC 3.4.22.11) (Burghen *et al.*, 1972; Duckworth & Kitabchi, 1981). This enzyme has been partly characterized and is reported to cleave insulin between residues B16 and B17 (Duckworth *et al.*, 1979). The role of insulin proteinase in physiological insulin degradation has yet to be established, although inhibitor studies in intact cells have suggested that this enzyme is responsible for at least some of the degradation observed (Duckworth *et al.*, 1981; Goldstein & Livingstone, 1981). Further characterization of the mechanism of degradation of insulin by this enzyme should help to clarify its physiological significance.

The degradation of insulin by its target tissues may simply serve to end the biological activity of the hormone. It has also been speculated that partially degraded insulin fragments may act as intracellular second messengers (Steiner, 1977). A knowledge of the mechanism of insulin degradation is therefore important to the understanding of metabolic control by this hormone.

The present paper describes the characterization of a product of the treatment of semisynthetically prepared insulin tracers in a cell-free system with insulin proteinase. The insulin was labelled either at position B1 (Halban & Offord, 1975) or at position A1 (Davies & Offord, 1985). We believe tritium to be preferable to radioactive iodine, as the latter gives a chemically altered form of the hormone. [[^{3}H]Phe^{B1}] insulin has been shown to be a useful tracer in all systems so far investigated (Halban *et al.*, 1976; Berger *et al.*, 1979; Morishima *et al.*, 1985).

MATERIALS AND METHODS

Materials

General chemical reagents were of analytical grade unless otherwise stated. Trypsin (250 units/mg) was purchased from Worthington Biochemical Co., Freehold, NJ, U.S.A., carboxypeptidase B (150 units/mg) from Boehringer, Mannheim, Germany, and bovine serum albumin (radioimmunoassay grade) from Sigma Chemical Co., St. Louis, MO, U.S.A. The scintillation-counting medium used was Biofluor, from New England Nuclear, Boston, MA, U.S.A.

Semisynthetic tritiated insulins

Semisynthetic tritiated insulins were prepared at a specific radioactivity of 10–20 Ci/mmol by a modified version of the method of Halban & Offord (1975) for material labelled at position B1 and by the method of Davies & Offord (1985) for material labelled at position A1.

Insulin proteinase

Insulin proteinase was a gift from Dr. W. Duckworth. The enzyme was obtained as a partially purified membrane preparation by the method of Duckworth *et al.* (1972), the procedure being taken only as far as the second $(NH_4)_2SO_4$ precipitation step. The enzyme

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preparation was dialysed against 20 mM-sodium acetate buffer, pH 6.2, frozen, transported to us packed in solid CO_2, and subsequently stored at −20 °C. At this point in the preparation insulin proteinase accounts for nearly all the proteinase activity observed (W. C. Duckworth, personal communication). The enzymic activity of the preparation does not, however, suffer from the extreme instability that develops on further purification from other, inert, membrane components (Duckworth *et al.*, 1972).

Analyses by h.p.l.c.

H.p.l.c. was carried out on a Spheri-5 C_{18} reversed-phase column (0.4 cm × 25 cm) from Brownlee (Santa Clara, CA, U.S.A.). Solvent A was made by mixing 100 ml of 3 M-$(NH_4)_2SO_4$ with 100 ml of acetonitrile, and making up to 1000 ml with water. Solvent B was made by mixing 100 ml of 3 M-$(NH_4)_2SO_4$ with 350 ml of acetonitrile, and making up to 1000 ml with water. Before use, the pH (glass electrode) of the 3 M-$(NH_4)_2SO_4$ solution had been adjusted to 2.7 by addition of conc. H_2SO_4. A Beckman model 344 gradient system was used to form a linear gradient at 1 ml/min over 15 min between 100% solvent A/0% solvent B and 0% solvent A/100% solvent B. For isocratic elution, the balance between the two solvents was adjusted so that an insulin standard emerged from the column after approx. 15 min. The flow rate was once again 1 ml/min.

Degradation of tritiated insulins

After purification by isocratic h.p.l.c., the A1- or B1-labelled material was passed down a column (0.8 cm × 60 cm) of Sephadex G-50 (fine grade), equilibrated and eluted with 0.1% bovine serum albumin/0.2 M-glycine adjusted to pH 8.8 with 1 M-NaOH. The insulin peak was pooled and the pH adjusted to 7.4 with 6 M-HCl. The insulin concentration of the resulting solution was adjusted to 80 nM by dilution with degradation buffer [bovine serum albumin (3.5 mg/ml) in a solution of Tris (6 g/l), adjusted to pH 7.5 with 6 M-HCl].

To the resulting solution of insulin was added the quantity of proteinase that had been shown by trial digestions to give an increase in trichloroacetic acid-soluble radioactivity of approx. 40% after 10 min incubation at 37 °C. The digestion was stopped by the addition of *N*-ethylmaleimide to a final concentration of 10 mM. Each set of duplicate digestions were accompanied by control tubes in which (*a*) the insulins were treated, not with proteinase, but by an equivalent volume of buffer, and (*b*) the insulins were treated with proteinase in the presence of 10 mM-*N*-ethylmaleimide.

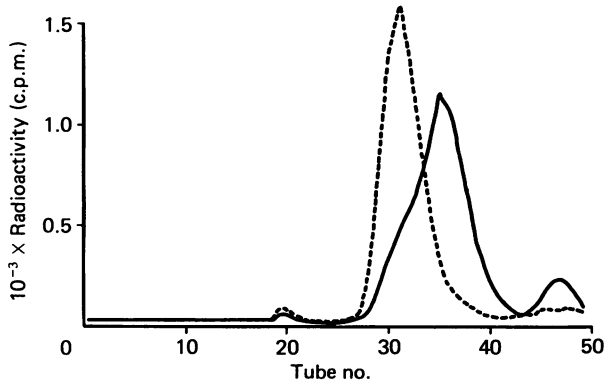

Fig. 1. Radioactivity profile of gel filtration (Sephadex G-50) of [[^{3}H]Phe^{B1}]insulin before degradation (——) and after degradation (——)

For conditions see the text.

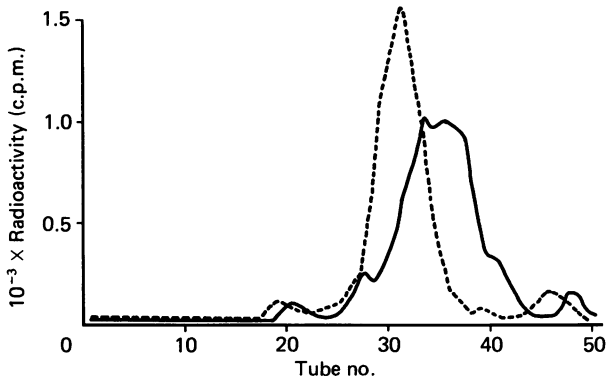

Fig. 2. Radioactivity profile of gel filtration (Sephadex G-50) of [[^{3}H]Gly^{A1}]insulin before degradation (——) and after degradation (——)

For conditions see the text.

After the degradation, unlabelled zinc-free insulin was added as carrier (500 μg/ml of incubation medium). After acidification by the addition of 1 M-acetic acid, the samples were subjected to gel filtration of 1 M-acetic acid on a Sephadex G-50 (fine grade) column (1 cm × 70 cm), with collection of 1 ml fractions, and radioactivity profiles were obtained. A further 500 μg of unlabelled insulin was added to any peaks of radioactivity that separated from insulin. After being freeze-dried, the preparations were each taken up in 100 μl 1% (w/v) NH_4HCO_3 and stored at 4 °C for periods of up to 2 weeks. Occasional paper-electrophoretic tests [in water/formic acid (7:3, v/v)] revealed no deterioration of samples stored in this way, though it is commonly suggested that macromolecules, when radioactively labelled, should not be stored frozen.

For subsequent chemical and enzymic treatments, samples containing sufficient radioactivity for the analytical procedures were added to sufficient unlabelled zinc-free insulin (1 mg/ml in 1% NH_4HCO_3) to adjust the total insulin content of each sample to 0.1 mg, and the mixtures were freeze-dried.

Performate oxidation. A 0.1 ml volume of 30% (w/v) H_2O_2 was added to 0.9 ml of formic acid at 0 °C, and 7 μl of this mixture was used to dissolve each sample. After 30 min at 0 °C, 8 M-urea was added to give a final concentration of 4 M, and the samples were immediately spotted on paper for electrophoresis (see below).

Trypsin treatment. Samples were dissolved in 30 μl of 1% NH_4HCO_3. Trypsin (3 μl of a 1 mg/ml aqueous solution) was then added, and the samples were incubated for 4 h at 37 °C. The digestion was stopped by freeze-drying.

Treatment with carboxypeptidase B. Samples were taken up in 10 μl of 2% NH_4HCO_3. Carboxypeptidase B (6 μl of a 5 mg/ml solution in 10 mM-Tris/HCl buffer, pH 7) was added, and the samples were incubated for 1 h at 37 °C. The reaction was stopped by freeze-drying.

Table 1. Paper-electrophoretic data (strong-acid system) for insulin and various degradation fragments

For experimental details see the text. Undigested insulin refers to unlabelled carrier insulin, added to the degraded product after inactivation of the insulin proteinase, but before the treaments described in the first column. Two major bands (observed and predicted) are shown for treatments that involve performate oxidation: in each case the upper mobility value of each pair relates to the oxidized B chain and the lower value to the oxidized A chain. For method of calculation of predicted *m* see the text. Abbreviation: N.D., not determined.

Treatment	Peak II (B1-labelled) *m* observed (^{3}H)	Peak II (A1-labelled) *m* observed (^{3}H)	Undigested insulin *m* observed (ninhydrin)	Undigested insulin *m* predicted
Material isolated from gel filtration, otherwise untreated	0.47	0.47	0.59	0.59
Trypsin	0.48 (0.6)*	N.D.	0.56	0.55
Carboxypeptidase B	0.48 (0.3) †	N.D.	0.59	0.59
Trypsin, then carboxypeptidase B	0.48 (0.3) †	N.D.	0.47	0.45
Performate oxidation	0.25 (0.48)		0.35	0.37
		−0.36	−0.37	−0.38
Performate oxidation, after trypsin	0.25 (0.48)‡	N.D.	0.27 −0.37	0.32 −0.38
Performate oxidation, after carboxypeptidase B	0.25	N.D.	0.34 −0.37	0.37 −0.38
Performate oxidation, after trypsin and carboxypeptidase B	0.27	N.D.	0.12 (0.27)§ (0.37)‖ −0.37	0.17 −0.38

* Minor band, attributed to contamination of the sample by undegraded insulin.
† Minor band, unexplained.
‡ Minor band, attributed to incomplete oxidation.
§ Minor band, attributed to incomplete digestion by carboxypeptidase B.
‖ Minor band, attributed to incomplete digestion by trypsin.

Paper electrophoresis

Chemically and enzymically treated samples were subjected to paper electrophoresis on Whatman 3MM paper (18 cm × 40 cm) in 30% (v/v) formic acid at 140 V for 15 h. Samples were applied to the paper in 15 μl of 4 M-urea. The electrophoretic mobilities of labelled peptides were determined by means of a Berthold linear analyser. Non-radioactive products were located with the cadmium/ninhydrin stain (e.g. Offord, 1969). Mobilities were measured relative to an internal fluorescent marker of 5-dimethylaminonaphthalene-1-sulphonyl-arginine. Each sample also had 5-dimethylaminonaphthalene-1-sulphonyl-phenylalanine as a secondary internal marker, and 5-dimethylaminonaphthalene-1-sulphonic acid was included as an indication of the true neutral point.

Predictions of electrophoretic mobilities on paper

Predictions of electrophoretic mobilities on paper were obtained by applying the equation:

$$m = k \cdot e \cdot M_r^{-\frac{2}{3}}$$

originally proposed for electrophoresis at pH 6.5 and pH 1.9 (Offord, 1966, 1977). Use was also made of the graph of mobilities at pH 1.9 by Bailey & Ramshaw (1973). It was found (see the Results section) that useful predictions could be directly obtained for positively charged peptides, even in the 30% formic acid system. It was, however, necessary to multiply the direct predictions at this pH by a correction factor of 0.83 when negatively charged peptides were involved. These are the peptides for which the mobilities are dominated by their cysteic acid residues, and the original published data exclude them. This normalization factor was obtained objectively, from the observed behaviour of authentic samples of the A-chain sulphonic acid. The value was checked against the observed mobilities of the sulphonic acid form of peptides Gly^{A1}–Tyr^{A14} and Gly^{A1}–Leu^{A13} and found to be correct within experimental error.

RESULTS

Figs. 1 and 2 show the profiles obtained on gel filtration of samples of the two tritiated insulins after degradation by insulin proteinase. In both these instances there is a peak of radioactive material of apparent M_r lower than that of insulin (peak II), and a peak of apparent M_r close to that of intact insulin (peak I). We have initially concentrated our attention on peak II, since it is a major product of interaction between insulin and the enzyme.

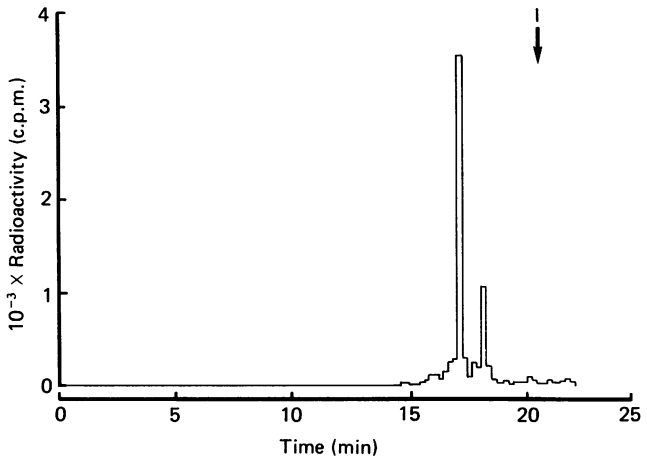

Fig. 3. Radioactivity profile on gradient h.p.l.c. of the A1-labelled peak-II material (see the text)

The elution position of intact insulin is shown by the arrow. Corresponding results (not shown) were obtained with B1-labelled material.

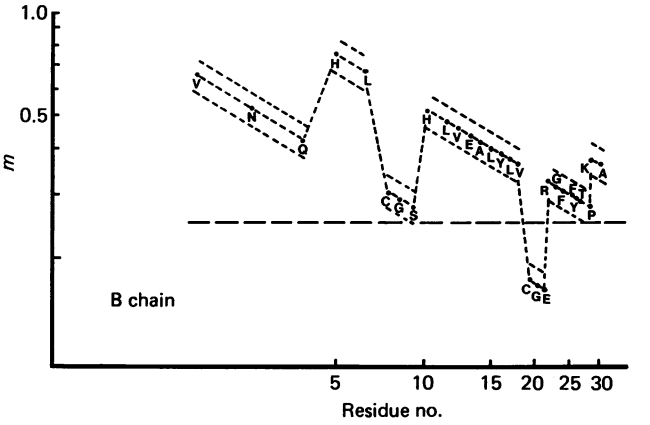

Fig. 4. Predicted *m* values for the oxidized B chain and all possible fragments that can be derived from it by truncation with retention of the B1 labelling

The *C*-terminal residue of each fragment (based on the sequence given by Ryle *et al.*, 1955) is indicated by the single-letter code adjacent to each point. The horizontal line at $m = 0.25$ indicates the observed *m* value of the oxidized form of the B1-labelled product of degradation by the proteinase. The broken lines on either side of the main curve indicate the approximate confidence limits for such predictions (Offford, 1977).

(1) Peak II is not insulin

The *m* value of the peak-II material, and the response of this quantity to various treatments, indicate that peak II is not insulin (Table 1). In addition, peak II material separates from insulin on reversed-phase h.p.l.c. (Fig. 3). The position of elution of peak II on gel filtration (unoxidized, Figs. 1 and 2; oxidized, results not shown) suggests that it has a lower M_r value than insulin. Therefore from now on we refer to peak-II material as 'the labelled fragment'.

(2) The labelled fragment retains at least part of both the A and the B chains

The fragment is labelled, whether or not the A1- or B1-labelled insulin is used as the starting material (Figs. 1 and 2).

(3) At least one interchain disulphide bridge exists in the labelled fragment

Until it is oxidized in performic acid, the fragment's properties are independent of whether the label is at A1 or B1. After performate oxidation, which converts cystine residues into cysteinesulphonic acid residues and therefore cleaves disulphide bridges, the different labels behave in different ways (e.g. compare the relevant parts of Table 1).

(4) The B chain no longer contains residues 22–30

The labelled fragment is unaffected by trypsin, carboxypeptidase B or these two enzymes in combination (Table 1). The two sites of action for these enzymes that exist in the insulin molecule are at positions B22 and B29, and thus both of these residues must be absent from the labelled fragment. Since there is no disulphide bridge that might link this region, or a part of it, to the rest of the molecule, the absence of residue B22 means that residues B23–B30 are absent as well.

(5) The labelled portion of the B chain runs from residue B1 to somewhere between residues B7 and B9 inclusive

For the electrophoretic evidence see Fig. 4. Further, the labelled B-chain fragment is co-eluted with an authentic sample of peptide Phe^{B1}–Ser^{B9} (an oxidized elastase fragment of insulin) on h.p.l.c. (Fig. 5) and Sephadex G-25 (results not shown). Although probably unlikely, it is not impossible that peptide Phe^{B1}–Gly^{B8} or Phe^{B1}–Cys^{B7} would be co-eluted on h.p.l.c. with Phe^{B1}–Ser^{B9}, and we prefer not to exclude these two other possibilities in the absence of definite evidence against them. The other possible conclusion suggested by Fig. 4 and Table 1, that the labelled portion runs from B1 past B22, has already been ruled out [point (4) above].

(6) The labelled portion of the A chain runs from residue A1 to beyond residue A11 and if it does not terminate close to residue A13 it must go on to end either at residue A20 or at residue A21

See Fig. 6.

(7) The labelled portion of the A chain does not extend as far as residue A20

The h.p.l.c. retention time of the oxidized A1-labelled fragment differs from that of the oxidized intact A chain and from that of Gly^{A1}–Cys^{A20}, the latter being prepared by the carboxypeptidase A treatment of the oxidized A chain.

(8) The labelled portion of the A chain is, with high probability, Gly^{A1}–Leu^{A13}

Points (6) and (7), together with the *m* value, give Gly^{A1}–Leu^{A13} as the most likely structure for the labelled A-chain fragment. The retention time on h.p.l.c. of an authentic sample of Gly^{A1}–Leu^{A13}, and an oxidized peptic fragment of insulin, is identical with that of the labelled A-chain fragment (Fig. 5). In contrast, Gly^{A1}–Tyr^{A14}, an

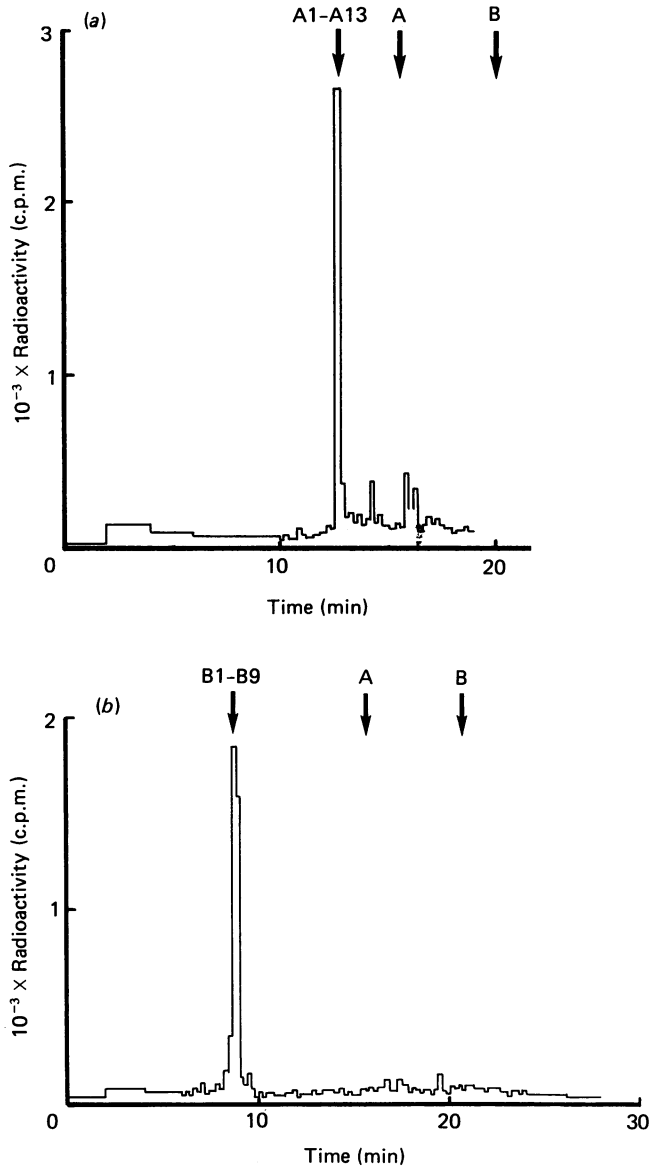

Fig. 5. H.p.l.c. elution of the labelled oxidized chains of the fragment, with positions of elution of the authentic standards mentioned in the text

(*a*) A1-labelled material; (*b*) B1-labelled material.

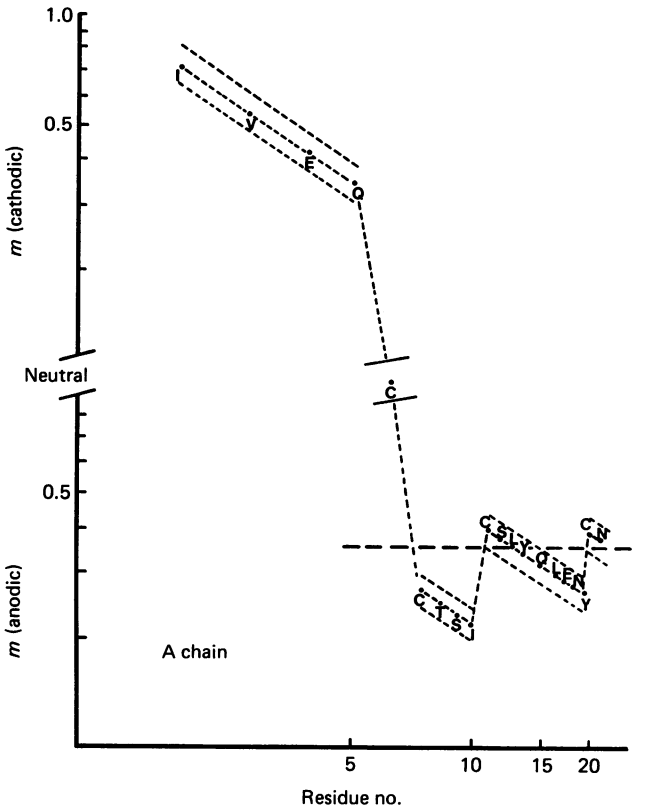

Fig. 6. Predicted *m* values for the oxidized A chain and all possible fragments that can be derived from it by truncation with retention of the A1 labelling

The conventions used are analogous to those described for Fig. 4. The horizontal line at $m = 0.36$ indicates the observed *m* value of the oxidized form of the A1-labelled product of degradation by the proteinase.

oxidized chymotryptic fragment of insuln, is eluted after the labelled A-chain fragment, and Gly^{A1}–Ser^{A12}, produced by the carboxypeptidase A treatment of Gly^{A1}–Leu^{A13}, emerges before it (results not shown). In the absence of an authentic marker, we must consider the possibility that the labelled A-chain fragment would also coincide with Gly^{A1}–Gln^{A15} in the separating sysems used. However, preliminary experiments (J. G. Davies, unpublished work) indicate that the increment of hydrophilic character conferred by Gln-A15 could not counterbalance the increment of hydrophobic character conferred by Tyr-A14, and that peptide Gly^{A1}–Gln^{A15} could not do as the labelled fragment does, and emerge with the Gly^{A1}–Leu^{A13} marker. Other A-chain peptides are excluded by the electrophoretic data.

(9) Disulphide bridge A7–B7 is intact, and is the only interchain bridge that remains

Points (5) and (7), above, show that the cysteine residues that give rise to the disulphide bridge A20–B19 are absent from the labelled portions of the chains. Point (3), above, established that an interchain bridge does exist, and it must be A7–B7. (Though it cannot be excluded that, in the fragment as isolated, this bridge may have undergone disulphide exchange with the intrachain bridge A6–A11, there seems no particular reason to expect that this event has occurred.)

DISCUSSION

Methodological aspects

Our approach to the characterization of the products of degradation was to rely for initial guidance on the values of the electrophoretic mobility (*m*) of the label, and on the manner in which these values responded to various chemical and enzymic treatments. Once the *m* values had drawn attention to the likely structure of a peptide, we required authentic samples of either the proposed peptide, or of one close to it in structure. Our methodology appears to give internally consistent results. It could usefully be applied to the study both of other

products of the action of insulin proteinase and of other forms of partly degraded insulin that might be recovered from '*in vivo*' and '*in-vitro*' systems of interest. A degraded form could be detected even if it had only undergone a single cleavage in between the interchain disulphide bridges, leaving the M_r unchanged. The charge due to the new carboxy group would only balance that of the new amino group at intermediate pH values.

We find a point of cleavage of insulin adjacent to Tyr-A14, which is one of the common sites of iodination of the molecule. Thus it is possible that iodination may affect the processing of insulin by this enzyme and even in intact cells, if this were to include cleavage next to the labelling site.

Specificity of insulin proteinase

The enzyme is known to cleave only a restricted range of substrates. As far as possible specificity of primary structure is concerned, the cleavages that we propose are at sites, not only of different sequence, but also of different general non-covalent character. Even if one looks for similarities of sequence or character at given distances from the proposed points of cleavage of the two chains, it is hard to see any strong similarities. In both chains the most likely points of cleavage are three peptide bonds to the *C*-terminal side of a disulphide bridge, but it is hard to attribute any significance to this fact.

Of the points of cleavage identified by other authors in glucagon (Baskin *et al.*, 1975) and insulin (see below), all but one resemble the cleavage of the Leu-A13–Tyr-A14 bond in having an apolar residue on the *C*-terminal side of the bond that is cleaved. The remaining cleavage, between Ser-11 and Lys-12 in glucagon (Baskin *et al.*, 1975), has obvious similarities to the cleavage between Ser-B9 and His-B10 that we have proposed above. However, it would be necessary to study other cleavages catalysed by the enzyme before being able to say a great deal about specificity. Among the points waiting to be explained is the fact that even the most highly purified form of the enzyme will progressively degrade insulin once it has been cleaved at one site, whereas the separated chains resist the enzyme (Duckworth *et al.*, 1975).

Biological importance of the fragment

With the exception of residues A1 and A2, the crucial portions of the insulin molecule that remain in our fragment are mainly concerned with the formation of correct tertiary structure around disulphide bridge A7–B7 in the intact hormone. Thus further interaction between the fragment and the receptor can effectively be ruled out.

Sites of cleavage

The points of cleavage that we find are exactly those proposed by Assoian & Tager (1982) to explain one of the fragments that they isolated after the degradation of iodinated insulin by isolated hepatocytes. In addition, they propose two other, earlier, cleavages in the B chain, one somewhere between residues B10 and B18 and the second somewhere between residue B10 and the new *C*-terminus produced by the first cleavage. For us not to have seen the fragments produced by these other cleavages, they would have to have a relatively transient existence. Duckworth *et al.* (1979) propose that the first cleavage carried out by insulin proteinase occurs between residues B16 and B17. We attribute their not having seen the A-chain cleavage to methodological factors. On the other hand, there is nothing incompatible between our conclusions regarding the B-chain cleavage and theirs, provided that one assumes that the cleavage that we propose takes place either before, or nearly concurrently with, their cleavage.

The similarity of our results obtained with a cell-free system to those of Assoian & Tager (1982) obtained with intact cells should encourage the further study of the enzyme as having potential importance in the physiological degradation of insulin.

Conclusion

We conclude that the labelled fragment, a major product of the attack on insulin of insulin proteinase, consists of truncated portions of the two chains, joined by disulphide bridge A7–B7. The A-chain component of the fragment is, with high probability, Gly^{A1}–Leu^{A13}. The B-chain component is most likely to be peptide Phe^{B1}–Ser^{B9} with a lesser probability of its being (in decreasing order of likelihood) Phe^{B1}–Gly^{B8} or Phe^{B1}–Cys^{B7}.

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