Biochem. J. (1986) 240, 609–612 (Printed in Great Britain)

Identification of some cleavage sites of insulin by insulin proteinase

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In a previous study [Muir, Offord & Davies (1986) Biochem. J. 237, 631–637] the chromatographic and electrophoretic behaviour of a major labelled fragment in the degradation of tritiated insulins by insulin proteinase were used to locate the probable sites of cleavage which had produced this fragment. In order to define these cleavage sites more precisely, authentic markers for the fragments which would be produced by cleavages at, or adjacent to, the most likely sites have now been synthesized. These markers were compared with labelled fragments of the A- and B-chains of insulin produced by insulin proteinase. The results, together with those of our previous study, show that in order to produce the observed major labelled fragment, the enzyme must have cleaved the insulin A-chain between leucine-A13 and tyrosine-A14 and the insulin B-chain between serine-B9 and histidine-B10. In addition, a minor component was observed in the labelled B-chain fragment which corresponded to a cleavage either between histidine-B10 and leucine-B11 or between leucine-B11 and valine-B12.

INTRODUCTION

The physiological mechanism of insulin's degradation by its target tissues remains unknown. Studies *in vitro* and *in vivo* have suggested that the enzyme insulin proteinase may be responsible for at least some of the degradation in intact tissues (Duckworth & Kitabchi, 1981). In order to define the activity of this enzyme it is necessary to characterize the intermediates and products of the degradation of insulin by this enzyme.

We have previously reported the characterization of a major product in the degradation of insulin by insulin proteinase (Muir *et al.*, 1986). By using semisynthetic tritiated insulin labelled at either phenylalanine-B1 (Halban & Offord, 1975) or at glycine-A1 (Davies & Offord, 1985), we have demonstrated that the structure of the labelled fragment was, with high probability, (Gly^{A1}–Leu^{A13})-peptide linked to (Phe^{B1}–Ser^{B9})-peptide by the disulphide bridge A7–B7. The analysis depended on measurements of electrophoretic and chromatographic behaviour both before and after chemical and enzymic treatment of the product. Despite the high probability that our conclusions were correct, the results left open the possibilities that the A-chain fragment might consist of (Gly^{A1}–Gln^{A15})-peptide and that the B-chain fragment is (Phe^{B1}–Cys^{B7})-peptide or (Phe^{B1}–Gly^{B8})-peptide. We now report the synthesis of unlabelled markers for these peptides. These markers were used to test our previous conclusions as to the nature of the fragments produced by insulin proteinase. The results confirm and extend our previous findings.

METHODS

All reagents were analytical grade. Chymotrypsin, elastase and carboxypeptidase A were obtained from Sigma, St. Louis, MO, U.S.A. L-Glutamine t-butyl ester was from Bachem, Bubendorf, Switzerland. Trifluoroacetic acid (sequence-analysis grade) was obtained from Fluka, Buchs, Switzerland.

H.p.l.c. was carried out as described by Muir *et al.* (1986). Paper electrophoresis and measurement of mobilities was carried out as described by Offord (1966) or Muir *et al.* (1986).

Insulin proteinase was the gift of Dr. W. Duckworth and was prepared by the method of Duckworth *et al.* (1972). Degradation of tritiated porcine insulins by insulin proteinase and purification of the labelled fragment and its performate oxidation (see below) were as described by Muir *et al.* (1986).

Synthesis of (Gly^{A1}–Gln^{A15})-peptide

(Gly^{A1}–Gln^{A15})-peptide was prepared by the use of chymotrypsin to catalyse the synthesis of a peptide bond between (Gly^{A1}–Tyr^{A14})-peptide and glutamine t-butyl ester. Removal of the ester then gives the desired product. (Gly^{A1}–Tyr^{A14})-peptide was prepared by dissolving performate-oxidized porcine insulin A-chain (5 mg) in NH_4HCO_3 (1%, w/v; 0.5 ml). Chymotrypsin was then added as a solution in 1% NH_4HCO_3 to give a final enzyme/substrate ratio of 1:200 (w/w). After 4 h at 37 °C the resulting peptides were purified by reversed-phase h.p.l.c. (Gly^{A1}–Tyr^{A14})-peptide was identified by its electrophoretic behaviour and by amino acid analysis.

(Gly^{A1}–Tyr^{A14})-peptide (0.5 mg) was then dissolved in dimethyl sulphoxide (8 μl) and 5 μl of water were added. A 0.5 M-solution of glutamine t-butyl ester hydrochloride was prepared in butane-1,4-diol and the pH adjusted to 6.8 by the addition of solid Tris base. A 28.5 μl portion of this solution were added to the peptide solution and the mixture was warmed to 37 °C. A 0.5 μl portion of a solution of chymotrypsin (100 mg/ml in water) was added. The reaction was allowed to proceed for 1 h at 37 °C and was then stopped by acidification to ~ pH 3.0 with acetic acid. The resulting peptides were purified by reversed-phase h.p.l.c. The eluted peaks were desalted by passing through a Sep-Pak cartridge equilibrated in acetonitrile/water/trifluoroacetic acid (100:900:1, by

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vol.). After washing the cartridge with 20 ml of this solvent the peptide was eluted with 10 ml of acetonitrile/water/trifluoroacetic acid (400:600:1, by vol.) and freeze-dried. A sample was removed for paper electrophoresis and the rest redissolved at 6 mg/ml in trifluoroacetic acid. After 40 min the trifluoroacetic acid was removed under vacuum and the peptides redisolved in 50 μl of 1% NH_4HCO_3.

Synthesis of (Phe^{B1}–Gly^{B8})-peptide

This was obtained from (Phe^{B1}–Ser^{B9})-peptide by removal of serine-B9 with carboxypeptidase A. In order to produce (Phe^{B1}–Ser^{B9})-peptide, the performate-oxidized B-chain of porcine insulin (7 mg) was dissolved in 2 ml of 0.05 M-Na_2CO_3, and elastase (1 mg/ml in water) was added to given an enzyme/substrate ratio of 1:350 (w/w). After 20 h at 37 °C the resulting peptide were purified by reversed-phase h.p.l.c. (Phe^{B1}–Ser^{B9})-peptide was identified by its electrophoretic mobility at pH 1.9 and 6.5 and by amino acid analysis. This peptide was dissolved at 2 mg/ml in 1% NH_4HCO_3. Carboxypeptidase A was added (1 μl of a 1000 units/ml solution in 2 M-NH_4HCO_3 to 10 μl of peptide solution) and the mixture incubated for 24 h at 37 °C. A further 1 μl of the carboxypeptidase A solution was added and the mixture left for a further 24 h at 37 °C. The release of serine was monitored by paper electrophoresis at pH 1.9, and on this basis the resulting cleavage product was taken to be (Phe^{B1}–Gly^{B8})-peptide. It was separated from any remaining (Phe^{B1}–Ser^{B9})-peptide by isocratic reversed-phase h.p.l.c.

RESULTS

Synthesis of (Gly^{A1}–Gln^{A15})-peptide

The elution profile of the peptides resulting from reaction of (Gly^{A1}–Tyr^{A14})-peptide with glutamine t-butyl ester in the presence of chymotrypsin is shown in Fig. 1. A peak of material was produced which has a greater retention time than (Gly^{A1}–Tyr^{A14})-peptide and which is not present in the reaction blank. This material was subsequently shown to have a reduced mobility on paper electrophoresis at pH 6.5, consistent with a blocked carboxy group. After treatment with trifluoroacetic acid, which removes t-butyl esters, the mobility of this material was the same as that of (Gly^{A1}–Tyr^{A14})-peptide within experimental error. This would be expected for (Gly^{A1}–Gln^{A15})-peptide as the predicted mobility values for the two peptides are too close to allow separation under these conditions.

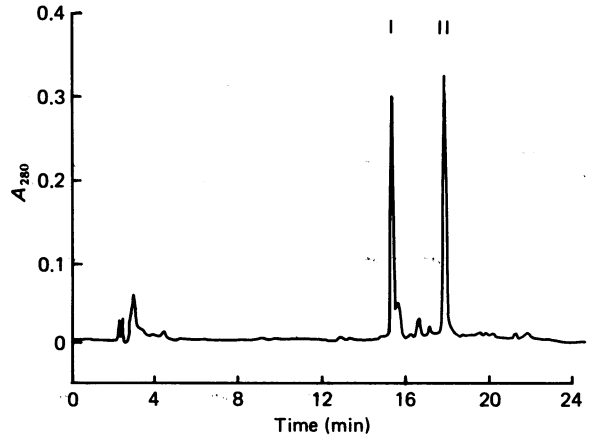

Fig. 1. Elution profile on reversed-phase h.p.l.c. of the result of the reaction between peptide (Gly^{A1}–Tyr^{A14}) and glutamine-butyl ester in the presence of chymotrypsin

Peaks: I, (A1–A14)-peptide; II, a more hydrophobic peptide produced by the reaction.

Separation of (Gly^{A1}–Gln^{A15})-peptide from (Gly^{A1}–Leu^{A13})-peptide

Fig. 2 shows the elution profile on h.p.l.c. of the radioactive peptide obtained by the performate oxidation of the major labelled A-chain fragment produced by insulin proteinase from [*Gly*^{A1}-^{3}H]insulin. In addition, the retention times of the insulin A-chain (Gly^{A1}–Leu^{A13})-, (Gly^{A1}–Tyr^{A14})- and (Gly^{A1}–Gln^{A15})-peptides and the intact oxidized A-chain are indicated. The radioactive peptide is co-eluted with (Gly^{A1}–Leu^{A13})-peptide, which separates from the other peptides.

Separation of (Phe^{B1}–Gly^{B8})-peptide from (Phe^{B1}–Ser^{B9})-peptide

Fig. 3 shows the elution profile on isocratic h.p.l.c. of the result of treatment of (Phe^{B1}–Ser^{B9})-peptide with

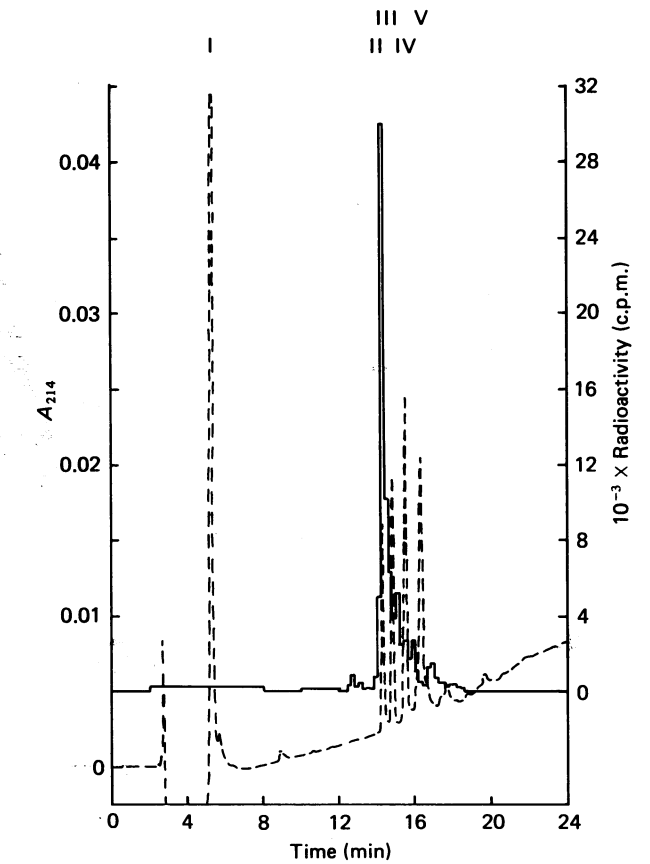

Fig. 2. Elution profile on h.p.l.c. of the radioactive peptide obtained by performate oxidation of the A1-labelled fragment produced by insulin proteinase, and a mixture of unlabelled insulin A-chain peptides

I, Solvents; II, (Gly^{A1}–Leu^{A13})-peptide; III, (Gly^{A1}–Gln^{A15})-peptide; IV, (Gly^{A1}–Tyr^{A14})-peptide; and V, (Gly^{A1}–Asn^{A21})-peptide. ——, Radioactivity; ----, A_{214}.

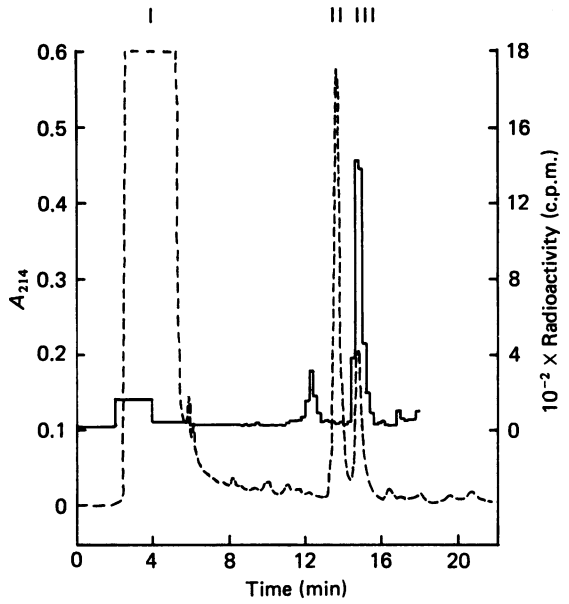

Fig. 3. Elution profile on h.p.l.c. of the radioactive peptides obtained by performate oxidation of the B1-labelled fragment produced by insulin protease, and the result of the treatment of (B1–B9)-peptide with carboxypeptidase A

I, Solvents; II, (Phe^{B1}–Gly^{B8})-peptide, III, (Phe^{B1}–Ser^{B9})-peptide. ——, Radioactivity; ----, A_{214}.

carboxypeptidase A. Also shown is the elution profile of the radioactive peptide obtained by performate oxidation of the major labelled B-chain fragment produced by insulin proteinase from [*Phe*^{B1}-^{3}H]insulin. This labelled peptide is co-eluted with (Phe^{B1}–Ser^{B9})-peptide and separates from (Phe^{B1}–Gly^{B8})-peptide. In addition a minor radioactive component was observed which was eluted before (Phe^{B1}–Ser^{B8})-peptide and was subsequently shown to have an electrophoretic mobility in water/formic acid (7:3, v/v) corresponding to (Phe^{B1}–His^{B10})-peptide or peptide (Phe^{B1}–Leu^{B11})-peptide [observed mobility (*m*) (relative to dansylarginine) = 0.51, predicted *m* values: (Phe^{B1}–His^{B10})-peptide, 0.51; (Phe^{B1}–Leu^{B11})-peptide, 0.49 (see Fig. 4 of Muir *et al.*, 1986)]. (Phe^{B1}–Asn^{B3})-peptide, which has a predicted mobility of 0.52, is excluded because it would have been separated from the labelled fragment during its preparation on Sephadex G-50 (Muir *et al.*, 1986). No other B1-labelled peptide has a predicted mobility close to this value.

DISCUSSION

In our previous observations, the labelled A-chain peptide (obtained by the performate oxidation of the A1-labelled insulin-proteinase fragment) corresponded to either (Gly^{A1}–Leu^{A13})-peptide or (Gly^{A1}–Gln^{A15})-peptide. Other possibilities had been excluded by the electrophoretic-mobility (*m*) value of the labelled A-chain fragment or by comparison of the h.p.l.c. retention time of the labelled A-chain fragment with that of standard peptides on h.p.l.c. These peptides were easily obtained as they are produced by proteolytic enzymes of known specificity. (Gly^{A1}–Gln^{A15})-peptide is not produced by such an enzyme, and it was necessary to synthesize this peptide. The method chosen was to use chymotrypsin to catalyse the formation of a peptide bond between (Gly^{A1}–Tyr^{A14})-peptide and glutamine t-butyl ester. The conditions were adapted from those of Rose *et al.* (1983) and were chosen such that the peptide and enzyme were soluble but the quantity of water was kept low (12%). After deprotection, the coupled product was used as a standard of (Gly^{A1}–Gln^{A15})-peptide on h.p.l.c. The results indicate that the labelled A-chain fragment is not (Gly^{A1}–Gln^{A15})-peptide, thus leaving (Gly^{A1}–Leu^{A13})-peptide as the only remaining possibility. The cleavage of insulin by insulin proteinase has thus occurred between residues A13 and A14.

The labelled B-chain peptide (obtained by performate oxidation of the B1-labelled insulin-proteinase fragment) had been shown by Muir *et al.* (1986) to correspond to (Phe^{B1}–cysteic acid^{B7})-, (Phe^{B1}–Gly^{B8})- or (Phe^{B1}–Ser^{B9})-peptide. The labelled B-chain fragment has now been shown to be co-eluted with (Phe^{B1}–Ser^{B9})-peptide in a system which separates this peptide from (Phe^{B1}–Gly^{B8})-peptide. The labelled fragment is therefore not (Phe^{B1}–Ser^{B8})-peptide. Although we did not find it easy to prepare an authentic marker for (Phe^{B1}–cysteic acid^{B7})-peptide, we have discarded this as a possible structure for the radioactive fragment on the following grounds. If the labelled fragment were (Phe^{B1}–cysteic acid^{B7})-peptide, then the latter would have to be co-eluted with (Phe^{B1}–Ser^{B9})-peptide, as is the radioactivity. This is most improbable, since it would imply that the removal of glycine from (Phe^{B1}–Gly^{B8})-peptide would have to render the product more hydrophobic.

An additional radioactive peptide was observed as a minor peak on the h.p.l.c. analysis of the labelled B-chain fragment. Its electrophoretic mobility corresponds to that of (Phe^{B1}–His^{B10})- or (Phe^{B1}–Leu^{B11})-peptide. This is of interest, as it corresponds to a peptide observed by Assoian & Tager (1982), who studied the degradation of radioiodinated insulin in intact hepatocytes. The equivalent peptide in their study was an intermediate in the processing of the B-chain which had previously been cleaved nearer to the *C*-terminus and was subsequently cleaved between the disulphide bridge A7–B7 and histidine–B10. As the cleavages observed for our major fragment also correspond with those seen in intact hepatocytes, these results should stimulate interest in the physiological role of insulin proteinase in insulin degradation.

In conclusion, a major product of the degradation of insulin with insulin proteinase is produced by a cleavage in the A-chain between residues A13 and A14 and a cleavage in the B-chain between residues B9 and B10. In addition, a peptide resulting from a cleavage in the B-chain between residues B10 and B11 or between residues B11 and B12 was observed.

We thank the Fonds National Suisse de la Recherche Scientifique, the Sandoz-Stiftung zur Fordering der Medizinisch-Biologischen Wissenschaften and the Stanley-Thomas Johnson Foundation for their financial support. We are grateful to Dr. K. Rose for invaluable help and advice with the use of chymotrypsin to synthesize peptide bonds and to Dr. G. Hughes for amino acid analyses. We thank Mr. C. Bradshaw for preparation of the tritiated insulins and Mr. F. Cerini for technical assistance.

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Received 30 July 1986/10 September 1986; accepted 29 September 1986