Semisynthetic human [$^3\text{H}_2$]Phe 1]proinsulin

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Biosynthetic human proinsulin (obtained by recombinant DNA techniques) was used as the starting material for the preparation, by semisynthetic methods, of [^3H]proinsulin with the label at the *N*-terminal phenylalanine residue. The labelled proinsulin was characterized by its retention time on reversed-phase h.p.l.c., by polyacrylamide-gel electrophoresis, by the time course of its enzymic conversion into insulin and by chromatographic analysis after extensive proteolytic degradation. The specific radioactivity of the product was 5 Ci/mmol. Experimental details of the preparation of human [^3H]Phe 1]proinsulin, the isolation of this product by isocratic h.p.l.c. and gel filtration, and further characterization of protein intermediates have been deposited as supplement SUP 50138 (12 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on prepayment [see Biochem. J. (1987) 241, 5].

INTRODUCTION

Proinsulin has long been recognized as a precursor of insulin (Steiner & Oyer, 1967). Unlike many precursor proteins, however, it already possesses a significant proportion (approximately one-eighth) of the biological activity of the activated form (Shaw & Chance, 1968). It is far from being established that its biological activity is simply due to activation of proinsulin to insulin between the beginning of the assay and the observation of the biological response (Lazarus *et al.*, 1970). Proinsulin, which circulates both as the intact 67-residue protein and in partially degraded forms (Gutman *et al.*, 1972), disappears from the circulation more slowly than does insulin (Sönksen *et al.*, 1973). This fact, together with its biological activity, offers the possibility that its use in the therapy of diabetes might complement that of insulin. The availability of human proinsulin through recombinant DNA techniques means that this possibility can now be studied in detail. The present paper describes the preparation by protein semisynthesis of a tritiated form of the prohormone and its characterization *in vitro*. This derivative is likely to be more authentic in its behaviour than a radioiodinated one, as well as safer for use in man.

MATERIALS AND METHODS

Materials

Biosynthetic human proinsulin was the gift of Eli Lilly and Co., Indianapolis, IN, U.S.A. L-Phenyl[2,3- ^3H]alanine was obtained from Amersham International, Amersham, Bucks., U.K. (code TRK 266). It was isotopically diluted before use with unlabelled L-phenylalanine (Sigma Chemical Co.). Radioactivities of labelled samples were counted in a Beckman liquid-scintillation counter (model LS 6800), with the use of Biofluor (New England Nuclear).

Experimental details of the preparation of human [^3H]Phe 1]proinsulin (X), the isolation of product (X) by isocratic h.p.l.c. and gel filtration, and further character-

ization of protein intermediates have been deposited as Supplement SUP 50138 at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K.

Tosyl-L-phenylalanylchloromethane-treated trypsin was obtained from Worthington Biochemical Corp., and carboxypeptidase B from Boehringer. Leucine aminopeptidase solution was prepared as described by Rose *et al.* (1984). Subtilisin (subtilopeptidase A) was from Sigma Chemical Co., Pronase (Pronase P) from Kaken Chemical Co., Tokyo, Japan, and carboxypeptidase Y from Carlsberg Biotechnology, Copenhagen, Denmark.

Analytical h.p.l.c.

Samples for analysis by reversed-phase h.p.l.c. were taken up in either aq. 0.1% (v/v) trifluoroacetic acid (solvent A) or 0.1% trifluoroacetic acid in 20% (v/v) acetonitrile (solvent B), and injected into the h.p.l.c. system described by Rose *et al.* (1984). The column was a 10 μm -particle-size Radialpak C $_{18}$ Bondapak in a Z-module. The flow of equilibration (starting) solvent was maintained for 5 min after injection of the sample, before the gradient was started. The flow rate was 2 ml/min. Trifluoroacetic acid was h.p.l.c./spectro grade from Pierce Chemical Co., Rockford, IL, U.S.A.

Determination of free amino groups in protein samples

Dansylation and hydrolysis of protein samples were carried out by using the method of Gray (1967) modified as described by Offord (1980). Hydrolysates were dissolved by adding 10 μl of water followed by 10 μl of pyridine, acidified by adding 30 μl of acetic acid and then diluted with 50 μl of water. A 40 μl sample of the solution was analysed for dansyl-amino acids by h.p.l.c. on a linear gradient of 1 h duration between solvent A and 0.1% trifluoroacetic acid in 40% (v/v) acetonitrile. Solutions of dansyl-amino acids (Sigma Chemical Co.): *N* $^{\epsilon}$ -dansyl-lysine, *O*-dansyltyrosine, dansylphenylalanine and dansylvaline [67 μM , dissolved in 0.1% trifluoroacetic acid in 1% (v/v) methanol] were run as standards. Detection was at 280 nm.

Abbreviations used: Boc, t-butyloxycarbonyl; Msc, methanesulphonylethylloxycarbonyl; Ptc, phenylthiocarbamoyl.

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Exhaustive proteolytic digestion of product (X)

A portion of the stock solution of product (X) (60 μ l) was allowed to equilibrate for at least 2 h with 60 μ g of compound (I). It was buffered with 2.5 mM-MgCl₂/1% (w/v) NH₄HCO₃ (to an external pH of 8.2). The protein was then exhaustively digested with subtilisin, Pronase, carboxypeptidase Y (1.2 μ g of each) and leucine aminopeptidase (12 μ l) at 37 °C.

A 40 μ l sample of the digest was diluted into a mixture of 100 μ l of solvent A and 5 μ l of acetic acid, and 130 μ l was separated by h.p.l.c. The starting solvent was solvent A, with a linear gradient of 10 min duration to 0.1% trifluoroacetic acid in 10% (v/v) acetonitrile, followed by a linear gradient of 5 min duration to 100% acetonitrile. The phenylalanine peak, which was eluted between 16.5 and 17.5 min, was collected and its radioactivity counted.

Enzymic conversion of product (X) into [³H]insulin

To 0.90 mg of compound (I) were added 12 μ l of 1 M-Tris/HCl buffer, pH 7.56, followed by 90.4×10^4 d.p.m. of product (X). The mixture was allowed to equilibrate for 4 h at room temperature. To the solution was added 2 μ l of trypsin (1.25 mg/ml) followed immediately by 2 μ l of carboxypeptidase B (0.625 mg/ml). The solution was stirred with a microcapillary. The time of addition of the carboxypeptidase B was taken as the start of the reaction. Samples (20 μ l) of the reaction mixture taken at 0.5, 2, 4 and 10 min (a 'zero-time' sample was also taken before the addition of the enzymes) were diluted into 0.26 ml of 0.1% trifluoroacetic acid in 5% (v/v) acetic acid and analysed by h.p.l.c. with a linear gradient of 20 min duration from 0.1% trifluoroacetic acid in 30% (v/v) acetonitrile to 0.1% trifluoroacetic acid in 37% (v/v) acetonitrile. For each sample, the proinsulin and insulin peaks were collected into tubes containing approx. 1 mg of bovine serum albumin.

RESULTS AND DISCUSSION

The general strategy for the semisynthesis of [³H]-Phe¹proinsulin is shown in Scheme 1. The numbering of intermediates corresponds to that used in the text.

Preparation of the protected truncated intermediate N^εN^{ε'}-diMsc-des-Phe¹-proinsulin (IV)

Brandenburg (1969) has shown that reaction of phenyl isothiocyanate with insulin can be largely limited to the phenylalanine-B1 amino group by using only a small molar excess of phenyl isothiocyanate in pyridine/water solution. Such conditions were extended to the specific reaction of the α -amino group of the starting material (I) (Fig. 1b).

After purification of compound (II) by h.p.l.c., dansylation and hydrolysis gave no detectable dansyl-phenylalanine, but large peaks of ϵ -dansyl-lysine and O -dansyltyrosine. Thus the N -terminus appears to have reacted with the Edman reagent whereas, as expected, at least a substantial proportion of the two ϵ -amino groups have not. Compound (II) is homogeneous on h.p.l.c., and, since zero-, mono- and di- ϵ -Ptc derivatives could not possibly migrate together, compound (II) must either be all zero- or all mono- ϵ -Ptc-substituted. We reject the latter hypothesis since, in various subsequent steps, strong acid treatment leaves no significant amount of derivative that behaves as though its ϵ -amino group is

Scheme 1. Semisynthesis of human [³H]proinsulin

blocked. It is well known that, in contrast with the α -Ptc group, strong acid treatment removes only a small proportion of the ϵ -Ptc group.

The Msc- N -hydroxysuccinimido ester reagent (Tesser & Balvert-Geers, 1975) was employed for protection of the ϵ -amino groups, based on the method of Geiger *et al.* (1975). Any trace of proinsulin that has escaped reaction with phenyl isothiocyanate [(I) in Fig. 1(b)] is converted into compound (V) (Scheme 1) and thus cannot participate in the subsequent coupling step.

After reaction of methanesulphonylethoxycarbonylated Ptc-proinsulin with trifluoroacetic acid (Fig. 1c) essentially no material was eluted at the position expected for compound (III), showing that cyclization and removal of the phenylthiohydantoin derivative of phenylalanine is complete. This suggests that the main peak is N^εN^{ε'}-diMsc-des-Phe¹-proinsulin (IV). Amino group determination indicated the presence of only N -terminal valine in the compound (IV)/compound (V) mixture, which was to be used for coupling. (O -Dansyltyrosine was detected, but there was no dansylphenylalanine or ϵ -dansyl-lysine.)

Coupling of compound (IV) with compound (VIII)

Gradient h.p.l.c. of the coupling reaction mixture before deprotection (Fig. 1d) showed that essentially all the compound (IV) had reacted. As a working hypothesis, the main peak that was eluted after compound (IV) and (V) was assigned to the protected coupled product (IX). The specific radioactivity of (VIII) was 6 Ci/mmol.

Isolation of product (X)

Analysis of deprotected coupled product by the gradient h.p.l.c. system, previously used for the analysis

Fig. 1. H.p.l.c. of human proinsulin and derivatives

(a) Biosynthetic human proinsulin; (b) Phenyl isothiocyanate-treated proinsulin (96 h); (c) $N^{\epsilon}N^{\epsilon}$ -diMsc-des-Phe 1 -proinsulin used for coupling: the positions of compounds (III) and (V) shown were determined by analysis of methanesulphonylethylloxycarbonylated h.p.l.c.-purified compounds (II) and (I) respectively; (d) coupling reaction mixture; (e) deprotected coupling reaction mixture. In all peaks shown that are off-scale, the top of the peak is located on a scale 5-fold reduced. A linear gradient of 1 h duration of 0.1% trifluoroacetic acid in 25% (v/v) acetonitrile to 0.1% trifluoroacetic acid in 45% (v/v) acetonitrile was used.

of human proinsulin and its derivatives, indicated only a poor separation of proinsulin from other products of the coupling reaction (Fig. 1e). Isocratic h.p.l.c. separation was therefore used for the isolation of product (X) (Fig. 2a).

H.p.l.c. separation of insulin derivatives can lead to a decrease in their biological activity (Welinder *et al.*, 1983a). This loss of activity is minimized if the protein is isolated by gel filtration after the h.p.l.c. separation (Welinder *et al.*, 1983b). H.p.l.c.-separated product (X) was therefore subjected to Sephadex G-50 gel filtration. Essentially all the radioactive material recovered was eluted in the same fraction as the biosynthetic starting material (I).

Product characterization

The radioactive product is seen (Fig. 2b) to be eluted on isocratic reversed-phase h.p.l.c. with the u.v.-absorbance peak due to co-injected unlabelled proinsulin.

The acid and alkali polyacrylamide gels (Fig. 3) show that product (X) migrates essentially as a single band in the same position as compound (I). Densitometry of the

Fig. 2. Isocratic h.p.l.c. of product (X)

(a) Deprotected coupling reaction mixture. The proinsulin peak (96 min) was eluted at the same position as a proinsulin standard applied in the same deprotection mixture. Only the latter half of the peak, fraction (X), was collected, in order to ensure complete separation from the earlier peak (82 min). (b) A sample of product (X) (2.7×10^5 d.p.m.) mixed with 50 μg of compound (I) was freeze-dried and taken up in 200 μl of solvent B for analysis by h.p.l.c. Each fraction was collected and the radioactivity determined. The column was eluted with 0.1% trifluoroacetic acid in 29% (v/v) acetonitrile at 1 ml/min. With isocratic runs of such a long duration, carried out on different days, the difference in elution time of product (X) between (a) and (b) cannot be regarded as significant. In any case an internal standard of proinsulin was added to run (b).

dye-stained protein bands shows a radiochemical purity on this criterion of about 95%. Very little deamidation of the purified product is evident.

Exhaustive digestion of product (X) with a mixture of alkaline proteinases enabled 94% of the applied radioactivity to be recovered in the phenylalanine position after separation of the digest by h.p.l.c. From this result we conclude that racemization during activation and coupling, if it occurred, must have been at a low level.

Kemmler *et al.* (1971) showed that enzymic conversion of proinsulin into insulin could be carried out with a combination of trypsin and carboxypeptidase B. The absorption trace of the h.p.l.c. separations of product (X) that had been treated with these enzymes shows that essentially all the starting proinsulin was converted into a single insulin peak after 10 min of reaction. Correspondingly, 99% of the radioactivity in the starting proinsulin peak was recovered in the insulin peak after 10 min of reaction, showing that the semisynthetic [^3H]proinsulin can be completely converted into [^3H]insulin by the enzymic reaction, in the same way as the biosynthetic proinsulin starting material. The rate of disappearance of proinsulin (Table 1) shows that the tritiated proinsulin behaves in a similar way to the unlabelled material in this system.

Fig. 3. Polyacrylamide-gel electrophoresis of product (X)

Samples (1.6×10^4 d.p.m.) of product (X) were freeze-dried in the presence of $10 \mu\text{l}$ of a solution of compound (I) (1 mg/ml). Analytical polyacrylamide-gel electrophoresis was carried out on slab gels as modified from the procedures of Poole *et al.* (1974) for acid gels (a) and Laemmli (1970) for alkali gels (b). Electrophoreses were performed at pH 3 with 20% polyacrylamide in the separating gel for acid gels and at pH 8.3 and 5% polyacrylamide in the separating gel for alkali gels, at a constant current of 20 mA per slab, with 8 M-urea present in both sample and gel. The gels were stained with Coomassie Blue. Radioactive material was then detected by autoradiography after enhancing with En³Hance autoradiography enhancer (New England Nuclear). The absorbance of the autoradiographs was measured with an Isco (model UA-5) detector.

Specific radioactivity and yield

On the basis of the area of the absorbance peak measured (Fig. 2a) and the corresponding radioactivity, the specific radioactivity of the product was 5 Ci/mmol. Recent experience with insulin suggests that higher specific radioactivities could be obtained if desired. The yield of product (X) from the protein precursor (IV) was 1%. The radiochemical yield for the conversion of compound (VI) into product (X) was 0.34%. It is possible to recycle the labelled amino acid.

Both the chemical and radiochemical yields are low, but such problems are commonplace in the semisynthetic labelling of proteins to high specific radioactivity. The causes of such low yields, which seem to have to do with the small quantities with which one is obliged to work, and with radiation-induced side reactions, have been discussed elsewhere (Offord, 1980). Nonetheless, the methods described in the present paper give relatively simply an amount of product, labelled at a specific site, sufficient for an extensive experimental programme.

Conclusion

We conclude that the radioactive product (X) has the wanted molecular structure, and that it behaves authen-

Table 1. Enzymic conversion of product (X) into [³H]insulin

The enzymic conversion of proinsulin into insulin was monitored by h.p.l.c. (for details see the Materials and methods section). The radioactivity in the proinsulin position is compared with the area of the proinsulin peak.

Time (min)	Radioactivity in proinsulin position relative to that at zero time (%)	Area of proinsulin peak relative to that at zero time (%)
0	100	100
0.5	34	46
2	23	21
4	7	3

tically *in vitro*. This material now requires further evaluation *in vivo*.

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