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A mass-spectrometric investigation of the mechanism of the semisynthetic transformation of pig insulin into an ester of insulin of human sequence

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In the trypsin-mediated semisynthetic transformation of pig insulin into an ester of insulin of human sequence, the B30 alanine residue of the pig hormone is replaced by an ester of threonine. The mechanism of this reaction was investigated by carrying out the transformation in a medium containing water enriched with ^{18}O. Subsequent analysis by combined g.l.c.–mass spectrometry demonstrated that the oxygen isotope is incorporated into the B29 carbonyl group of the insulin ester product. This result, together with those of appropriate controls, supports the conclusion that the transformation occurs, in the system studied, by a mechanism involving hydrolysis followed by coupling, and not by direct transpeptidation as has been previously found the case for another such system [Markussen & Schaumberg (1983) in *Peptides 1982* (Bláha & Maloň, eds.), pp. 387–394, W. de Gruyter, Berlin and New York].

The semisynthetic transformation of pig insulin into insulin of human sequence is a subject of considerable academic and commercial interest (see the references cited by Rose *et al.*, 1983*a*). Several different semisynthetic approaches have been tried, but the most promising in terms of simplicity, speed and efficiency involves incubation of pig insulin, in a medium containing an ester of threonine, with an enzyme catalyst (usually trypsin). Such an approach leads to accumulation of an ester of insulin of human sequence and avoids the need to prepare and isolate des-Ala-B30-insulin or des-octapeptide-B23-30-insulin (see Rose *et al.*, 1983*a*, and references cited therein) or other truncated insulins (Gregory & Walton, 1976; Seikagaku Kogyo, 1982).

A partial reaction scheme for the enzyme-catalysed transformation is shown in Scheme 1, and is

Abbreviations used: Thr(Bu^t)-OBu^t, *O*-t-butylthreonine *t*-butyl ester; h.p.l.c., high-pressure liquid chromatography.

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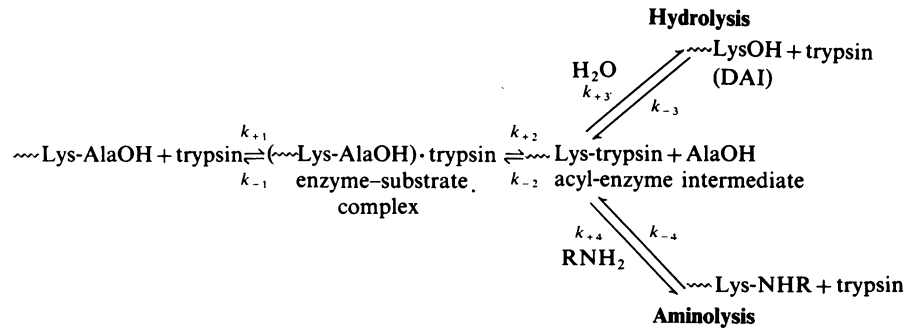

~~ Lys-AlaOH = pig insulin
RNH_2 = Thr(Bu^t)-OBu^t
DAI = des-Ala-B30-insulin

Scheme 1. *Partial reaction scheme for the trypsin-catalysed transformation of pig insulin into an ester of insulin of human sequence*

adapted from Gattner *et al.* (1980) and Widmer *et al.* (1981). Gattner *et al.* (1980), assuming that $k_{+3} \gg k_{-3}$ and $k_{+4} \gg k_{-4}$, state that the ratio of aminolysis to hydrolysis depends on the ratio of k_{+4}/k_{+3} (as well as on the concentration of the nucleophile RNH_2 and on the proportion of organic solvent, which diminishes the concentration of water). This is indeed the case, although it should be made clear that the statement refers to partitioning of the acyl-enzyme intermediate and not to product accumulation.

Under the range of coupling conditions generally employed, the reaction, which is under thermodynamic control and subject to the Law of Mass Action (Petkov, 1982), leads to high yields of coupled product whether insulin or des-Ala-B30-insulin is used as starting material. Also, the des-Ala-B30-insulin couples more rapidly (Rose *et al.*, 1983*a*; Markussen & Schaumburg, 1983). This means that were the acyl-enzyme intermediate to partition strongly towards hydrolysis, such that des-Ala-B30-insulin were to be an intermediate on the reaction pathway, the concentration of des-Ala-B30-insulin would be low.

For the semisynthetic transformation of pig insulin into insulin of human sequence, three extreme mechanistic cases may now be considered: (i) the reaction proceeds via aminolysis of the acyl-enzyme intermediate (i.e. via transpeptidation) without prior hydrolysis to des-Ala-B30-insulin; (ii) pig insulin is hydrolysed to des-Ala-B30-insulin, which then undergoes immediate coupling to give product; (iii) pig insulin, and thereafter acyl-enzyme intermediate, is hydrolysed reversibly to des-Ala-B30-insulin, and on average only after many cycles of acyl-enzyme$\rightleftharpoons$des-Ala-B30-insulin is the acyl-enzyme aminolysed to product.

Evidence, based on ^{17}O-n.m.r. studies, has been presented to support mechanism (i) (Markussen & Schaumburg, 1983).

The three mechanisms outlined above may be distinguished by observing the incorporation of ^{18}O into product (and starting material). The results of such experiments are reported in the present paper.

Materials and methods

Enzyme-assisted couplings

These were carried out by using techniques and materials as previously described (Rose *et al.*, 1983*a*). A mixture of Thr(Bu^t)-OBu^t (231 mg), butane-1,4-diol (710 μl) and acetic acid (40 μl) was prepared, ensuring thorough mixing. To 10 mg of zinc-free pig insulin was added 150 μl of this mixture followed by 5 μl of $H_2{}^{18}O$ (99 atom% ^{18}O; ICN), and the stoppered tube was incubated at 37°C for 10 min with occasional agitation: the insulin disperses but does not dissolve fully. During this time a solution of trypsin (1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated; 125 mg/ml) was prepared in $H_2{}^{18}O$, and 5 μl was added to the reaction mixture with immediate and rapid mixing. The tube was again stoppered and incubated at 37°C with occasional mixing: the sample soon dissolves completely. After 10 min, 75 μl of the sample solution was withdrawn and quenched with an equal volume of acetic acid. The insulin fraction was then isolated by gel filtration on a column (60 cm × 1 cm diam.) of Sephadex G-50 (fine grade; Pharmacia) eluted with 1% (v/v) acetic acid at a flow rate of 50 ml/h, and was recovered by freeze-drying. The yield was 4.4 mg. Similar experiments were performed, for control purposes, with des-Ala-B30-insulin [prepared in NH_4HCO_3 solution by the procedure of Schmitt & Gattner (1978)] and with a sample of pure semisynthetic human insulin ether ester previously prepared with unlabelled water. Portions of all three samples were then deprotected with trifluoroacetic acid (no scavenger present) as previously described (Rose *et al.*, 1983*a*). Analytical electrophoresis on cellulose acetate (Rose *et al.*, 1983*a*) before and after deprotection showed in the case of pig insulin starting material that the coupling reaction had proceeded, in the 10 min reaction time, to an extent of about 50%, and almost quantitatively in the case of des-Ala-B30-insulin starting material; the preformed ether ester sample appeared unchanged by the incubation with Thr(Bu^t)-OBu^t, as expected.

Isolation of the C-terminal peptide of the B-chain

A 1.2 mg portion of each of the three deprotected samples was dissolved separately in 200 μl of 1% (v/v) formic acid, and 12 μl of pepsin solution (1 mg/ml in water was added). After 2 h at 37°C a further 12 μl of pepsin solution was added, and digestion continued for a further 2 h. Analytical high-voltage paper electrophoresis at pH 6.5 of 5 μl of each digest showed the presence of a single basic spot of relative mobility (Offord, 1966) $m_{Asp} = 0.33$, staining red with cadmium/ninhydrin and giving a positive reaction with the α-nitroso-β-naphthol test for tyrosine (Offord, 1969). [Tyr-Thr-Pro-Lys-Thr/Ala, m_{Asp} = approx. 0.37, calculated by the method of Offord (1966).] An amount (20 μl) of leucine aminopeptidase suspension (5.41 mg/ml, approx. 200 units/mg; Serva, Heidelberg, Germany) was centrifuged and the pellet was dissolved in 200 μl of 1% (w/v) NH_4HCO_3 containing $MgCl_2$ (1 mM in Mg^{2+}). The peptic digests were dissolved in 200 μl of 1% (w/v) NH_4HCO_3 (no $MgCl_2$), and 10 μl of enzyme solution was added to each sample. After incubation for 22 h at 37°C, analytical high-voltage paper electrophoresis at pH 6.5 showed the

presence of a basic spot staining first yellow with cadmium/ninhydrin and later turning red. This spot, present in all three samples, had a relative mobility of $m_{Asp} = 0.41$ [Thr-Pro-Lys-Thr/Ala, m_{Asp} = approx. 0.48, calculated by the method of Offord (1966)]. The corresponding peptides from all three samples were obtained by preparative paper electrophoresis at pH6.5 (staining guide strips with cadmium/ninhydrin) followed by centrifugal elution with 1% (v/v) acetic acid and freeze-drying. The dry samples were then dissolved in aq. 0.1% trifluoroacetic acid (100μl), and approx. 40μl was injected into an h.p.l.c. system (Waters) consisting of a model U6K injector, M6000A and M45 pumps, model 680 programmer, a Radialpak C_{18} cartridge (10μm particles) in a Z module, and a model 440 absorbance detector with a prior pass through an extended wavelength module (214nm). The column, equilibrated with aq. 0.1% trifluoroacetic acid, was eluted at 2ml/min for 5min, after which time a linear gradient of pure acetonitrile was started (1%/min for 10min), with detection at 214nm. Peptide fractions were collected manually at the detector-cell exit and freeze-dried. A second portion of the sample derived from the pig insulin coupling (which gave rise to two h.p.l.c. fractions, see below) was injected, and fractions were collected as before, with pooling of the two appropriate fractions before freeze-drying. The dry samples were dissolved in 100μl of 1% NH_4HCO_3, and 50μl portions were taken for g.l.c.–mass-spectrometric analysis. This would represent about 20nmol of peptide if all steps had been quantitative, and takes into account the coupling yield of about 50% in the case of the reaction in which the starting material was pig insulin.

G.l.c.–mass-spectrometric analysis

Sample and reagent preparation and g.l.c.–mass-spectrometric equipment and operating parameters were as previously described (Rose *et al.*, 1983*b*). *N*-Trifluoroacetyl-*NO*-permethyl derivatives were employed. For flame ionization detection, one-fiftieth of the sample was injected on to a fused silica column (6m × 0.32mm internal diam.) coated with the immobilized stationary phase CP Sil 5 CB (film thickness 0.38μm; Chrompak, Middelburg, The Netherlands). At 1 min after

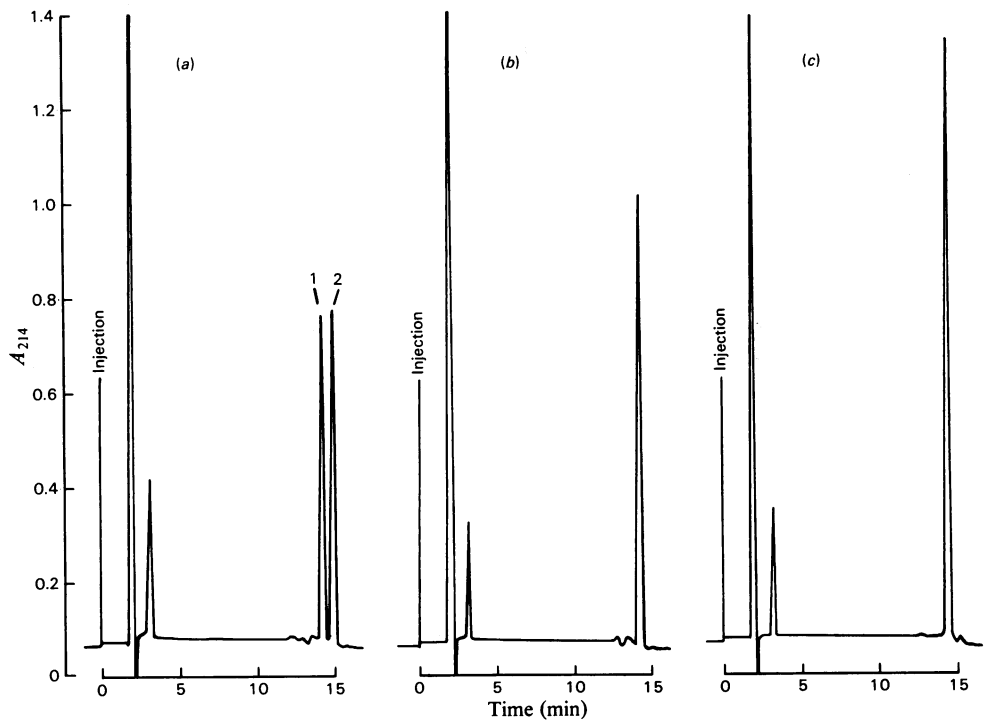

Fig. 1. *H.p.l.c. of the C-terminal peptides of the insulin B-chain*

(*a*) Thr-Pro-Lys-Thr (peak 1) and Thr-Pro-Lys-Ala (peak 2), from the coupling reaction with pig insulin as starting material. The chromatographic conditions for this and the following separations are given in the Materials and methods section. (*b*) Thr-Pro-Lys-Thr from the coupling with des-Ala-B30-insulin as starting material. (*c*) Thr-Pro-Lys-Thr from the incubation, under coupling conditions, of human insulin ether ester previously prepared in $H_2{}^{16}O$.

injection, the oven temperature was programmed linearly from an initial 80°C to 320°C at 6°C/min.

Results and discussion

Isolation of the C-terminal peptide of the B-chain

In the case of the coupling starting with pig insulin, which proceeded to an extent of about 50% in the 10min reaction time, the action of pepsin on the deprotected material produced the peptides Tyr-Thr-Pro-Lys-Ala and Tyr-Thr-Pro-Lys-Thr as the only basic peptides. These are not separable by electrophoresis under the conditions used. In the case of the other two samples, in which the coupling had started either with des-Ala-B30-insulin or with previously prepared human insulin ether ester, pepsin produced Tyr-Thr-Pro-Lys-Thr. Digestion with leucine aminopeptidase of the peptic products removed the tyrosine residue and left the corresponding tetrapeptides, further digestion being inhibited by the X-Pro sequence. As expected, no separation of Thr-Pro-Lys-Ala and Thr-Pro-Lys-Thr was achieved by electrophoresis under the conditions used, and the preparative electrophoresis step served merely to remove unwanted amino acids, peptides and salts. A preparative separation of the two peptides was achieved by h.p.l.c. (Fig. 1*a*), with the alanine-containing peptide being eluted later, as expected from its less-polar nature. The ratio of the peak areas of these two peptides, approx. 1:1, agrees well with the estimate of the coupling yield based on cellulose acetate electrophoresis.

G.l.c.–mass-spectrometric analysis

The mass spectra obtained are shown in Figs. 2–5. In the Figure legends and in the discussion of the mass spectra that follows, it is to be understood that the conventional three-letter code is used to denote *NO*-permethylated amino acid residues, and that the α- and ε-amino groups of the peptide derivatives carry the trifluoroacetyl group (Rose *et al.*, 1983*b*).

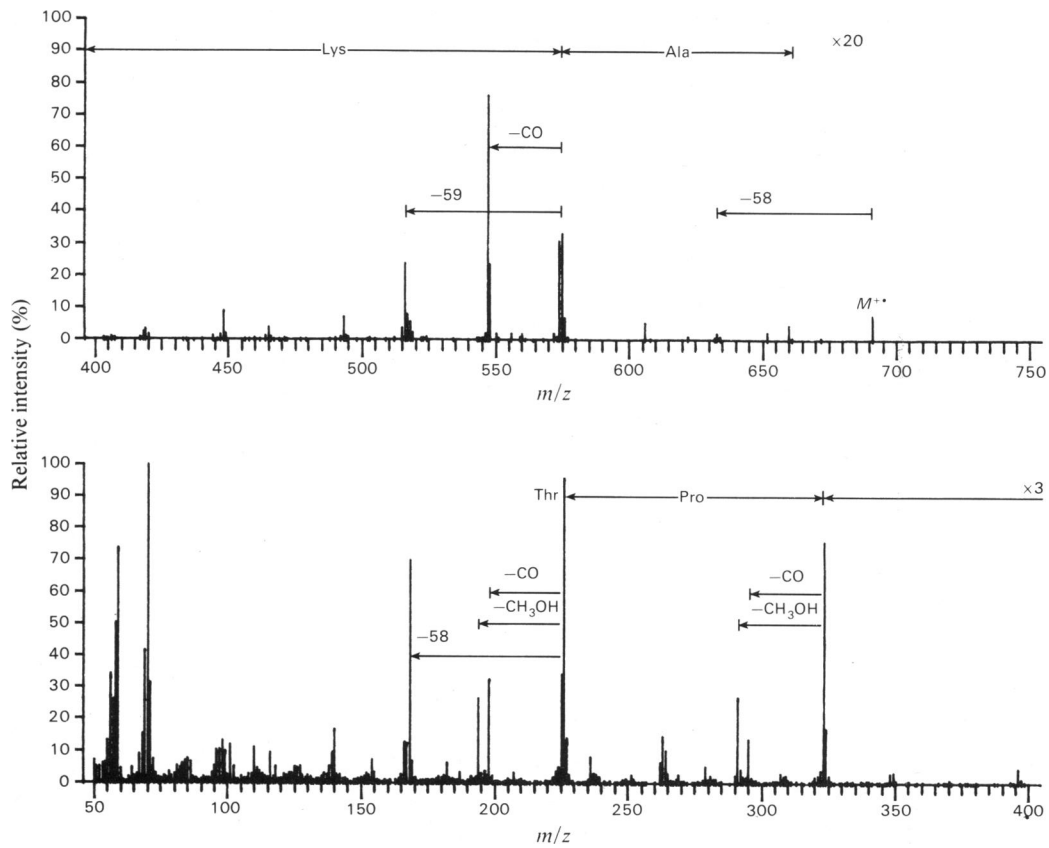

Fig. 2. *Mass spectrum of Thr-Pro-Lys-Ala*

The peptide was recovered as peak 2 by h.p.l.c. (Fig. 1*a*) and originated from the coupling employing pig insulin as starting material. Interpretation of the spectrum is straightforward and follows well-established rules (e.g. Rose, 1979; Dell & Morris, 1981).

Mechanistic interpretation

Fig. 2 is the mass spectrum of Thr-Pro-Lys-Ala recovered as the second h.p.l.c. fraction from the pig insulin coupling. The coupling reaction had been deliberately stopped short of completion (10min reaction time, corresponding approximately to $t_{\frac{1}{2}}$ for the reaction) in order to collect a significant amount of this unchanged material. Since in the same time and under the same conditions coupling to des-Ala-B30-insulin is essentially quantitative, it is clear that the rate-limiting step for the semisynthetic conversion of pig insulin into human insulin ether ester precedes the partitioning of the acyl-enzyme intermediate, and is probably (Petkov, 1982) the formation of this intermediate (see Scheme 1). Fig. 2 shows essentially no incorporation of ^{18}O into the carbonyl group of the lysine residue of Thr-Pro-Lys-Ala, and serves as a control. Incorporation of ^{18}O would have shifted upwards by 2 mass units the signals at *m/z* 575, 633, 660 and 691. Relative intensities beyond the scale-expansion factor of 20 at *m/z* 675 are not very reliable, so we shall confine our attention, in the discussion that follows, to the strong signals in the region of *m/z* 575. Accompanying the signal at *m/z* 575, which corresponds to the sequence Thr-Pro-Lys, is a signal of similar intensity at *m/z* 574. This lower-mass species, formally due to loss of H from *m/z* 575 (Rose, 1979; Dell & Morris, 1981), contributes to the intensity at *m/z* 575 through its ^{13}C isotope peak. The intensity of the signal at *m/z* 575 therefore represents the sample concentration (*a*) as the function $a(1+c)$, where *c* is the ^{13}C contribution due to *m/z* 574. Similarly, formal loss of H from *m/z* 577 (Thr-Pro-[^{18}O]Lys) leads to a signal at *m/z* 576 similar in intensity to that at *m/z* 577 (see below), and contributes to the intensity at *m/z* 577 through its ^{13}C isotope peak. The intensity of the signal at *m/z* 577 therefore represents the ^{18}O-labelled sample concentration (*b*) as the function $b(1+c)$. Since the factor $(1+c)$ cancels, we may simplify the discussion that follows and consider the relative intensities of the signals at *m/z* 575 and 577 to represent the relative amounts of Thr-Pro-Lys and Thr-Pro-[^{18}O]Lys sequences present respectively.

Fig. 3 is the mass spectrum of Thr-Pro-Lys-Thr,

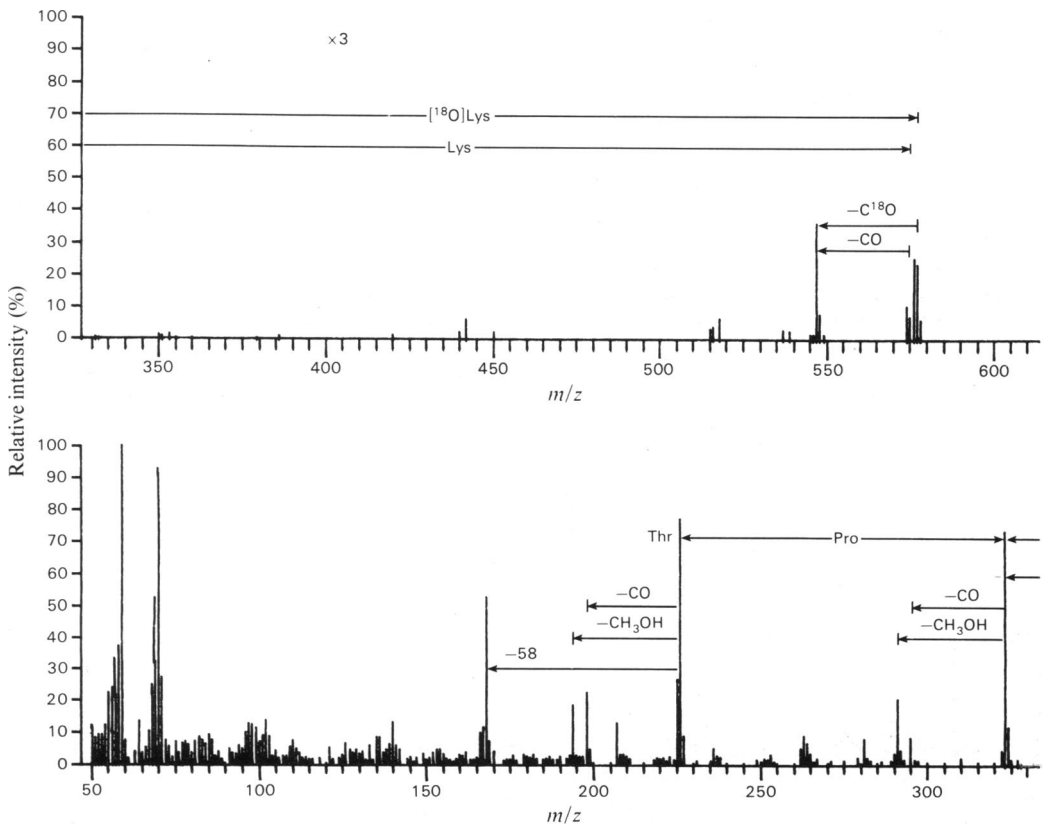

Fig. 3. *Mass spectrum of Thr-Pro-Lys-Thr*

The peptide was recovered as peak 1 by h.p.l.c. (Fig. 1*a*) and originated from the coupling with pig insulin as starting material. The molecular-ion region was too weak to be recorded.

recovered as the first h.p.l.c. fraction from the pig insulin coupling. The relative intensities of the signals at *m/z* 575 and 577 show that the incorporation of ^{18}O into the carbonyl group of the lysine residue is about 75%; this value did not increase on leaving the reaction to proceed for 90min (results not shown). Since the amount of $H_2{}^{18}O$ added to the reaction mixture is so small [1 μl per mg of insulin and per 15 μl of diol/Thr(Bu^t)-OBu^t/acetic acid], the residual amount of $H_2{}^{16}O$ that would have been associated with the insulin solution is significant and serves to dilute the label. Nevertheless, we are able to decide between the mechanistic extremes mentioned in the introduction.

We exclude mechanism (i), since it would not lead to any ^{18}O incorporation. We exclude mechanism (ii), since it would lead to a maximum of about 50% incorporation (the labelled oxygen atom incorporated on hydrolysis is one of two, and one is subsequently lost on product formation). Mechanism (iii) explains the high incorporation of ^{18}O observed and is taken to be the major route to the product.

Two further experiments were performed that confirmed this proposal. In one, des-Ala-B30-insulin, a presumed intermediate, was found to give rise to product containing ^{18}O in the carbonyl group of Lys-B30 (Fig. 4). The extent of incorporation, about 75% as judged by the relative intensities of the signals at *m/z* 575 and 577, is similar to that obtained with pig insulin as starting material (Fig. 3), as expected if mechanism (iii) is the correct one. In the other experiment, human insulin ether ester that had been produced and isolated with the use of $H_2{}^{16}O$ was then incubated under coupling conditions containing $H_2{}^{18}O$. Some incorporation of ^{18}O into the Lys-B30 residue was observed (Fig. 5), this being about 32% as judged by the relative intensities of *m/z* 575 and 577. This shows that the product of the coupling is itself a substrate and that in the course of exchange of the Thr(Bu^t)-OBu^t at least part of the material passes via des-Ala-B30-insulin. [Had the Thr(Bu^t)-OBu^t used carried a different label, say $^2H_5$, it would have been possible to compare the rate of exchange of this derivative with the rate of incorporation of ^{18}O

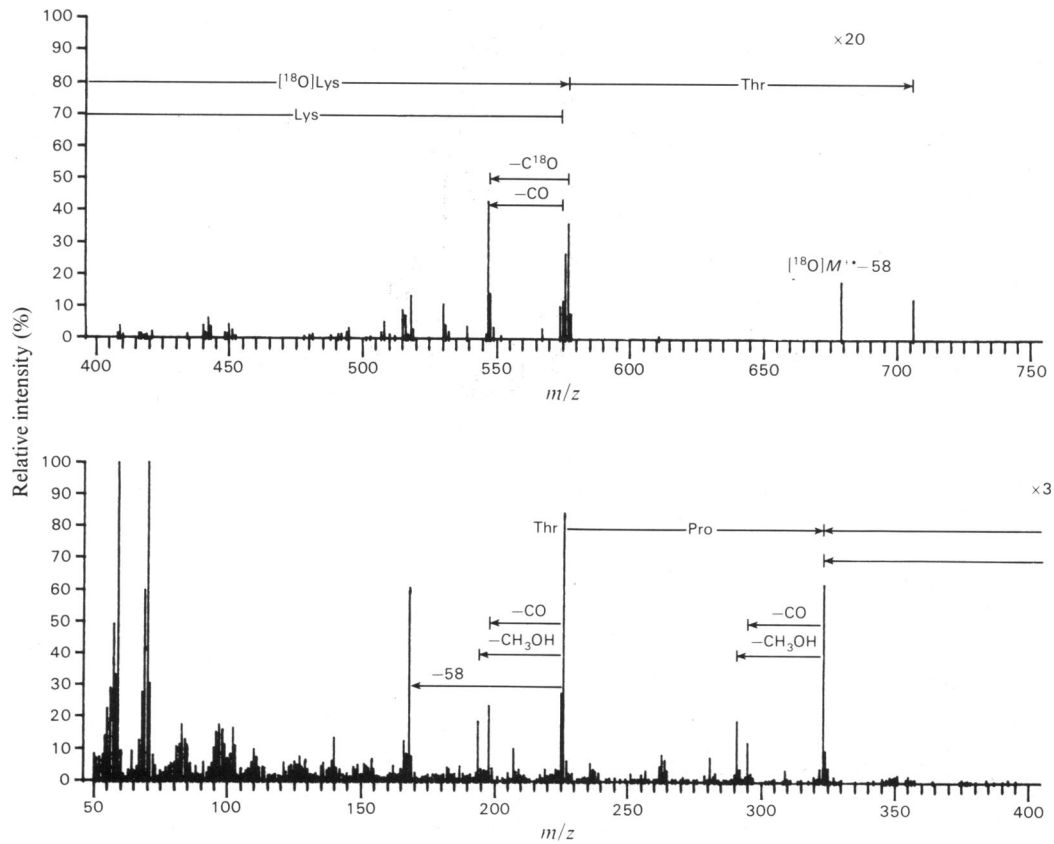

Fig. 4. *Mass spectrum of Thr-Pro-Lys-Thr*

The peptide was isolated by h.p.l.c. (Fig. 1*b*) and originated from the coupling employing des-Ala-B30-insulin as starting material.

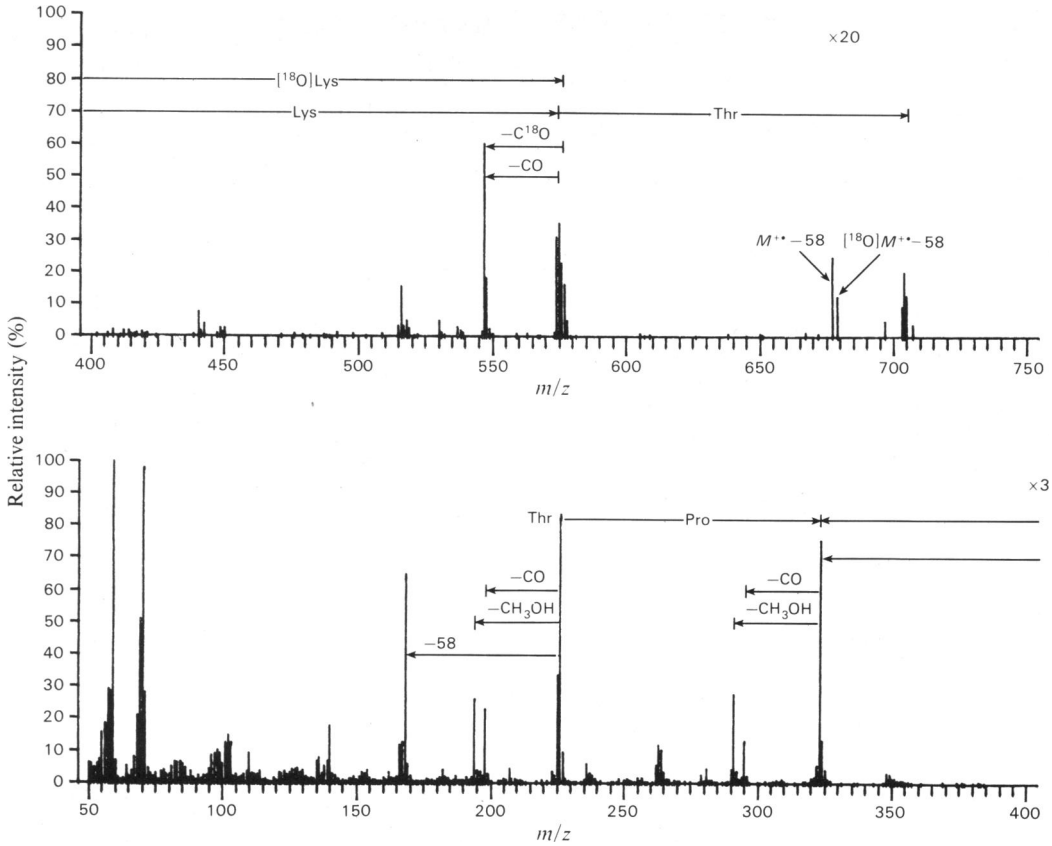

Fig. 5. *Mass spectrum of Thr-Pro-Lys-Thr*

The peptide was isolated by h.p.l.c. (Fig. 1*c*) and originated from the incubation, under coupling conditions, of human insulin ether ester previously prepared in $H_2{}^{16}O$.

into Lys-B30.] From Fig. 5 it is clear that the incorporation of ^{18}O into human insulin ether ester observed in Figs. 3 and 4 cannot be explained by exchange into product once formed. Mechanism (iii) therefore seems well established as the major route to product under the conditions described. These conditions, described in the present paper, were exactly those used to produce human insulin ether ester in very high yield (see Fig. 2 of Rose *et al.*, 1983*a*). It would appear that the present work constitutes an exception to the proposal by Markussen & Schaumburg (1983) that, for such couplings to occur in high yield, the rate of hydrolysis of the acyl-enzyme intermediate must obligatorily be much lower than that of its aminolysis.

Conclusion

We have established that, under a defined set of conditions, the enzyme-assisted semisynthetic transformation of pig insulin into human insulin ether ester does not proceed via direct transpeptidation, but rather via the hydrolysed intermediate des-Ala-B30-insulin. By using ^{17}O-n.m.r., Markussen & Schaumburg (1983) found, for a different solvent system and a different ester of threonine, that the mechanism is transpeptidation in this case. Until the mechanism has been established for any particular set of conditions, we suggest that the term 'transpeptidation' (e.g. Jonczyk & Gattner, 1981; Oka *et al.*, 1983) be replaced by the term 'transformation'.

In addition to providing mechanistic information, the present work permits a ready synthesis of [^{18}O]Lys-B30-labelled human insulin. Exhaustive drying, or exchanging with $H_2{}^{18}O$, of proteins and solvents before reaction would be expected to increase the degree of enrichment (about 75%) still further. ^{17}O could also be incorporated, for n.m.r. purposes, and the synthesis is economical, since 0.8–1 mg of protein is obtained/μl of labelled water.

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