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The preparation of porcine (Tyr-A[14]-[2]H) insulin and its characterization by mass spectrometry

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The title compound was prepared by catalytic deiodination of (Tyr-A[14]-3-I) insulin with deuterium gas. Under special conditions (large excess of PdO in CH_3OD/D_2O at pH 9.2) developed to minimize problems due to poisoning of the catalyst, deuterated insulin was produced in yields of about 35% after purification by reversed phase HPLC. For analysis, the deuterated insulin was digested with V8 protease and was shown by mass spectrometry to have incorporated deuterium to an extent of 96.6 atom %, exclusively in a pentapeptide containing Tyr A[14]. The title compound should prove useful to those workers studying insulin by mass spectrometry, and use of the method with tritium gas in place of deuterium should permit the preparation of a specifically labelled radioactive insulin analogue which behaves identically to the natural hormone.

Key words: deuteration/fast atom bombardment mass spectrometry/insulin/isotopic labelling

Introduction

In comparison with polypeptides substituted with exogenous elements (e.g. ^{125}I), polypeptides which differ only from the natural form in possessing a different isotopic distribution can be expected to behave more authentically. In the case of insulin, replacement of ^1H by ^3H using semisynthetic techniques has yielded analogues which have provided a considerable amount of information on the pharmacokinetics of the hormone (Berger *et al.*, 1979). In addition, both radioactive analogues and analogues possessing stable isotope substitutions have proved useful in studying the degradation of the hormone *in vitro* in the presence of insulin proteinase (Savoy *et al.*, 1988). The sensitivity of detection of peptides by mass spectrometry is now approaching levels which would permit the use of stable-isotope labelled analogues in pharmacokinetics experiments. Up to now, specific isotope labelling of insulin has been confined to positions Gly-A^1, Ile-A^2, Ile-A^{10}, Phe-B^1, Phe-B^{24}, Phe-B^{25}, Tyr-B^{26} and Lys-B^{29}, and has involved semisynthesis or biosynthesis (Berger *et al.*, 1979; Misbin *et al.*, 1981; Kaufmann *et al.*, 1985; Savoy *et al.*, 1988). Therefore, methods are required which permit the production of insulin analogues labelled with tritium and deuterium in positions other than those easily accessible by semisynthetic techniques, particularly in the A chain.

The metal-catalyzed replacement of iodine with hydrogen isotopes is a frequently used method of preparing analogues of peptides containing tyrosine (Brundish and Wade, 1981). Unfortunately, when sulphur-containing amino acid residues are present, catalyst poisoning and side reactions (desulphurization) are to be expected. We find, however, that with increasing excess of palladium catalyst, the deiodination reaction is favoured, so that a practical preparation of (Tyr-A^{14}-3-^2H) insulin in a substantial yield becomes feasible.

A preliminary presentation of part of this work has been given at the 10th American Peptide Symposium (Halstrøm *et al.*, 1987).

Materials and methods

All solvents and reagents were of analytical grade or better, and were used without further purification unless otherwise stated.

Porcine insulin (monocomponent grade) was the product of Novo, Denmark, palladium (II) oxide ('Gold label', 99.999%, lot no. TM 04616LM) was from Aldrich, methanol-d_1 (>99.5 atom % D) from Sigma, deuterium gas (>99.5 atom % D) and deuterium oxide (>99.75 atom % D) were both from Merck.

Catalytic deiodination

(Tyr-A^{14}-3-I) insulin was prepared by iodination of porcine monocomponent insulin by the lactoperoxidase method (Thorell and Johanson, 1971), and purified by a combination of anion exchange chromatography on QAE-Sephadex A-25 and reversed phase HPLC on C18 silica, as used earlier for isolation of highly purified (Tyr-A^{14}-3-I) insulin (Jørgensen *et al.*, 1982). The material was found to be ~99% pure by analytical reversed phase HPLC.

Prior to catalytic deiodination, exchangeable hydrogen was exchanged for deuterium. Thus, the iodo insulin (10 mg) was dissolved in D_2O (3 ml) by addition of dilute trifluoroacetic acid to pH 3, and the solution was freeze-dried.

Catalytic deiodination was performed by dissolving the freeze-dried product in CH_3OD (1 ml), followed by 0.6 M $NaHCO_3$ in D_2O (1.6 ml) in a 5 ml round-bottom flask. After addition of a teflon-coated magnetic stirring bar and PdO (1000 mg), atmospheric air was removed by applying a slight vacuum. After connection to the deuterium gas supply, the mixture was vigorously stirred at ambient pressure and temperature for 1 h. The catalyst was allowed to settle, and the supernatant immediately filtered on Millex GV. The catalyst was washed once with 1 ml 50% methanol. Removal of methanol *in vacuo* from the combined filtrates was followed by purification of the deuterated insulin by semi-preparative isocratic reversed phase HPLC using a 7 μm C18 100 Å 25 × 0.8 cm column and 36 vol. % ethanol acidified with 0.06 vol. % trifluoroacetic acid. After concentrating and freeze-drying the insulin-containing fraction, the purity of the product (3–4 mg) was found to be ~98% by analytical reversed phase HPLC on a Novapak C18 column, using 0.15 M ammonium sulphate, pH 3, with an acetonitrile gradient (18–45%). The deuterated insulin co-eluted with porcine monocomponent insulin.

Deuterium content

In separate experiments, deuterated insulin and control (undeuterated insulin), freed from zinc, were dissolved at a

Table I. Incorporation of deuterium (^2H) as measured by FAB/MS

Peptide	Atom % $^2H^a$
A^{1-4}	−0.12 ± 0.11 (15)
A^{5-12}	−0.54 ± 0.28 (13)
A^{13-17}	96.60 ± 0.08 (15)
$A^{18-21}-B^{14-21}$	0.89 ± 0.79 (15)
B^{1-13}	1.26 ± 1.99 (15)
B^{14-21}	0.20 ± 0.65 (15)
B^{22-30}	1.30 ± 0.40 (15)

The table shows values obtained for deuterium incorporation into various peptides isolated by HPLC after digestion of deuterated insulin with V8 protease. The conditions of deuteration were those giving highest incorporation (see Table II).
[a]Values are given ± the standard error of the mean (SEM). The number of spectra used in each calculation is given in parentheses.

Table II. Incorporation of deuterium (^2H) into Tyr-A^{14} as a function of conditions

Conditions	Yield (mol % insulin)	Atom % ^2H
pH = 4.2 Protic solvent	25−35	41
pH 9.2 Protic solvent	30−40	56
pH 9.2 Deuterated solvent	30−40	96.6

(Tyr-A^{14}-3-I) insulin was catalytically deiodinated in the presence of deuterium gas, under 3 sets of conditions: (i) As in Materials and methods, but instead of 0.6 M $NaHCO_3$, 0.5 M triethylammonium acetate was used, and the mixture with methanol was adjusted to an apparent pH of 4.2 by addition of acetic acid. Also, non-deuterated solvents were used. (ii) As in Materials and methods, but using non-deuterated solvents. (iii) As in Materials and methods. pH values are apparent pH, measured in the aqueous-methanolic solution before and after the reaction, with a combined glass electrode, adjusted with aqueous standard buffers. The yield of deuterated insulin (isolated by reversed phase HPLC) is given based on starting (Tyr-A^{14}-3-I) insulin. The degree of incorporation of deuterium into Tyr-A^{14} is also shown.

concentration of 200 μM in ammonium bicarbonate solution (1% w/v) and were digested with *Staphylococcus aureus* V8 protease (Cooper Biomedical) at an enzyme:substrate ratio of 1:60 (w/w) at 37°C for 4 h. After removal of solvent and ammonium bicarbonate in a vacuum centrifuge (Speed Vac, Savant Instruments), samples were subjected to reversed phase HPLC and to analysis by fast atom bombardment mass spectrometry (FAB/MS).

HPLC was performed on equipment previously described (Rose *et al.*, 1988) using either a μBondapak C_{18} cartridge in a Z module (Waters) or a Nucleosil 5 μm C8 300 Å 25 × 0.4 cm column (Macherey Nagel) operated at a flow rate of 1 ml/min. Solvent A was 1 g HPLC-grade trifluoroacetic acid (Pierce) dissolved in 1 l HPLC-grade water (MilliQ system), and Solvent B was HPLC-grade acetonitrile (Merck). The column was equilibrated with Solvent A, the sample was injected, and after 5 min at 100% Solvent A, a linear gradient (1%/min for 50 min) of Solvent B was applied. The effluent was monitored at 214 nm, and fractions absorbing at this wavelength were collected manually. After solvent removal in the vacuum centrifuge, samples were examined by mass spectrometry.

FAB/MS was performed as previously described (Savoy *et al.*, 1988). Bioassay was carried out by the mouse blood glucose assay, using the international standard.

Results and discussion

Deuterium content

From the method of deuteration employed, it was expected that deuterium would be incorporated upon deiodination of the (Tyr-A^{14}-3-I) insulin. However, owing to the unfavourable kinetic isotope effect, the atomic abundance of deuterium in the product was expected to be lower than that of the deuterium gas used. In addition, non-specific incorporation in other residues, in particular tyrosine and histidine, could not be excluded *a priori*. Mass spectrometry was employed to localize the label and to quantify the degree of incorporation.

Digestion with V8 protease followed by HPLC permits isolation of peptides covering all of the sequence of porcine insulin (Rose *et al.*, 1988). In order to obtain sufficient precision in the measurement of isotopic abundance, the peptides produced by V8 digestion were examined separately byFAB/MS, taking 13 or 15 spectra over the molecular ion region. Each fragment from the deuterated insulin and from the control (undeuterated) was examined twice in this way. The extent of deuteration was calculated from these spectra. Table I shows the extent of incorporation of deuterium into the peptides examined. The only peptide found to have incorporated appreciable amounts of deuterium was that containing residues A^{13}-A^{17}, which includes Tyr-A^{14}. The sequence of the peptide, Leu-Tyr-Gln-Leu-Glu, contains no other residues capable of incorporating deuterium under the conditions employed, so we may deduce that incorporation into Tyr-A^{14} proceeded specifically. The possibility that the mass increase (1 a.m.u.) of this peptide might have been due to deamidation of residue Gln-A^{15} was excluded by subjecting the peptide to electrophoresis at high voltage on paper at pH 6.5: the peptide migrated with a relative mobility of −0.25 relative to aspartic acid = −1. This value is close to that calculated (Offord, 1966) for the nondeamidated peptide (relative mobility −0.34) and very different from that calculated for the deamidated sequence (−0.64).

Catalytic deiodination

Table II shows the degree of incorporation of deuterium into Tyr-A^{14} found under various conditions. Under the best conditions tried, specific incorporation of 96.6 atom % ^2H was achieved.

The most important parameters affecting the yield appear to be the quantity and the quality of the PdO employed. The most consistent yields were obtained using the grade of PdO specified in Materials and methods. Lower ratios of PdO to iodo insulin, such as the 1:1 ratio often employed in catalytic hydrogenations, invariably resulted in extended reaction times and low yields. Furthermore, if the reaction is allowed to continue beyond the point where about 10% of iodo insulin remains (HPLC), the insulin peak will decline, and eventually disappear altogether. This indicates the occurrence of competing reactions, e.g. irreversible adsorption to the catalyst, possibly after reduction of disulphide bridges. In the semi-preparative HPLC purification the only major impurity observed is remaining iodo insulin. One explanation for the apparently very simple composition of the reaction mixture might be the removal of degraded insulins by catalyst adsorption.

The potency of the product showing the highest incorporation of deuterium was assayed on an aliquot of a 1 mg/ml solution, the exact insulin concentration of which was determined by quantitative amino acid analysis. Potency found: 29.3 IU/mg (95% confidence limits: 34.2−25.1 IU/mg). The corresponding value for anhydrous and salt-free porcine monocomponent insulin is 29.0 IU/mg.

Conclusion

Using a special palladium catalyst under particular conditions, it is possible to produce insulin deuterated specifically at Tyr A^{14} by deiodination of the appropriate precursor. It would be expected that similar conditions, with appropriate precursors, would permit the preparation of insulin analogues deuterated specifically at any of the other three tyrosine residues.

By using tritium gas in place of deuterium, it should be possible to prepare specifically labelled insulin analogues of relatively high specific activity (15 Ci/mmol for 50% incorporation of one ^{3}H), analogues which should behave authentically.

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Note added in proof

By using tritium gas in place of deuterium, a labelled insulin analogue of relatively high specific activity (10–15 Ci/mmole) has been prepared (Halstrøm, Jørgensen and Kovacs, 1988, Abstract no.45, presented at 20th European Peptide Symposium Tubingen).