CROATICA CHEMICA ACTA CCACAA 57 (2) 289—294 (1984)

YU ISSN 0011—1643
CCA-1443 UDC 547.9
Original Scientific Paper

Crystalline Suspension of Bovine Zinc Des-AlaB30-Insulin with Prolonged Hypoglycaemic Activity

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Received July 11, 1983

The reversible reaction of cyclohexane-1,2-dione with the guanidino function of Arg-B22 in bovine insulin gave the dihydroxycyclohexylene [DHCH]-Arg-B22-insulin complex (I) which, after selective tryptic cleavage at the Lys-B29 residue and regeneration of the guanidino group, afforded bovine des-AlaB30--insulin (III) in an overal 85.8% yield. The truncated insulin III was converted into the regular zinc salt and, under conditions that favour binding of more than 2 Zn atoms per insulin hexamer unit, into a crystalline suspension. When tested for their hypoglycaemic activity, the two forms of III exhibited significantly different durations of the effect in mice: the blood glucose curves obtained for the III-Zn salt and the III-crystalline suspension were indistinguishable from those exerted by regular- and long--acting bovine zinc insulin, respectively.

INTRODUCTION

In a previous paper[1], we have shown that cyclohexane-1,2-dione, the bifunctional reagent used by Patthy and Smith[2–4] in a reversible modification of the arginyl residues in proteins, reacts specifically and completely with the guanidino function of the arginyl-B22 residue in porcine insulin to give the N^{7},N^{8}-(1,2-dihydroxycyclohex-1,2-ylene)-arginine-borate insulin complex ([DHCH-Arg-B22]-insulin) which, upon treatment with hydroxylamine, regenerates insulin of unchanged biological acticity. Incubation of the complex with trypsin led to selective cleavage at the Lys-B29 residue to give, after removal of the DHCH group, porcine des-AlaB30-insulin in high yield.[1] Morihara et al.[5] claimed that des-Ala-B30-insulin, obtained[6] by incubation of insulin with carboxypeptidase A, reacted with cyclohexane-1,2-dione to produce des-Ala--B30-[DHCH-Arg-B22]-insulin; the latter was converted by trypsin-catalyzed coupling into semisynthetic human insulin. In a study on the role of the Arg-B22 residue in insulin action, Rose et al.[7] prepared porcine [DHCH-Arg--B22]-insulin complex and examined its ability to lower blood sugar concentration.

In the presence of zinc ions, depending on the ionic strength and the nature of anions present in the solution, insulin gives two crystalline forms

which contain per insulin hexameric[8] unit 2 and 4 zinc atoms, respectively; crystallographic studies have established[9,10] that the *C*-terminal Ala-B30 is not included into regions of the hexamer packed helices. The effect of added zinc ions in the timing of insulin hypoglycaemic action has been investigated in detail, and preparations possessing a low solubility in tissue fluids and a prolonged duration of action have been introduced into insulin therapy[8]. Studies from several laboratories have shown that the Ala-B30 residue of insulin is not essential for biological activity of the molecule[6,11–13].

In view of the above findings it was of interest to synthesize bovine des-Ala-B30-insulin, to examine its ability to bind zinc under different experimental conditions, and to find out whether the products formed exhibit some different physical and biological properties.

RESULTS AND DISCUSSION

The reaction of cyclohexane-1,2-dione with the arginyl-B22 residue of bovine insulin to give the [DHCH-Arg-B22]-insulin complex (I) proceeded smoothly when either the Zn-free form or the 2Zn crystals were used as the starting material. However, bovine zinc insulin required a considerably higher proportion of pH = 9 buffer to attain dissolution than did the corresponding porcine insulin salt. Therefore, in order to avoid concentrations of large volumes of the solvent, we used the more soluble Zn-free form in preparative-scale experiments.

Variation of the reaction conditions previously employed[1] in the synthesis of porcine des-Ala-B30-insulin led to simplification and improvement of the whole procedure. The most notable changes were: (*a*) a decrease in reaction time required for the formation of the complex I (from 12 to 3 hr), (*b*) a decrease in the trypsin incubation-time needed for quantitative cleavage of the LysB29-AlaB30 peptide bond in I (from 20 to 8 hr), and (*c*) the use of even milder conditions for the removal of the DHCH group from I and des-AlaB30-[DHCH-ArgB22]-insulin (II) (0.1 M NH_2OH at room temperature, instead of 0.5 M NH_2OH at 37 °C).

The homogeneity of the DHCH-complex I was established by amino acid analysis (Table I) and electrophoresis; treatment of a sample with hydroxylamine regenerated bovine insulin of the original activity (mouse fall test). Trypsin digestion of I gave the des-Ala-B30-insulin complex II (Table I) which, after deblocking and chromatography of the product on a Sephadex G-50 column (fine), afforded bovine des-AlaB30-insulin (III, Table I) in an overall 85.8% yield, calculated on the starting Zn-free bovine insulin. The biological activity of III was equipotent to that of the parent insulin (mouse fall test).

Conversion of the truncated insulin III into the 2Zn form was effected by the standard procedure[14] used in the preparations of regular insulin zinc salts. Treatment of III under conditions that favour binding of more than two zinc atoms per hexamer unit was carried out by following essentially the sodium chloride-sodium acetate crystallisation method elaborated by Schlichtkrull[8]. Compound III behaved rather similarly to the identically treated bovine insulin and deposited at pH = 7.4 a crystalline suspension of rhombohedral crystals (10—40 μm diameter). The zinc content (2.17% w/w) of these crystals indicated the presence of additionally bound zinc atoms per unit cell.

The blood sugar-lowering activity of the two types of bovine des-Ala-B30-insulin zinc crystals was tested on alloxan-induced diabetic mice and

TABLE I

Amino Acid Ratios of Bovine [DHCH-Arg-B22]*-insulin Complex (I), Des-Ala-B30--insulin Complex II, and Bovine Des-Ala-B30-insulin (III)*[a]

Amino Acid Residue[b]		Found: I	II	III
Asp	(3)	3.00	3.00	3.00[c]
Thr	(1)	0.97	0.89	1.11
Ser	(3)	2.78	2.83	2.99
Glu	(7)	7.11	7.04	7.04
Pro	(1)	0.93	1.02	1.01
Gly	(4)	4.02	4.01	4.03
Ala	(3)	2.94	1.97	1.90
Cys	(6)	4.27	4.37	4.13
Val	(5)	3.78	3.79	3.60
Ile	(1)	0.19	0.21	0.17
Leu	(6)	5.97	6.02	5.92
Tyr	(4)	3.83	3.89	3.75
Phe	(3)	2.94	2.93	2.87
His	(2)	2.01	2.03	2.01
Lys	(1)	0.99	0.97	0.96
Arg	(1)	0.00	0.00	1.00

[a] Ratios are calculated with respect to Asp = 3.00, and the values are not corrected for destruction. Hydrolysis of samples was performed in 6 M HCl and in the presence of thioglycolic acid, if not stated otherwise. [b] The values in brackets refer to the number of amino acid residues in bovine insulin. [c] Hydrolysis performed without addition of thioglycolic acid.

compared in parallel experiments with the effects given by regular bovine zinc insulin and the long-acting insulin Novo Ultralente. As can be seen from Figure 1., the blood glucose curves exhibited by the standardly prepared III-zinc salt and bovine insulin were closely similar and typical of short-acting

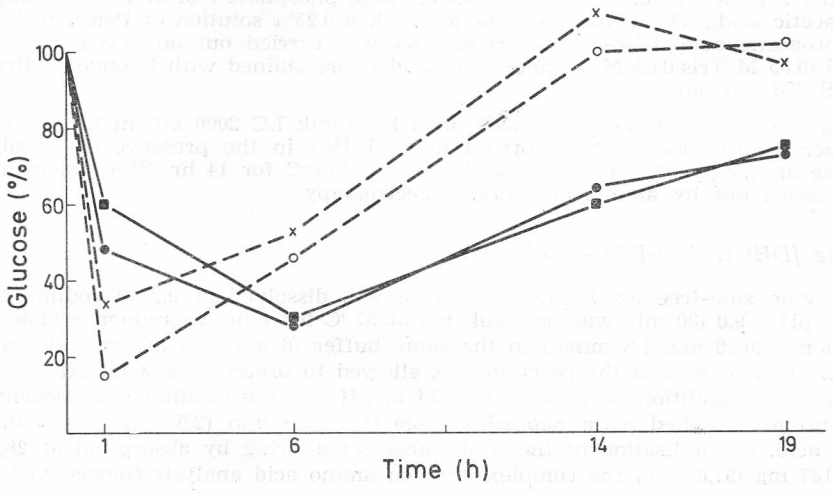

Figure 1. A comparison of the blood glucose response following the administration of: bovine des-Ala-B30-insulin (III)-zinc salt ○———○; bovine insulin zinc salt (PLIVA) ×———×; bovine des-Ala-B30-insulin (III)-crystalline suspension ●———●; insulin Novo Ultralente ■———■. Insulins were administered subcutaneously to alloxan-diabetic mice in doses of 4 I. U. per mouse; the values represent the mean response of 10 mice in each group.

insulin preparations. On the other hand, the glucose curve observed after injection of the III-crystalline suspension differed substantially from the curves given by the two former preparations and paralleled that exhibited by the insulin Novo Ultralente, at least for the times investigated.

From the present investigation, it can be concluded that removal of the C-terminal alanine residue from bovine insulin does not affect the ability of the molecule to exhibit two types of zinc binding. Moreover, conditions used for conversion of the intact molecule into insulin preparation with prolonged action showed to be also valid for the formation of a high-zinc des-Ala-B30--insulin crystalline suspension of very low solubility. The fact that the latter preparation exhibited a remarkably long duration of blood sugar-lowering effect in mice can be considered as additional evidence for the C-terminal alanine not being essential to insulin hypoglycaemic activity.

EXPERIMENTAL

Materials

Bovine insulin, crystalline zinc salt, was obtained from PLIVA (lot No. 1,431,124, 24.4 I.U./mg) and was used throughout. Bovine zinc-free insulin was prepared after Sluyterman[15] as already described[16] for porcine insulin. Insulin Novo Ultralente was from Novo, Denmark. Cyclohexane-1,2-dione and trypsin (bovine, Type XI, chymotrypsin free) were from Sigma, hydroxylamine hydrochloride p. a. from Merck, and thioglycolic acid from Fluka. Other chemicals were high-purity preparations from commercial sources.

Methods

All separations on columns were carried out at 4 °C. Products were recovered by evaporation of the eluates (by concentration, rotary evaporator) in a bath temp. 30—33 °C, followed by lyophilisation.

Electrophoresis on cellulose acetate was performed on strips (2.5 × 14 cm) in a standard Chemetron apparatus at 200 V at pH 4.8 (0.1 M pyridine/6 M urea buffer adjusted with acetic acid), and at pH 8.0 (0.065 M phosphate/7 M urea buffer adjusted with acetic acid). The strips were stained with a 0.25% solution of Ponceau S in 3% trichloroacetic acid. Disc-gel electrophoresis was carried out on acrylamide gel at pH 9.5 (0.05 M Tris/0.03 M glycine); the bands were stained with Coomassie Brilliant Blue R-250 (Sigma).

Amino acid analyses were made on a Biotronik LC 2000 automatic amino acid analyzer. Hydrolyses were performed in 6 M HCl in the presence (20 μmol) and absence of thioglycolic acid, in sealed tubes at 110 °C for 14 hr. Zinc determination was carried out by atomic absorption spectroscopy.

Bovine [DHCH-Arg-B22]-insulin (I)

Bovine zinc-free insulin (200 mg, 35 μmol), dissolved in 0.2 M sodium borate buffer, pH = 9.0 (30 ml), was preincubated at 37 °C for 1 hr whereupon cyclohexane--1,2-dione (201.6 mg, 1.8 mmol) in the same buffer (6 ml) was added under a slow stream of nitrogen and the reaction was allowed to proceed for 3 hr. After cooling (ice-bath) and addition of 15% acetic acid to pH = 3, the solution was concentrated and chromatographed on a Sephadex G-50 (fine) column (2.5 × 90 cm) with 1 M acetic acid. Lyophilisation of the peak tubes (monitoring by absorption at 280 nm) gave 187 mg (91.0%) of the complex I which amino acid analysis showed to be free of the starting insulin.

In the same treatment of bovine zinc insulin (50 mg, 8.7 μmol), 18 ml of the buffer was required for dissolution; the isolated I (52 mg) was identical to the product obtained from zinc-free insulin.

Bovine Des-Ala-B30-[DHCH-Arg-B22]-insulin (II)

A solution of freshly prepared complex I (180 mg, 31 μmol) in 0.1 M sodium borate buffer, containing 10 mmol $CaCl_2$, pH = 8.0 was incubated with trypsin (3.6 mg in 6.0 ml of the same buffer) in two equal portions at 37 °C; after the third hour of incubation, the second half of the enzyme solution was added (final ratio E : S = 1 : 50), and the digestion was allowed to process for an additional 8 hr. After cooling (ice-bath) and acidification (15% AcOH) to pH = 3, the digest was concentrated and fractionated on a Sephadex G-50 (fine) column (2.5 × 90 cm) with 1 M acetic acid. Lyophilisation of the main peak fractions gave II (170 mg, 95.6%) as a fluffy mass which was characterised by amino acid analysis.

Bovine Des-Ala-B30-insulin (III)

The complex II (170 mg, 29.8 μmol) was dissolved in 0.1 M hydroxylamine (34 ml), and the solution, adjusted with 0.1 M sodium hydroxide to pH = 7.0, was kept under a slow stream of nitrogen at room temperature overnight. After addition of 15% acetic acid to pH = 3, the solution was concentrated and chromatographed on a Sephadex G-50 (fine) column (2.5 × 90 cm) with 1 M acetic acid. Lyophilisation of the peak tubes gave 168 mg, 80% of III as a fluffy mass which was characterised by amino acid analysis.

A sample of III (15 mg) was converted into the 2 Zn salt by the procedure given by Petersen.[14]

Regeneration of Bovine Insulin From the Complex I

Treatment of a sample (5 mg) of I with 0.1 M hydroxylamine (1 ml) as already described, yielded 4.5 mg (90%) of bovine insulin whose electrophoretic mobility and biological activity (mouse fall test) were indistinguishable from those of the untreated bovine insulin.

Preparation of Bovine Des-Ala-B30-insulin Crystalline Zinc Suspension

Bovine des-Ala-B30-insulin (III, 120 mg) was dissolved in 0.015 M HCl (5.6 ml), and to the solution were added $ZnCl_2$ (2.5 mg, quantity equivalent to a total of 1% Zn^{2+} by weight of III) and 0.4 M sodium acetate, containing 28% NaCl and 0.4% methyl *p*-hydroxybenzoate, pH = 10 (1.9 ml). The turbid solution was adjusted to pH = 5.75, and the crystallisation medium was stirred at room temperature for 24 hr. The resulting crystalline suspension was diluted with 0.1% aq. solution (w/w) of methyl *p*-hydroxybenzoate (65 ml), and a second portion of $ZnCl_2$ (10 mg, quantity equivalent to 4% of Zn^{2+} by weight of III) was then added. The final preparation was obtained by addition of 0.1% solution of methyl *p*-hydroxybenzoate, with simultaneous adjustment of pH with 0.1 M NaOH, to make the volume up to 75 ml and the pH = 7.4; this preparation contained 40 I. U. of insulin/ml. The filtered crystals, dried in a vacuum desiccator to constant weight, contained 2.17% of Zn.

Biological Assays

Biological activity of insulins and derivatives was tested either by the mouse fall test, or by using *in vivo* blood glucose assay on alloxan diabetic mice[17]. Tetrahydric alloxan in saline (0.5% solution) was administered to mice intravenously (75 or 100 mg/kg) at least 2 days before subcutaneous injection of 4 I. U./mouse (groups of 10) of insulin preparations[18]. Blood samples were taken before, 1, 6, 14 and 19 hr after the injection, and glucose analyses were performed by the glucose oxidase/4--aminophenazone method[19].

Acknowledgement. — We are indebted to Dr. M. Slijepčević and Dr. M. Hadžija, Department of Biology, »Ruđer Bošković« Institute for carrying out the mouse fall tests.

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SAŽETAK

Kristalna suspenzija goveđeg cink des-Ala-B30-insulina sa produženom hipoglikemičkom aktivnošću

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Reverzibilna reakcija cikloheksan-1,2-diona i guanidino grupe Arg-B22 ostatka goveđeg insulina dala je kompleks I koji je nakon inkubacije sa tripsinom te regeneracije guanidino grupe dao goveđi des-Ala-B30-insulin kao jedini produkt. Pokazano je da se ovakova »osakaćena« molekula insulina može lako prevesti u regularnu 2 Zn sol, a da pod uvjetima koji pogoduju vezanju dodatnih atoma cinka daje suspenziju kristala (sadržaj 2.17% cinka) kao i nativni goveđi insulin. Ispitivanja hipoglikemičke aktivnosti ovih dviju preparacija u miševa pokazala su da je trajanje hipoglikemičkog efekta des-Ala-B30-insulin 2 Zn soli jednako onome nativnog goveđeg insulina, dok je efekt kristalne suspenzije des-Ala-B30-insulina mnogo duži i jednak onome koji izaziva insulin produženog djelovanja (Ultralente).