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Preparation of Des-alanine-B^{30}-insulin *via* the Tryptic Hydrolysis of Porcine Insulin Modified at the Arginyl Residue by Cyclohexane-1,2-dione

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The feasibility of the selective modification of the arginyl residue in insulin by the method of Patthy and Smith (*J. Biol. Chem.* **250** (1975) 557—564; *ibid.* 565—569), involving the reversible reaction of cyclohexane-1,2-dione with the guanidino function of arginine in a borate buffer, was studied. It was found that cyclohexane-1,2-dione reacts specifically and completely with the arginyl residue of porcine insulin in 0.2 M borate buffer at pH = 9.0 to form a single addition product ([DHCH-Arg-B^{22}]-insulin complex I) which, upon treatment with 0.5 M hydroxylamine at pH = 7.0, regenerated insulin of unchanged biological activity. Incubation of I with trypsin led to specific cleavage at the Lys-B^{29} residue to give des-Ala-B^{30}-[DHCH-Arg-B^{22}]-insulin complex II, which, upon hydroxylamine treatment afforded des-Ala-B^{30}-insulin (III) in high yield.

INTRODUCTION

Porcine, bovine and human insulin contain a single arginyl residue occurring at position 22 of the B chain. Among various bifunctional reagents used[1] in reversible modification of the arginyl residues in proteins, cyclohexane-1,2-dione has attracted considerable attention[2]. Patthy and Smith[3,4] demonstrated the reagent that reacts completely with the guanidino function of arginine at pH 8—9 in sodium borate buffer to give a single product, N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene (DHCH) arginine-borate complex, which could be regenerated quantitatively by strong nucleophiles, such as hydroxylamine, under mild reaction conditions. They also showed that the DHCH-Arg residue in modified proteins is resistant to the action of trypsin, thus restricting hydrolysis to the peptide bonds involving lysine. The procedure has been repeatedly employed for the recognition of functionally significant arginyl residues in enzymes[2,5], parathyroid hormone[6], serum albumin[7] and lipoproteins[8], however, to our knowledge, it has not been used so far in modification of the

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* We thank one of the referees of drawing our attention to the paper by K. Morihara, T. Oka, H. Tsuzuki, K. Inouye, and S. Sakakibara, presented at the 6th American Peptide Symposium (Summer 1979), in which the reaction of cyclohexane-1,2-dione with des-alanine-B^{30}-insulin to produce des-Ala-B^{30}-[DHCH-Arg-B^{22}]-insulin is reported.

insulin molecule, or as a preparative method for polypeptide fragments suitable in semisynthetic work.*

In the present paper we show that cyclohexane-1,2-dione reacts selectively with the arginyl residue in the insulin molecule to give the corresponding DHCH-complex I which, after tryptic digestion and regeneration, gives des--Ala-B^{30} insulin in high yield.

RESULTS AND DISCUSSION

Treatment of porcine insulin with 0.05 M cyclohexane-1,2-dione (a 100 fold excess) in 0.2 M borate buffer under nitrogen at 37 °C for 12 hr, resulted in a single product which was characterised as 1,2-dihydroxylcyclohex-1,2-ylen (DHCH)-Arg-B^{22}-insulin complex (I) as follows. On cellogel electrophoresis, the product was relatively more negative and migrated slower toward the cathode than the untreated insulin (Figure 2.), thus suggesting the loss of the positive

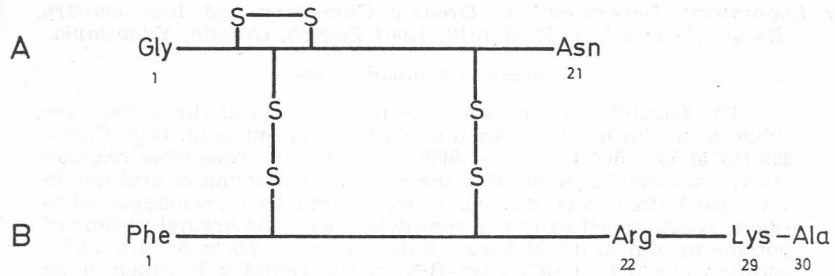

Figure 1. Schematic drawing of porcine insulin.

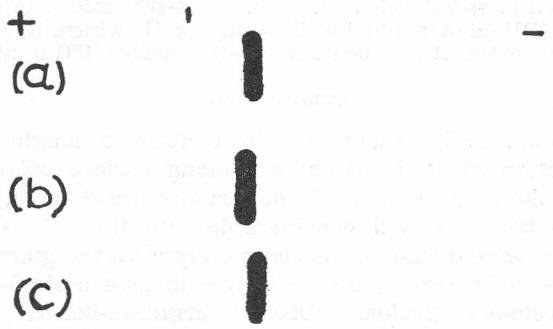

Figure 2. Electrophoresis on cellulose acetate (pH = 4.8, 0.1 *M* pyridine/6 *M* urea) of (*a*) untreated (native) porcine insulin, (*b*) insulin treated with cyclohexane-1,2-dione, and (*c*) insulin from which cyclohexane-1,2-dione was removed. Conditions for the modification and regeneration were as described in the Experimental part. The strips were stained with Ponceau S (0.25% in 3% trichloroacetic acid).

charge from the arginyl side chain. Patthy and Smith[2,3] have demonstrated that under conditions of conventional acid hydrolysis the DHCH-arginine-borate complex regenerated up to 20% of arginine, whereas regeneration was prevented completely when hydrolysis was performed in the presence of thioglycolic acid. Accordingly, it was possible to determine the extent of modification of the insulin molecule by amino acid analysis of the isolated product. The finding that the amino acid composition (Table I) of DHCH-insulin complex I lacked arginine but otherwise was identical to that of untreated insulin, indicates (*a*)

complete reaction of the reagent with the guanidino function of the arginyl residue, and (b) no alterations in the other amino acid residues.

Treatment of the DHCH-insulin complex I with 0.5 M hydroxylamine at pH = 7.0 under nitrogen at 37 °C for 1 hr, led to regeneration of the insulin molecule, as proved by electrophoretic mobility (Figure 2.) and amino acid analysis (Table I) of the product isolated in 87% yield. The biological activity of the regenerated insulin was indistinguishable, as determined by the mouse fall test performed with two separate preparations, from that of the starting (untreated) insulin. When Zn-free insulin was subjected to subsequent treatment with cyclohexane-1,2-dione and hydroxylamine, under conditions described above, the electrophoretic properties and amino acid analyses of the products were the same as those of DHCH-insulin complex I and the starting insulin, respectively.

The extent of the modification of the insulin molecule with cyclohexane-1,2--dione as a function of the reaction time, was followed by measuring the amount of unmodified arginine in the hydrolysates of samples withdrawn from the reaction mixture at timed intervals. As shown in Figure 3., the time course of the reaction of the arginine guanidino function with the reagent

TABLE I

Amino Acid Composition of [DHCH-Arg-B22]-insulin Complex (I), Insulin Regenerated from I (Ins), Des-Ala-B30-[DHCH-Arg-B22]-insulin Complex (II), and Des-Ala-B30--insulin (III)[a]

Amino Acid Residue[b]		Found: (I)		(Ins)	(II)	(III)
Asp	(3)	3.00	3.00[c]	3.00	3.00[c]	3.00
Thr	(2)	2.05	1.72	3.13	1.67	1.79
Ser	(3)	2.64	2.80	2.72	2.75	2.74
Glu	(7)	6.97	7.03	7.04	7.06	7.13
Pro	(1)	0.94	1.04	1.06	1.09	1.04
Gly	(4)	4.10	3.98	4.13	3.91	4.01
Ala	(2)	*2.25*	*2.01*	2.27	*1.12*	*1.13*
Cys	(6)	4.96	5.48	4.79	6.05	4.68
Val	(4)	3.22	2.94	3.17	2.97	3.19
Ile	(2)	0.95	1.06	1.09	1.00	1.11
Leu	(6)	5.82	5.97	6.03	5.93	6.14
Tyr	(4)	3.23	3.65	3.49	3.78	3.47
Phe	(3)	2.67	2.87	2.87	2.94	2.78
His	(2)	1.85	1.95	2.03	1.91	2.04
Lys	(1)	1.00	1.00	0.97	0.96	1.13
Arg	(1)	*< 0.2*	*0.00*	*1.04*	*0.00*	*0.92*

[a] The values found are given as molar ratios with respect to aspartic acid = 3.00 and they are not corrected for destruction. Hydrolysis of samples was performed in 6 M HCl, if not stated otherwise.

[b] The values in brackets refer to the number of amino acid residues in native porcine insulin.

[c] Hydrolysis performed in the presence of thioglycolic acid.

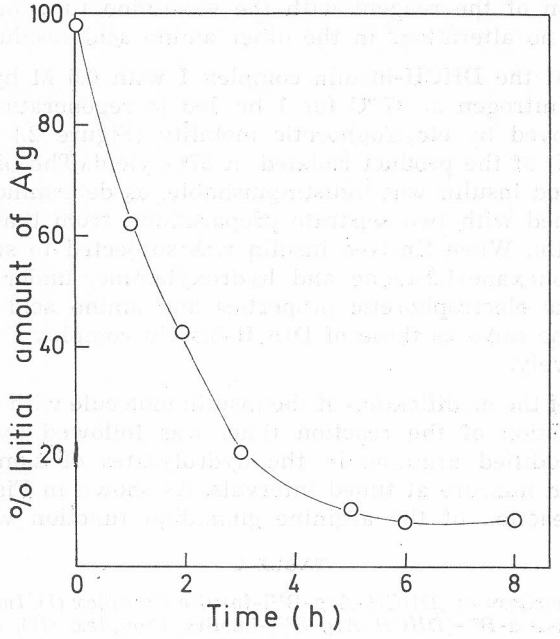

Figure 3. Plot of the percentage of unmodified arginine in hydrolysates of cyclohexane-1,2-dione-treated insulin as a function of the reaction time. The reaction was performed as described in the Experimental part, and aliquots (250 µl) were removed at each time point, acidified to pH = 3.5 and desalted on Sephadex G-50 (superfine) with 0.1 M acetic acid. After lyophilisation of the peak tubes, the residues were subjected to acid hydrolysis (without addition of thioglycolic acid) and amino acid determination.

indicates that most of the complex I formation occurred by 6 hr; for preparative work, a 12 hr (overnight) incubation period was found to be most suitable.

The tryptic incubation of the freshly prepared DHCH-insulin complex I was performed at pH = 8.0 and 37 °C for 20 hr. Fractionation of the digest on a Sephadex G-50 column is shown in Figure 4; no small peptides were detectable in the low-molecular weight fractions. Amino acid analysis (Table I) of the isolated (81%) product revealed that it contained one alanyl residue less than the starting DHCH-complex I, thus indicating that the enzyme action was limited to hydrolysis of the Lys-B^{29} residue to give des-Ala-B^{30}-[DHCH--Arg-B^{22}]-insulin complex (II) as the sole product .Treatment of II with hydroxylamine, followed by ultrafiltration and lyophilisation of the reaction mixture, afforded (85% yield) des-Ala-B^{30}-insulin (III) the amino acid composition of which (Table I) was fully consistent with the structure proposed.

Des-alanine-B^{30}-insulin was obtained[9,10] by the digestion of porcine insulin with carboxypeptidase A in Tris/HCl buffer, followed by separation from the preponderantly formed des-Ala-B^{30}, des-Asn-A^{21}-insulin on DEAE Sephadex. Schmitt and Gattner[11] improved the method by performing incubation in 0.1 M ammonium hydrogen carbonate at pH = 8.2; under these conditions, only alanine was liberated to give, upon desalting and fractionation of the product on DEAE Sephadex ,des-Ala-B^{30}-insulin in high yield. The present route, involving prevention of tryptic cleavage at the arginyl residue by reversible

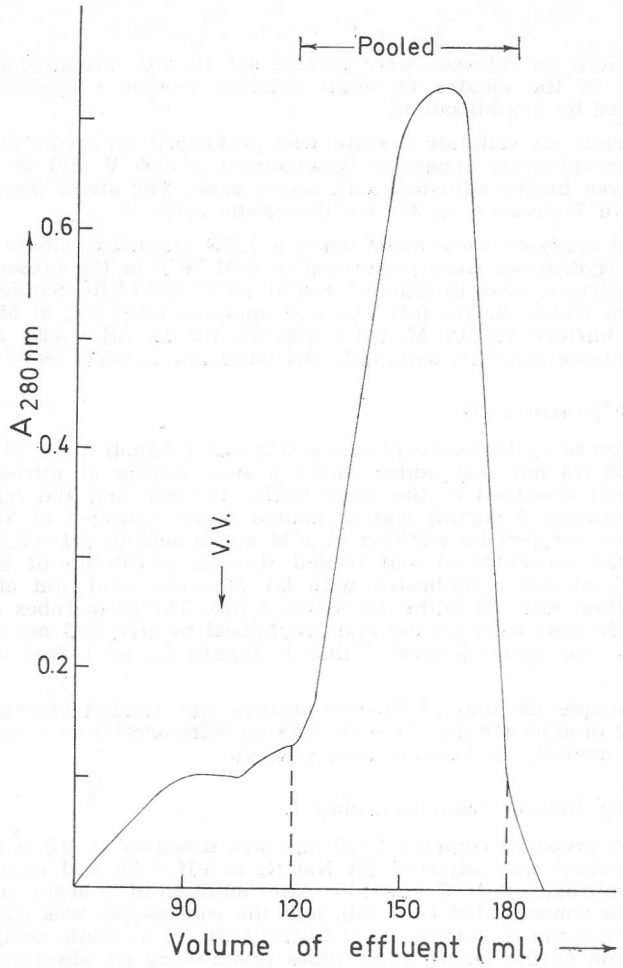

Figure 4. Sephadex G-50 (superfine, 2.5 × 70 cm) elution pattern of the tryptic digest of [DHCH-Arg-B^{22}]-insulin with 0.1 M acetic acid (flow rate: 15 ml/hr, fractions: 3 ml). The peak fractions were pooled as indicated, concentrated and lyophilised to give des-alanine-B^{30}--[DHCH-Arg-B^{22}]-insulin (II, 32.4 mg, 81%). v. v. =-void volume.

blocking with cyclohexane-1,2-dione, offers a new and simple means for preparing des-Ala-B^{30}-insulin. The mild conditions used in this procedure, the specificity of the reaction and homogeneity of the products, suggest its possible usefulness for preparing fragments suitable for the semisynthesis of human insulin.

EXPERIMENTAL

Materials

Porcine insulin, crystalline, zinc-salt, was obtained from PLIVA (lot No 4,047,022, 24.4 I. U./mg) and was used throughout. Zn-free insulin was prepared after Slyterman[12] as already described[13]. Cyclohexane-1,2-dione and trypsin (bovine, Type XI, chymotrypsin free) were obtained from Sigma, hydroxylamine hydrochloride p. a. from Merck, and thiglycolic acid puriss. from Fluka.

Methods

All separations on columns were carried out at 4 °C. Products were recovered by evaporation of the eluates to small volumes (rotary evaporator, bath temp. < 35 °C), followed by lyophilisation.

Electrophoresis on cellulose acetate was performed on strips (2.5 × 14 cm) in a standard electrophoresis apparatus (Chemetron) at 200 V and at pH 4.8 (0.1 M pyridine/6 M urea buffer adjusted with acetic acid). The strips were stained with 0.25% solution of Ponceau S in 3% trichloroacetic acid.

Amino acid analyses were made using a LKB automatic amino acid analyzer, model BC 200. Hydrolyses were performed in 6 M HCl, in the presence (20 µl) and absence of thioglycolic acid, in sealed tubes at 110 °C for 14 hr. Samples were added in 0.2 M sodium citrate buffer (pH 2.2), and analyses were run at 55 °C with three sodium citrate buffers: (1) 0.2 M, pH = 3.25, (2) 0.2 M, pH = 4.25, and (3) 1.2 M, pH = 6.45. In kinetic measurements only the basic amino acids were determined.

[DHCH-Arg-B^{22}]-insulin (I)

To a solution of cyclohexane-1,2-dione (112 mg, 1 mmol) in 0.2 M sodium borate buffer, pH = 9.0 (18 ml) was added under a slow stream of nitrogen, Zn-insulin (50 mg, 9.4 µmol) dissolved in the same buffer (18 ml), and the reaction mixture (insulin concentration 3 mg/ml) was incubated under nitrogen at 37 °C for 12 hr. The reaction was stopped by addition of 1 M acetic acid to pH = 3.5 (~ 5 ml), and the solution was concentrated and passed through a column of Sephadex G-50 (superfine, 2.5 × 40 cm) equilibrated with 0.1 M acetic acid and eluted with the same solvent (flow rate: 20 ml/hr. fractions: 3 ml). The peak tubes (monitoring by absorption at 280 nm) were pooled and lyophilised to give 46.3 mg (92%) of fluffy material which was characterised (Table I, Figure 2.) as I free of the starting insulin.

When a sample (20 mg) of Zn-free insulin was treated exactly as described above, the final product (18 mg, 90%) showed no difference from I, as judged by the electrophoretic mobility and amino acid analysis.

Regeneration of Insulin from Complex I

The freshly prepared complex I (20 mg) was dissolved in 0.5 M hydroxylamine (15 ml), the solution was adjusted (M NaOH) to pH = 7.0 and incubated under a slow stream of nitrogen at 37 °C for 12 hr. After addition of M acetic acid to pH = 3.5, the solution was concentrated (~ 5 ml), and the concentrate was passed through a Sephadex G-50 column (superfine, 2.5 × 40 cm) with 0.1 M acetic acid. Concentration and lyophilisation of the pooled peak tubes (monitoring by absorption at 280 nm), afforded insulin (17.5 mg, 87%) the electrophoretic mobility (Figure 2.), amino acid analysis (Table I) and biological activity of which were indistinguishable from those of the starting (untreated) insulin.

Tryptic Hydrolysis of I Into des-Ala-B^{30}-[DHCH-Arg-B^{22}]-insulin (II)

A solution of the freshly prepared complex I (40 mg, 7.5 µmol) in 0.1 M borate buffer, pH = 8.0 (8 ml) was incubated with trypsin (0.8 mg, ratio enzyme : substrate 1 : 50) at 37 °C for 20 hr. The digestion was arrested by adding M acetic acid to pH = 3.5, and, after concentration, the material was subjected to fractionation on Sephadex G-50 as described in Figure 4. Lyophilisation of the peak tubes afforded II (32.4 mg, 81%) as a fluffy mass which was characterised by amino acid analysis (Table I).

Des-Ala-B^{30}-insulin (III)

The complex (II) (30 mg) was treated with 0.5 M hydroxylamine (25 ml) as described for the regeneration of insulin from I. The digest, after acidification to pH = 3.5, was diluted to 30 ml with 15% acetic acid, and the solution was freed from small molecules and concentrated (~ 5 ml) in an Amicon stirred cell with a UM-2 membrane (nominal cut off at 1,000 daltons) by subsequent filtration with 15, 10 and

6% acetic acid (25 ml each). Lyophilisation of the concentrate left 25.6 mg (85%) of a fluffy mass which was characterised (Table I) as the title compound (III).

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SAŽETAK

Priprava des-Ala-B^{30}-insulina putem triptičke hidrolize svinjskog insulina kojemu je blokiran arginil ostatak sa cikloheksan-1,2-dionom

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Pokazano je da cikloheksan-1,2-dion reagira selektivno, i pod vrlo blagim reakcionim uvjetima, sa guanidino funkcijom arginil ostatka u molekuli insulina. Reakcija se provodi u 0.2 M borat puferu, pH = 9.0 kod 37 °C, a iz nastalog kompleksa tretiranjem sa hidroksilaminom (0.5 M, pH = 7.0) regenerira se insulin nepromijenjene biološke aktivnosti. Inkubacija izoliranog kompleksa I sa tripsinom rezultirala je u isključivom cijepanju veze Lys-B^{29}—Ala-B^{30} pošto je reverzibilno blokiranje arginil ostatka (B^{22}) sa cikloheksan-1,2-dionom spriječilo normalnu ataku enzima na položaj Arg-B^{22}—Gly-B^{23}. Tretiranjem enzimskog produkta sa hidroksilaminom dobiven je des-Ala-B^{30}-insulin u čistom stanju i visokom iskorištenju. Produkti opisanih reakcija karakterizirani su analizom aminokiselina, elektroforetskom pokretljivošću, i u slučaju regeneriranog insulina biološkom aktivnošću.

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