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A highly potent insulin: Des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin(human)

GERALD P. SCHWARTZ, G. THOMPSON BURKE, AND PANAYOTIS G. KATSOYANNIS*

Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029

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ABSTRACT **An insulin analogue that embodies two distinct structural modifications, each of which independently increases insulin activity, has been synthesized and evaluated for biological activity. The analogue, des-(B26–B30)-[Asp^{B10}, Tyr^{B25}-NH$_2$]insulin is the most potent insulin analogue yet described; it displays an 11- to 13-fold higher activity than natural insulin. The findings are discussed with regard to the receptor-binding domains of insulin.**

In a recent publication, we described the synthesis of [Asp^{B10}]insulin, an analogue of human insulin that displays a potency *ca.* 4–5 times greater than that of insulin (1). This finding in conjunction with previous studies carried out in our laboratory involving modifications of insulin at the B10 position (2–4) suggests that this site may be important in the expression of the biological activity of the hormone. Extensive studies in various laboratories have also demonstrated that elements of the C-terminal region of the B chain of insulin are importantly involved in the biological activity of the hormone. Modifications to this region resulted in analogues varying in potency from nearly zero to greater than natural insulin itself (5–14), including des-(B26–B30)-[Tyr^{B25}-NH$_2$]-insulin and its His-B25 analogue (5, 7, 8), which exhibit 2–3 times greater potency than insulin. These studies indicate the important role of the B25 site in the expression of the biological activity of insulin (5, 7, 8). The present communication describes the synthesis and biological evaluation of des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin, an analogue embodying modifications at both the aforementioned sites of the insulin molecule, which appear to play a pivotal role in the expression of the biological activity of the hormone.

EXPERIMENTAL PROCEDURES

Materials and Analytical Procedures. Commercial reagents were as follows: *t*-butoxycarbonyl amino acid derivatives (Bachem), *t*-butoxycarbonyl aspartic acid α-cyclohexyl ester and the corresponding glutamic acid derivative (Peninsula Laboratories), 4-methylbenzhydrylamine resin (0.5 mmol of amine per g; Bachem), dicylohexycarbodiimide and 1-hydroxybenzotriazole (Fluka). The latter reagent was recrystallized from 70% (vol/vol) ethanol. All solvents and reagents were of analytical or HPLC grade. Antibodies and ^{125}I-labeled insulin (^{125}I-insulin) for radioimmunoassay of insulin were obtained from Amersham. ^{125}I-insulin for receptor binding assays and [3-^{3}H]glucose for lipogenesis were products of Dupont–NEN, and cellulose acetate filters were obtained from Sartorius. The scintillation fluids, Soluscint-O and Filtron-X, were purchased from National Diagnostics (Manville, NJ). Details of analytical procedures used in this study have been described (15). The sodium tetrathionate used was freshly prepared (16). Crystalline porcine insulin was a gift from R. E. Chance (Eli Lilly).

Biological Evaluation. The activity of the synthetic compound was compared with that of natural insulin in three types of assays: (*i*) competition with ^{125}I-insulin in binding to insulin receptors in a rat liver membrane preparation, (*ii*) stimulation of the conversion of [3-^{3}H]glucose into organic-extractable material in isolated adipocytes (lipogenesis), and (*iii*) radioimmunoassay—competition with ^{125}I-insulin in binding to guinea pig antibodies raised against insulin. A commercial kit supplied reagents for a double antibody method for radioimmunoassay. The data were analyzed by the method of Hales and Randle (17). Complete details of receptor binding and lipogenesis assays were recently described (18).

Synthesis. Des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin was prepared by the combination of the S-sulfonated form of human A chain with the synthetic S-sulfonated human des-(B26–B30)-[Asp^{10},Tyr^{25}-NH$_2$] B chain in the presence of dithiothreitol (19). The S-sulfonated human A chain, which is identical with the respective chain of porcine insulin (20), was prepared by oxidative sulfitolysis of porcine insulin and separation of the resulting S-sulfonated A and B chains by column chromatography (21). The S-sulfonated doubly substituted des-(B26–B30) B chain was assembled by stepwise solid-phase synthesis (22, 23) by using 4-methylbenzhydrylamine resin as the solid support (0.5 mmol of amine per g; 1 g). The *t*-butoxycarbonyl group was used for N$^{\alpha}$ protection except for the N-terminal phenylalanine residue, which was protected by the benzyloxycarbonyl group. Side-chain protecting groups were as follows: benzyl for serine, 2,6-dichlorobenzyl for tyrosine, N^{G}-*p*-toluenesulfonyl for arginine, cyclohexyl for glutamic and aspartic acids, benzyloxymethyl for histidine, and 4-methylbenzyl for cysteine. A manual double-coupling protocol was followed (24) with activated protected amino acids (1-hydroxybenzotriazole/dicyclohexylcarbodiimide in dimethylformamide) in 3-fold excess. The completion of the reaction was monitored by the qualitative ninhydrin test (25) and was negative after each double coupling.

After the chain was assembled, the peptide-resin was washed extensively with methylene chloride and methanol and dried: weight, 3.0 g. A portion of this product (700 mg) was deprotected by the low/high hydrogen fluoride procedure (26). In the first step the peptide-resin was treated with a mixture consisting of *p*-cresol (1 ml), dimethyl sulfide (6.5 ml), and liquid hydrogen fluoride (2.5 ml). After 2 hr at 0°C, the mixture was concentrated under reduced pressure, and the residue was treated with liquid hydrogen fluoride (10 ml) for 1 hr at 0°C. The hydrogen fluoride was then removed, and the residue was triturated with ethyl acetate and petroleum ether. To a suspension of this product in 8 M guanidine hydrochloride (20 ml) buffered with 0.1 M Tris·HCl (pH 8.8) were added sodium sulfite (700 mg) and sodium tetrathionate (500 mg). After 3 hr at room temperature, the reaction mixture was filtered to remove the resin, and the filtrate was placed in Spectra/por membrane tubing no. 3 and dialyzed against four changes of

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Abbreviation: ^{125}I-insulin, ^{125}I-labeled insulin.

*To whom reprint requests should be addressed.

distilled water (4 liters each) at 4°C for 24 hr. Lyophilization of the dialysate afforded the crude S-sulfonated B-chain analogue as a white powder: weight, 250 mg.

The lyophilized material was dissolved in a mixture of water/2-propanol, 2:1 (vol/vol) containing 0.02 M Tris·HCl (pH 7.5) (5) and purified by high-performance liquid ion-exchange chromatography on a Synchropak AX 300 column (1 × 25 cm) connected to an LKB liquid chromatography system. Batches (*ca.* 70 mg of protein each) were chromatographed at a flow rate of 1.5 ml/min with a 0–80% linear gradient of 0.5 M sodium chloride in the above-mentioned solvent over 140 min. The chromatographic pattern is shown in Fig. 1. The effluent under the main peak (*ca.* 60 min) was concentrated under reduced pressure to *ca.* 50% of its original volume, dialyzed against distilled water (Spectra/por membrane tubing no. 3), and lyophilized. From the 250 mg of crude material, 150 mg of purified product was obtained as a white fluffy powder. Amino acid analysis of the purified S-sulfonated B-chain analogue after acid hydrolysis gave the following composition, expressed in molar ratios, which is in good agreement with the theoretically expected values (shown in parentheses): $Asp_{1.9(2)}Ser_{1.0(1)}Glu_{3.0(3)}Gly_{3.0(3)}$-$Ala_{1.1(1)}Val_{2.7(3)}Leu_{3.8(4)}Tyr_{1.9(2)}Phe_{2.0(2)}His_{0.9(1)}$-$Arg_{1.0(1)}$ (cysteine was not determined).

Synthesis and Isolation of Des-(B26–B-30)-[Asp^{B10},Tyr^{B25}-NH$_2$]Insulin(Human). To a solution of S-sulfonated porcine (identical with human) A chain (20 mg) and S-sulfonated human des-(B26–B30)-[Asp^{10},Tyr^{25}-NH$_2$] B chain (10 mg) in 6 ml of 0.1 M glycine buffer (pH 10.6) cooled to 4°C was added dithiothreitol (3.5 mg). After 24 hr at 4°C, the mixture was diluted with glacial acetic acid (1 ml), and the resulting precipitate was removed by centrifugation (International HN; 3000 rpm). The supernatant, containing the active material, was passed through a 0.45-μm cellulose acetate filter (Sartorius) and was subjected to reversed-phase HPLC on a Vydac 218 TP column (0.45 × 25 cm) connected to an LKB liquid chromatography system. Batches (*ca.* 5 mg of protein each) were chromatographed at a flow rate of 0.5 ml/min with a 10–50% linear gradient of 2-propanol in 0.1% trifluoroacetic acid over 70 min. The chromatographic pattern is shown in Fig. 2*A*. The fraction containing the active material, as was determined by insulin assays, eluted at *ca.* 36.8 min. It was concentrated and rechromatographed on the same column with a 20–35% linear gradient of 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min over 110 min. The chromatographic pattern is shown in Fig. 2*B*. The fraction containing the active material, eluting at *ca.* 63.1 min, was collected and lyophilized. From the mixture of A and B chains described above, 1.4 mg of highly purified product was obtained. Amino acid analysis of the synthetic analogue after acid hydrolysis gave the following composition, expressed in molar ratios, which is in good agreement with the theoretically expected values: $Asp_{4.1(4)}Thr_{0.9(1)}$-$Ser_{3.0(3)}Glu_{7.0(7)}Gly_{3.8(4)}Ala_{1.1(1)}Val_{3.3(4)}Ile_{1.4(2)}Leu_{6.0(6)}$-$Tyr_{3.8(4)}Phe_{2.0(2)}His_{1.0(1)}Arg_{0.9(1)}$ (cysteine was not determined).

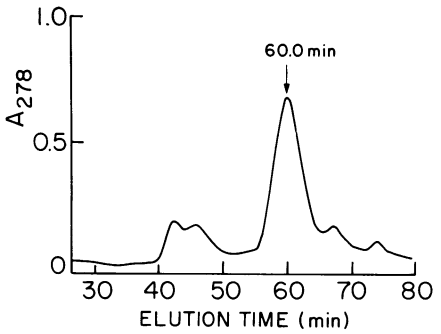

FIG. 1. High-performance liquid ion-exchange chromatography of crude human des-(B26–B30)-[Asp^{10},Tyr^{25}-NH$_2$] B chain S-sulfonate on a Synchropak AX 300 column (1 × 25 cm) with a 0–80% linear gradient of 0.5 M sodium chloride in 2-propanol/water, 1:2 (vol/vol) containing 0.02 M Tris·HCl (pH 7.5) over 140 min. The purified chain was recovered from the effluent under the main peak (60 min) after dialysis and lyophilization.

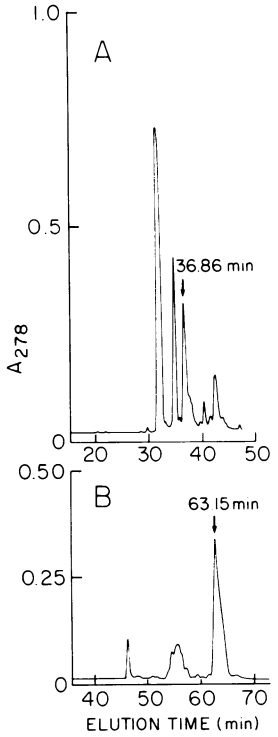

FIG. 2. (*A*) Reversed-phase HPLC of the combination mixture of human S-sulfonated A chain and human des-(B26–B30)-[Asp^{10}, Tyr^{25}-NH$_2$] B chain S-sulfonate on a 0.45- × 25-cm Vydac 218 TP column at 0.5 ml/min with a 10–50% linear gradient of 2-propanol in 0.1% trifluoroacetic acid over 70 min. (*B*) Rechromatography of the material eluting at 36.86 min in *A* with the same column and a 20–35% linear gradient of 2-propanol in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min over 110 min. The active material eluting at 63.15 min was obtained by concentration of the effluent.

RESULTS AND DISCUSSION

Competition with ^{125}I-insulin in binding to insulin receptors in rat liver membranes was compared for bovine insulin and synthetic human des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin. Fig. 3 shows that, in a typical experiment, the synthetic compound displaced 50% of the specifically bound ^{125}I-insulin at a concentration more than 10-fold lower than the concentration required for natural insulin; the calculated potency for the analogue was 11.7 (± 0.3)-fold relative to bovine insulin.

Des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin and bovine insulin were tested for their ability to stimulate lipogenesis in isolated fat cells. Fig. 4 depicts the results of a typical experiment, which indicated that the synthetic compound produced half-maximal stimulation of lipogenesis at a much lower concentration than that required for natural insulin, resulting in a calculated potency of 13.5 (± 1.1)-fold relative to insulin. The maximum stimulation of lipogenesis was the same for both compounds.†

†C. R. Kahn (Joslin Diabetes Center, Boston) has independently confirmed that des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin displays greater potency in lipogenesis in isolated rat adipocytes than [Asp^{B10}]insulin, which itself displays greater potency than natural insulin (personal communication).

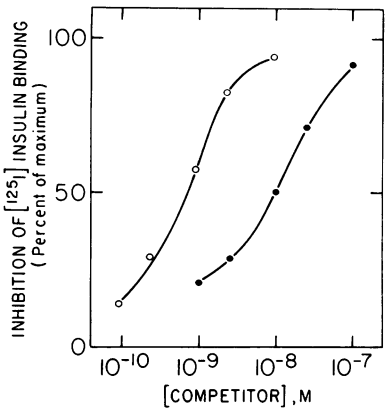

FIG. 3. Effect of bovine insulin (●) and des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin (○) upon the binding of ^{125}I-insulin to insulin receptors in a rat liver plasma membrane preparation. The inhibition of binding of ^{125}I-insulin, after subtraction of nonspecific binding, is presented as a function of the molar concentration of the competing compound. Data points are the means of triplicate determinations in a representative experiment, which was performed four times using three different preparations of the synthetic compound. In this experiment, binding of ^{125}I-insulin in the absence of competitor amounted to 9.1% of input radioactivity, and nonspecific binding amounted to 9.6% of total binding.

In radioimmunoassays (data not shown), bovine insulin and des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin were indistinguishable in potency. Apparently, the structural differences that give rise to the stronger binding to the insulin receptor and the concomitantly higher *in vitro* insulin-like activity displayed by the synthetic compound do not greatly affect the immunogenic determinants recognized by the polyclonal guinea pig anti-insulin antiserum preparation used in these studies.

We have recently reported that the substitution of the histidine residue at position B10 in insulin, a surface residue, by aspartic acid led to an analogue, [Asp^{B10}]insulin, which displays *in vitro* potency *ca.* 4–5 times greater than that of natural insulin in binding assays to the insulin receptor and in stimulating lipogenesis (1). We suggested that the superactivity of this analogue might be due to either of two factors: (*i*) a direct interaction of the ligand, bearing a negative charge

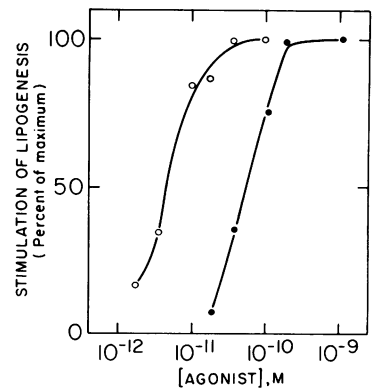

FIG. 4. Effect of bovine insulin (●) and des-(B26–B30)-[Asp^{B10},Tyr^{B25}-NH$_2$]insulin (○) upon the stimulation of lipogenesis in isolated rat fat cells. The stimulation of lipogenesis, expressed as percent of maximum, is presented as a function of the molar concentration of agonist. The data points represent the means of triplicate incubations in a representative experiment, which was performed four times with three different preparations of the synthetic compound. In this experiment, 0% and 100% stimulation refer, respectively, to 11.4 and 78.8 nmol of [3-^{3}H]glucose converted to organic-extractable material per hr per mg of cells.

at the B10 position, with a complementary site on the surface of the receptor, bearing a positive charge; or (*ii*) a change in conformation of the ligand, which is more favorable for binding to the receptor, resulting from intramolecular interactions involving the negative charge at position B10. Such an interaction could involve a salt bridge between the negatively charged Asp-B10 and a positively charged residue, for example His-B5. Salt bridge formation involves a conformational change that also results in a change in charge distribution with a concomitant change in solvation, which enhances hydrophobic interactions in the immediate area.

In reversed-phase HPLC, [Asp^{B10}]insulin did, in fact, appear more apolar than the natural hormone; it displayed a significant increase in retention time. This suggests that, at least under the acidic conditions employed in reversed-phase HPLC, [Asp^{B10}]insulin exists in a conformation different from that of insulin. It is thus reasonable to assume that conformational differences could explain the stronger binding of [Asp^{B10}]insulin to the insulin receptor, which is reflected in its higher activity in lipogenesis.

Elimination of the C-terminal pentapeptide sequence of the B-chain moiety of insulin and amidation of the carboxyl group of the newly formed C terminus, Phe-B25, results in an analogue, des-(B26–B30)-[Phe^{B25}-NH$_2$]insulin, which displays comparable potency with the natural hormone (5–7). Substitution of Phe-B25 with several other amino acid residues, as well as various modifications of the B26–B30 segment of these substituted insulins, led to analogues varying in potency from almost total inactivity to potency higher than natural insulin (5, 7, 8). Among these, des-(B26–B30)-[Tyr^{B25}-NH$_2$]insulin and its His-B25 analogue display a potency *ca.* 270–300% relative to insulin. Based upon these studies, it has been suggested that the B25 amino acid residue of insulin interacts with the receptor, thereby initiating conformational changes in as yet undefined areas of the insulin molecule that are involved in hormone–receptor binding. The B25-residue–receptor interaction may be modulated in a positive or negative manner by the C-terminal B-chain domain (5, 7, 8), depending on the nature of the modifications to the B25 residue and on the extent to which the B-chain C-terminal domain has been altered.

In the present analogue, we have incorporated two modifications that, when individually introduced into the insulin molecule, led to analogues displaying higher potency than the natural hormone. We have (*i*) eliminated the B26–B30 segment and substituted Phe-B25 with Tyr-NH$_2$ and (*ii*) replaced His-B10 with Asp. This analogue, des-(B26–B30)-[Asp^{B10}, Tyr^{B25}-NH$_2$]insulin, is the most potent insulin analogue yet described. Its biological activity is greater than the sum of the enhanced potencies displayed by analogues containing either modification alone. This finding suggests that the B25 and B10 sites may modulate the conformation of distinct receptor-binding regions of insulin to high-affinity receptor binding states. In fact, the high association constant of the insulin–receptor complex in various tissues (*ca.* 10^9 M^{-1}) would appear to require the concerted action of several binding species. The histidine residue at position B10 is not one of the residues proposed to contribute to the recognition of insulin by its receptor (27–29). However, the increased potency of [Asp^{B10}]insulin, as well as the reduced potency of [Leu^{B10}]insulin, [Asn^{B10}]insulin, and [Lys^{B10}]insulin (2–4) demonstrates that substitutions at this position can have profound effects upon the ability of the resulting molecules to interact with the insulin receptor and initiate a biological response. Whether the B10 site is an element of a receptor-binding region or whether the modifications at that site affect a distal binding region cannot be unambiguously determined from the available data. Many models can be proposed to account for the high potency of the present analogue. We suggest one model, which is in accord with the observed

properties of the present analogue. The B25 and B10 sites could, in fact, be elements of distinct receptor-binding regions of insulin, which may be individually modulated by the modifications at the B25 and B10 positions, respectively, leading, in the present analogue, to high-affinity states for receptor binding. In addition, the B25 and B10 modifications may independently and in concert favorably affect the conformation of other distinct regions that constitute important receptor-binding domains of insulin. Such a model need not contradict the concept of a single receptor-binding region made up of noncontiguous amino acid residues distributed throughout the A- and B-chains of insulin (27–29). The model simply allows for consideration of these and perhaps other amino acid residues acting singly or in clusters to form individual binding entities whose contribution to the total binding affinity may vary.

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