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Semisynthesis and biological activity of porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin

(solid-phase peptide synthesis/desoctapeptide insulin/insulin analog/insulin antagonist/rat adipocytes)

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ABSTRACT **Two analogs of porcine insulin with substitutions of leucine for phenylalanine in the COOH-terminal region of the insulin B chain have been prepared by a combination of solid-phase synthesis and semisynthesis. Solid-phase synthesis of the substituted octapeptides B23–B30 bearing the trifluoroacetyl group on lysine-B29, enzymatic coupling of the octapeptides to bis(*tertiary*-butyloxycarbonyl)desoctapeptide insulin by trypsin, and deprotection of the corresponding adducts in formic acid and piperidine resulted in two insulin derivatives, one with leucine at position B24 and the other with leucine at position B25. These analogs had only about 10% and 1%, respectively, of the activity of porcine insulin in competing for the binding of [^{125}I]iodoinsulin to both rat adipocytes and human IM-9 lymphocytes. The relative potencies of the analogs in stimulating glucose oxidation by rat adipocytes decreased in the order porcine insulin > [Leu^{B24}]insulin > [Leu^{B25}]insulin. However, at high concentrations both analogs had full agonist activity. Experiments in which the semisynthetic insulins were mixed with the native hormone showed that [Leu^{B24}]insulin, but not [Leu^{B25}]insulin, was an active antagonist of insulin action. These results suggest that the antagonistic activity of a human insulin variant having leucine at position B24 or B25 can be assigned to the molecule with the sequence Gly-Leu-Phe-Tyr (residues B23–B26) in its active site.**

Studies of naturally occurring and chemically modified insulins have shown that the COOH-terminal region of the B chain lies within the site conferring activity to the hormone (1–5). Although residues B23–B26 (Gly-Phe-Phe-Tyr) have remained invariant during animal evolution, we recently identified an abnormal human insulin in which this portion of the molecule had been modified: the variant, isolated in equimolar amounts with normal insulin from the pancreas of a diabetic but hyperinsulinemic patient, appeared to contain a substitution of leucine for phenylalanine at position 24 or 25 of the insulin B chain (6). The mixture of normal and abnormal hormones had lower than expected activity both in competing for the binding of [^{125}I]iodoinsulin to membrane receptors on rat adipocytes and in stimulating 2-deoxyglucose uptake and glucose oxidation by the same cells (6). Importantly, the biological activity of the hormone preparation (15% of normal) was much lower than its apparent binding activity (45% of normal). These results, and others on the biological effectiveness of the insulin mixture present in plasma (7) and of the insulin variant purified by cellular adsorption (8), were consistent with the patient's clinical features (7) and suggested that the abnormal hormone was an active antagonist of insulin action. Further studies on placing the amino acid substitution in sequence and on determining the mechanism by which the variant might interfere with a cellular response to insulin were prevented, however, by the small amount of material available.

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The pioneering work of Inouye *et al.* (9), which describes the semisynthesis of insulin derivatives by trypsin-catalyzed peptide bond formation at Arg-B22, now permits the preparation of analogs containing amino acid substitutions in the COOH-terminal region of the insulin B chain. In this report we describe the solid-phase synthesis of the [Leu^{B24}, trifluoroacetyl (TFA)-Lys^{B29}] and [Leu^{B25}, TFA-Lys^{B29}] derivatives of the porcine insulin octapeptide B23–B30, the semisynthesis of the corresponding insulin analogs by the action of trypsin on the synthetic octapeptides and bis(*tertiary*-butyloxycarbonyl) (*t*-BOC)-desoctapeptide porcine insulin, and the binding and biological activities of the deprotected insulin analogs. Our studies show that the analog with leucine at position B24 has the antagonistic properties of the previously identified human insulin variant.

MATERIALS AND METHODS

Solid-Phase Synthesis of Peptides Gly-Leu-Phe-Tyr-Thr-Pro-TFALys-Ala ([Leu^{B24}, TFA-Lys^{B29}]Octapeptide) and Gly-Phe-Leu-Tyr-Thr-Pro-TFALys-Ala ([Leu^{B25}, TFA-Lys^{B29}]Octapeptide). Solvents and reagents were purified as described (10). Chloromethylated styrene–divinylbenzene copolymer (1% crosslinked) was obtained from Pierce and the α-*t*-BOC-amino acid derivatives of L-Ala, Gly, L-Leu, L-Phe, L-Pro, *O*-benzyl-L-Thr, and *O*-2,6-dichlorobenzyl-L-Tyr were obtained from Bachem (Torrance, CA). ϵ-TFA-Lys was prepared by the method of Schallenberg and Calvin (11) and was converted to α-*t*-BOC-ϵ-TFA-Lys by a modification of the method of Anfinsen *et al.* (12) with *t*-BOC-anhydride. The product melted at 102°C, as did a sample of the authentic material obtained from Bachem. *t*-BOC-L-Ala was attached to the chloromethylated, styrene–divinylbenzene copolymer support by the method of Gisin and Merrifield (13). Hydrolysis of a portion of the derivatized resin in 12 M HCl/propionic acid, 1:1 (vol/vol), and subsequent amino acid analysis on a Beckman model 121 analyzer showed the level of amino acid substitution to be 0.46 mmol/g.

The B-chain octapeptides were synthesized with the aid of a Beckman model 990 peptide synthesizer by using a program slightly modified from that described by Yamashiro and Li (14). Coupling steps were performed with symmetric anhydrides (15) generated with a 6.5-fold molar excess of the *t*-BOC-amino acid and a 3.0-fold molar excess of dicyclohexylcarbodiimide. The completeness of each coupling reaction was determined by the ninhydrin color test (16) before proceeding. Completed peptides were cleaved from the resin with hydrofluoric acid (10) and the products were extracted with ethyl acetate. The crude [Leu^{B24}, TFA-Lys^{B29}]octapeptide was extracted from the residue with dimethylformamide and the solvent was re-

Abbreviations: TFA, trifluoroacetyl; *t*-BOC, *tertiary*-butyloxycarbonyl.

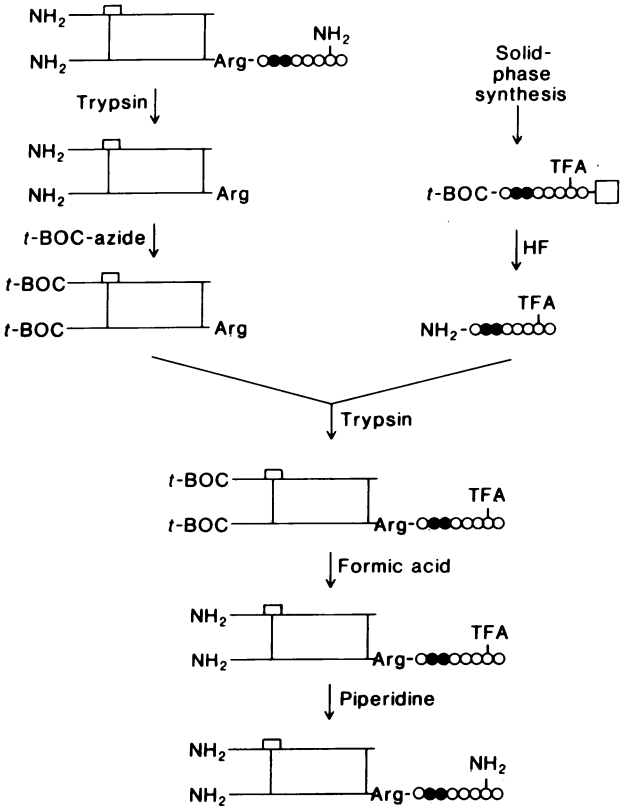

FIG. 1. Flow chart for the semisynthesis of insulin derivatives. The highly schematic figure illustrates at the upper left the preparation of bis(*t*-BOC)-desoctapeptide insulin and at the upper right the solid-phase synthesis of B-chain octapeptides containing TFA-lysine at position B29. The open square in the upper right signifies the resin on which the octapeptides were synthesized. The lower part of the figure illustrates peptide bond formation through the action of trypsin and the sequential deprotection of the semisynthetic derivatized insulin. The arginine residue at position B22 of insulin and desoctapeptide insulin (the site for enzymatic formation of the peptide bond) is shown explicitly. Amino acid residues comprising the COOH-terminal octapeptide (B23–B30) are illustrated by circles. Residues corresponding to positions B24 and B25 (sites for replacement of phenylalanine by leucine) are shown as solid forms.

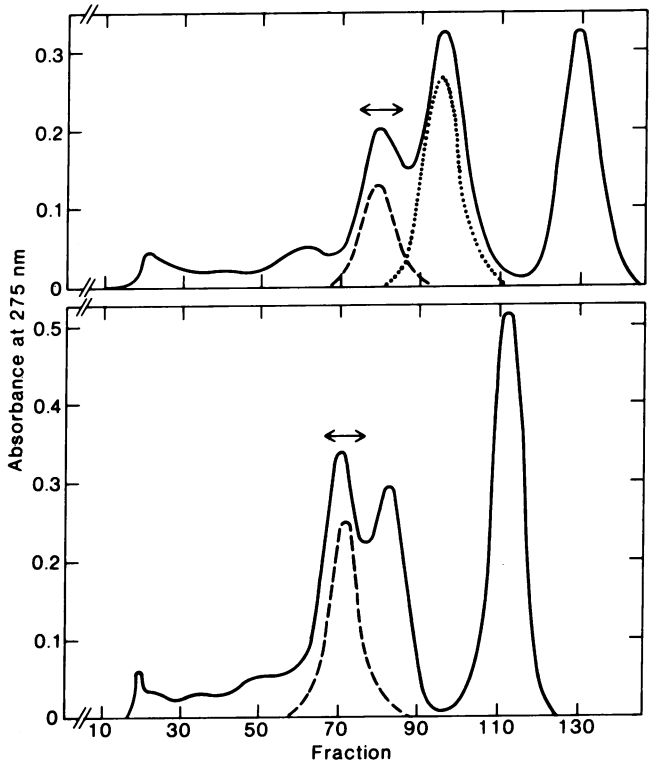

FIG. 2. Purification of protected, semisynthetic insulins by gel filtration. (*Upper*) [Leu^{B24}]Insulin, 2.5 × 90 cm column; (*Lower*) [Leu^{B25}]insulin, 2.5 × 75 cm column. Profiles were obtained by application of the diluted reaction mixtures from the trypsin-catalyzed coupling of octapeptide derivatives and bis(*t*-BOC)-desoctapeptide insulin to columns of Bio-Gel P-10 in 3 M acetic acid. Peaks appearing in the upper panel at fractions 79, 95, and 130 and in the lower panel at fractions 71, 83, and 113 represent semisynthetic insulin, unreacted desoctapeptide insulin, and unreacted octapeptide, respectively. ·····, Elution profile for bis(*t*-BOC)-desoctapeptide insulin. Fractions of the semisynthetic insulins as indicated by horizontal arrows were pooled, concentrated, and reapplied to the separate, respective columns yielding the profiles shown (– –). Fractions, again as indicated by the horizontal arrows, were pooled and further treated for removal of the *t*-BOC and TFA protecting groups.

moved under reduced pressure. The product was dissolved in the minimum amount of dimethylformamide and was crystallized at −20°C by the addition of 10 ml of methanol and 12 ml of diethyl ether. This poorly soluble peptide was then dissolved in acetic acid; 4 vol of water were added and the resulting solution was gel-filtered on Bio-Gel P-2 (Bio-Rad), with 20% (vol/vol) acetic acid as the solvent. The purified material was dried under reduced pressure and was lyophilized from acetic acid. The [Leu^{B25}, TFA-Lys^{B29}]octapeptide was extracted from the resin with 20% acetic acid and the solvent was removed under reduced pressure. The residue was dissolved in 30% acetic acid and was gel-filtered on Sephadex G-15 (Pharmacia) in 30% acetic acid. The peptide was then dried under reduced pressure and was lyophilized from dilute acetic acid.

Preparation of Bis(*t*-BOC)-desoctapeptide Porcine Insulin. Monocomponent porcine insulin (300 mg; gift of J. Schlichtkrull of Novo Pharmaceuticals, Copenhagen, and D. F. Steiner of The University of Chicago) was dissolved in 150 ml of 0.1 M *N*-ethylmorpholine containing 2 mM EDTA brought to pH 8 with acetic acid. The solution was diluted with an equal volume of the *N*-ethylmorpholine buffer containing both 20 mM $CaCl_2$ and 45 mg of tosylphenylalanine chloromethylketone-treated trypsin (Worthington) and the mixture was incubated at 37°C for 3 hr. The solution was diluted with 75 ml of acetic acid and was brought to dryness. The residue was dissolved in 50 ml of 3 M acetic acid and the solution was applied to a column (2 × 10 cm) of SP-Sephadex equilibrated with the same solvent. Peptides were eluted by a linear gradient of NaCl dissolved in 3 M acetic acid (0–0.5 M NaCl, 750 ml of each) at a flow rate of 60 ml/hr. Fractions contining desoctapeptide insulin were pooled, desalted by chromatography on Bio-Gel P-2, concentrated, and gel-filtered in three portions over a column (2.5 × 95 cm) of Bio-Gel P-10 in 3 M acetic acid. Fractions from the midpoint of the leading edge of the peak of desoctapeptide insulin through the trailing edge were pooled, concentrated, and gel-filtered again. The lyophilized product (170 mg) was dissolved in 20 ml of dimethylformamide and was treated with *t*-BOC-azide as described (17). The yield of bis(*t*-BOC)-desoctapeptide insulin, after isolation of the product by ether precipitation, was 150 mg.

Semisynthesis of [Leu^{B24}]Insulin and [Leu^{B25}]Insulin. Bis(*t*-BOC)desoctapeptide insulin (20 mg) and either 8 mg of the [Leu^{B24}, TFA-Lys^{B29}]octapeptide or 13 mg of the [Leu^{B25}, TFA-Lys^{B29}]octapeptide were dissolved in 0.1 ml of dimethylformamide and the solutions were diluted with 0.1 ml of 0.25 M Tris brought to pH 7.5 with HCl. Although the final pH was

Table 1. Amino acid compositions of insulin-related peptides*

Amino acid	Porcine insulin	Desocta-peptide insulin	Synthetic [Leu^{B24}]octapeptide	Synthetic [Leu^{B25}]octapeptide	Semisynthetic [Leu^{B24}]insulin	Semisynthetic [Leu^{B25}]insulin
Asp	3.0 (3)	3.0 (3)			3.1 (3)	3.2 (3)
Thr	1.8 (2)	0.9 (1)	0.9 (1)	0.9 (1)	1.8 (2)	1.9 (2)
Ser	2.3 (3)	2.4 (3)			2.8 (3)	2.7 (3)
Glu	6.8 (7)	6.8 (7)			6.7 (7)	6.7 (7)
Pro	1.1 (1)		0.9 (1)	0.9 (1)	1.2 (1)	1.2 (1)
Gly	4.0 (4)	3.0 (3)	1.0 (1)	1.0 (1)	4.2 (4)	4.0 (4)
Ala	1.9 (2)	1.0 (1)	1.0 (1)	1.0 (1)	2.1 (2)	2.2 (2)
Val	3.5 (4)	3.7 (4)			3.7 (4)	3.8 (4)
Ile	1.8 (2)	1.9 (2)			2.0 (2)	1.9 (2)
Leu	6.2 (6)	6.1 (6)	1.1 (1)	1.0 (1)	7.1 (7)	7.1 (7)
Tyr	3.9 (4)	2.8 (3)	0.9 (1)	1.0 (1)	3.7 (4)	3.8 (4)
Phe	3.2 (3)	0.9 (1)	1.0 (1)	1.0 (1)	1.8 (2)	2.0 (2)
His	2.1 (2)	2.1 (2)			2.1 (2)	2.0 (2)
Lys	1.0 (1)		1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Arg	1.0 (1)	1.0 (1)			1.0 (1)	1.0 (1)

Peptides were hydrolyzed under reduced pressure at 110°C for 20 hr in 6 M HCl containing a small amount of phenol. Analyses were performed on a Durrum amino acid analyzer. Relative amino acid concentrations were converted to nearest integer values to obtain the compositions reported. Values in parentheses indicate the amino acid compositions expected.

* Values are reported as mole of amino acid per mole of peptide.

6.5 in both cases, the solution with the Leu-B24 peptide became quite turbid. Tosylphenylalanine chloromethylketone-treated trypsin (1 mg) was added and the solutions were incubated at 37°C for 8 hr. Another 1-mg portion of trypsin was added and the solutions were incubated for an additional 12 hr. The mixtures were cooled and dissolved in 1 ml of acetic acid. The resulting solutions were diluted with 4 ml of water and were gel-filtered on Bio-Gel P-10 with 3 M acetic acid. (See *Results* and Fig. 2 for further details.) Fractions corresponding to the semisynthetic insulin were pooled, concentrated, and gel-filtered again. Insulin-containing fractions were brought to dryness under reduced pressure, the residue was dissolved in 2.5 ml of 99% formic acid, and the resulting solution was incubated at 21°C for 3 hr for removal of the *t*-BOC protecting group. The solution was then dried and the residue was twice lyophilized from water. The product was dissolved in 4 ml of ice-cold 10% piperidine and the solution was incubated at 4°C for 3 hr for removal of the TFA protecting group. Cool acetic acid (1.5 ml) was added and the resulting solution was gel-filtered on Bio-Gel P-10 in 3 M acetic acid. Insulin-containing fractions were dried and lyophilized from water. The yields were 2.5 mg for porcine [Leu^{B24}]insulin and 4.0 mg for porcine [Leu^{B25}]-insulin.

Biological Assays. Methods for studies of insulin binding to membrane receptors with cultured human IM-9 lymphocytes (18) and for studies of insulin binding and insulin stimulation of [1-^{14}C]glucose oxidation with isolated rat adipocytes (19–21) have been described.

RESULTS

Our strategy and procedure for the semisynthesis of porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin, as illustrated in Fig. 1, closely followed those suggested by Inouye *et al.* (9), with several exceptions dictated by our choice of solid-phase methods for the synthesis of the protected COOH-terminal octapeptides B23–B30. Incorporation of Tyr-B26 as the *O*-2,6-dichlorobenzyl derivative, Thr-B27 as the *O*-benzyl derivative, and Lys-B29 as the ε-TFA derivative permitted simultaneous cleavage of the peptide from the supporting resin and deprotection of Tyr-B26 and Thr-B26 with anhydrous hydrofluoric acid and retention of the trypsin-insensitive TFA-Lys as the penultimate amino acid residue. After their purification by gel filtration, the synthetic octapeptides Gly-Leu-Phe-Tyr-Thr-Pro-TFALys-Ala and Gly-Phe-Leu-Tyr-Thr-Pro-TFALys-Ala yielded single spots on thin-layer chromatography both on silica and cellulose, and their amino acid compositions (Table 1, columns 4 and 5) were consistent with theory. The purified desoctapeptide in-

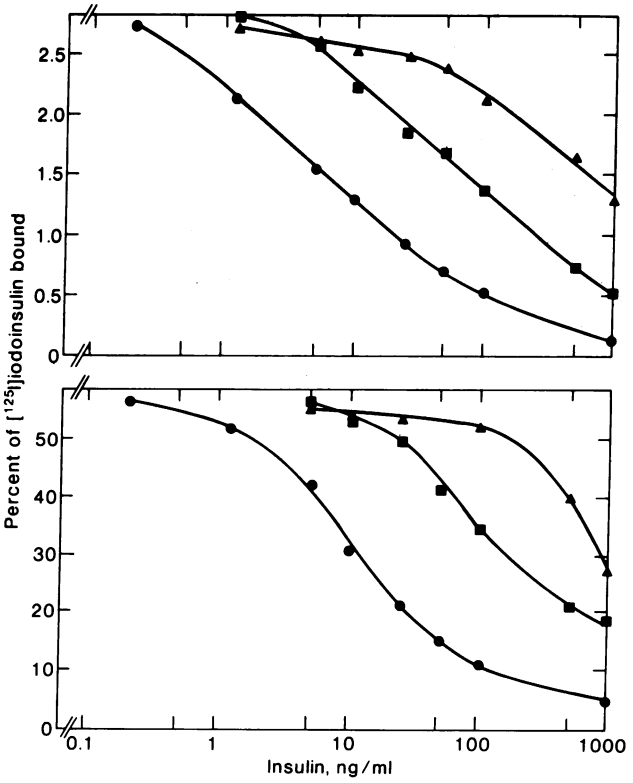

FIG. 3. Competition for the binding of [^{125}I]iodoinsulin to plasma membrane receptors by porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin. (*Upper*) Binding to rat adipocytes; (*Lower*) binding to cultured human IM-9 lymphocytes. In each case, the potency of porcine insulin (●) is compared to the potency of the [Leu^{B24}] analog (■) and to the potency of the [Leu^{B25}] analog (▲) in competing for the binding of the radiolabeled hormone.

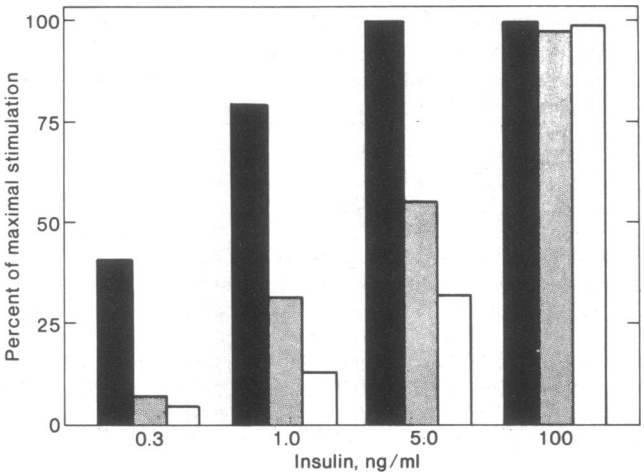

FIG. 4. Stimulation of [^{14}C]glucose oxidation by porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin in rat adipocytes. The figure compares the biological activities of porcine insulin (solid bars), porcine [Leu^{B24}]insulin (stippled bars), and porcine [Leu^{B25}]insulin (open bars) at the concentrations shown as a percent of maximal response (obtained in the presence of 25 ng of porcine insulin per ml).

sulin contained a small amount of its monodesamido form but no detectable insulin, as assessed by polyacrylamide gel electrophoresis at pH 8.7 (22) or pH 4.5 (23) or by amino acid analysis (Table 1, column 2).

As shown in Fig. 2, the products of the trypsin-catalyzed reaction between bis(*t*-BOC)-desoctapeptide insulin and the protected octapeptides yielded complex gel filtration profiles. Identifiable components included products of tryptic autolysis, semisynthetic insulin, residual desoctapeptide insulin, and residual octapeptide. Efficiencies of correct coupling (estimated from the chromatographic profiles) were 35% for bis(*t*-BOC)-[Leu^{B24}, TFA-Lys^{B29}]insulin (Fig. 2 *Upper*) and 60% for bis(*t*-BOC)-[Leu^{B25}, TFA-Lys^{B29}]insulin (Fig. 2 *Lower*). The relatively low yield of the former analog undoubtedly results from the poor solubility of the corresponding protected octapeptide in the coupling buffer.

Deprotection of the blocked insulin derivatives followed the course outlined in the lower part of Fig. 1. Incubation of the leucine-substituted bis(*t*-BOC)-[TFA-Lys^{B29}]insulins in formic acid resulted in loss of the *t*-BOC protecting group. Subsequent removal of the TFA group by 10% piperidine gave the deprotected, semisynthetic insulin analogs. Although 10% piperidine maintains a pH of 12.5, we found no evidence of disulfide bond interchange on incubation of insulin in the solvent at 4°C.

Table 2. Effects of porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin on insulin-stimulated glucose oxidation by rat adipocytes

Hormone and analog concentrations, ng/ml			Response, % of maximal stimulation
Porcine insulin	[Leu^{B24}]-Insulin	[Leu^{B25}]-Insulin	
0.3	—	—	36 ± 5
—	0.3	—	6 ± 2
—	—	0.3	5 ± 1
0.3	0.3	—	16 ± 3
0.3	—	0.3	40 ± 3

Rat adipocytes and [^{14}C]glucose were incubated with porcine insulin, porcine [Leu^{B24}]insulin, and porcine [Leu^{B25}]insulin, either alone or in combination, under standard conditions and $^{14}CO_2$ was collected. The data show the mean ± SEM from seven experiments and are reported as percent of maximal stimulation obtained in the presence of 25 ng of porcine insulin per ml.

Control incubations of the hormone in phosphate buffer at the same pH resulted in considerable damage to the insulin molecule, and the protective effect of piperidine is incompletely understood. Polyacrylamide gel electrophoresis of the purified, semisynthetic insulins at pH 8.7 (22) and 4.5 (23) showed that they contained between 5% and 8% monodesamido and mono-undeblocked forms. Neither preparation contained detectable amounts of multiply blocked forms, desoctapeptide insulin, or other contaminants. The amino acid compositions of the semisynthetic insulin analogs agree well with those expected from theory (Table 1, columns 6 and 7) and indicate that the preparations are free from possible polymeric or other contaminants.

A comparison of the abilities of porcine insulin, porcine [Leu^{B24}]insulin, and porcine [Leu^{B25}]insulin to compete for the binding of [^{125}I]iodoinsulin to plasma membrane receptors is illustrated in Fig. 3. As shown in Fig. 3 *Upper*, the [Leu^{B24}] and [Leu^{B25}] analogs had only 9% and 1%, respectively, of the activity of native insulin in rat adipocytes. Fig. 3 *Lower* shows that the two analogs had only 8% and 1%, respectively, of the activity of the native hormone in competing for insulin binding to cultured human IM-9 lymphocytes. At higher analog concentrations, [Leu^{B24}]insulin and [Leu^{B25}]insulin completely inhibited the binding of iodoinsulin to both cell types (data not shown). These results suggest that both analogs are capable of interacting with insulin receptors of normal and cultured cells and that their relative affinities for binding to the insulin receptor are independent of the cell type used.

The biological potencies of [Leu^{B24}]insulin and [Leu^{B25}]-insulin in stimulating glucose oxidation by rat adipocytes are compared in Fig. 4. Although these analogs show decreased potency relative to porcine insulin, both derivatives show full agonist activity at high concentrations. In addition, the greater potency of [Leu^{B24}]insulin relative to that of [Leu^{B25}]insulin parallels the results of Fig. 3 on the abilities of these analogs to interact with the insulin receptor. Experiments designed to assess potential antagonist effects of the insulin analogs further documented the activities of [Leu^{B24}]insulin and [Leu^{B25}]insulin during their interaction with rat adipocytes (Table 2). Porcine insulin, at a concentration resulting in about 40% of maximal stimulation of glucose oxidation, was mixed with equimolar amounts of [Leu^{B24}]insulin or [Leu^{B25}]insulin to mimic the mixture of normal and variant human insulins previously reported (6). Although the minimal activity of [Leu^{B25}]insulin and the activity of porcine insulin were additive, the addition of [Leu^{B24}]insulin to porcine insulin decreased its biological potency by 55%. Thus the analog containing a substitution of leucine for phenylalanine at position B24 can be identified as an antagonist of insulin action. Importantly, porcine insulin sequentially incubated in 99% formic acid and 10% piperidine to mimic conditions of deprotection maintained full activity in all of the biological systems, whereas deprotected desoctapeptide insulin had no activity at any of the concentrations tested ($\leq$1000 ng/ml for binding competition studies and $\leq$100 ng/ml for biological activity studies).

DISCUSSION

Our results illustrate the effectiveness of solid-phase peptide synthesis and the semisynthetic approach of Inouye *et al.* (9) in the preparation of insulin analogs with amino acid substitutions in the COOH-terminal region of the B chain. The full biological activity of insulin treated with formic acid and piperidine documents the stability of the hormone to these chemical deprotecting agents. The absence of biological activity in our preparation of desoctapeptide insulin (at the concentrations tested) also precludes the possibility of any important

contamination by the native hormone. Thus the activities of the final insulin preparations can be assigned solely to the semisynthetic hormone analogs. Although the synthesis of an insulin derivative containing alanine at positions B24, B25, and B26 has been reported (24), the two insulin analogs described here represent molecules with single amino acid substitutions in the invariant region B23–B26. Use of the TFA group for protection of lysine and of piperidine for deprotection should be readily applicable to the preparation of additional insulin analogs and other peptides by the combination of solid-phase and semisynthetic techniques.

The lower than normal potencies of porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin in competing for insulin binding to membrane receptors and in stimulating glucose oxidation by adipocytes confirms the importance of both Phe-B24 and Phe-B25 in the biologically active site of the hormone. The full agonist activities of the [Leu^{B24}] and [Leu^{B25}] analogs at high concentrations suggest, however, that neither Phe-B24 nor Phe-B25 is absolutely required for biological activity when they are replaced singly. Nevertheless, the differential biological activities of the [Leu^{B24}] and [Leu^{B25}] positional isomers indicates that the two sites in the insulin molecule are not biological equivalent: substitution of Phe-B25 by leucine produces the more functionally perturbed effector. The question of whether this perturbation (or the less marked one induced by substitution at position B24) results from the loss of positive qualities associated with phenylalanine or from the acquisition of negative qualities associated with leucine cannot be answered at this time. It is probable that the hydrophobic character of leucine permits insulin activity in a molecule which, otherwise lacking phenylalanine, might be without activity.

Previous findings on the insulin isolated from the pancreas of a diabetic patient with marked hyperinsulinemia showed that the hormone was an approximately equimolar mixture of normal insulin and a variant containing a substitution of leucine for phenylalanine at position B24 or B25 (6). Our results suggested that the variant had full agonist activity at high concentrations, but that it antagonized the action of insulin at lower concentrations. Although porcine [Leu^{B24}]insulin and [Leu^{B25}]insulin both have full agonist properties (Fig. 4), only the [Leu^{B24}] analog possesses antagonist activity (Table 2). The unique inhibition of insulin action afforded by the admixture of [Leu^{B24}]insulin with normal insulin identifies the analog as an active antagonist of insulin action. It is thus likely that the sequence of residues B23–B26 in the previously identified human insulin variant is Gly-Leu-Phe-Tyr. The combined antagonistic and agonistic properties of [Leu^{B24}]insulin represent unusual and interesting qualities. Determination of whether these properties reflect interactions between hormone and receptor, interactions between occupied receptors, interactions between occupied receptors and other cellular components involved in insulin action, or specific interactions distal to receptor binding will require further study. Material obtained by the semisynthetic method now permits a careful assessment of the mechanism by which the [Leu^{B24}]analog interferes with the normal cellular response to insulin.

Note Added in Proof. As a control, we recently prepared normal porcine insulin by solid-phase synthesis of the [TFA-Lys^{B29}]octapeptide B23–B30 and enzymic coupling of the octapeptide derivative to bis(*t*-BOC)desoctapeptide insulin. Deprotection and purification of the product followed the course described. Based on its immunoreactive insulin and protein content, the semisynthetic hormone had 75–100% of the binding and biological activity of native insulin in the assay systems reported here.

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