Decreased Biologic Activity and Degradation of Human [Ser^{B24}]-Insulin, a Second Mutant Insulin

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SUMMARY

A second mutant insulin, identified as [Ser^{B24}]-insulin, has a highly hydrophilic character. To determine the biologic activity and the degradation of this mutant insulin, human [Ser^{B24}]- and [Ser^{B25}]-insulin analogues were semisynthesized from porcine insulin by an enzyme-assisted coupling method. All of the following studies on isolated rat adipocytes were performed at 37°C to directly correlate the binding potency and the biologic activity. The ability of these insulins to displace ^{125}I-porcine insulin bound to adipocytes was 0.5–2% and 1–4%, respectively, of porcine insulin. When the ability of these insulins to stimulate glucose transport and glucose oxidation was measured, both analogues had full activity at high concentrations (250 ng/ml). However, ED_{50} of the porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to stimulate glucose transport was 0.37 ± 0.05, 46.3 ± 5.4, and 23.3 ± 5.5 ng/ml, respectively. Similarly, for glucose oxidation, ED_{50} was 0.38 ± 0.06, 33.8 ± 3.6, and 16.6 ± 3.4 ng/ml, respectively. Thus, the biologic activity of [Ser^{B24}]- and [Ser^{B25}]-insulins was reduced to 0.5–2% and 1–4% of that of porcine insulin, which was compatible with our previous studies under different conditions. No antagonistic effects were observed for either analogue. Degradation of ^{125}I-labeled [Ser^{B24}]- and [Ser^{B25}]-insulins was also decreased to 62.8% and 55.8%, respectively, of ^{125}I-porcine insulin. These results confirm the importance of the hydrophobic residues at B24 and B25 in the biologic activity of insulin; the patient having this hydrophilic insulin was considered to be in an insulinopenic state despite the hyperinsulinemia due to decreased degradation of the mutant insulin. DIABETES 1985; 34:568–73.

Mutant insulins have been found in three unrelated diabetic subjects and their families.[1] The most characteristic clinical finding in these patients was fasting hyperinsulinemia without any evidence of insulin resistance.[2,3] Evaluation of the biologic activities of these mutant insulins is of importance not only to evaluate the clinical manifestations and the cause of glucose intolerance in these patients, but to understand the structure-function relationship of the insulin itself. Insulins isolated from the sera showed markedly reduced receptor binding ability and biologic activity.[2,3] However, because the serum insulins of these patients are the mixture of normal and mutant insulins[1] and the total amount of the insulin that can be isolated from the sera is limited, further detailed studies required purified materials or synthetic insulin analogues. Thus, human [Leu^{B25}]-insulin, the mutant insulin of the first patient, has been semisynthesized and was demonstrated to have low biologic activity, 1–4% of that of normal insulin.[4–7]

Substitution of serine for phenylalanine at the position of B24 has recently been identified in the second patient by sequence analysis of the cloned insulin gene.[8] Human [Ser^{B24}]- and [Ser^{B25}]-insulins have been semisynthesized by Shoelson et al. and the identity of [Ser^{B24}]-insulin and the second mutant insulin was confirmed by HPLC analysis.[9] Since serine is a polar amino acid, substitution of this amino acid for a hydrophobic amino acid, Phe, leads to a hydrophilic insulin. It is of interest whether this hydrophilic insulin similarly demonstrates decreased biologic activity. Thus, we have semisynthesized the [Ser^{B24}]-analogue of human insulin and compared its biologic activity and degradation with that of semisynthesized human [Ser^{B25}]- and porcine insulin. Because our previous results, which have indicated that [Ser^{B24}]-insulin is less potent than [Ser^{B25}]- and human insulins,[10] are discrepant with the results obtained by Shoelson et al.,[9] we have chosen more physiologic conditions to correlate the receptor binding potency and biologic activity. The results indicate that human [Ser^{B24}]-insulin has extremely low (0.5–2%) biologic activity and that the receptor-mediated degradation is impaired, which may explain the hyperinsulinemia seen in the patient with this mutant insulin.

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MATERIALS AND METHODS

Materials. Porcine monocomponent insulin was kindly supplied by Eli Lilly and Company, Indianapolis, Indiana. $Na^{125}I$, [1-^{14}C]-D-glucose, and [U-^{14}C]-D-glucose were purchased from New England Nuclear, Boston, Massachusetts; collagenase (type I) was from Worthington Biochemical Corp., Freehold, New Jersey; albumin (bovine, fraction V) was from Armour Pharmaceuticals, Kankakee, Illinois; and osmium tetraoxide was from Nakarai Chemicals, Kyoto, Japan.

Synthesis of octapeptides. The two octapeptides, H-Gly-X-Y-Tyr-Thr-Pro-Lys(Boc)-Thr-OH (I: X = Ser, Y = Phe; II: X = Phe, Y = Ser), corresponding to positions B23-B30 of the [Ser^{B24}]- and [Ser^{B25}]-analogues of human insulin were synthesized by conventional solution methods. Z-Gly-Ser-Phe-Tyr-OMe (III, Z = benzyloxycarbonyl) and Z-Gly-Phe-Ser-Tyr-NHNH-Boc (IV, Boc = *t*-butoxycarbonyl) were prepared stepwise from the C-terminal using the Z group for temporary protection of the α-amino function. Couplings were performed by the N-hydroxysuccinimide ester method except for the serine residue, which was introduced by the azide method. III was treated with hydrazine hydrate and IV with trifluoroacetic acid-anisole to give hydrazides Z-Gly-X-Y-Tyr-$NHNH_2$. These hydrazides were then treated with isoamyl nitrite and the azides formed were coupled with H-Thr-Pro-Lys(Boc)-Thr-OH[11] to yield Z-Gly-X-Y-Tyr-Thr-Pro-Lys(Boc)-Thr-OH, from which the Z group was removed by catalytic hydrogenolysis. The purification of the products by partition chromatography on a column of Sephadex LH-20 with ethyl acetate:acetic acid:water (4:1:1) as solvent gave the desired octapeptides I ($[\alpha]_D^{26}$ − 37.3 ± 0.8° [c 1.0, acetic acid]) and II ($[\alpha]_D^{26}$ − 34.2 ± 0.7° [c 1.0, acetic acid]). The sufficient purity of I and II was assessed by TLC, HPLC, and amino acid analysis of acid hydrolysates and aminopeptidase-M digests.[11]

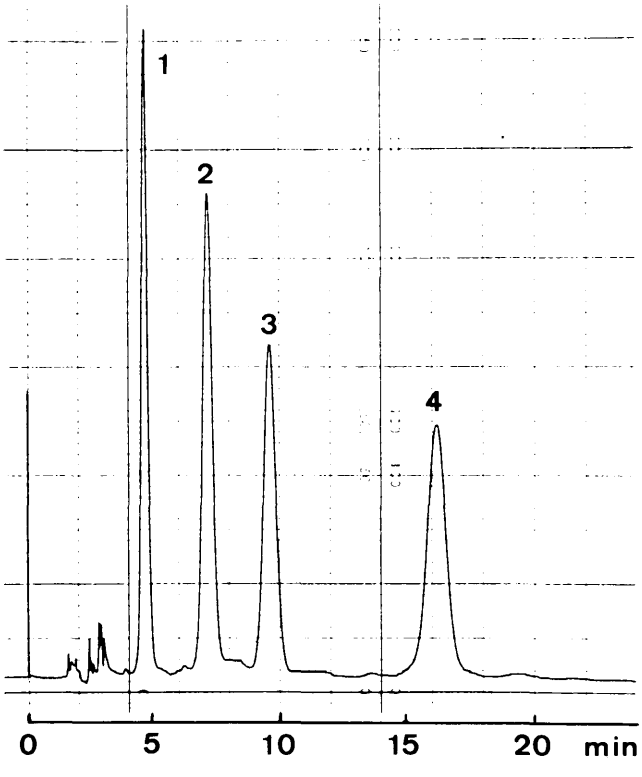

FIGURE 1. HPLC separation of human insulin and its analogues. Approximately the same amounts of human [Ser^{B25}]-insulin (1), human [Ser^{B24}]-insulin (2), human [Ala^{B24}]-insulin (3), and normal human insulin (4) were combined and subjected to HPLC under the following conditions: column: Nucleosil 5C_{18}, 0.46 × 15 cm; mobile phase: 50 mM sodium phosphate buffer (pH 3.0) containing 100 mM sodium sulfate and 28.5% acetonitrile, 1.125 ml/min; and detection: 220 nm (range 0.08).

Semisynthesis of human [Ser^{B24}]- and [Ser^{B25}]-insulins. $N^{\alpha A1}$,$N^{\alpha B1}$-$(Boc)_2$-DOI (50 mg, DOI = desoctapeptide[B23-B30]-insulin), derived from porcine insulin (Eli Lilly and Company, lot 1FJ91),[12] and octapeptide I (100 mg) were dissolved in a mixture of N,N-dimethylformamide (DMF):1,4-butanediol:water (35:35:30, 0.5 ml) containing 0.25 M Tris(hydroxymethyl)aminomethane (Tris) acetate and 10 mM $CaCl_2$. To this was added tosyl-L-phenylalanine chloromethylketone-treated trypsin (5 mg) and the solution was incubated at 37°C for 20 h; the apparent pH of the incubation mixture was 6.5. At the end of the incubation, the mixture was subjected to gel filtration on a Sephadex LH-20 column (3.6 × 41 cm) equilibrated with DMF:0.5 M acetic acid (1:1). Fractions corresponding to the first peak were pooled and evaporated in vacuo. To remove the protecting groups, the resulting material was treated with trifluoroacetic acid-anisole at 0°C for 60 min. Most of the solvent was removed by evaporation and the residue was treated with ether. The crude product thus obtained was again gel filtered through a Sephadex G-50 column (superfine, 2.7 × 106 cm) in 0.5 M acetic acid and then chromatographed on a QAE-Sephadex A-25 column (1.4 × 17 cm) in 0.06 M Tris-HCl buffer (pH 8) containing 60% ethanol and 0.08–0.17 M NaCl.[13] The insulin-containing fractions were collected, dialyzed against 0.01 M HCl and then against distilled water at 4°C, and lyophilized to give a preparation of human [Ser^{B24}]-insulin (24 mg, 42%). In exactly the same manner as above, except for using octapeptide II in place of I, human [Ser^{B25}]-insulin was prepared in a yield of 49% (28 mg).

Iodination of insulins. ^{125}I-porcine, ^{125}I-[Ser^{B24}]-, and ^{125}I-[Ser^{B25}]-human insulins with a specific activity of 100–150 μCi/μg were prepared according to the method of Freychet.[14]

Preparation of the isolated adipocytes. Male Sprague-Dawley rats (180–220 g) were stunned by a blow to the head and decapitated, and epididymal fat pads were removed. Isolated fat cells were prepared by shaking at 37°C for 60 min in Krebs-Ringer bicarbonate buffer containing collagenase (3 mg/ml) and bovine serum albumin (40 mg/ml), as described by Rodbell.[15] Cells were filtered through 250-μm pore-size nylon mesh, centrifuged at 30 × *g* for 4 min, and washed twice in buffer. Adipocyte counts were performed by modified method III of Hirsch and Gallian,[16] in which the cells were fixed by 2% OsO_4 in 0.05 M collidine buffer for 24 h at 37°C and then taken up in a known volume of 0.154 M NaCl for counting with a Coulter counter.

The following experiments were all performed at 37°C using these isolated adipocytes in Krebs-Ringer-phosphate-Hepes buffer (NaCl 135 mM, KCl 2.2 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.25 mM, Na_2HPO_4 2.5 mM, KH_2PO_4 2.5 mM, and Hepes 10 mM, pH 7.4).

Insulin binding study. The ability of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to displace the binding of ^{125}I-porcine insulin to adipocytes was measured by the method of Foley et al.[17]

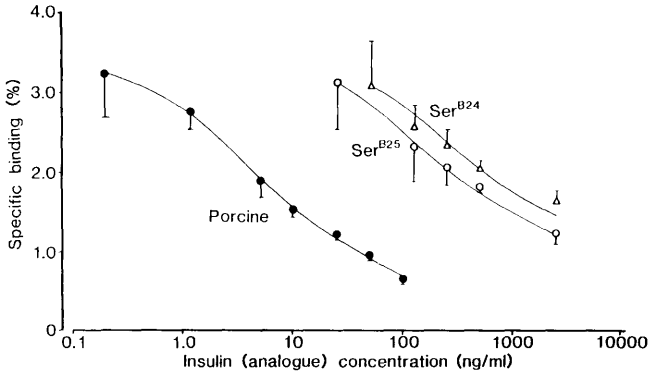

FIGURE 2. Ability of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to displace ^{125}I-porcine insulin from the isolated rat adipocytes. Data represent specific binding (%)/2 × 10^5 adipocytes (mean ± SEM, N = 4).

with slight modification. The isolated adipocytes were incubated with labeled (0.2 ng/ml) and various concentrations of unlabeled insulin in the buffer described above, containing 1% BSA, 0.5 mg/ml bacitracin, and 10 mM glucose at 37°C for 60 min. After the incubation, cells were separated by centrifugation through silicon oil. Data were corrected by subtracting the nonspecific binding, which was assessed by incubating the cells with 20 μg/ml of unlabeled porcine insulin.

Glucose transport study. The glucose transport rate was assessed by measuring the glucose clearance rate. This method has been developed on the premise that glucose uptake provides a measurement of glucose transport when studied at very low glucose concentrations.[18] It has been reported that the glucose clearance rate correlates well with the 3-O-methylglucose transport rate.[18] Isolated adipocytes (2% lipocrit) were preincubated in 500 μl of the buffer described above with 1% BSA containing various concentrations of insulin (or analogue) at 37°C for 30 min. A tracer amount (300 nM) of [U-^{14}C]-D-glucose was then added and the incubation was continued at 37°C for 60 min. After the incubation, cells were separated by centrifugation through silicon oil and the radioactivities associated with the cells were determined in a liquid scintillation counter. The results were calculated in femtoliters (fl)/s/cell.

Glucose oxidation study. The ability of porcine insulin and insulin analogues to stimulate the glucose oxidation in isolated rat adipocytes was determined according to the method of Rodbell.[15] Adipocytes were incubated at 37°C for 60 min with [1-^{14}C]-D-glucose at a total glucose concentration of 2 mM and with various concentrations of insulin (or analogue) in the buffer containing 4% BSA. After the incubation, the generated $^{14}CO_2$ was collected and counted in a liquid scintillation counter.

Degradation study. Adipocytes (5–6 × 10^5 cells) were allowed to associate with ^{125}I-porcine, ^{125}I-[Ser^{B24}]-, or ^{125}I-[Ser^{B25}]-insulin in the presence or absence of 20 μg/ml unlabeled porcine insulin at 37°C for 30 min. Cells were separated by centrifugation through silicon oil, collected, and solubilized in a solution containing 8 M urea, 0.1% Triton X-100, and 1.5 mM acetic acid for 20 min at 4°C. The mixture was then centrifuged for 5 min at 10,000 × *g* at 4°C and the infranatant was applied to a 1 × 50-cm Bio-Gel P-30 column. The column was eluted with 3 M acetic acid containing 0.005% BSA. After the separation of the cells, the degradation of ^{125}I-insulin (or analogues) in the incubation buffer was assessed by trichloroacetic acid (TCA) precipitation and gel filtration.

RESULTS

Characterization of analogues. Amino acid ratios in acid hydrolysates (6 M HCl, 110°C, 20 h in the presence of phenol) were as follows: [Ser^{B24}]-insulin: Asp 3.00 (3), Thr 2.84 (3), Ser 3.73 (4), Glu 6.99 (7), Pro 1.03 (1), Gly 4.10 (4), Ala 1.09 (1), Val 3.54 (4), Ile 1.66 (2), Leu 6.00 (6), Tyr 3.95 (4), Phe 1.85 (2), Lys 0.99 (1), His 1.96 (2), and Arg 1.38 (1); and [Ser^{B25}]-insulin: Asp 2.96 (3), Thr 2.75 (3), Ser 3.73 (4), Glu 6.93 (7), Pro 0.95 (1), Gly 4.03 (4), Ala 1.07 (1), Val 3.41 (4), Ile 1.52 (2), Leu 6.00 (6), Tyr 4.04 (4), Phe 1.93 (2), Lys 0.93 (1), His 1.99 (2), and Arg 0.98 (1). These semisynthetic insulins were shown to be at least 94% pure by analytic HPLC. Retention times of human [Ser^{B25}]- and [Ser^{B24}]-insulins were 4.7 and 7.2 min, respectively (for HPLC conditions, see Figure 1). Under the identical HPLC conditions, human [Ala^{B24}]-insulin and normal human insulin showed retentions of 9.6 and 16.3 min, respectively (Figure 1).

Receptor binding. The ability of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to displace ^{125}I-porcine insulin bound to the isolated rat adipocytes is shown in Figure 2. Human [Ser^{B24}]- and [Ser^{B25}]-insulins had 0.5–2% and 1–4% the potency of porcine insulin, respectively. These results are compatible with the binding potency to IM-9 lymphocytes at 15°C.[10]

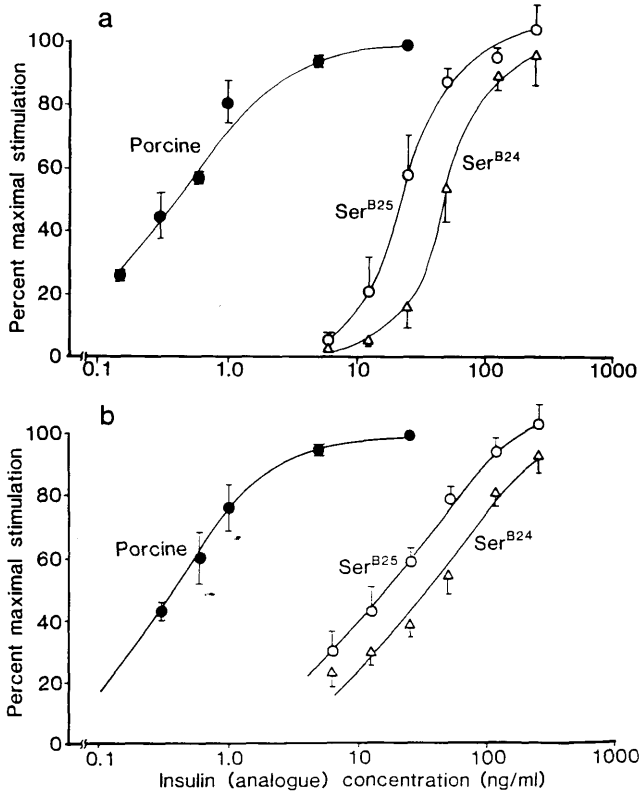

FIGURE 3. Ability of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to stimulate the glucose transport (a) and the glucose oxidation (b). Data represent percent maximal stimulation when the stimulation by 25 ng/ml porcine insulin was considered to be 100% (mean ± SEM, N = 4).

Biologic activity. The ability of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to stimulate the glucose transport rate, as assessed by the glucose clearance rate, and to stimulate glucose oxidation in isolated rat adipocytes is shown in Figure 3, a and b. Basal glucose clearance rate, 8.5 ± 2.0 fl/s/cell, was stimulated to the maximal level of 171.3 ± 9.7 fl/s/cell by porcine insulin. The maximal stimulation of human [Ser^{B24}]- and [Ser^{B25}]-insulins (at 250 ng/ml) was 168.7 ± 21.0 and 188.0 ± 6.6 fl/s/cell, respectively. These levels were not different from those of porcine insulin. Similarly, the maximal effect of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to stimulate glucose oxidation was 53.8 ± 3.4, 51.2 ± 2.5, and 55.3 ± 3.2 nmol CO_2 produced/60 min/2 × 10^5 cells, respectively, from the basal level of 13.5 ± 0.7. Thus, both analogues demonstrated full activity to stimulate glucose metabolism at high analogue concentrations. However, the ED_{50} of porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins to stimulate glucose transport was 0.37 ± 0.05, 46.3 ± 5.4, and 23.3 ± 5.5 ng/ml, respectively. Similarly, in the glucose-oxidation study, the ED_{50} was 0.38 ± 0.06, 33.8 ± 3.6, and 16.6 ± 3.4 ng/ml, respectively. Thus, human [Ser^{B24}]- and [Ser^{B25}]-insulins were considered to have 0.5–2% and 1–4% of the biologic activity, respectively, in glucose metabolism. These activities are consistent with the binding potency described above. The reason for the lack of the parallelism of the dose-response curve of the analogues to that of the porcine insulin in the glucose transport study (Figure 3a) is not clear, but it might be possible that the incubation time was not long enough for low concentrations of the analogues to elicit their full activities in this experimental condition.

Since the [Leu^{B24}]-insulin analogue was once reported to have an antagonistic effect on normal insulin,[4] although not reproducibly so,[7,19,20] the effects of human [Ser^{B24}]- and [Ser^{B25}]-insulins on the stimulatory activity of porcine insulin in glucose transport were examined. As shown in Figure 4, neither analogue had an antagonistic effect at low concentration, but both had agonistic effects at their submaximal concentrations on porcine insulin.

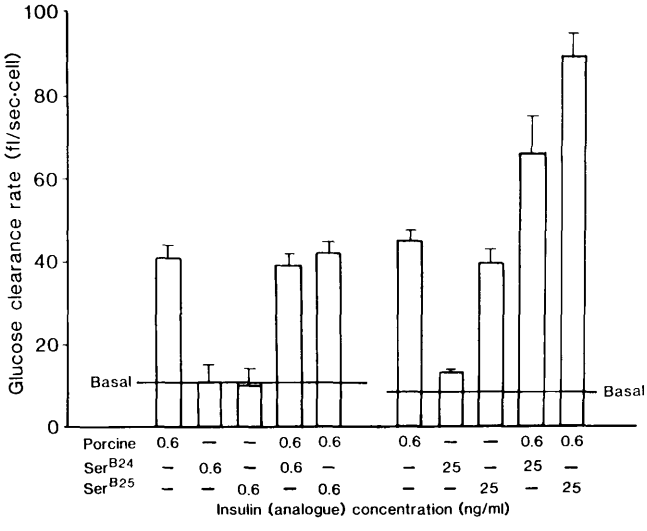

FIGURE 4. Effects of [Ser^{B24}]- and [Ser^{B25}]-insulins on insulin-stimulated glucose transport by rat adipocytes (mean ± SEM, N = 4).

Degradation study. After the labeled insulin (or analogue) was incubated with the adipocytes, radioactive materials associated with the cells and in the incubation buffer were analyzed by gel filtration. A representative profile of the gel filtration is shown in Figure 5, a and b. Peak I was found at the void volume and peak II at the fraction where native porcine insulin was eluted. Peaks III and IV were considered to be the degraded materials. Peak IV is probably ^{125}I-Tyr and peak III might be an intermediate having some more amino acids. The percent degradation was calculated from the peak III + IV/peak I + II + III + IV ratio, and from the TCA precipitability of the radioactive materials in the incubation buffer. These results are summarized in Table 1. By either method, the degraded materials of ^{125}I-[Ser^{B24}]- and ^{125}I-[Ser^{B25}]-insulins in the incubation buffer were less than those of ^{125}I-porcine insulin. Total cell-associated radioactivities were much greater in ^{125}I-porcine insulin than in ^{125}I-analogues. However, the cell-associated degraded materials of these labeled insulins were all approximately 50% of the total radioactivity, indicating that both analogues might have been degraded in the same way as porcine insulin after binding to the cell receptors.

DISCUSSION

In this study, we evaluated the receptor binding ability, biologic activity, and degradation of human [Ser^{B24}]-insulin, the second mutant insulin,[8,9] and compared these measurements with those of porcine and human [Ser^{B25}]-insulins. Results indicate that human [Ser^{B24}]-insulin has extremely low receptor binding ability and biologic activity (0.5–2% of that of porcine insulin) and that these activities are in the order: porcine > Ser^{B25} > Ser^{B24}. These results are compatible with our previous results in different systems (binding to IM-9 lymphocytes at 15°C and stimulation of 2-deoxyglucose uptake in isolated rat adipocytes at 24°C).[10] On the contrary, Shoelson et al., who showed the identity of this mutant insulin and semisynthesized human [Ser^{B24}]-insulin, have reported that [Ser^{B24}]-insulin has almost 16% activity of human insulin in stimulating glucose oxidation and is more active than [Ser^{B25}]-insulin.[9] Because of this discrepancy, we further examined the ability of our semisynthesized analogues to stimulate glucose transport and glucose oxidation as well as receptor binding potency. To directly correlate the binding potency with the biologic activity, we performed these studies under physiologic conditions (pH 7.4, 37°C). In selected studies, we used the semisynthetic insulin preparations further purified by reverse-phase, liquid chromatography using a LiChroprep RP-18 column (size B, E. Merck) with 50 mM sodium phosphate buffer (pH 3.0) containing 100 mM sodium sulfate and a linear gradient (15–40%) of acetonitrile (total volume 200 ml). These preparations had a purity of >99% and showed the same receptor binding potency and biologic activity (data not shown). Thus, the apparent discrepancy (i.e., lower activity of our [Ser^{B24}]-insulin analogue) is not due to the purity of our analogues. The only apparent difference between the two groups was in the method of synthesizing the octapeptides employed in the course of insulin semisynthesis, i.e., the conventional solution method being used in this communication and the solid-phase method in Shoelson's report.[9] In the case of another insulin analogue with substitution at B24 (Leu^{B24}), the reported activity of the

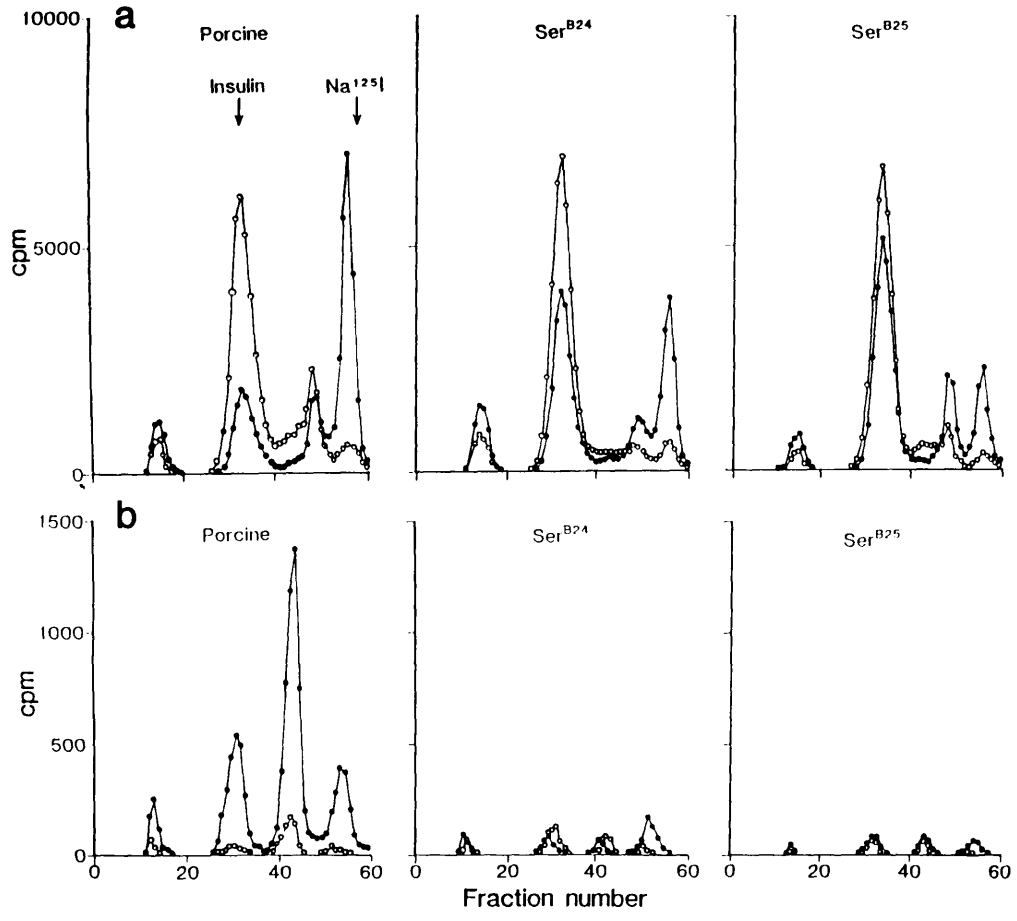

FIGURE 5. Gel filtration profile of the radioactive materials in the incubation buffer (a) and the cell-associated radioactivities (b). Adipocytes were incubated with ^{125}I-porcine, [Ser^{B24}]-, and [Ser^{B25}]-insulins for 30 min at 37°C in the presence (○) or absence (●) of unlabeled porcine insulin (20 μg/ml). Cells were separated, solubilized, and applied to a Bio-Gel P-30 column. After taking aliquots for TCA precipitation, the incubation buffer was also applied to the column.

[Leu^{B24}]-insulin analogue varies from laboratory to laboratory (i.e., 10%,[4] 20.9 ± 2.8%,[6] and 30–48%[7]). This discrepancy again might be due to the difference in the method of peptide synthesis, although it is unclear how this difference in the method results in an apparent discrepancy of the biologic activity of the final product.

Residues from B23 to B26 have been proposed to be an active site of the insulin molecule.[21,22] Among them, Phe^{B24} and Phe^{B25} may play different roles in the structure-function relationship of the insulin molecule. Since the substitution of Phe^{B25} by other amino acids markedly decreased the binding and biologic activity of insulin$^{4-7.9-11.23}$ without changing the structural integrity of the insulin molecule,[6,11] and since the aromatic ring of Phe^{B25} protrudes outside from the molecule, Phe^{B25} could be considered to play a central role in the receptor binding.

On the other hand, substitution of Phe^{B24} appears to change the receptor binding ability by changing the structure of the insulin molecule. From the results of this communication, the hydrophobicity of the amino acid at B24 is considered to be important in maintaining the structural integrity, because the receptor binding ability is of the order: D-Phe^{B24} > Phe^{B24} > Leu^{B24} > Ala^{B24} ≧ Ser^{B24} insulin$^{4-7.11.23.24}$ and the hydrophobicity index of these amino acids is Phe > Leu > Ala > Ser.[25] Similar to [Ala^{B24}]-insulin,[24] [Ser^{B24}]-insulin may decrease the interaction between X^{B24} and Leu^{B15}, resulting in a great flexibility of the C-terminal region of the insulin molecule and a decrease in the receptor binding ability. Thus, the hydrophobicity at the B24 residue could play an important role in the structure-function relationship of the insulin molecule.

The degradation of ^{125}I-[Ser^{B24}]- and [Ser^{B25}]-insulins in the incubation buffer was about 62.8% and 55.8% of that of ^{125}I-porcine insulin, respectively. Since the degradation in the incubation buffer is mainly due to the release of the degraded materials from the cells,[26] this difference in degradation appears to be due to the decreased binding of ^{125}I-analogues to the cell receptors. However, the relatively small difference in degradation compared with binding between porcine in-

TABLE 1
Degradation of ^{125}I-insulin (or analogues) in the incubation buffer (%)

	Porcine	Ser^{B24}	Ser^{B25}
TCA precipitation (mean ± SEM, N = 3)	32.8 ± 0.48	20.6 ± 1.60	18.3 ± 3.45
Gel filtration (mean of 2 determinations)	43.6	26.3	23.5

sulin and both analogues might be attributable to the leakage of the degrading enzyme(s) from the cells. Total cell-associated radioactivities were much greater in ^{125}I-porcine insulin than in ^{125}I-analogues, but the percent cell-associated degradation was the same among them, indicating that both analogues could have the same affinity to the intracellular degradation process as does porcine insulin. However, in contrast to the binding ability, the degradation of ^{125}I-[Ser^{B24}]-insulin in the incubation buffer was slightly greater than that of ^{125}I-[Ser^{B25}]-insulin. Thus, there might be a small difference in the affinity to the degrading enzyme(s) between the two analogues. Further study is necessary to prove this.

A distinct feature of the patients with mutant insulin is hyperinsulinemia.[2,3] This study indicates that hyperinsulinemia is considered to be due to the decreased degradation of the mutant insulin, which is a reflection of the decreased receptor binding. Although hyperinsulinemia exists in the patients, the net insulin activity is not increased, because the amount of the mutant insulin with extremely low biologic activity exceeds 90% of total immunoreactive insulin in the peripheral circulation.[1] Thus, about 100 μU/ml (4 ng/ml) of "immunoreactive" insulin found in the patient's fasting sera[3] is the sum of 0.4 ng/ml normal and 3.6 ng/ml mutant insulins, and, thus, could be calculated to be as potent as 11–12 μU/ml of the "bioactive" normal insulin. Similarly, about 200 μU/ml immunoreactive insulin in the stimulated state[3] is as potent as 21–24 μU/ml of the bioactive normal insulin. The patient is thus considered to be in an insulinopenic state. The antagonistic effect of the mutant insulin on the normal insulin[27] is an attractive hypothesis for explaining the glucose intolerance in these patients. However, [Ser^{B24}]-insulin was found to be an agonist in this study. Therefore, because the effector system of the patient was entirely normal,[3] the glucose intolerance found in the patient and her family members with [Ser^{B24}]-insulin might be due to the impairment of insulin release from the pancreas.

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