A Novel Approach for a Water-Soluble Long-Acting Insulin Prodrug: Design, Preparation, and Analysis of [(2-Sulfo)-9-fluorenylmethoxycarbonyl]3-insulin

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In this study we designed, prepared, and analyzed a water-soluble, long-acting insulin derivative whose protracted action in vivo is based on a new principle rather than on slower absorption rates of suspended insulin formulations. To this end, we have prepared (9-fluorenylmethoxy-carbonyl-SO_3H)3-insulin ((FMS)3-insulin), a derivative having three 9-fluorenylmethoxycarbonyl-SO_3H (FMS) moieties covalently linked to the three amino side chains of insulin. (FMS)3-insulin is soluble in aqueous buffers at neutral pH, at a concentration range of 0.15−0.60 mM, and has about 1% of both the biological potency and the receptor-binding affinity of the native hormone. Upon incubation at pH 7.4 and 37 °C, it undergoes a slow hydrolysis with linear regeneration of insulin possessing full biological potency. A single subcutaneous administration of (FMS)3-insulin to streptozocin-treated rats lowered circulating glucose levels for a prolonged period ($t_{1/2} = 30$ h). Similarly, intraperitoneal administration of (FMS)3-insulin to healthy rats had a prolonged glucose-lowering effect. In this experimental system, recovery from hypogly-cemia proceeded with a $t_{1/2}$ value of 14 ± 1 h, as compared with $t_{1/2} = 8.0 \pm 1$ h for native insulin and $t_{1/2} = 10.0 \pm 1$ h for NPH-insulin. (FMS)3-insulin is more resistant to proteolysis and appears to be nonimmunogenic. On the whole, (FMS)3–insulin represents a prototype version of a water-soluble, long-acting preparation of insulin. It is nearly inactive at the time of administration, and therefore can be administered, at high dose, with no concern for hypoglycemia. Because of its decreased receptor-binding affinity, (FMS)3-insulin evades receptor-mediated endocytosis and degradation and, hence, persists for a long period in the circulation. The insulin constituent of the (FMS)3-insulin conjugate undergoes a slow, spontaneous activation in the circulatory system, manifesting a prolonged glucose-lowering action in vivo. According to the data presented here, (FMS)3-insulin represents a typical prodrug: a compound which by itself shows only marginal activity but over time it is chemically hydrolyzed to the fully active hormone.

Introduction

Insulin is a peptide hormone that regulates glucose and fatty acid metabolism. Diabetic patients, particularly those with Type I, require daily subcutaneous administrations of insulin preparations in order to maintain normoglycemia. Prolonged-acting insulin formulations for basal delivery are currently administered as suspensions of insulin crystals complexed with zinc or protamine.[1] To produce a protracted effect, these formulations are designed to be less soluble in aqueous solutions and thereby to dissolve slower at the injection site.[1–3] Yet, none of the available insulin preparations has sufficiently prolonged action to provide a basal insulin supply throughout the day.[2,4] Moreover, by using insulin suspensions, the accuracy of dosing is limited, and absorption profiles and kinetics are widely variable—both among and within individuals.[5,6] Therefore, insulin preparations that combine water solubility with pro-tracted action are highly desirable.

In recent years, several approaches have been attempted to develop soluble, long-acting insulin deriva-tives with a more reliable and prolonged effect than the zinc−protamine insulin suspensions. Some approaches were based on lowering the isoelectric point of insulin by amino acid substitutions that produce analogues that are soluble in their formulation at pH 3.0 and crystallize after injection when the pH rises to about 7.4.[5,7,8] In another approach, the soluble Co^{3+}−insulin hexamer was used as an alternative to the insoluble Zn^{2+}−insulin hexamer.[9] A different proposal took advantage of soluble, fatty acid acylated insulin that binds to serum albumin. The slow rate by which acylated insulin is released from albumin to the bloodstream produces a prolonged ef-fect.[6,10] While some of these approaches failed to show any improved clinical results, others are still under investigation.[3]

In this study, we have designed, synthesized, and characterized a novel water-soluble derivative of insulin with a protracted action in vivo. For this purpose, we relied on our earlier methodology for a long-acting insulin derivative, represented by (Fmoc)2-insulin.[11]

Results

Preparation of FMS-OSu. The procedure described in the Experimental Section was found to be optimal. Fmoc-OSu was initially subjected to sulfonation with 0.9 equiv of chlorosulfonic acid in CH_2Cl_2. Under these experimental conditions, sulfonation occurs preferen-tially by electrophilic substitution of the fluorene ring

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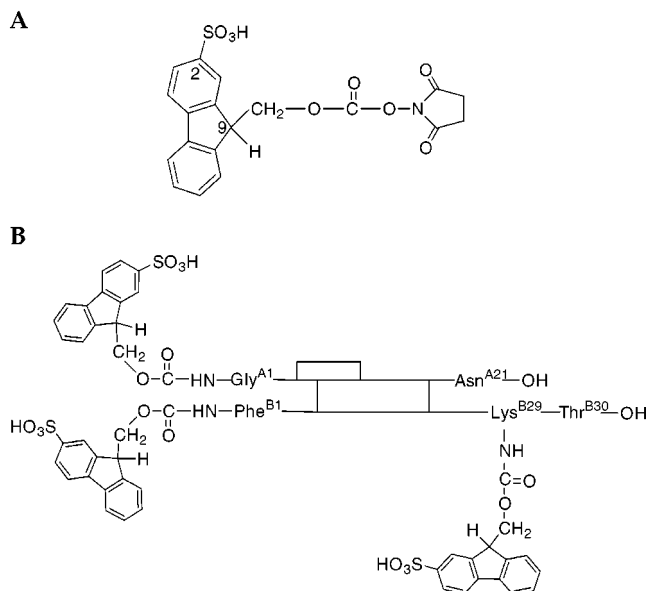† Department of Biological Chemistry.
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B

Figure 1. (A) Structure of FMS-OSu. The position of the sulfonyl group on the fluorene ring is indicated. (B) Structure of (FMS)$_3$-insulin. The positions of the amino groups that were modified are indicated.

at position 2 (Figure 1A). The substituent at position 9 (Figure 1A) had no effect on the orientation of the sulfonation.[12] The product has been obtained in high yield (86%). Unlike Fmoc-OSu, FMS-OSu is water-soluble. Its molar extinction coefficient values in neutral aqueous media are $\epsilon_{280} = 21\ 200$ and $\epsilon_{301} = 10\ 100$.

Synthesis and Biological Potency. Following synthesis of FMS-OSu, the three amino groups of human insulin were covalently modified by reacting with an excess of FMS-OSu. The product was further purified by HPLC (see Experimental Section). The primary structure of (FMS)$_3$-insulin is depicted in Figure 1B. (FMS)$_3$-insulin was extensively characterized chemically and biologically. Figure 2 and Table 1 summarize the characteristic features of this compound relevant to this study. (FMS)$_3$-insulin is a water-soluble derivative at concentrations ranging from 1 mg/mL at pH 6.0 to 4 mg/mL at pH 7.5. It contains 3 mol of FMS/mol of insulin, covalently linked to the α-amino functions of PheB1 and GlyA1 and to the ε-amino function of LysB29. (FMS)$_3$-insulin is characterized by high absorbance at 280 nm ($\epsilon_{280} = 69\ 600 \pm 3000$) and at 301 nm ($\epsilon_{301} = 33\ 300 \pm 2000$), where the native hormone absorbs negligibly. The representative UV absorption spectrum given in Figure 2A shows an absorption maximum at 275 nm with a shoulder at 301 nm. Using analytical HPLC procedures, (FMS)$_3$-insulin emerged as a single symmetric peak (Figure 2B, $t_R = 25.59$ min). (FMS)$_3$-insulin activates lipogenesis in rat adipocytes with a half-maximal effect (ED$_{50}$) of 20 $\pm$ 3 ng/mL (as compared with ED$_{50}$ = 0.2 $\pm$ 0.02 ng/mL for native insulin). Also, compared with native insulin, about 100 times higher concentrations of (FMS)$_3$-insulin are required for half-maximal displacement of bound [^{125}I]-insulin from intact rat adipocytes. Thus, before incubation under physiological conditions (see the next paragraph), (FMS)$_3$-insulin has about ~1% receptor binding affinity and 1% the biological potency of the native hormone.
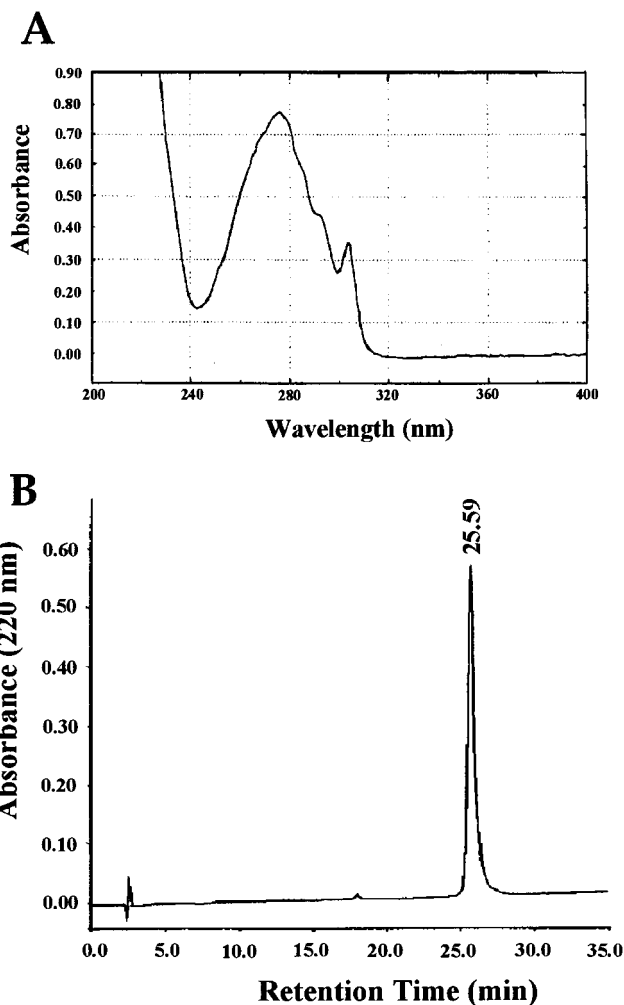
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Figure 2. (A) UV absorption spectrum of (FMS)$_3$-insulin. The absorption spectrum was recorded for (FMS)$_3$-insulin (1.15 $\times$ 10^{-5} M) in H$_2$O, pH 7.0, at 25 °C. (B) HPLC analysis of the purified synthetic product (FMS)$_3$-insulin. A, 0.1% TFA in H$_2$O; B, 0.1% TFA in acetonitrile$-$H$_2$O, 75:25. HPLC was conducted in a linear gradient of 30% to 100% of solution B over 50 min, at a flow rate of 0.8 mL/min, using a C$_{18}$ (5 μm, 250 $\times$ 4 mm) column. The effluent was monitored at 220 nm. Insulin elutes under the same experimental conditions with $t_R = 20.27 \pm 0.1$ min.

Table 1. Chemical and Biological Features of (FMS)$_3$-insulin

characteristic	(FMS)$_3$-insulin
amino acid composition	identical to insulin
mol FMS/mol insulin[a]	3.1
retention time[b] (min)	25.59
MS[c] (*m/z*) calcd	6713
MS (*m/z*) found	6716
solubility in aqueous buffer, pH 7.4 (mg/mL)	4 $\pm$ 0.3
relative receptor binding potency[d] (%)	1.2 $\pm$ 0.3
relative biological potency[e] (%)	1.0 $\pm$ 0.2

[a] Determined spectroscopically (experimental part). [b] Established with analytical HPLC procedure (Experimental Section). Insulin elutes under the same experimental conditions with $t_R = 20.27 \pm 0.1$ min. [c] Mass spectra was determined using electrospray ionization technique. [d] Receptor-binding capacity was determined by a binding assay and displacement of [^{125}I]insulin from rat adipocytes.[19,24] [e] Biological potency was determined by a lipogenic assay with rat adipocytes.[17,18]

Rate of Insulin Reactivation upon Incubation of (FMS)$_3$-insulin at Physiological pH and Temperature. In a preliminary experiment, we found that
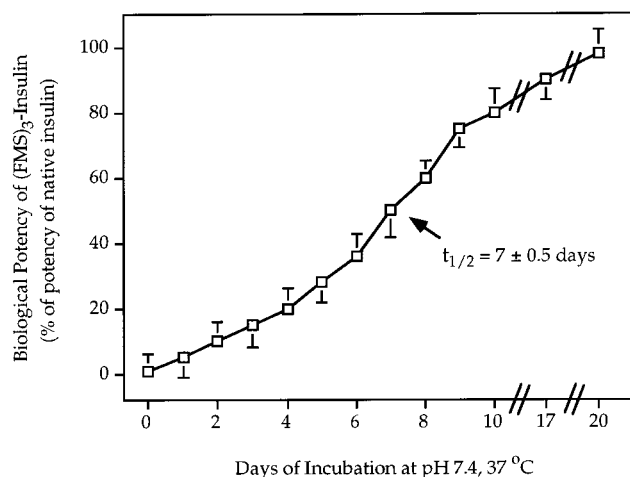
Figure 3. Time course of reactivation of $(FMS)_3$-insulin. $(FMS)_3$-insulin (1 mg/mL in 50 mM Hepes buffer) was incubated at pH 7.4, 37 °C. Aliquots were drawn at the indicated time points and analyzed for biological activity in a lipogenic assay with rat adipocytes.[17,18] Under our assay conditions, insulin activates lipogenesis 4−5-fold above the basal level with an ED_{50} value = 0.2 ± 0.02 ng/mL. An insulin derivative exhibiting ED_{50} = 20 ± 2 ng/mL in this assay is considered having 1% the native biological potency.[25]

$(FMS)_3$-insulin (1 mg/mL), upon incubation in 0.1 M $NaHCO_3$ (pH 8.5) for 40 h, resumed the full activity of the native hormone. This was confirmed both by HPLC and by a lipogenic assay (not shown). Subsequently, $(FMS)_3$-insulin (1 mg/mL dissolved in 50 mM Hepes buffer, pH 7.4) was incubated in a water bath at 37 °C, and aliquots were withdrawn daily during this 20-day study, frozen, and examined both for their biological potency by a lipogenic assay (Figure 3) and for the regeneration of native insulin by an HPLC procedure (Figure 4). Under these incubating conditions, the insulin constituent of the $(FMS)_3$-insulin conjugate is activated slowly ($t_{1/2} = 7.0 \pm 0.5$ days) in a nearly linear fashion. Thus, starting from about 1% of the biological potency of insulin, $(FMS)_3$-insulin increased its potency during incubation to 5 ± 0.5%, 11 ± 2%, 16 ± 3%, 21 ± 3%, and 27 ± 4% on days 1 to 5, respectively. On day 20, $(FMS)_3$-insulin returned to almost the full (96%) potency of native insulin, as judged by the lipogenic assay. HPLC analyses (Figure 4) revealed a decrease in the level of $(FMS)_3$-insulin (t_R = 25.59 min) side by side with the appearance of native insulin (t_R = 20.27 min), monomodified intermediates, and dimodified intermediates (t_R = 21.07−24.71 min), starting shortly after incubation (day 3). The large peak at 13.14 min represents the released FMS moiety in the form of 2-sulfodibenzofulvene (verified by mass spectra analysis, ES^-: m/z 257 (100%, M − 1)). The level of native insulin kept increasing along the hydrolysis in parallel to the decline in $(FMS)_3$-insulin and the intermediates (days 7 and 14). Almost full generation of native insulin was observed on day 20, with the complete disappearance of the $(FMS)_{1,2}$-insulin intermediates, which correlated well with the biological potency obtained.

Effect of a Single Subcutaneous Administration of $(FMS)_3$-insulin to Diabetic Rats. Because streptozocin-treated rats (STZ-rats) are hyperglycemic (>300 mg/dL glucose), hypoinsulinemic, and catabolic,[13] they provide an appropriate model for testing the protracted

action of insulin derivatives. In the set of experiments presented in Figure 5, a group of STZ-rats was treated with a single subcutaneous dose of $(FMS)_3$-insulin (3 mg/rat) and then monitored for the circulating glucose levels and daily weight gains over a period of 3 days. Another group of STZ-rats was treated similarly with native insulin. $(FMS)_3$-insulin reduced blood glucose levels of STZ-rats within 2 h after administration (Figure 5). Glucose levels were further decreased and normoglycemia was attained after ∼12 h and maintained for an additional 12 h; hyperglycemia then reoccurred ($t_{1/2}$ = 48 ± 3 h, Figure 5). In native insulin-treated rats (3 mg/rat), hyperglycemia reoccurred within 7 ± 1 h after administration. Evidently, $(FMS)_3$-insulin has a significantly longer lasting glucose-lowering effect ($t_{1/2}$ = 48 ± 3 h) as compared with native insulin ($t_{1/2}$ = 7 ± 1 h).

Also, STZ-rats showed a higher rate of daily weight gain when treated with $(FMS)_3$-insulin (9.5 ± 1.5 g/rat/day) as compared with native insulin (5.1 ± 1.1 g/rat/day).

Effect of a Single Intraperitoneal Administration of $(FMS)_3$-insulin to Normal Healthy Rats. Recently, we have established an in vivo experimental model in rats for evaluating the long-acting capacity of a given insulin derivative applied downstream to the subcutaneous compartment.[11] Accordingly, insulins are administered intraperitoneally, and $t_{1/2}$ (in hours) for recovery from hypoglycemia is determined. This assay reflects the protracted action in the circulation, independent of the rate of insulin absorption from the site of subcutaneous administration to the circulatory system.

In the experiments presented in Figure 6, groups of healthy rats received intraperitoneally either native insulin, NPH-insulin, or $(FMS)_3$-insulin. Blood glucose levels were then monitored over a period of 24 h. $(FMS)_3$-insulin reduced circulating glucose levels over a period of 20 h, with a $t_{1/2}$ of recovery of 14 ± 1 h. As expected for this experimental system, native insulin and the commercial long-acting NPH-insulin had close $t_{1/2}$ values ($t_{1/2}$ = 8 ± 1 h and $t_{1/2}$ = 10 ± 1 h, respectively).

Stability toward Proteolysis by Trypsin and α-Chymotrypsin. Resistance to proteolysis in the bloodstream or tissue is an additional contributing factor to prolonging the half-life of peptide and protein hormones. We therefore estimated the susceptibility of $(FMS)_3$-insulin to proteolysis by a mixture of trypsin and α-chymotrypsin at 37 °C (see Experimental Section). Whereas native insulin was fully degraded within 6 h with a $t_{1/2}$ value of 0.8 h, $(FMS)_3$-insulin was highly resistant to proteolysis, having a $t_{1/2}$ value of 8.5 h (Figure 7). The observed lower trypsin sensitivity of $(FMS)_3$-insulin is due to the implemented side chain Fmoc-modification of LysB29, resulting in ArgB22 remaining the only trypsin-cleavable residue. With α-chymotrypsin, all the aromatic amino acid residues are available for cleavage. The resistance of $(FMS)_3$-insulin to proteolysis probably stems from steric hindrance of the bulky FMS moieties leading to reduced binding of the derivative to the proteases.

Antigenicity of $(FMS)_3$-insulin. Bearing in mind the putative usage of $(FMS)_3$-insulin in the future care of diabetes, we determined its antigenic capacity in
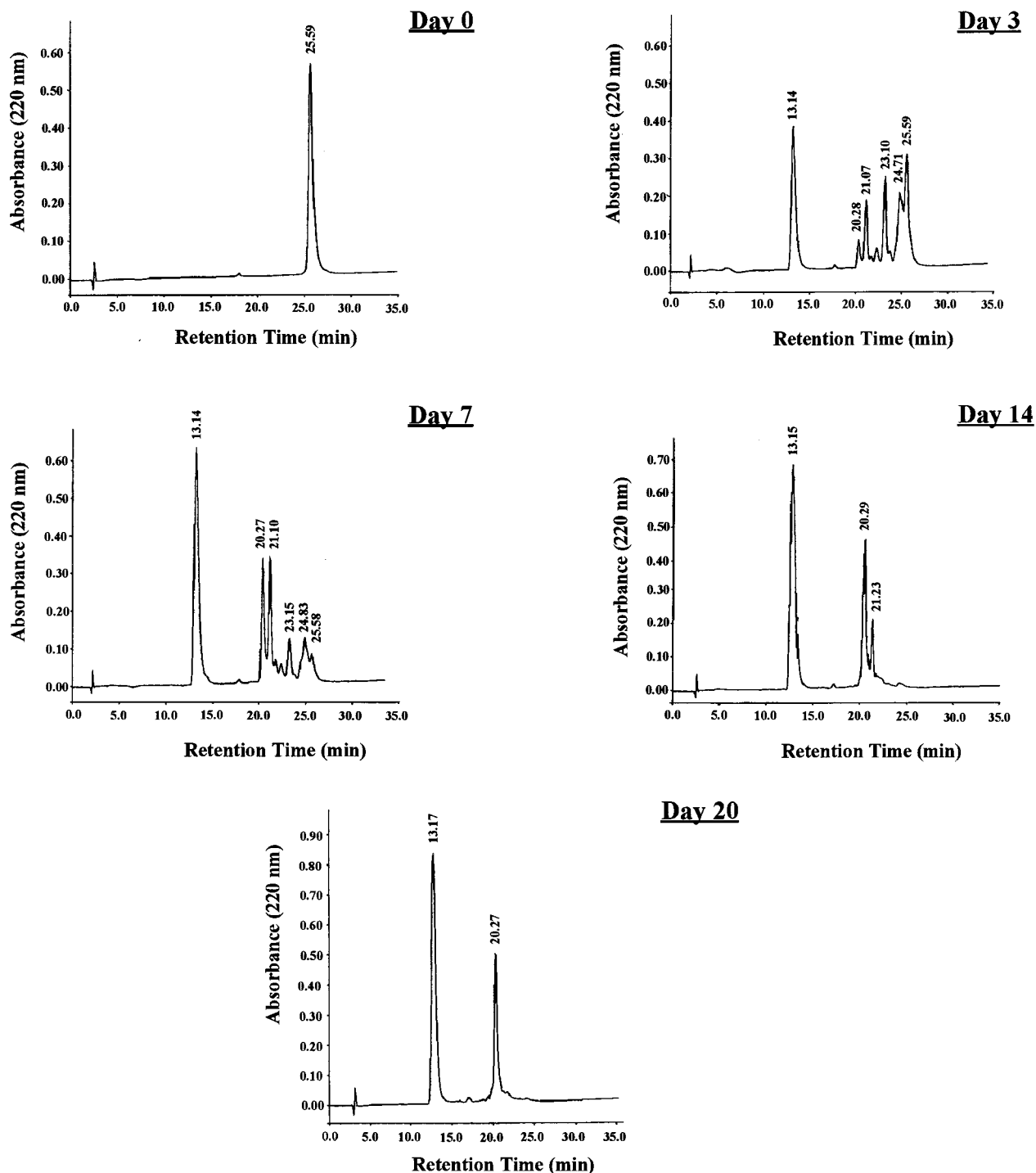
Figure 4. Time dependent regeneration pattern of native insulin from (FMS)$_3$-insulin as analyzed by an HPLC procedure. (FMS)$_3$-insulin (1 mg/mL in 50 mM Hepes buffer) was incubated at pH 7.4, 37 °C. Aliquots were drawn at the indicated days and subjected to HPLC analysis. HPLC conditions were the same as in Figure 2B. (FMS)$_3$-insulin, native insulin, and 2-sulfodibenzofulvene elute with retention times of 25.59, 20.27, and 13.14 min, respectively.

comparison to the native hormone. Figure 8 shows the levels of antibodies elicited after treating mice with native insulin or with (FMS)$_3$-insulin in complete Freund's adjuvant oil. Measurements of antibody levels were performed in an ELISA assay. Both the native hormone and the derivative induced antibodies to insulin to a similar extent. The sera of (FMS)$_3$-insulin-treated mice manifested even a lower incidence of antibodies to (FMS)$_3$-insulin as compared with insulin-treated mice (Figure 8, *P* < 0.01); sera of untreated control mice had a negligible level of antibodies to

insulin (OD$_{405}$ < 0.025, not shown). On the whole, (FMS)$_3$-insulin appears to be less immunogenic than the native hormone. In a separate set of experiments, we found that antibodies to insulin insignificantly cross-reacted with (FMS)$_3$-insulin (~4%, not shown).

Discussion

A new conceptual approach for long-acting insulins is a scientific challenge with obvious clinical implications. This is particularly valid if the long-acting preparation is *soluble* in aqueous media.[5-7] Previously,
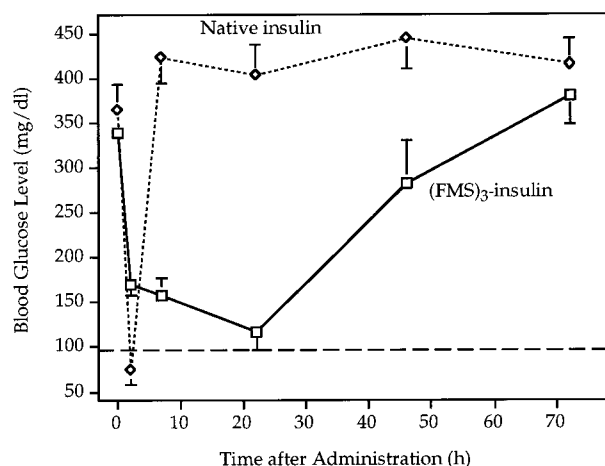
Figure 5. Effect of a single subcutaneous administration of (FMS)$_3$-insulin or native insulin on blood glucose levels of STZ-rats. STZ-rats received either (FMS)$_3$-insulin or native insulin (3 mg/rat, dissolved in 0.75 mL of 50 mM Hepes buffer, pH 7.4). Blood glucose levels were determined at the time points indicated (i.e., 0, 2, 7, 22, 46, and 72 h following administration). Each point represents the arithmetic mean of blood glucose levels of five rats. The horizontal dashed line indicates the arithmetic mean of blood glucose of control healthy rats.
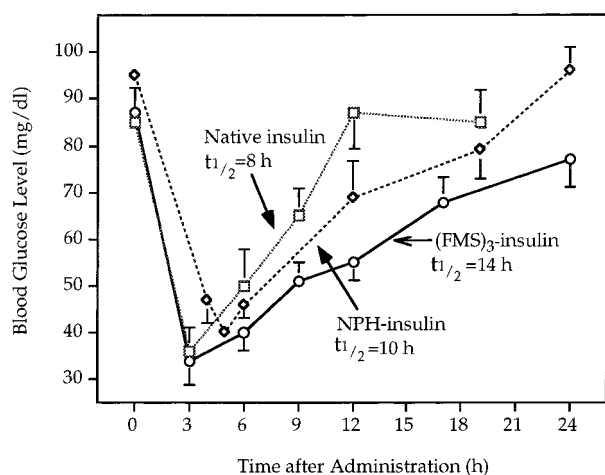

Figure 6. Effect of a single intraperitoneal administration of (FMS)$_3$-insulin on blood glucose levels of normal healthy rats. Groups of five normal, healthy rats were administered (FMS)$_3$-insulin, native insulin, or NPH-insulin (3 mg/rat dissolved in 0.75 mL of 50 mM Hepes buffer, pH 7.4). Blood glucose levels, determined at the time points indicated, are the arithmetic means of five rats. Arrows indicate the half-life of recovery from hypoglycemia for each treatment.

we have introduced such a long-acting prototype—intrinsically *a water-insoluble* derivative of insulin.[11] In the present study we described an aqueous-soluble long-acting preparation that acts through a mechanism distinctive of suspended preparations of insulin. The available insulin preparations suffer from variable absorption profiles, increased risks of macrophage attack and hypoglycemic episodes at unpredicted time points after administration or, alternatively, from insufficient basal glucose lowering effect over prolonged periods.[3,5,6] Combining rapid-acting and long-acting insulin preparations raises another problem, namely, the possible interaction of rapid insulin with the free zinc and protamine of the long-acting preparation.[1]

In this study we have designed and prepared a water-soluble insulin derivative and examined whether it is
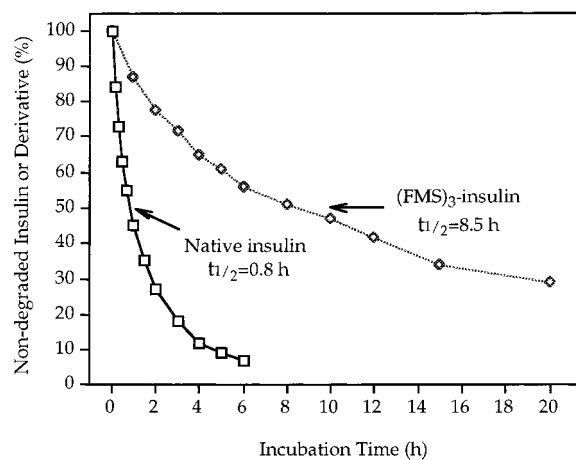
Figure 7. Stability of (FMS)$_3$-insulin toward enzymatic degradation. (FMS)$_3$-insulin and native insulin were incubated with a mixture of trypsin and α-chymotrypsin at 37 °C. At the indicated time points, aliquots were subjected to analytical HPLC. The quantity of the hormone (peak area) at time = 0 was assigned 100%.
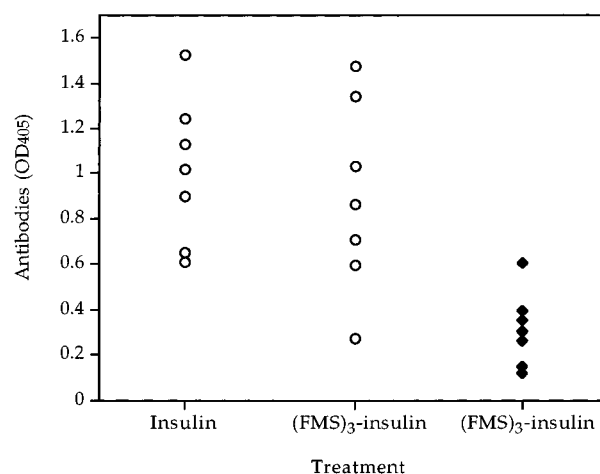

Figure 8. Antigenic capacity of (FMS)$_3$-insulin in mice. Both insulin and (FMS)$_3$-insulin induced antibodies to insulin to the same extent. Lower levels of antibodies to (FMS)$_3$-insulin were found in the sera of (FMS)$_3$-insulin-treated mice. Groups of seven BALB/C mice (3-month-old) were treated with 100 μg of native insulin or with 100 μg of (FMS)$_3$-insulin emulsified in complete Freund's adjuvant oil, boosted at 2 weeks, and analyzed for antibodies to insulin or to (FMS)$_3$-insulin in an ELISA assay at 6 weeks (Experimental Section). A significant amount of antibody was defined as an OD$_{405}$ nm reading >0.10, which is 5 SD over the mean ELISA reading obtained in the sera of six normal mice. $P < 0.01$ by the Kruskal–Wallis nonparametric test; ○, antibody to native insulin; ♦, antibody to (FMS)$_3$-insulin.

long-acting in vivo. To do so, we have introduced an acidic ionizable sulfonic acid moiety into the fluorene ring of Fmoc-OSu. Three molecules of FMS were then introduced to the insulin molecule, yielding a water-soluble derivative in neutral and slightly alkaline media (Table 1). Other than being soluble in aqueous media, (FMS)$_3$-insulin resembles our previous prototype, having 1% the binding affinity and the biological potency of the native hormone (Table 1). Since receptor-mediated endocytosis is a major degradative pathway for insulin,[14] naturally, an insulin derivative with greatly reduced receptor-binding affinity is a longer lived species in the circulation, as well as pharmacologically inactive. The insulin constituent of the (FMS)$_3$-insulin

conjugate, however, is capable of regaining its biological potency upon incubation at pH 7.4, in a slow, spontaneous linear fashion, with a $t_{1/2}$ value = 7 ± 0.5 days (Figure 3). HPLC analyses confirmed that native insulin is fully generated following hydrolysis under these conditions (Figure 4). A single subcutaneous administration of (FMS)$_3$-insulin lowered blood glucose levels of STZ-rats over a period of 2 days (Figure 5). We have also confirmed that (FMS)$_3$-insulin has a prolonged action within the circulation by administering the derivative intraperitoneally. Thus, recovery from hypoglycemia proceeded with a $t_{1/2}$ value of 14 ± 1 h as compared with 8 ± 1 and 10 ± 1 h for native or NPH-insulin, respectively (Figure 6). We also found that (FMS)$_3$-insulin is resistant to proteolysis by trypsin and chymotrypsin (Figure 7) and ascertained that it is relatively nonimmunogenic (Figure 8).

The in vivo results agree with the accelerated rate of activation of (FMS)$_3$-insulin previously found in vitro. Thus, the sulfonic acid group at position 2 of the fluorene ring increased the rate of proton abstraction at position 9 and, therefore, the β-elimination and deprotection process of the FMS moiety by 2–4-fold. Earlier studies performed on the lability of the FMS group to various bases showed greater base sensitivity than the corresponding parental system.[12] Thus, for example, the rate constant for the release of the FMS group from the α-amino function of glycine was much higher than for the Fmoc group (by a factor of about 30). Therefore, by introducing the FMS group into insulin, the increase in solubility decreases reciprocally the long-lasting effect of this derivative. To compensate for this characteristic, we introduced three groups of FMS into insulin, as opposed, for example, to the two groups of our previous long-acting (Fmoc)$_2$-insulin derivative.[11] Conversion into the native, active insulin is therefore extended. Nevertheless, the principle of long-lasting antidiabetic effect through escaping receptor-mediated endocytosis and degradation holds for the (FMS)$_3$-insulin derivative as well.

Our previous long-acting prototype, (Fmoc)$_2$-insulin, and the present one, (FMS)$_3$-insulin, resemble each other in having about 1% the biological potency and the receptor-binding affinity of the native hormone. They differ, however, in two distinct parameters: first, (FMS)$_3$-insulin is soluble in aqueous, neutral media, whereas (Fmoc)$_2$-insulin is insoluble; second, at pH 7.4 (37 °C), the insulin of the (FMS)$_3$-insulin conjugate is activated at a faster rate ($t_{1/2} = 7 \pm 0.5$ days) than (Fmoc)$_2$-insulin ($t_{1/2} = 12 \pm 1.0$ days). As both derivatives have ~1% of the native receptor-binding affinity, the rate of their clearance through receptor-mediated degradation is expected to be similar. We could not compare these two derivatives through subcutaneous administration, as the long-acting effect of the insoluble (Fmoc)$_2$-insulin is attributable both to a slower rate of absorption and to its extended survival efficacy in the circulation. We compared, however, these two derivatives by intraperitoneal administration. In this experimental system, recovery from hypoglycemia occurred with a $t_{1/2}$ value = 26 h for (Fmoc)$_2$-insulin[11] and $t_{1/2}$ = 14 h for (FMS)$_3$-insulin (Figure 6). This agrees well with the rate of activation of these two prototypes at pH 7.4 and 37 °C. However, the rate of hydrolysis of (FMS)$_3$-insulin in

vivo, or in rat's serum in vitro, is apparently 5–7 times faster than that obtained in PBS buffer, pH 7.4, 37 °C (Figures 3–6). This is most likely because of the high nucleophilic capacity of the serum, contributed by the free ions, amines, peptides, and proteins.

In summary, we have described here a novel concept for a water-soluble, long-acting insulin. Three acidic (FMS) moieties were covalently coupled to insulin to obtain a water-soluble derivative having negligible receptor-binding capacity. By avoiding receptor-mediated degradation, this derivative persists longer in circulation. (FMS)$_3$-insulin undergoes slow, spontaneous regeneration of the native insulin biological activities, yielding the desired kinetics of glucose-lowering action in vivo. In addition, the stability of (FMS)$_3$-insulin toward proteolysis further prolongs its half-life in the circulatory system. Using this prodrug approach, we also avoid the dilemma of permanently altering the primary structure and related conformational consequences of insulin and thus its properties (which directly affect its hormonal activity). In fact, derivatization of the insulin molecule via reversible chemical modification will abolish its biological activity. Under physiological conditions, however, (FMS)$_3$-insulin is capable of reverting back to the native, fully biologically active hormone over time. Finally, we confirmed that this derivative is nonimmunogenic, making it an attractive candidate as a therapeutical agent. The finding that Fmoc-amino acids are nontoxic in model animals[15] enhances the potential clinical application of this derivative. We are currently synthesizing a battery of Fmoc moieties with diverse ionizable functions and polarities to enable the preparation of insulin derivatives with varying solubilities and rates of activation. Engineering the type and number of substituents attached to insulin can be directly reflected in their onset of action, duration, and solubility. This approach can lead to an optimal soluble formulation for basal insulin release that is characterized by rapid onset, protracted action, and reproducibility, following a single administration.

Experimental Section

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide; FMS, (2-sulfo)-9-fluorenylmethoxycarbonyl; (FMS)$_3$-insulin, an insulin derivative having three FMS moieties covalently attached to the amino side chains of Lys B29, PheB1, and GlyA1; NPH, neutral protamine Hagedorn, a commercial long-acting insulin preparation; OD, optical density; t_R, retention time; RT, room temperature; STZ-rats, streptozocin-treated diabetic rats.

Materials. Human (Zn^{2+}-free) insulin, kindly donated by Novo Nordisk and by Biotechnology General, was used without further purification. D-[U-^{14}C]Glucose (4–7 mCi/mol) was obtained from Du Pont-NEN (Boston, MA). Collagenase, type I (134 units/mg), was purchased from Worthington (Freehold, NJ). 9-Fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu) is a product of Novabiochem (Laüfelfingen, Switzerland). All other materials used in this study were of analytical grade.

Chemical Procedures. Ultraviolet spectra were obtained with a Beckman Du 7500 spectrophotometer in 1 cm path length UV cuvettes. Thin-layer chromatography was performed on Merck Kieselgel 60-F254, precoated aluminum plates. Mass spectra were determined with a VG AutoSpec Q (EBE-qQ) mass spectrometer (VG Fisons, Altrincham, UK)

using the electrospray ionization technique. Reverse-phase HPLC was performed with a Spectra-Physics SP8800 liquid chromatography system (Spectro-Physics, San Jose, CA) equipped with an applied Biosystem 757 variable wavelength absorbance detector. The column effluents were monitored by UV absorbance at 220 nm and chromatograms were recorded on a chrom-Jet integrator (Thermo-Separation, Riviera Beach, FL). HPLC prepacked columns used in this study included LiChroCART 250−10 mm containing LiChrosorb RP-18 (7 μm) and LiChrospher 100 RP-18 (5 μm), 250−4 mm (Merck, Rahway, NJ). Linear gradients were used between solution A (0.1% TFA in H_2O) and solution B (0.1% TFA in acetonitrile−H_2O, 75:25). For analytical HPLC procedures, a linear gradient between 30 and 100% of solution B was run for 50 min at a flow rate of 0.8 mL/min. Amino acid analyses were performed following 6 N HCl acid hydrolysis at 110 °C for 24 h using a Dionex automatic amino acid analyzer HP 1090 (Palo Alto, CA).

Preparation of (2-Sulfo)-9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide [Fmoc(SO_3H)-OSu]. This compound was prepared by modifying the procedure of Merrifield and Bach.[12] Briefly, Fmoc-OSu (337.4 mg, 1 mmol) was dissolved in 4 mL of CH_2Cl_2 and cooled to 0 °C. A solution of $ClSO_3H$ (60 μL, 0.9 mmol) in 2 mL of CH_2Cl_2 was added with constant stirring and cooled over a period of 15 min. The solution, which turned yellow, was allowed to warm to room temperature (RT). A white precipitate was formed within 1 h. At 2 h, cyclohexane (4 mL) was added to dissolve the unreacted Fmoc-OSu. The suspension was centrifuged and washed four times with 6 mL of 1:1 cyclohexane−CH_2Cl_2. The white solid thus formed was dried under P_2O_5 in vacuo for 24 h and had the following characteristics: yield of 290 mg (86%); mp 140−146 °C; TLC (1-butanol−acetic acid−water, 8:1:1), R_f 0.31; Mass spectrum (ES⁻), m/z 416 (100%, M − 1).

Synthesis of (FMS)₃-insulin. A solution of Fmoc(SO_3H)-OSu (108 mg, 260 μmol) in 2 mL of water was added gradually to a stirred solution of insulin (100 mg, 17.2 μmol) and $NaHCO_3$ (43 mg, 512 μmol) in acetonitrile−water (1:1 v/v; 4 mL) at 0 °C. The final pH of the mixture, 7.5−8, was kept in this range by a slight addition of 0.1 M $NaHCO_3$ during synthesis. The reaction mixture was stirred for 2 h at RT and then completely dried under vacuo. Excess reagent was removed by dilution with acidified H_2O (pH 2.0) and centrifugation. The (FMS)₃-insulin thus obtained was further purified by a preparative HPLC procedure to yield 40 mg of pure (FMS)₃-insulin. Amino acid analysis verified the correct amino acid composition of native insulin. No free amino functional groups were detected using Sanger's procedure with dinitrofluorobenzene, trinitrobenzenesulfonic acid, or dansyl chloride.[16] Absorption at 301 nm (molar extinction coefficient for FMS moiety: $\epsilon_{301} = 10\,100$) indicated that (FMS)₃-insulin had three FMS moieties attached to insulin. Mass spectra verified the correct molecular weight (see Table 1).

Biological Methods. Rat adipocytes were prepared from fat pads of male Wistar rats (100−200 g) by collagenase digestion.[17] Lipogenesis (incorporation of [U-¹⁴C]glucose into lipids) was performed according to the method of Moody et al.[18] Displacement of ¹²⁵I-labeled insulin from rat adipocytes was carried out at 7 °C, essentially as described by Shechter et al.[19] Diabetes was induced by a single intravenous injection of a freshly prepared solution of streptozocin (STZ, 55 mg/kg body weight) according to the work of Meyerovitch et al.[20] Rats were maintained at 24 °C under controlled lighting conditions and fed ad libitum. Blood samples for the analysis of blood glucose were taken from the tail veins and measured with a glucose analyzer (Beckman Instruments Inc., with the glucose oxidase method). Groups consisted of five rats, and the data are presented as means ± SE.

Enzymatic Degradation. Native insulin and (FMS)₃-insulin (1 mg) were dissolved in 1 mL of Hepes buffer (50 mM, pH 7.4) containing 5% DMSO and 4-nitrophenol (0.2 mg; used as an inert internal marker). Trypsin and chymotrypsin, dissolved in 0.1 mM HCl (10 μL, 1 mg/mL), were added and the solutions were incubated at 37 °C. At the indicated time

points, aliquots were withdrawn (25 μL), acidified with 0.5 mL mixture of solutions A and B (60:40 v/v, the initial gradient ratio used for HPLC separations), and then analyzed by HPLC. Each sample (containing 25 μg of protein at time 0′) was eluted at a flow rate of 1 mL/min with a gradient as described above for insulin derivatives. The area corresponding to the protein peak (in percent, calculated by the integrator) is referred to as the internal peak of 4-nitrophenol. The protein peak area at $t = 0$ was designated 100%.

Antigenicity. Twelve-week-old BALB/C mice were initially treated with 100 μg of native insulin or with 100 μg of (FMS)₃-insulin emulsified in Complete Freund's Adjuvant oil (Difco, Detroit, MI) and then boosted at 2 weeks using Incomplete Freund's Adjuvant (Difco).[21,22] At 6 weeks, the mice were individually bled and the sera diluted up to 1:1000 and tested for antibodies to native insulin or to (FMS)₃-insulin in an ELISA assay.[23] Briefly, the two antigens (10 μg) were applied to assay plates that bind the antigens and the plates were further incubated with the tested sera. Antibodies bound to the adhered antigens were detected with alkaline phosphatase-anti-mouse IgG + IgM conjugate. A significant antibody binding has been defined as having an absorbance >0.10 at 405 nm, which is 5 SD over the mean ELISA reading obtained in the sera of six normal mice.

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