Synthesis of Human [9-Leucine-B]Insulin†

Gerald Schwartz and Panayotis G. Katsoyannis*

ABSTRACT: The synthesis and isolation in purified form of [Leu^9-B]insulin, a biologically active analogue of human insulin, is described. This analogue differs from the parent molecule in that the polar residue, serine, occupying position 9 in the B chain and located on the outside of the insulin molecule, has been replaced with the hydrophobic leucine residue. For the synthesis of this analogue the [Leu^9]B chain of human insulin was chemically synthesized by the fragment condensation approach and isolated in the S-sulfonated form. Combination of this compound with the sulfhydryl form of human A chain afforded [Leu^9-B]insulin. Separation of this analogue from the combination mixture and isolation as the hydrochloride in purified form were accomplished by chromatography on a carboxymethylcellulose column with an acetate buffer (pH 3.3) and an exponential sodium chloride gradient. [Leu^9-B]Insulin possesses a potency of 13–14 IU/mg when assayed by the mouse convulsion method and 11–12 IU/mg by the radioimmunoassay method as compared to 23–25 IU/mg possessed by the natural hormone.

It is now established that the primary interaction of many hormones with the cells of their target organs entails binding of the hormone with specific sites (receptors) on the cell surface. Insulin does not appear to be an exception, and all the existing evidence indicates that the primary event of its action is its interaction with a receptor. Obviously then, in such a process amino acid residues found on the surface of the insulin molecule play an important part in the hormone–receptor interaction and hence, in the expression of the biological profile of the hormone. Consequently, the determination of the contribution of amino acid residues, located on the surface of the insulin molecule, to its biological activity is one of the primary goals in our efforts to elucidate structure–activity relationships in this hormone. The elucidation of such relationships is being pursued in our laboratory through the chemical synthesis of insulin analogues and evaluation of their biological properties. The present report describes the synthesis and isolation of such an analogue related to human insulin. In this analogue the polar amino acid residue at position B^9, serine, which is located on the outside of the insulin molecule (Blundell et al., 1971), was replaced with the hydrophobic residue leucine.

Experimental Procedures and Results

Materials and Techniques. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. Capillary melting points were determined for the peptide derivatives and are not corrected. The homogeneity of the intermediate peptide derivatives used in this study was ascertained, after deblocking at the amino end, by thin-layer chromatography on 6060 silica gel. The solvent systems used were 1-butanol–acetic acid–water (4:1:1) and 1-butanol–pyridine–acetic acid–water (30:20:6:24). Thin-layer electrophoresis was performed by a method developed in this laboratory (Tometsko and Delihas, 1967) and was carried out with a Wieland–Pfleiderer pherograph (Brinkman Instruments). Amino acid analyses were performed in a Beckman-Spinco amino acid analyzer equipped with a digital readout system (Model 12AB, Infotronics Corp., Houston, Texas) according to the method of Spackman et al. (1958). Acid hydrolysis and calculations of molar ratios were carried out as previously described (Katsoyannis et al., 1967c). Enzymatic digestion with aminopeptidase M (Henley and Co., New York, N.Y.) was performed by the method of Pfleiderer et al (1964). Preswollen microgranular carboxymethylcellulose (CM[1]-cellulose) (Whatman CM 52/1) was used in this investigation. The washing of the resin and the preparation of the columns used were described previously (Katsoyannis et al., 1967a,c). Desalting of the synthetic B chain analogue after column chromatography was performed by the hollow fiber technique in a Bio-Fiber 50 beaker, as described in bulletin no. 1004 of the Bio-Rad Laboratories (Richmond, Calif.). Protein determinations were carried out by the method of Lowry et al. (1951). Biological assays were carried out by the mouse convulsion method as has been described previously (Katsoyannis et al., 1967b). For radioimmunoassays, the double-antibody technique of Hales and Randle (1963) was employed using an "insulin immunoassay kit" (Amersham/Searle Co.). Crystalline porcine insulin was generously provided by Eli Lilly and Co. Sodium tetrathionate was prepared as described by Gilman et al. (1946).

General Aspects of the Synthesis and Isolation of [Leu^9-B]Insulin. This analogue was prepared by combination of the thiol form of the human A chain with the S-sulfonated form of the human [Leu^9]B chain. The thiol form of the A chain of human insulin, which is identical with the respective chain of porcine insulin (Nicol and Smith, 1960), was prepared by reduction with 2-mercaptoethanol of the S-sulfonated derivative of that chain. The latter compound was prepared by oxidative sulfitolysis of porcine insulin followed by separation of the resulting S-sulfonated derivatives of the A and B chains. The sulfitolysis of insulin and the separation of the S-sulfonated A and B chain derivatives have been described previously (Katsoyannis et al., 1967c). The synthesis of the S-sulfonated human [Leu^9]B chain was patterned after that of our recent novel synthesis of human B chain (Schwartz and Katsoyannis, 1973b). The key intermediate in this synthesis is the protected

† From the Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029. *Received February 13, 1976.* This work was supported by the National Institute for Arthritis, Metabolism, and Digestive Diseases, United States Public Health Service (AM 12925). For the previous paper of this series, see Cosmatos et al. (1975).

[1] Abbreviations used are: CM, carboxymethyl, TLC, thin-layer chromatography.

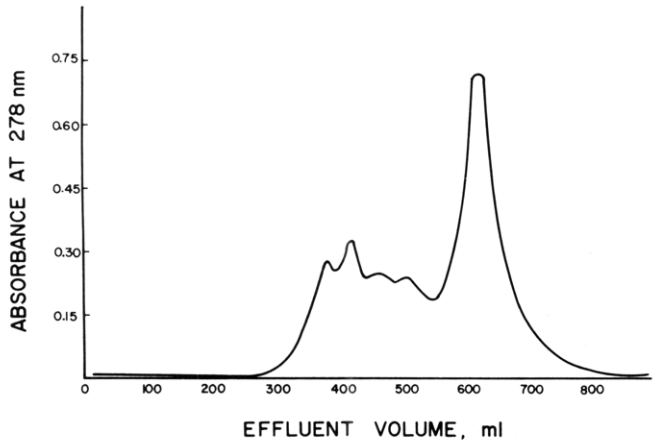

FIGURE 1: Elution pattern from chromatography on a CM-cellulose column (4 × 60 cm) with urea–acetate buffer (pH 4.0) of a crude synthetic S-sulfonated human [Leu^9]B chain obtained by hydrogen fluoride deblocking of the protected triacontapeptide followed by oxidative sulfitolysis, desalting, and lyophilization. From the effluent (560–680 ml), 61 mg of purified [Leu^9]B chain *S*-sulfonate was obtained.

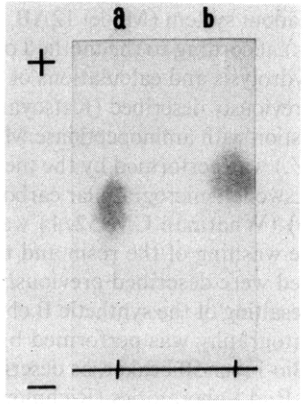

FIGURE 2: High-voltage thin-layer electrophoresis of the S-sulfonated derivatives of natural porcine (bovine) B chain (a) and synthetic human [Leu^9]B chain (b); 0.01 M NH_4HCO_3 adjusted to pH 10.0 with NH_4OH (2500 V, 25 min).

triacontapeptide embodying the entire amino acid sequence of the human [Leu^9]B chain. The overall synthesis of this polypeptide involves the coupling of the C-terminal hexadecapeptide (sequence B^{15}–B^{30}) with the adjacent hexapeptide (sequence B^9–B^{14}) to produce the C-terminal docosapeptide (sequence B^9–B^{30}), which in turn is condensed with the *N*-terminal octapeptide (sequence B^1–B^8) to produce the protected B chain analogue. All the protecting groups were chosen so that they can be removed in one final step, namely, exposure to liquid hydrogen fluoride. The reduced product was converted to the S-sulfonated derivative by oxidative sulfitolysis. Purification of this material was achieved by the procedures we have described previously (Katsoyannis et al., 1967a,c, 1971a,b). Briefly, the crude [Leu^9]B chain S-sulfonate was chromatographed on a CM-cellulose column equilibrated and eluted with a urea–acetate buffer, pH 4.0. The chromatographic pattern obtained (Figure 1) indicates the presence of one major component, which is eluted at the position where natural B chain *S*-sulfonate emerges in this chromatographic system (Katsoyannis et al., 1967c; Schwartz and Katsoyannis, 1973b), and small amounts of other components that were not characterized. The fractions under the main peak were pooled and desalted in a hollow fiber device. This technique replaced our original procedure that consisted of two chromatographic steps on Sephadex G-15 and a picric acid salt precipitation (Katsoyannis et al., 1967c; 1971a,b) and led to an excellent recovery of the purified chain. The desalted solution was lyophilized to give the purified [Leu^9]B chain *S*-sulfonate as a white fluffy material in an overall yield (based on the crude protected B chain used) of 40%.

TABLE I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Aminopeptidase M) of the S-Sulfonated [Leu^9]B Chain of Human Insulin.

Amino Acid	Acid Hydrolysis		Enzymatic Hydrolysis	
	Theory	Found	Theory	Found
Lysine	1	1.0	1	1.1
Histidine	2	1.9	2	1.8
Arginine	1	1.0	1	1.0
Aspartic acid	1	1.0	0	0
Glutamine	0	0	1	*d*
Asparagine	0	0	1	*d*
Threonine	2	2.1	2	1.6
Glutamic acid	3	3.2	2	1.9
Proline	1	1.0	1	0.8
Glycine	3	3.1	3	3.0
Alanine	1	1.1	1	1.0
Half-cystine	2	1.3[b]	0	0
Valine	3	3.1	3	2.9
Leucine	5	4.8	5	5.1
Tyrosine	2	1.7[b]	2	2.0
Phenylalanine	3	3.0	3	3.1
S-Sulfocysteine	0	0	2	2.1[c]

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction. [c] Eluted from the long column of the Beckman-Spinco analyzer after 26 ml of effluent. [d] Emerge on the same position and not determined.

Amino acid analysis of the purified material after acid hydrolysis gave a composition in good agreement with the theoretically expected values (Table I). The synthetic chain was completely digested with aminopeptidase M (Table I). On high-voltage thin-layer electrophoresis at pH 10 (Figure 2) the synthetic material exhibited a single Pauly-positive spot.

The conversion of the S-sulfonated human (porcine) A chain to its thiol form and its combination with the S-sulfonated human [Leu^9]B chain to produce the human insulin analogue were accomplished by a modification of our original procedure used in the synthesis of human and sheep insulin (Katsoyannis and Tometsko, 1966; Katsoyannis et al., 1967a,b). The S-sulfonated A chain was reduced in a Tris-HCl buffer at pH 8.3 with 2-mercaptoethanol. The reduced product was isolated by precipitation with a mixture of 2-propanol–ethyl acetate, washed with petroleum ether, and allowed to react with the S-sulfonated [Leu^9]B chain at pH 10.6. The amounts of A and B chains used were in a ratio of 2:1 instead of 4:1 employed in our original procedure (Katsoyannis and Tometsko, 1966; Katsoyannis et al., 1967b). These modifications made it easier to work with larger quantities of chains without a loss in combination efficiency.

Isolation of the insulin analogue from the combination mixture in a highly purified form was achieved by chromatography on a CM-cellulose column with an acetate buffer (pH 3.3) and an exponential sodium chloride gradient, as described previously (Katsoyannis et al., 1967a,b) (Figure 3). From the effluent of the column, the insulin analogue was isolated via picrate as the hydrochloride by the procedure that we have described previously (Katsoyannis et al., 1967a,b). Amino acid

TABLE II: Amino Acid Composition[a] of an Acid Hydrolysate of the Human [Leu^9-B]Insulin.

Amino Acid	Theory	Found
Lysine	1	1.0
Histidine	2	1.8
Arginine	1	0.9
Aspartic acid	3	2.9
Threonine	3	2.6
Serine	2	1.8
Glutamic acid	7	6.8
Proline	1	1.1
Glycine	4	4.1
Alanine	1	1.1
Half-cystine	6	3.4[b]
Valine	4	3.6
Isoleucine	2	1.4
Leucine	7	6.6
Tyrosine	4	2.9[b]
Phenylalanine	3	2.8

[a] Number of amino acid residues per molecule. [b] Uncorrected for destruction.

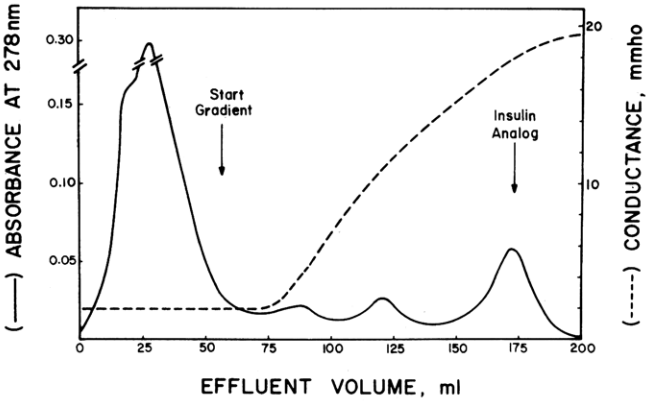

FIGURE 3: Chromatography of a combination mixture (see Experimental Procedures and Results) of synthetic human [Leu^9]B chain *S*-sulfonate with the sulfhydryl form of human (porcine) A chain on a CM-cellulose column (0.9 × 23 cm) with acetate buffer (pH 3.3; [Na^+] 0.024 M) and an exponential NaCl gradient. The column eluate was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). The purified human [Leu^9-B]insulin (150–195 ml of eluate) was recovered via picrate as the hydrochloride (1.1 mg).

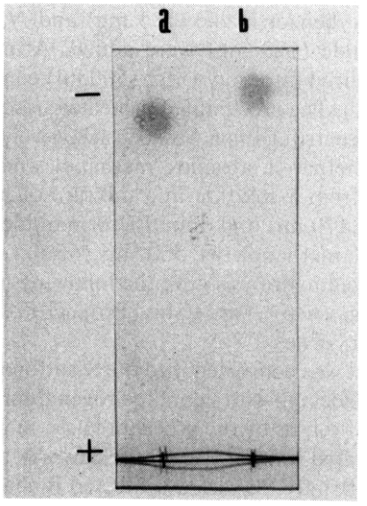

FIGURE 4: High-voltage thin-layer electrophoresis of natural porcine insulin (a) and synthetic human [Leu^9-B]insulin (b): 0.5 N acetic acid, 3400 V, and 15 min.

analysis after acid hydrolysis (Table II) and thin-layer electrophoresis (Figure 4) were employed to ascertain its homogeneity. By the mouse convulsion assay method [Leu^9-B]insulin was found to possess a potency of 13–14 IU/mg, ca. 55% of that of the natural hormone (23–25 IU/mg); the radioimmunoassay method gave a value of 11–12 IU/mg, ca. 47% of that of insulin.

N-tert-Butyloxycarbonyl-L-leucyl-N^{im}-tosyl-L-histidyl-L-leucyl-L-valyl-γ-benzyl-L-glutamyl-L-alanine (*I*). A solution of *N-tert*-butyloxycarbonyl-*N*^{im}-tosyl-L-histidyl-L-leucyl-L-valyl-γ-benzyl-L-glutamyl-L-alanine *tert*-butyl ester (Schwartz and Katsoyannis, 1973b) (3.4 g) in trifluoroacetic acid (20 ml) was stored at room temperature for 1 h and then poured into ether (500 ml). The precipitate was collected, washed with ether, and dried. To a solution of this product in dimethylformamide (20 ml) containing triethylamine (0.5 ml) was added *N-tert*-butyloxycarbonyl-L-leucine *p*-nitrophenyl ester (Vogler et al., 1965). After 48 h, the mixture was poured into ether (700 ml) and the precipitate was reprecipitated from dimethylformamide–ether and dimethylformamide–water: wt 2.9 g (80%), mp 152–153 °C, $[\alpha]_D^{26}$ −21.6° (c 1, dimethylformamide). After deblocking with trifluoroacetic acid, the peptide showed a single spot on TLC in two solvent systems (Eastman Chromatogram Sheet, Eastman Kodak Co., Rochester, N.Y.; solvent systems: 1-butanol–acetic acid–water, 4:1:1; 1-butanol–pyridine–acetic acid–water, 30:20:6:24). Anal. Calcd for $C_{50}H_{74}N_8O_{13}S$: C, 58.4; H, 7.3; N, 10.9. Found: C, 58.3; H, 7.3; N, 11.0. Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $His_{0.9}Glu_{1.1}Ala_{1.0}Val_{1.1}Leu_{1.9}$.

N-tert-Butyloxycarbonyl-L-leucyl-N^{im}-tosyl-L-histidyl-L-leucyl-L-valyl-γ-benzyl-L-glutamyl-L-alanyl-L-leucyl-O-benzyl-L-tyrosyl-L-leucyl-L-valyl-S-diphenylmethyl-L-cysteinylglycyl-γ-benzyl-L-glutamyl-N^{ω}-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-O-benzyl-L-tyrosyl-L-threonyl-L-prolyl-N^{ε}-benzyloxycarbonyl-L-lysyl-O-benzyl-L-threonine Benzyl Ester (*II*). A solution of *N-tert*-butyloxycarbonyl-L-leucyl-*O*-benzyl-L-tyrosyl-L-leucyl-L-valyl-*S*-diphenylmethyl-L-cysteinylglycyl-γ-benzyl-L-glutamyl-*N*^{ω}-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-*O*-benzyl-L-tyrosyl-L-threonyl-L-prolyl-*N*^{ε}-benzyloxycarbonyl-L-lysyl-*O*-benzyl-L-threonine benzyl ester (Schwartz and Katsoyannis, 1973a) (800 mg) in 98% formic acid (30 ml) was stored at room temperature for 3 h and subsequently concentrated under reduced pressure. The residue was triturated with ether and dissolved in dimethylformamide (25 ml). This solution, cooled to 0 °C, was diluted with 1 N NH_4OH (5 ml) and immediately poured into cold saturated aqueous sodium chloride (300 ml). The pH of the mixture was adjusted to 9.5 with 1 N NH_4OH and the precipitated free base of the partially protected hexadecapeptide was collected, washed (water, 2-propanol and petroleum ether) and dried. This material was dissolved in a mixture of hexamethylphosphoramide (5 ml) and dimethylformamide (5 ml) and to this solution, cooled to 4 °C, the hexapeptide derivative (I) (1.5 g) was added. The mixture was stirred for a few minutes and to the resulting solution 1-hydroxybenzotriazole (200 mg) and *N*,*N*′-dicyclohexylcarbodiimide (300 mg) were added. After 48 h at 4 °C, the mixture was poured into cold saturated aqueous sodium chloride (400 ml) containing 2 M Na_2CO_3 (20 ml). The precipitated protected docosapeptide was col-

lected by filtration, washed (water, acetone, and ether), and reprecipitated from a solution in dimethylformamide by the addition of 2-propanol: wt 750 mg (70%). Amino acid analysis after acid hydrolysis showed the following ratios: $Lys_{1.1}$-$His_{0.9}Arg_{0.7}Thr_{1.8}Glu_{2.0}Pro_{1.0}Gly_{2.0}Ala_{1.1}Cys_{0.4}Val_{2.0}$-$Leu_{4.0}Tyr_{0.6}Phe_{1.8}$.

L-Phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-S-sulfo-L-cysteinylglycyl-L-leucyl-L-histidyl-L-leucyl-L-valyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-sulfo-L-cysteinylglycyl-L-glutamyl-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysyl-L-threonine ([*Leu*^{9}-*B*] *Human Insulin B Chain S-Sulfonate*) (*III*). The protected docosapeptide (II) (600 mg) was deblocked with 98% formic acid as described in the synthesis of (II). The resulting free base of the deblocked docosapeptide was dissolved in a mixture of hexamethylphosphoramide (5 ml) and dimethylformamide (5 ml) and to this solution the octapeptide *N-tert*-butyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-diphenylmethyl-L-cysteinylglycine (Schwartz and Katsoyannis, 1973b) (900 mg) was added. The mixture was stirred for a few minutes and to the resulting solution 1-hydroxybenzotriazole (135 mg) and *N*,*N*′-dicyclohexylcarbodiimide (170 mg) were added. After 48 h, the mixture was poured into cold water (500 ml) containing 1 N NH_4OH (10 ml). The precipitated protected triacontapeptide was isolated by centrifugation, washed successively with water, 50% aqueous methanol, absolute methanol, and ether, and reprecipitated from a solution in a mixture of hexamethylphosphoramide (10 ml) and dimethylformamide (10 ml) by the addition of methanol: wt 500 mg (65%). Amino acid analysis after acid hydrolysis gave the following molar ratios: $Lys_{1.1}His_{1.9}Arg_{0.8}Asp_{1.0}Thr_{1.9}Glu_{3.0}Pro_{1.0}Gly_{3.3}Ala_{1.0}Cys_{1.3}$-$Val_{2.7}Leu_{4.8}Tyr_{0.6}Phe_{2.8}$.

This material was converted into the S-sulfonated B chain analogue by deblocking with liquid hydrogen fluoride followed by oxidative sulfitolysis by the procedure used in the synthesis of the S-sulfonated human B chain (Schwartz and Katsoyannis, 1973b). Briefly, the dried protected B chain analogue (200 mg) and anisole (1 ml) were placed in a Daiflon reaction vessel of a hydrogen fluoride apparatus (Toho Co., Japan) and treated (10 °C, 1 h) with liquid hydrogen fluoride (10 ml; distilled over cobalt trifluoride) as described by Sakakibara (1971). The hydrogen fluoride was then removed by use of a vacuum pump with a liquid nitrogen trap. The residue was dried in high vacuum (over KOH) for 24 h and then triturated with ethyl acetate. This product was subsequently dissolved in 8 M guanidine hydrochloride (20 ml) and to this solution, adjusted to pH 8.9 with dilute NH_4OH, were added sodium sulfite (1.2 g) and freshly prepared sodium tetrathionate (0.7 g). The mixture was stirred at room temperature for 3 h and then placed in a Visking 18/32 dialysis tubing and dialyzed against four changes of distilled water (4 l each) at 4 °C for 24 h. Upon lyophilization of the dialysate the crude S-sulfonated human B chain analogue was obtained as a white powder.

For purification, the lyophilized material was dissolved in 6 ml of urea–acetate buffer (0.04 M acetate–8 M urea, pH 4.0) and applied to a CM-cellulose column (4 × 60 cm) equilibrated and eluted with the same buffer. The preparation of this column has been described in previous reports (Katsoyannis et al., 1967a,c). The elution pattern of this column, as determined by continuously monitoring the effluent by a Gilford recording spectrophotometer, is shown in Figure 1. The eluate under the major peak was collected and desalted at 4 °C in two 60-ml batches by the hollow fiber technique. Briefly, each batch (60 ml) was placed in a Bio-Fiber-50 beaker and desalted by forcing through the fiber bundle (25–50 ml/min) successively 1 l of sodium acetate buffer (0.05 M, pH 5.0) containing 0.15 M sodium chloride, 1 l of distilled water, and 6 l of 0.05 M ammonium hydrogen carbonate. Lyophilization of the desalted samples afforded the S-sulfonated [Leu^9]B chain as a white fluffy material: wt 61 mg (40%, based on the crude protected B chain analogue used).

Amino acid analysis of the purified material after acid hydrolysis gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table I). The synthetic material was completely digested by aminopeptidase M (Table I). On high-voltage thin-layer electrophoresis in 0.01 M ammonium hydrogen carbonate (adjusted to pH 10.0 with NH_4OH) and 2500 V the synthetic chain analogue moved as a single component (Pauly reaction) (Figure 2).

S-Sulfonated Derivatives of A and B Chains of Insulin. These chain derivatives were prepared by oxidative sulfitolysis of porcine insulin followed by separation of the resulting S-sulfonated chains as described previously (Katsoyannis et al., 1967c).

Synthesis and Isolation of Human [*Leu*^{9}-*B*]*Insulin.* The synthesis was accomplished by interaction of the thiol form of the A chain of human (porcine) insulin and the S-sulfonated form of [Leu^9]B chain by a modification of the procedure we have reported previously (Katsoyannis and Tometsko, 1966; Katsoyannis et al., 1967a,b). The ratio of the A to B chain used in the combination experiments has been changed from 4:1 to 2:1 and the thiol form of the A chain has been isolated by precipitation with organic solvents. In a typical experiment, 20 mg of S-sulfonated human (porcine) A chain was dissolved in 4 ml of a 0.1 M Tris-HCl buffer (which has been previously adjusted to pH 8.3 at 37 °C) in a 50-ml glass-stoppered centrifuge tube. The solution was cooled to 4 °C, flushed with nitrogen for 20 min, and, after adding 2-mercaptoethanol (0.08 ml), was heated at 37 °C for 6 min. The reaction mixture was then cooled to 4 °C and diluted successively with glacial acetic acid (0.2 ml), 2-propanol (6 ml), and ethyl acetate (10 ml). After cooling for 40 min, the mixture was centrifuged and the supernatant was decanted. The precipitated thiol form of the A chain was treated again with 2-propanol (0.5 ml) and ethyl acetate (40 ml), isolated by centrifugation, washed successively with two 40-ml portions of ethyl acetate and two 40-ml portions of petroleum ether, and dried under vacuum for 10 min. This product was then mixed with the S-sulfonated [Leu^9]B chain (10 mg) and cold water (4 ml). After adjusting the pH of the reaction mixture to 10.4 with 1 N NaOH, the resulting solution was diluted with 0.1 M glycine buffer (0.5 ml; pH 10.6) and stirred for 16 h at 4 °C. The combination mixture was then treated as described previously (Katsoyannis et al., 1967a,b). Isolation and purification of the insulin analogue from the combination mixture was carried out by chromatography on a 0.9 × 23 cm CM-cellulose column with an acetate buffer (Na^+ 0.024 M, pH 3.3) and an exponential NaCl gradient, according to the procedure we have reported previously (Katsoyannis et al., 1967a,b). The chromatographic pattern obtained is shown in Figure 3. The effluent containing the synthetic analogue (150–195 ml) was processed as we have described previously (Katsoyannis et al., 1967b) and the human [Leu^9-B]insulin was isolated via picrate as the hydrochloride (1.1 mg).

Amino acid analysis of this analogue after acid hydrolysis gave a composition expressed in molar ratios, shown in Table

II, in good agreement with the theoretically expected values. On thin-layer electrophoresis in 0.5 N acetic acid and 3500 V (Figure 4) the insulin analogue moved as a single component (Pauly reaction). This analogue was found to possess a potency of 13–14 IU/mg by the mouse convulsion assay method and 11–12 IU/mg by the radioimmunoassay method. Crystallization of the analogue was not attempted.

Discussion

X-ray analysis of the three-dimensional structure of insulin (Adams et al., 1969) has shown that the region of the B chain located between the amino acid residues at positions 9 and 19 forms a classical α helix. In addition, the residues B^{20} to B^{23} form a U-turn within the insulin molecule so that the carboxyl terminal segment of the B chain (sequence B^{24}–B^{30}) is folded back and lies in antiparallel fashion against this helix (Blundell et al., 1971; 1972). As a result, several nonpolar amino acid residues from the aforementioned helical segment are brought into juxtaposition with the C-terminal region of the B chain. This nonpolar area together with other nonpolar amino acid residues of the A chain form a topochemical region that is an important structural feature of the insulin molecule. An abundance of data indicate that modification of this nonpolar core leads to substantial changes of the biological profile of the hormone (for a review see Blundell et al., 1972). Evidently, the biological activity of insulin is dependent upon maintaining the integrity of this region.

The disposition of the amino acid residues of the B^9–B^{19} helical segment is best illustrated (Blundell et al., 1971) by the Schiffer–Edmundson (1967) helical wheel. This is a two-dimensional projection of amino acid side chains onto a plane perpendicular to the axis of the helix, in which the perimeter corresponds to the polypeptide chain backbone and the spokes to the amino acid side chains. Adjacent amino acid residues in the peptide chain are placed in 100°-arc intervals on this wheel. In the helical wheel representing the B^9 to B^{19} segment shown in Figure 5 the nonpolar residues (B^{16}–B^{18}) are clustered on one side and are members of the hydrophobic core of the aforementioned topochemical region. The polar residues (B^9, B^{10}, B^{13}) are clustered on the other side of the helical wheel and are on the outside of the insulin molecule (Blundell et al., 1971). Since the B^9–B^{19} segment contributes to the biologically important hydrophobic core, distortion of the helix might well result in deleterious effects on the biological activity of insulin. It can be anticipated that selective substitution of amino acid residues of the B^9–B^{19} helical segment may change the biological profile of the hormone. However, substitution of the polar residue B^9 (Ser), since it is the first member of the helical segment and further is on the outside of the protein molecule, would not be expected to significantly distort the helix and change the biological activity of insulin. Indeed, rat I (Smith, 1966) and mouse I (Markussen, 1971; Burr et al., 1971) insulins, both of which contain a proline residue at B^9, are not any different biologically from the other mammalian hormones. In the present investigation, we have found, however, that substitution of the polar serine at B^9 with the nonpolar leucine causes a ca. 48% loss of the biological activity of the molecule. It thus appears that the presence of an amino acid residue, with a bulky hydrocarbon side chain, occupying a position on the outside of the insulin molecule and thus exposed to the aqueous environment may itself have a considerable effect on the biological profile of the hormone. It is tempting to speculate that the partial loss of biological activity of [Leu^9-B]insulin might be attributed to a perturbing influence, in the aqueous environment, of B^9 Leu on the hydrophobic interactions of that region or to steric effects that come into play during the hormone–receptor interactions.

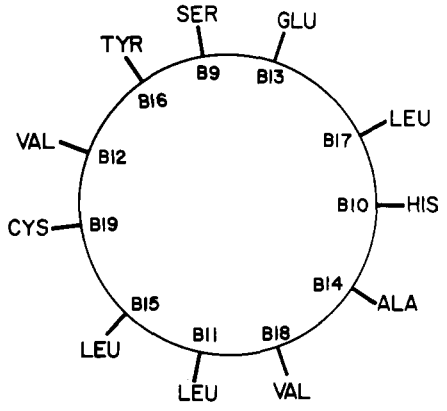

FIGURE 5: Helical wheel as suggested by Schiffer and Edmundson (1967) for the residues B^9 to B^{19}.

Acknowledgments

The authors express their appreciation to Dr. Agnes Horvat and Mrs. Edith Li for the radioimmunoassays, to Mrs. Mei-Hing Liu for the biological assays and technical assistance, and to Mrs. Kay Tai for the amino acid analyses and enzymatic analysis.

References

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S. (1969), *Nature (London) 224,* 491.

Blundell, T. L., Dodson, G. G., Dodson, E. J., Hodgkin, D. C., and Vijayan, M. (1971), *Recent Prog. Horm. Res. 27,* 1.

Blundell, T. L., Dodson, G. G., Hodgkin, D. C., and Mercola, D. (1972), *Adv. Protein Chem. 26,* 279.

Burr, I. M., Stauffacher, W., Cameron, D. P., Buenzli, H. F., Humber, R. E., and Renold, A. E. (1971), *Endocrinology 88,* 17.

Cosmatos, A., Johnson, S., Breier, B., and Katsoyannis, P. G. (1975), *J. Chem. Soc. Perkin Trans. 1,* 2157.

Gilman, A., Philips, F. S., and Koelle, E. S. (1946), *Am. J. Physiol. 146,* 348.

Hales, C. N., and Randle, P. J. (1963), *Biochem. J. 88,* 137.

Katsoyannis, P. G., and Tometsko, A. (1966), *Proc. Natl. Acad. Sci. U.S.A. 55,* 1554.

Katsoyannis, P. G., Trakatellis, A., Johnson, S., Zalut, C., and Schwartz, G. (1967a), *Biochemistry 6,* 2642.

Katsoyannis, P. G., Trakatellis, A., Zalut, C., Johnson, S., Tometsko, A., Schwartz, G., and Ginos, J. (1967b), *Biochemistry 6,* 2656.

Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S., and Trakatellis, A. C. (1967c), *Biochemistry 6,* 2635.

Katsoyannis, P. G., Ginos, J., Zalut, C., Tilak, M., Johnson, S., and Trakatellis, A. (1971a), *J. Am. Chem. Soc. 93,* 5877.

Katsoyannis, P. G., Zalut, C., Tometsko, A., Tilak, M., Johnson, S., and Trakatellis, A. (1971b), *J. Am. Chem. Soc. 93,* 5871.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Markussen, J. (1971), *Int. J. Protein Res. 3,* 149.

Nicol, D. S. H. W., and Smith, L. F. (1960), *Nature (London)*

187, 483.
Pfleiderer, G., Celliers, P. G., Stanulovic, M., Wachsmuth, E. D., Determann, H., and Braunitzer, G. (1964), *Biochem. Z. 340,* 552.
Sakakibara, S. (1971), Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Weinstein, B., Ed., New York, N.Y., Marcel Dekker, p 51.
Schiffer, M., and Edmundson, A. B. (1967), *Biophys. J. 7,* 121.
Schwartz, G., and Katsoyannis, P. G. (1973a), *J. Chem. Soc. Perkin Trans. 1,* 2890.
Schwartz, G., and Katsoyannis, P. G. (1973b), *J. Chem. Soc. Perkin Trans. 1,* 2894.
Smith, L. F. (1966), *Am. J. Med. 40,* 662.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Tometsko, A., and Delihas, N. (1967), *Anal. Biochem. 18,* 272.
Vogler, K., Studer, R. O., Lanz, P., Lergier, W., and Böhne, W. (1965), *Helv. Chim. Acta 48,* 1161.

Synthesis of Two Biologically Active Insulin Analogues with Modifications at the N-Terminal and N- and C-Terminal Amino Acid Residues†

Alexandros Cosmatos, Yoshio Okada, and Panayotis G. Katsoyannis*

ABSTRACT: The synthesis and isolation in purified form of two analogues of insulin is described. [21-Isoasparagine-A] ([Iasn^{21}-A]) insulin differs from the parent molecule in that the amino acid residue, asparagine, found at the C terminus of the A chain (A^{21}) has been replaced by isoasparagine. [Sar^1,Iasn^{21}-A]insulin differs from insulin in that both the A^1 and A^{21} amino acid residues, glycine and asparagine, have been substituted by sarcosine and isoasparagine, respectively. The synthesis of these analogues followed the pattern employed in this laboratory for the synthesis of insulin and its analogues. The S-sulfonated derivatives of the A chain analogues were chemically synthesized, converted to their sulfhydryl forms, and then combined with the S-sulfonated B chain to produce the respective insulin analogues. Isolation of the insulin analogues from the combination mixtures was effected by chromatography on a carboxymethylcellulose column with an exponential sodium chloride gradient. By the mouse convulsion assay method [Iasn^{21}-A]insulin possessed a potency of 21 IU/mg and [Sar^1,Iasn^{21}-A]insulin 15 IU/mg. The radioimmunoassay method gave values of 16 IU/mg for the former and 7 IU/mg for the latter analogue. The natural hormone has a potency of 23–25 IU/mg by both assay methods. These data indicate that the α- and β-carboxyl groups of the A^{21} amino acid residue are nearly equivalent in terms of their contribution to the expression of the biological activity of insulin. Furthermore, these data strengthen the speculation (Cosmatos, A., Johnson, S., Breier, B., and Katsoyannis, P. G. (1975), *J. Chem. Soc. Perkin Trans. 1,* 2157) that the change in the relative positive charge at the N-terminal amino acid residue of the A chain is responsible for the considerable decrease in the immunoreactivity observed in such modified insulins.

A program has been initiated in this laboratory directed to the synthesis of insulin analogues in an attempt to understand the relationship between chemical structure, biological activity, and immunoreactivity of this hormone. One aspect of these studies has been an attempt to elucidate the role of the amino acid residues found at the amino- and carboxyl-terminal positions of the A chain (A^1 and A^{21}, respectively) in the expression of the biological activity of insulin. We have found that replacement of the α-amino group of the A^1 residue by hydrogen results in a substantial loss (ca. 65%) of the biological activity of the hormone (Katsoyannis and Zalut, 1972). However, substitution of one hydrogen of the α-amino group of the same residue by a methyl group results in a small decrease of the biological activity (ca. 17%) and a pronounced decrease (ca. 63%) of the immunoreactivity of insulin (Okada and Katsoyannis, 1975). On the other hand, substitution of the A^{21} residue, L-asparagine, by its optical isomer (Cosmatos et al., 1975) results in a substantial loss of the biological potency and immunoreactivity of insulin (ca. 67 and 82%, respectively). In the present communication, we describe the synthesis and biological evaluation of two insulin analogues with modifications either at the A^1 position or at both A^1 and A^{21} positions.

Experimental Procedures and Results

Materials and Techniques. Details of the materials and techniques used are given in the preceding paper of this issue (Schwartz and Katsoyannis, 1976). In all synthetic steps, coupling of the fragments was followed by detection of the amino component present with ninhydrin; completion of the reaction was indicated by a negative ninhydrin test. The homogeneity of all intermediate peptide derivatives, deblocked at the amino end, was ascertained by thin-layer chromatography (whenever solubility properties allowed it) on 6060 silica gel (Eastman Chromagram Sheet, Eastman Kodak Co.,

† From the Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029. *Received February 13, 1976.* This work was supported by the National Institute of Arthritis, Metabolism, and Digestive Diseases, United States Public Health Service (AM 12925). For the previous paper of this series, see Schwartz and Katsoyannis (1976).