Steric Requirements at Position B12 for High Biological Activity in Insulin†

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ABSTRACT: The α-helix formed by the amino acid residues 9–19 of the B-chain of insulin is involved in the stabilization of its three-dimensional structure. We have shown that modification at positions B9, B10, B12, and B16 results in analogues possessing biological activities ranging from ca. 0.2% to ca. 500% relative to that of natural insulin. The lowest potency was displayed by [B12 Asn]insulin, in which the hydrophobic B12 Val residue was replaced by the hydrophilic Asn residue. We now report the synthesis of four insulin analogues in which hydrophobicity is retained, and only the spatial arrangement of atoms in the B12 region is altered. Substitution of B12 Val with α-aminoisobutyric acid (Aib), D-Ala, and Phe led to analogues possessing biological activities, in lipogenesis assays, of 8.5%, 2%, and 0.2%, respectively, relative to that of natural insulin. Inversion of the B11–B12 sequence, -Leu-Val-, led to an analogue displaying 3.3% activity. A synthetic B-chain in which the B11 Leu–B12 Val sequence was replaced by B11 Ala–B12 Ile was incapable of combining with the natural A-chain. We conclude that the Val residue in the B12 position in insulin fulfills special side-chain packing requirements involved in the stability of the structure of insulin. Even slight steric alteration at position B12 results in a distortion of the overall conformation of the B-chain which affects its ability to combine with the natural A-chain. This distortion is retained in the corresponding analogue, which is reflected in diminished biological potency.

Positions 11 and 12 of the B-chain of insulin are occupied by leucine and valine, respectively, in insulins found in many mammals, birds, and fish. These residues form part of an α-helical segment extending from residue B9 to residue B19, the integrity of which has been postulated to be required for the maintenance of a three-dimensional structure commensurate with high biological activity. This α-helical segment is in turn part of a hydrophobic patch on the insulin monomer, which participates in dimer formation. We have reported previously that the substitution of a hydrophilic amino acid residue, Asn, for B12 Val results in an analogue, [B12 Asn]-insulin, which displays very low biological activity (Schwartz et al., 1981). In the present communication, we describe the synthesis and biological properties of four insulin analogues in which residues B11 and/or B12 have/has been replaced by other hydrophobic amino acid residues, with the aim of exploring the spatial requirements of this region.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and Techniques. Commercially obtained reagents were (butoxycarbonyl)amino acids and derivatives and (butoxycarbonyl)alanine–PAM resin, which was the starting material for all solid-phase syntheses described (Bachem, Inc., Torrance, CA). All solvents were high-performance liquid chromatography (HPLC) grade. Amino acid analysis of the synthetic chains and insulin analogues after acid hydrolysis under standard conditions was done on a Beckman System 6300 high performance analyzer. Details of the materials and analytical procedures were given in a previous publication (Kitagawa et al., 1984). The sources of radioisotopes and other materials for assay were given, and the methods for assay of the insulin analogues were described in a recent publication (Joshi et al., 1990). Two types of assay were employed. In the first type of assay, the ability of the analogues, relative to that of porcine insulin, to compete with ^{125}I-insulin in binding to insulin receptors prepared from rat liver was determined. In the second type of assay, the stimulation by an insulin analogue of the conversion of [3-^{3}H]-glucose into an organic extractable form by isolated rat adipocytes (lipogenesis) was observed, again relative to porcine insulin.

General Aspects of the Synthesis of [B12 Aib]-, [B12 D-Ala]-, [B12 Phe]-, [B11 Val, B12 Leu]Insulins and of the Attempted Synthesis of [B11 Ala, B12 Ile]insulin. The first four analogues were prepared by the interaction of S-sulfonated bovine A-chain with the S-sulfonated forms of [12 Aib]B-, [12 D-Ala]B-, [12 Phe]B-, and [11 Val, 12 Leu]B-chains, respectively, at pH 10.6 in the presence of dithiothreitol (Chance et al., 1981). Attempts to prepare [B11 Ala, B12 Ile]insulin by the interaction of the S-sulfonated [11 Ala, 12 Ile]B-chain and the S-sulfonated bovine A-chain under similar conditions failed to yield a measurable quantity of the desired insulin analogue. The S-sulfonated bovine A-chain was obtained by oxidative sulfitolysis of bovine insulin, followed by separation of the resulting S-sulfonated A- and B-chains by CM-cellulose chromatography as described previously (Katsoyannis et al., 1967a), with the only difference being that sulfitolysis was performed for 3.5 h instead 24 h. The key intermediates in the synthesis of the B-chain analogues were the protected triacontapeptides, each containing the amino acid sequence of the respective B-chain. Except for [12 Phe]B-chain, the protected triacontapeptides were constructed by stepwise solid-phase synthesis (Barany & Merrifield, 1980), using PAM resin as the solid support (Mitchell et al., 1978). The *tert*-butoxycarbonyl group was used for N^{α} protection. Side-chain protecting groups were *p*-toluenesulfonyl for Arg, 4-methylbenzyl for Cys, cyclohexyl for Glu, ((2-chlorobenzyl)oxy)carbonyl for Lys, (benzyloxy)methyl for His, benzyl for Ser and Thr, and 2,6-dichlorobenzyl for Tyr. A manual double-coupling protocol (Merrifield, 1982) was

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followed. With the exception of the Val residue at position B2 and the Asn residue at position B3, which were incorporated from preformed symmetric anhydrides (Hagenmaier & Frank, 1972), the protected amino acids were incorporated from preformed 1-hydroxybenzotriazole esters in 3-fold excess. Active esters were prepared with *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in dimethylformamide. The completion of the reactions was monitored by the qualitative ninhydrin test (Kaiser et al., 1970), which was negative after each coupling step. The protected triacontapeptide corresponding to the [12 Phe]B-chain was constructed by fragment condensation methods in solution [for a review, see Katsoyannis and Schwartz (1977)]. The synthesis followed essentially the pattern used previously in the construction of several B-chain analogues (Wang et al., 1991). Specifically, the C-terminal hexadecapeptide (sequence 15–30), which was prepared by fragment condensation, was condensed with the adjacent hexapeptide (sequence 9–14) to give the C-terminal docosapeptide (sequence 9–30). This compound was condensed with the adjacent hexapeptide (sequence 3–8) to yield the C-terminal octacosapeptide (sequence 3–30), which was subjected to two stepwise elongations to produce the protected triacontapeptide. The *tert*-butoxycarbonyl group was used for N^{α} protection. Side-chain protecting groups were *p*-toluenesulfonyl for Arg, diphenylmethyl for Cys, benzyl or cyclohexyl for Glu, benzyl for Ser and Thr, benzyl for Tyr, 2-bromobenzyloxycarbonyl for Lys, and (benzyloxy)methyl for His.

The protected triacontapeptides were deblocked by treatment with liquid hydrogen fluoride and subjected to oxidative sulfitolysis as described previously (Wang et al., 1991; Chu et al., 1992). All five crude S-sulfonated B-chain analogues were purified by CM-cellulose chromatography with a urea–acetate buffer, pH 4.0, as described (Katsoyannis et al., 1967a; Wang, et al., 1991).

The synthesis of the insulin analogues was carried out by the interaction of 20 mg of S-sulfonated bovine A-chain and 10 mg of S-sulfonated synthetic B-chain in 5 mL of 0.1 M glycine buffer, pH 10.6, in the presence of 4 mg of dithiothreitol (Wang et al., 1991; Chu et al., 1992). For the isolation of [B12 Aib]-, [B12 D-Ala]-, and [B11 Val, B12 Leu]insulins from the combination mixture of the corresponding S-sulfonated A- and B-chains, the procedure employed previously in the isolation of other insulin-like compounds was used (Chen et al., 1988). Briefly, after 24 h at pH 10.6 and 4 °C, the mixture was acidified with 1 mL of glacial acetic acid, and the precipitated material was removed by centrifugation. The supernatant was chromatographed on a Sephadex G-50 column (2.5 × 113 cm) equilibrated and eluted with 1 N acetic acid. The effluent representing the monomer fraction, which contains the biologically active material (localized by precalibration of the column with insulin), was collected and lyophilized. This product was subjected to reversed-phase HPLC on a Vydac 218 TP column (0.45 × 25 cm), at a flow rate of 0.5 mL/min, with a 20–80% linear gradient of 80% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 70 or 80 min. For final purification, the effluent containing active material (insulin assays) was concentrated to a small volume and rechromatographed under the same conditions as just described. All three analogues, [B12 Aib]-, [B12 D-Ala]-, and [B11 Val, B12 Leu]insulins, exhibited single sharp peaks. A somewhat different route was followed for the isolation of [B12 Phe]insulin from the combination mixture of the corresponding S-sulfonated A- and B-chains. After acidification of the mixture and removal of precipitated material, the supernatant was directly subjected to reversed-phase HPLC as described above, with a linear gradient of 10–50% acetonitrile containing 0.1% trifluoroacetic acid over 70 min. Upon concentration of the active fraction and rechromatography under the same conditions as above, the analogue exhibited a single sharp peak. From a combination mixture containing S-sulfonated bovine A-chain and S-sulfonated synthetic [11 Ala, 12 Ile]B-chain, no product exhibiting the appropriate amino acid analysis for an insulin analogue was obtained, using any of the methods described above, and the combination mixture was devoid of insulin-like activity.

[B12 Aib]Insulin. The purified [12 Aib]B-chain S-sulfonate after acid hydrolysis gave the following amino acid ratios, in good agreement with the theoretically expected values shown in parentheses: $Asp_{1.1(1)}$ ($Thr_{1.0(1)}$ $Ser_{1.0(1)}$ $Pro_{1.1(1)}$ $Glu_{3.3(3)}$ $Gly_{3.0(3)}$ $Ala_{1.9(2)}$ $Val_{1.8(2)}$ $Leu_{3.7(4)}$ $Tyr_{1.8(2)}$ $Phe_{2.8(3)}$ $Lys_{1.0(1)}$ $His_{1.7(2)}$ $Arg_{1.0(1)}$. Cys and Aib were not determined. The HPLC-purified [B12 Aib]insulin (1.18 mg) gave the following amino acid ratios after acid hydrolysis: $Asp_{3.4(3)}$ $Thr_{0.8(1)}$ $Ser_{2.8(3)}$ $Pro_{1.0(1)}$ $Glu_{7.7(7)}$ $Gly_{3.7(4)}$ $Ala_{2.8(3)}$ $Val_{3.8(4)}$ $Leu_{5.4(6)}$ $Ile_{1.0(1)}$ $Tyr_{3.7(4)}$ $Phe_{2.4(3)}$ $Lys_{1.0(1)}$ $His_{2.0(2)}$ $Arg_{1.0(1)}$. Cys and Aib were not determined.

[B12 D-Ala]Insulin. The purified [12 D-Ala]B-chain S-sulfonate gave the following amino acid ratios following acid hydrolysis: $Asp_{1.1(1)}$ $Thr_{1.0(1)}$ $Ser_{1.0(1)}$ $Pro_{1.3(1)}$ $Glu_{3.3(3)}$ $Gly_{2.9(3)}$ $Ala_{3.1(3)}$ $Val_{2.0(2)}$ $Leu_{3.6(4)}$ $Tyr_{1.9(2)}$ $Phe_{2.8(3)}$ $Lys_{1.1(1)}$ $His_{1.8(2)}$ $Arg_{1.0(1)}$. Cys was not determined. The HPLC-purified [B12-D-Ala]insulin (540 μg) gave the following amino acid ratios after acid hydrolysis: $Asp_{2.8(3)}$ $Thr_{1.1(1)}$ $Ser_{2.7(3)}$ $Pro_{1.4(1)}$ $Glu_{7.4(7)}$ $Gly_{4.3(4)}$ $Ala_{4.2(4)}$ $Val_{3.3(4)}$ $Leu_{5.3(6)}Ile_{0.5(1)}$ $Tyr_{3.1(4)}$ $Phe_{2.6(3)}$ $Lys_{1.1(1)}$ $His_{1.9(2)}$ $Arg_{0.9(1)}$. Cys was not determined.

[B11 Val, B12 Leu]Insulin. The S-sulfonated [11 Val, 12 Leu]B-chain gave the following amino acid ratios after acid hydrolysis: $Asp_{1.1(1)}$ $Thr_{1.0(1)}$ $Ser_{0.9(1)}$ $Pro_{1.0(1)}$ $Glu_{3.2(3)}$ $Gly_{2.9(3)}$ $Ala_{2.0(2)}$ $Val_{2.8(3)}$ $Leu_{3.8(4)}$ $Tyr_{1.9(2)}$ $Phe_{2.8(3)}$ $Lys_{1.2(1)}$ $His_{1.9(2)}$ $Arg_{1.1(1)}$. Cys was not determined. The HPLC-purified [B11 Val, B12 Leu]insulin (590 μg) gave the following amino acid ratios after acid hydrolysis: $Asp_{2.7(3)}$ $Thr_{1.0(1)}$ $Ser_{2.3(3)}$ $Pro_{1.0(1)}$ $Glu_{6.7(7)}$ $Gly_{4.2(4)}$ $Ala_{3.2(3)}$ $Val_{4.6(5)}$ $leu_{6.2(6)}$ $Ile_{0.9(1)}$ $Tyr_{3.8(4)}$ $Phe_{2.9(3)}$ $Lys_{1.4(1)}$ $His_{2.2(2)}$ $Arg_{1.1(1)}$. Cys was not determined.

[B12 Phe]Insulin. The S-sulfonated [12 Phe]B-chain gave the following amino acid ratios after acid hydrolysis: $Asp_{1.0(1)}$ $Thr_{1.0(1)}$ $Ser_{1.0(1)}$ $Pro_{1.0(1)}$ $Glu_{3.0(3)}$ $Gly_{3.0(3)}$ $Ala_{1.9(2)}$ $Val_{1.9(2)}$ $Leu_{4.0(4)}$ $Tyr_{1.7(2)}$ $Phe_{3.8(4)}$ $Lys_{1.0(1)}$ $His_{1.9(2)}$ $Arg_{0.9(1)}$. Cys was not determined. The HPLC-purified [B12 Phe]insulin (215 μg) gave the following amino acid ratios after acid hydrolysis: $Asp_{3.0(3)}$ $Thr_{1.0(1)}$ $Ser_{3.0(3)}$ $Pro_{1.4(1)}$ $Glu_{6.7(7)}$ $Gly_{4.3(4)}$ $Ala_{3.7(3)}$ $Val_{4.0(4)}$ $Leu_{6.2(6)}$ $Ile_{0.9(1)}$ $Tyr_{4.3(4)}$ $Phe_{3.9(4)}$ $Lys_{0.8(1)}$ $His_{1.9(2)}$ $Arg_{1.0(1)}$. Cys was not determined.

Attempted Synthesis of [B11 Ala, B12 Ile]Insulin. The purified, S-sulfonated [11 Ala, 12 Ile]B-chain gave the following amino acid analysis after acid hydrolysis, in good agreement with theoretically expected values: $Asp_{1.0(1)}$ $Thr_{1.0(1)}$ $Ser_{0.9(1)}$ $Pro_{1.1(1)}$ $Glu_{3.1(3)}$ $Gly_{2.9(3)}$ $Ala_{3.0(3)}$ $Val_{1.9(2)}$ $Ile_{1.0(1)}$ $Leu_{2.9(3)}$ $Tyr_{1.8(2)}$ $Phe_{2.7(3)}$ $Lys_{1.0(1)}$ $His_{1.6(2)}$ $Arg_{1.0(1)}$. Cys was not determined. Several attempts were made to synthesize [B11 Ala, B12 Ile]insulin by interaction of the S-sulfonated synthetic B-chain with the S-sulfonated A-chain of bovine insulin. Application of the chromatographic techniques described above for the isolation of insulin analogues present in the combination mixture were unsuccessful. Also, unsuccessful were attempts to isolate the insulin analogue from the combination mixture by CM-cellulose chromatography with acetate buffer, pH 3.3, and an exponential NaCl gradient

Table I: Assay Values for B11 and B12 Insulin Analogues

name of insulin analogue	assay value (natural insulin = 100)	
	receptor binding	lipogenesis
[B12 Aib]insulin	9.3	8.5
[B12 D-Ala]insulin	0.7	2.0
[B11 Val, B12 Leu]insulin	5.9	3.3
[B12 Phe]insulin		0.2

(Katsoyannis et al., 1967b), a procedure used successfully in this laboratory for the isolation of several insulin analogues. Material corresponding to all of the peaks resulting from the various chromatographic techniques was purified and subjected to amino acid analysis. All materials were identifed as A- and B-chain derivatives devoid of insulin-like activity. Insulin assays also revealed no trace of activity in the combination mixture. We therefore conclude that the S-sulfonated [11 Ala, 12 Ile]B-chain cannot combine with the S-sulfonated bovine A-chain to yield an insulin analogue under the conditions employed.

Biological Evaluation of B11 and B12 Insulin Analogues. The potency of the B11 and B12 insulin analogues, relative to that of porcine insulin, are given in Table I. It should be noted that all of the insulin analogues in this table are full agonists in lipogenesis assays, reaching the same maximum stimulation as that seen with natural insulin. No value for potency in the receptor-binding assay of [B12 Phe]insulin is presented, because too little of the material was available for use in this type of assay.

DISCUSSION

The X-ray model of insulin indicates that the amino acid residues B9–B19, -Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-, form a classical α-helix. Furthermore, the residues B20–B23 form a β-turn within the insulin molecule, resulting in the placement of the C-terminal B24-B30 segment in an antiparallel fashion against the B9–B19 α-helix (Blundell et al., 1972). As a result, nonpolar residues of the B9–B19 helix are brought into juxtaposition with nonpolar residues at the C-terminal region of the B-chain, forming a largely apolar area on the surface of the insulin monomer. This apolar area is involved in the monomer–monomer interaction in the formation of the insulin dimer and is important for the stabilization of the three-dimensional structure of insulin (Blundell et al., 1972; Cutfield et al., 1981).

Early studies on structure–activity relationships of insulin suggested that the surface residues of the dimer-forming region of the insulin monomer are part of the region involved in the binding of insulin to its receptor (Blundell et al., 1972; Cutfield et al., 1981; Pullen et al., 1976). The integrity of the B9–B19 helical segment would thus be of considerable importance for hormone–receptor interaction and consequently for the biological activity of insulin.

More recently, however, data obtained from the synthesis of a number of insulin analogues, as well as from crystallographic studies of certain insulins and modified insulins, have required a revision of these original ideas. The newer data indicate that the conformation of the 2 Zn insulin crystal is not the same as that of the monomer when it binds to its receptor and, further, that several residues which are part of the dimer interface are not part of the biologically active surface of insulin [see Baker et al. (1988), and Derewenda et al. (1991) and references therein].

Regardless of the extent to which the dimer-forming surface of the insulin monomer overlaps with the active surface of the hormone, substitutions of amino acid residues in the B9–B19 segment do result in profound changes in its biological activity. Indeed, as shown by our own work (Table II), modifications at positions B9, B10, B12, and B16 result in analogues displaying biological activity ranging from ca. 0.2% to ca. 500% relative to that of natural insulin. It is thus apparent that the integrity of the conformation of the B9–B19 α-helical segment is of considerable importance for the expression of the biological activity of insulin.

Table II: Assay Values for Selected Insulin Analogues Containing Substitutions in the B9–B19 α-Helical Segment

name of insulin analogue	assay value (natural insulin = 100)		
	receptor binding	lipogenesis	mouse convulsion
[B9 Leu]insulin[a]			54
[B10 Leu]insulin[b]			45
[B10 Lys]insulin[c]	17	14	
[B10 Asn]insulin[d]		35	
[B10 Asp]insulin[e]	ca. 530	ca. 450	
[B12 Asn]insulin[f]	0.29	0.14	
[B16 Gln]insulin[g]	9	17	

[a] Schwartz and Katsoyannis (1976). [b] Schwartz and Katsoyannis (1977a,b). [c] Schwartz et al. (1982). [d] Burke et al. (1984). [e] Schwartz et al. (1987). [f] Schwartz et al. (1981). [g] Schwartz et al. (1985).

The present communication focuses on the importance of one of the residues of this segment, B12 Val, which is an invariant residue in insulins from various species. This residue is part of the apolar surface of insulin; it is involved in dimerization, and it has been postulated to be of critical importance to the structure of the hormone (Baker et al., 1988).

We have shown that the replacement of B12 Val with Asn leads to an insulin analogue which displays very low potency relative to that of natural insulin, 0.29% in receptor binding and 0.14% in lipogenesis assays (Schwartz et al., 1981). Most interestingly, this analogue was shown to have full agonist activity in the lipogenesis assay, reaching the same maximum stimulation of lipogenesis as that seen with natural insulin, albeit at vastly higher concentrations. We concluded that the substitution of the hydrophobic Val residue with the hydrophilic Asn at the B12 position results in a profoundly disturbed structure which is not consistent with proper receptor binding characteristics.

More recent studies have shown that replacement of B12 Val with Ile results in an analogue, [B12 Ile]insulin, possessing ca. 29% of the activity of the natural hormone in lipogenesis assays (Brange et al., 1988). Evidently, the replacement of B12 Val even by a similarly hydrophobic residue, Ile, has deleterious effects on the biological activity of insulin.

The data we present in this communication further emphasize the critical importance of B12 Val to the structure of insulin. Substitution of B12 Val with Aib results in an insulin analogue, [B12 Aib]insulin, which displays a considerable loss in biological activity, 8.5–9.3% relative to that of natural insulin (Table I). In this analogue, as in the aforementioned [B12 Ile]insulin, hydrophobicity is retained, but the hydrogen atom and the isopropyl group provided by B12 Val in natural insulin are replaced with two methyl groups, altering the side-chain packing and, consequently, the van der Waals interactions in the immediate area of position B12.

A comparable decrease in activity is observed when the naturally occurring sequence at positions B11 and B12, Leu-Val, is inverted. The resulting analogue, [B11 Val, B12 Leu]-insulin, was found to possess even lower activity than [B12 Aib]insulin, 3.3–5.9% relative to natural insulin. Again in this analogue, hydrophobicity is retained and only the spatial

Table III: Comparison of Potency of B12-Modified Insulin Analogues and Yield of Product Obtained by Combination of A- and B-Chains

names of insulin chains employed	yield of product[a]	assay value: receptor binding	assay value: lipogenesis
bovine insulin A- and B-chains	3.4 mg	(100)	(100)
bovine A-chain/[B12 Aib]B-chain	1.18 mg	9.3	8.5
bovine A-chain/[B11 Val, B12 Leu]B-chain	590 μg	5.9	3.3
bovine A-chain/[B12 D-Ala]B-chain	540 μg	0.7	2.0
bovine A-chain/[B12 Phe]B-chain	215 μg		0.2

[a] See Experimental Procedures and Results section.

properties have been modified in the B11–B12 region of the molecule. Taken together, the biological behavior of these two insulin analogues suggests that the spatial requirements in the immediate vicinity of residues B11 and B12 are highly constrained; very small changes in configuration are accompanied by profound losses in biological activity. Further weight is given to this interpretation by our failure to synthesize [B11 Ala, B12 Ile]insulin by combination of the appropriate synthetic B-chain analogue with the A-chain of bovine insulin. We had theorized that the low activity reported for [B12 Ile]-insulin might be due to the sequence B11 Leu–B12 Ile placing two bulky residues in positions within the molecule which result in steric hindrance and distortion of the α-helical B9–B19 segment of the synthetic B-chain. Accordingly, we sought to relieve any such hindrance by replacing B11 Leu with Ala. Although, as shown in the Experimental Procedures section, the appropriate B-chain was synthesized, it failed to combine with the A-chain of bovine insulin, suggesting that this synthetic [11 Ala, 12 Ile]B-chain cannot achieve the appropriate structure to combine with the bovine A-chain.

The very low potency of [B12 D-Ala]insulin, 0.7–2.0% relative to that of natural insulin, was anticipated, since the replacement of Val with D-Ala introduces a severe stereochemical alteration at that position.

Substitution of the B12 Val residue with Phe results in an analogue, [B12 Phe]insulin, which is the least active analogue in this series, displaying a potency of 0.2% relative to that of natural insulin. It is evident that introduction of the large Phe residue at the B12 position profoundly alters the side-chain packing in this region, resulting in an analogue displaying very low potency.

Table III shows a striking correlation between the biological activity of each insulin analogue in this series and the yield of product upon combination of the synthetic B-chains with the A-chain of bovine insulin. It appears that the modifications introduced into the synthetic B-chains affect the side-chain packing in the B11–B12 region, probably altering the overall conformation of the B-chain. This would be expected to result in a reduced capability of the modified B-chain to combine with the A-chain of bovine insulin. Further, it is possible that this distortion alters the conformation of any insulin analogue which is actually produced, resulting in reduced affinity in binding to the insulin receptor and concomitantly reduced biological activity.

As previously noted, all of the insulin analogues described here are full agonists in the stimulation of lipogenesis, reaching the same maximum as is seen with natural insulin. Thus, although a higher concentration of the each analogue is required to achieve interaction with the insulin receptor than is required for natural insulin, the analogue–receptor complex is fully capable of stimulating insulin-like metabolic activity.

In summary, it appears that the Val residue in the B12 postion plays a crucial role in the overall structure of insulin. The spatial and stereochemical choices for this position appear to be extremely limited, and even slight alterations in the configuration of the B12 residue have a profound effect on the structure and, consequently, the biological activity of the hormone. It is not necessary to postulate gross distortion of the B9–B19 α-helical region of insulin to explain our observations. Subtle changes in side-chain packing could affect the refolding pathway of the B-chain as reflected by its diminished ability to combine with the natural A-chain (Table III). In the fully folded insulin analogue, these changes in side-chain packing could be carried through to a change in conformation which affects receptor binding. Nonetheless, the conformation of the insulin receptor when the analogue is bound cannot differ appreciably from that when natural insulin is bound, because the same maximum stimulation of lipogenesis is obtained with natural insulin and with each of these insulin analogues.

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