Interaction between the A^2 and A^{19} Amino Acid Residues Is of Critical Importance for High Biological Activity in Insulin: [19-Leucine-A]insulin†

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ABSTRACT: The replacement of tyrosine at position A^{19} by leucine in the insulin molecule led to an analogue, [19-leucine-A]insulin ([Leu^{19}-A]insulin), displaying insignificant receptor binding affinity and in vitro biological activity less than 0.1 and 0.05%, respectively, compared to the natural hormone. This analogue along with the previously reported [2-glycine-A]-, [2-alanine-A]-, and [2-norleucine-A]insulins is the least potent insulin analogue we have examined. Circular dichroic studies showed that all these analogues are monomeric at concentrations at which insulin is primarily dimeric. We conclude that an aromatic ring at position A^{19} and the presence of the side chain of isoleucine at position A^2 are each of critical importance for high biological activity in insulin. It appears that the van der Waals interaction between the side chain of isoleucine A^2 and tyrosine A^{19}, present in crystalline insulin, is among the most important determinants for high biological activity in insulin.

In a recent paper (Kitagawa et al., 1984), we described the properties of two insulin analogues that differ from the parent molecule in that the naturally occurring isoleucine residue at position A^2 is replaced by glycine ([Gly^2-A]insulin) or by alanine ([Ala^2-A]insulin). Both compounds exhibit deeply reduced potency in biological assays relative to bovine insulin and somewhat reduced potency in radioimmunoassay. The data indicate that the isoleucine side chain is of critical importance in maintaining a structure commensurate with high biological activity in insulin. In a previous paper (Ferderigos et al., 1983), we described the synthesis and biological behavior of sheep [Phe^{19}-A]insulin, an analogue differing from the parent molecule in that the tyrosine residue at position A^{19} is replaced with phenylalanine. This analogue displayed ca. 8% of the biological activity of the natural hormone, demonstrating the importance of the hydroxyl function at position A^{19} in biological activity of insulin. Danho et al. (1980) reported similar results with porcine [Phe^{19}-A]insulin. We report here the synthesis and properties of [Leu^{19}-A]insulin, an analogue in which the tyrosine residue in position A^{19} is replaced by the bulky hydrophobic, but nonaromatic, leucine. We discuss the importance of the interaction of A^2 isoleucine and A^{19} tyrosine in contributing to a structural feature that appears to be of great importance to the biological activity of insulin.

Experimental Procedures

Capillary melting points were determined for all compounds and are not corrected. Optical rotations were taken with a Zeiss photoelectric precision polarimeter. 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 the intermediate peptide derivatives was ascertained by thin-layer chromatography (TLC)[1] on 6060 silica gel (Eastman chromagram sheet). For all peptides, the solvent systems used were chloroform–methanol–water (80:30:10 and 90:20:2). Detection of the peptides on the chromatogram was accomplished by sequential spraying of the chromagram sheet with dilute HCl and ninhydrin or by spraying with 1% solution of cerium sulfate in 10% H_2SO_4 followed by gentle heating on a hot plate. Amino acid analyses were performed in a Beckman 119-CL analyzer equipped with a Model 126 data system. Acid hydrolyses were carried out with 6 N HCl under nitrogen in the presence of phenol at 110 °C for 24 h. Enzymatic digestion with aminopeptidase M (Sigma Chemical Co.) was performed by the method of Pfleiderer et al. (1964). Preswollen microgranular CM-cellulose (Whatman CM-52) was used in this study. The washing of the resin and the preparation of the columns and the buffers used were described previously (Katsoyannis et al., 1967a,b). The preparation of the Cellex-E (Bio-Rad Laboratories) column and the washing of the resin were performed as described previously in the preparation of the comparable Ecteola-cellulose column (Ferderigos et al., 1979). Isoelectric focusing on thin-layer plates was carried out as described previously (Schwartz et al., 1981). Dialysis after column chromatography was performed in "Spectrapor" membrane tubing No. 3 (Fisher Scientific). Sodium tetrathionate was prepared as described by Gilman (1946). Protein determinations were carried out by the method of Lowry et al. (1951).

Receptor binding was assayed as a function of the displacement of ^{125}I-insulin (3×10^{-10} M) with rat liver membranes as described previously (Burke et al., 1980; Kitagawa et al., 1984). Lipogenesis, defined as the conversion of [3-^{3}H]glucose into organic solvent extractable material, was carried out in rat adipocytes as described previously (Burke et al., 1980; Kitagawa et al., 1984). Radioimmunoassay was performed with antibodies from Calbiochem-Behring, La Jolla, CA. Immunoprecipitates were filtered and washed on cellulose–acetate membranes, dried, and counted in Filtron X.

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[1] Abbreviations: CM, carboxymethyl; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; TEA, triethylamine; TLC, thin-layer chromatography; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol. Compounds designated by Roman numerals are described fully in the supplementary material (see paragraph at end of paper regarding supplementary material).

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Potency was calculated from the slopes of C_0/C_i plots, as described by Hales & Randle (1963). Circular dichroism (CD) measurements of the analogue and zinc-free insulin were obtained with a JASCO J-500A recording spectrophotometer equipped with a data processor, as described previously (Schwartz et al., 1983).

General Aspects of Synthesis of Sheep [Leu^{19}-A]insulin. This analogue was synthesized by the interaction of the S-sulfonated form of bovine (sheep) B chain with the thiol form of sheep insulin [Leu^{19}]A chain. The latter compound was obtained by reduction with 2-mercaptoethanol of the S-sulfonated A chain. The synthesis of the S-sulfonated [Leu^{19}]A chain was accomplished by the procedures employed in this laboratory for the construction of A-chain analogues [i.e., Ferderigos et al. (1983)]. The key intermediate in the procedure involves the synthesis of the protected heneicosapeptide (VII) containing the entire amino acid sequence of the A-chain analogue. Removal of the protecting groups by sodium in liquid ammonia and sulfitolysis of the resulting reduced product lead to the S-sulfonated A-chain analogue. In the present case, the synthesis of the protected heneicosapeptide VII involved the azide coupling of the C-terminal pentapeptide (sequence 17–21) with the adjacent pentapeptide (sequence 12–16) to yield the protected decapeptide (sequence 12–21). This, in turn, was coupled with the adjacent tripeptide azide (sequence 9–11) to yield the C-terminal tridecapeptide (sequence 9–21), which was condensed with the adjacent tetrapeptide azide (sequence 5–8) to produce the C-terminal heptadecapeptide (sequence 5–21). In the final step, the aforementioned heptadecapeptide was coupled with the N-terminal tetrapeptide azide (sequence 1–4) to produce the desired protected heneicosapaptide (VII).

*Glycyl-*L*-isoleucyl-*L*-valyl-*L*-glutamyl-*L*-glutaminyl-S-sulfo-*L*-cysteinyl-S-sulfo-*L*-cysteinyl-*L*-alanylglycyl-*L*-valyl-S-sulfo-*L*-cysteinyl-*L*-seryl-*L*-leucyl-*L*-tyrosyl-*L*-glutaminyl-*L*-leucyl-*L*-glutamyl-*L*-asparaginyl-*L*-leucyl-S-sulfo-*L*-cysteinyl-*L*-asparagine [Sheep Insulin [Leu^{19}]A Chain S-Sulfonate (VIII)].* Removal of the protecting groups of the heneicosapeptide VII with sodium in liquid ammonia and conversion of the reduced product to the S-sulfonated form by oxidative sulfitolysis was carried out as described previously (Katsoyannis et al., 1966). Briefly, the thoroughly dried product VII (284 mg) was dissolved in anhydrous liquid ammonia (200 mL) in a 500-mL round-bottom flask fitted for magnetic stirring. Deblocking was carried out at the boiling point of the solution by dipping a fine glass tube containing sodium into the solution until a faint blue color appeared throughout. The blue color was allowed to persist for 30 s and was then discharged by adding a few crystals of NH_4Cl. After evaporation of the ammonia, the residue was dissolved in 8 M guanidine hydrochloride (25 mL), and to this solution, adjusted to pH 8.9 with 1 N NH_4OH or acetic acid (depending on the pH of the solution), was added sodium sulfite (1.18 g) and freshly prepared sodium tetrathionate (0.56 g). After being stirred at 25 °C for 3.5 h, the mixture was placed on Spectrapor membrane tubing No. 3 and dialyzed against four changes of distilled water (4 L each) at 4 °C for 24 h. The crude [Leu^{19}]A chain *S*-sulfonate obtained by lyophilization of the dialyzate was dissolved in 0.015 M NH_4HCO_3 (4 mL) and chromatographed on a Sephadex G-15 column (4.5 × 49 cm) equilibrated and eluted with 0.015 M NH_4HCO_3. The effluent corresponding to the main peak, as monitored by an ISCO spectrophotometer (Model UA-5), was lyophilized, and the [Leu^{19}]A chain *S*-sulfonate was obtained as a white powder, 207 mg. For purification, this material (68 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0; 3 mL) and applied on a Cellex-E column (1.2 × 40 cm) that was equilibrated with the same buffer. The column was eluted with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient as described previously (Ferderigos et al., 1979). The elution pattern, as monitored with an ISCO spectrophotometer and a conductivity meter (Radiometer, Copenhagen), is shown in Figure 1. The effluent under the major peak (360–425 mL) was collected, dialyzed in Spectrapor membrane tubing No. 3 as described above, and lyophilized. The material obtained (55 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0; 3 mL) and rechromatographed on the same Cellex-E column under identical conditions as described above. the elution pattern of this column is shown in Figure 1. The effluent under the peak was collected, dialyzed, and lyophilized, and the purified [Leu^{19}]A chain S-sulfonate was obtained as a white fluffy powder (34 mg).

On thin-layer electrophoresis (precoated TLC plates, 10 × 20 cm, silica gel 60, EM Laboratories) in 2 N acetic acid–0.6 N formic acid (1:1 v/v), pH 2.0 and 480 V, the synthetic chain analogue moved as a single band (data not shown). Amino acid analysis of [Leu^{19}]A chain *S*-sulfonate after acid hydrolysis gave the molar ratios shown in Table I, in good agreement with the expected values. Digestion of the synthetic chain analogue with aminopeptidase M and amino acid analysis of the digest gave the molar ratios shown in Table

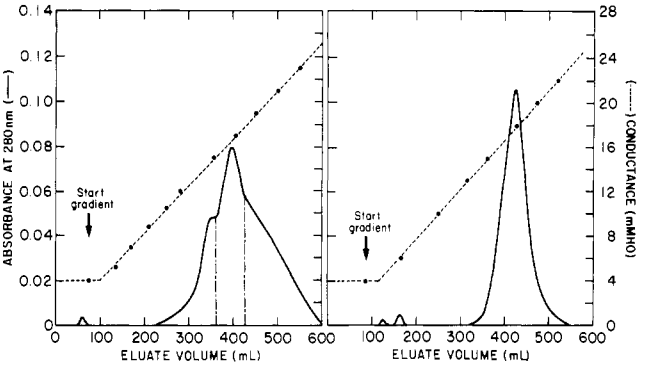

FIGURE 1: (Left) Chromatography of crude [Leu^{19}]A chain *S*-sulfonate on a 1.2 × 40 cm Cellex-E column with 0.1 M Tris-HCl buffer (pH 7.0) and a linear NaCl gradient. The column was monitored by an ISCO recording spectrophotometer and by a conductivity meter. (Right) Rechromatography of the product obtained after dialysis and lyophilization of the main peak effluent (360–425 mL) depicted in the left panel.

Table I: Amino Acid Composition[a] of an Acid Hydrolysate and an Enzymatic Digest (Aminopeptidase M) of the S-Sulfonated [Leu^{19}]A Chain of Sheep Insulin

	acid hydrolysis		enzymatic hydrolysis	
amino acid	theory	found	theory	found
aspartic acid	2	1.8	0	0
glutamine	0	0	2	*c*
asparagine	0	0	2	*c*
serine	1	0.9	1	*c*
glutamic acid	4	4.0	2	1.8
glycine	2	1.9	2	1.9
alanine	1	1.0	1	1.1
half-cystine	4	nd[b]	0	0
valine	2	1.7	2	2.1
isoleucine	1	0.6	1	0.9
leucine	3	3.1	3	3.0
tyrosine	1	1.0	1	1.0
S-sulfocysteine	0	0	4	4.1

[a] Number of amino acid residues per molecule. [b] nd, not determined. [c] Emerge at the same position and not determined.

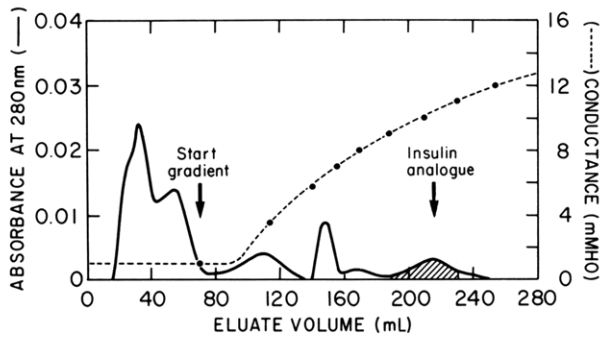

FIGURE 2: Chromatography of a combination mixture (see Experimental Procedures) of the thiol form of sheep [Leu^{19}]A chain with the S-sulfonated sheep (bovine) B chain on a 0.9 × 23 cm CM-cellulose column with acetate buffer ([Na^+] of 0.024 M, pH 3.3) and an exponential NaCl gradient. The column effluent was monitored by an ISCO spectrophotometer and a conductivity meter (Radiometer, Copenhagen). The insulin analogue (190–230 mL of effluent) was recovered as the hydrochloride.

Table II: Amino Acid Composition[a] of an Acid Hydrolysate of the Sheep [Leu^{19}-A]insulin

amino acid	theory	found	amino acid	theory	found
lysine	1	1.0	glycine	5	5.1
histidine	2	2.0	alanine	3	2.8
arginine	1	1.0	half-cystine	6	nd[b]
aspartic acid	3	3.1	valine	5	4.1[c]
threonine	1	0.8	isoleucine	1	0.5[c]
serine	2	2.2	leucine	7	7.4
glutamic acid	7	7.2	tyrosine	3	3.0
proline	1	1.0	phenylalanine	3	3.0

[a] Number of amino acid residues per molecule. [b] nd, not determined. [c] Recovery after acid hydrolysis of natural insulin: 70–78% for Val; 40–50% for Ile.

I. It is apparent that the synthetic material was completely digested by the enzyme, indicating that the optical configuration of the constituent amino acids was preserved during the synthetic processes.

S-Sulfonated Derivative of the B Chain of Sheep Insulin. This compound was prepared by oxidative sulfitolysis of bovine insulin followed by separation of the resulting S-sulfonated A and B chains by CM-cellulose chromatography as we have reported previously (Katsoyannis et al., 1967a). The B chains of bovine and sheep insulins are identical (Sanger & Tuppy, 1951; Brown et al., 1955).

Synthesis and Isolation of Sheep [Leu^{19}-A]insulin. This analogue was synthesized by the combination of the thiol form of [Leu^{19}]A chain with the S-sulfonated form of bovine (sheep) B chain by the procedure we have described previously (Katsoyannis & Tometsko, 1966; Katsoyannis et al., 1967b,c; Schwartz & Katsoyannis, 1976). In a typical experiment, 20 mg of [Leu^{19}]A chain *S*-sulfonate was converted to the thiol form upon treatment with 2-mercaptoethanol (in 5 mL of 0.1 M Tris-HCl buffer, pH 8.3, at 37 °C, 8 min) and then allowed to react with 5 mg of B chain *S*-sulfonate for 20 h at pH 10.6 and 4 °C. The reaction mixture was then processed as described previously (Katsoyannis et al., 1967b,c). Isolation of the insulin analogue from the combination mixture was carried out by chromatography on a CM-cellulose column (0.9 × 23 cm) with an acetate buffer ([Na^+] of 0.024 M; pH 3.3) and an exponential NaCl gradient as described previously (Katsoyannis et al., 1967c). The elution pattern, as monitored by an ISCO spectrophotometer and a conductivity meter, is shown in Figure 2. The eluate containing the analogue (190–230 mL of effluent) was processed as described previously (Katsoyannis et al., 1967c), and the purified sheep [Leu^{19}-A]insulin was isolated via picrate as the hydrochloride (1.05 mg).

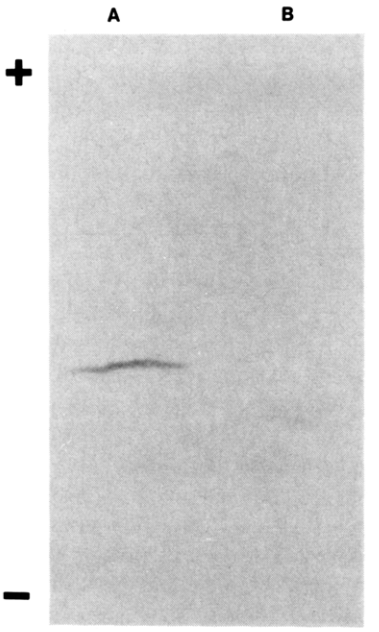

FIGURE 3: Paper print of thin-layer isoelectric focusing of natural bovine insulin (A) and synthetic [Leu^{19}-A]insulin (B) in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes on a 20-cm separation distance. Focusing was at a constant power of 8 W for 4 h.

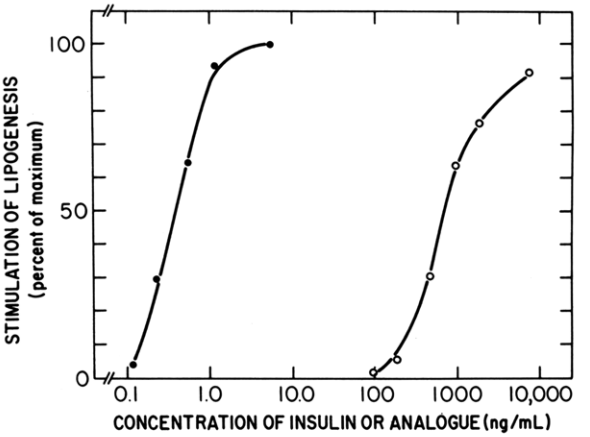

FIGURE 4: Effect of bovine insulin (●) and [Leu^{19}-A]insulin (○) on lipogenesis in rat adipocytes. The stimulation of lipogenesis, expressed as percent of maximum, is plotted as a function of the concentration of insulin and analogue. Data points represent the mean of triplicate determinations in a typical experiment that was performed 3 times.

Amino acid analysis of this analogue after acid hydrolysis gave a composition expressed in molar ratios (Table II) in good agreement with the expected values. On isoelectric focusing on thin-layer plates in a 1:1 mixture of pH 3–10 and pH 4–6 ampholytes, [Leu^{19}-A]insulin focused on one band (Figure 3).

Biological Evaluation of Sheep [Leu^{19}-A]insulin. Figure 4 depicts the stimulation of lipogenesis in rat adipocytes by bovine insulin and by [Leu^{19}-A]insulin. The analogue shows full agonist behavior, and the two dose–response curves are parallel. [Leu^{19}-A]insulin, however, reaches half-maximum stimulation of lipogenesis at a concentration more than 2 orders of magnitude higher than is required for bovine insulin. The calculated relative potency of the analogue is 0.05%, which makes it, along with [Gly^2-A]insulin (Kitagawa et al., 1984), the least potent insulin analogue we have examined. In receptor binding assays, [Leu^{19}-A]insulin was unable to displace 50% of specifically bound ^{125}I-insulin at a concentration of 25 μg/mL, the highest level tested (data not shown), which is consistent with a potency of less than 0.1%. Radioimmunoassay of [Leu^{19}-A]insulin resulted in a calculated relative

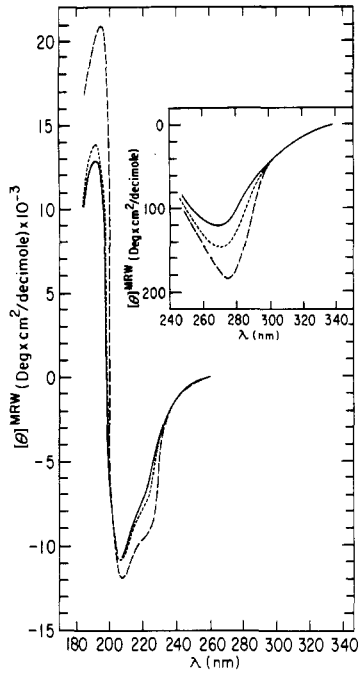

FIGURE 5: The near- and far-UV CD spectra of [Leu^{19}-A]insulin at 12.9×10^{-6} M (—) and zinc-free natural insulin at 11.4×10^{-6} (--) and 1.14×10^{-6} M (···). (Inset) Analogue (—) and insulin at 16.8×10^{-6} (--) and 1.68×10^{-6} M (···), all in 0.001 M HCl.

potency of 7.3% (data not shown), a striking illustration of the lack of correlation between this assay and biological activity.

Circular Dichroic Studies. Figure 5 illustrates the near- and far-ultraviolet circular dichroic spectra of [Leu^{19}-A]insulin and zinc-free insulin in 0.001 M HCl. At concentrations of 12.9×10^{-6} M for the analogue and 1.14×10^{-6} M for insulin, the far-ultraviolet CD spectra are very nearly the same, and in the near-ultraviolet spectra, the difference in their molar ellipticities may be attributed primarily to the fact that the analogue has three tyrosine residues as compared to the four in insulin. [Leu^{19}-A]insulin (12.9×10^{-6} M) exhibits a maximum at 193 nm and minima at 207, 220 (shallow sh), and 269 nm (br), whereas insulin (1.14×10^{-6} M) exhibits comparable maximum and minima very slightly red shifted to 193.5, 207.5, 221, and 270 nm. Insulin at a concentration of 11.4×10^{-6} M (0.001 M HCl) exhibits maxima and minima that are red shifted to 195, 208, 221, and 274 nm, with enhanced molar ellipticities especially evident at 195, 221, and 274 nm (Figure 5).

Results and Discussion

The X-ray model of insulin (Blundell et al., 1972) indicates that the A^2–A^8 segment is an irregular α-helix and that the side chain of isoleucine in position A^2 is in van der Waals contact with the tyrosine residue in position A^{19}. In a previous paper (Ferderigos et al., 1983), we showed that the replacement of tyrosine at position A^{19} of insulin by phenylalanine resulted in an analogue displaying only about 8% potency relative to bovine insulin. We suggested that the hydroxyl group of this tyrosine is necessary for high biological activity. We have also reported that a seemingly minor alteration at position A^2, the substitution of norleucine for isoleucine, produced an analogue ([Nle^2-A]insulin) with profoundly reduced potency, less than 1% relative to the natural hormone (Okada et al., 1981). Recently, we described the synthesis and biological activity of two analogues that have glycine or alanine substituted for the naturally occurring isoleucine at position A^2. Both analogues are extremely weak agonists, with potencies of 0.05 and 0.4%, respectively, as compared to insulin (Kitagawa et al., 1984).

In the present paper, we shown that the substitution of leucine for the naturally occurring tyrosine at position A^{19} produces a compound displaying biological potency very similar to the A^2-substituted analogues. It is striking, in fact, that the elimination of the side chain of isoleucine in [Gly^2-A]-insulin and the removal of aromaticity in [Leu^{19}-A]insulin result in compounds displaying indistinguishable potency. This similarity argues that these residues contribute to a common structural element, which appears to be among the strongest requirements yet described for high biological activity in insulin.

Insulin at 1.14×1^{-6} M in 0.001 M HCl, pH 3.2, exists primarily in the monomeric form, whereas at a concentration of 11.4×10^{-6} M (0.001 M HCl) it exists primarily as a dimer (Lord et al., 1973). The CD spectrum of [Leu^{19}-A]insulin at 12.9×10^{-6} M (0.001 M HCl) is shown in Figure 5. The calculated molar ellipticity (Figure 5) at 195 nm and the ratio of molar ellipticities $[\theta]_{195}/[\theta]_{208}$ indicate that this analogue is monomeric (Kitagawa et al., 1984) at a concentration at which insulin exists primarily as a dimer. It has previously been demonstrated by ultracentrifugal studies that [Nle^2-A]insulin is monomeric at the relatively high concentration of 1×10^{-4} M (Okada et al., 1981). The CD spectrum of the present analogue is virtually superimposable upon that of [Nle^2-A]insulin (Okada et al., 1981), as well as those of [Gly^2-A]insulin and [Ala^2-A]insulin (Kitagawa et al., 1984), and differs little from that of insulin solutions containing predominantly the monomeric form of the hormone (Figure 5).

It may reasonably be presumed that the conformation of the [Leu^{19}-A]insulin analogue, as well as that of [Gly^2-A]-, [Ala^2-A]-, and [Nle^2-A]insulin, is very similar to that of monomeric insulin. However, these monomeric analogues, in contrast to insulin, have little if any propensity to dimerize. The substantial decrease in molar ellipticity at wavelengths 195, 207, and 222 nm, displayed by insulin monomer and the analogues in comparison with the insulin dimer, is characteristically associated with a loss of the α-helical conformation. This fact, in conjunction with the position of the substitution (A^2) for the analogues, strongly suggests that the A^2–A^8 segment, helical in crystalline insulin and dimeric insulin, is distorted in the analogues and in monomeric insulin. This would account in large measure for the observed reduced ellipticities. It may be reasonably concluded then that in the case of the present analogue, [Leu^{19}-A]insulin, the A^2–A^8 segment is also not in an α-helical conformation. We have suggested that the transition to, and stabilization of, the helical conformation of this segment (Okada et al., 1981; Kitagawa et al., 1984) is dependent on the establishment of van der Waals interaction between the side chain of isoleucine A^2 and the phenolic ring of tyrosine A^{19}. This interaction apparently does not occur in any of these analogues.

From our previous findings with A^2-substituted analogues, together with the present data, it is reasonable to suppose that the binding of the insulin monomer to the insulin receptor is, as in dimer formation, dependent upon favorable interaction between residues A^2 and A^{19}. This interaction is absent in the A^2-substituted analogues and in [Leu^{19}-A]insulin, which results in compounds unable either to dimerize or to interact normally with the insulin receptor. Monomeric insulin, in interacting with the insulin receptor, appears able to assume a conformation favoring the van der Waals contact between isoleucine

A^2 and tyrosine A^{19}, with concomitant stabilization of the helical segment A^2–A^8. [Leu^{19}-A]insulin, in common with the A^2-substituted insulin, has a small probability for adaptation to the conformation required for receptor binding. The interaction between A^2 and A^{19} appears to be among the most important determinants for high biological activity in insulin.

Acknowledgments

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Supplementary Material Available

Complete synthetic details including references (6 pages). Ordering information is given on any current masthead page.

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5,10-Methenyl-5,6,7,8-tetrahydromethanopterin, a One-Carbon Carrier in the Process of Methanogenesis†

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ABSTRACT: 5,10-Methenyl-5,6,7,8-tetrahydromethanopterin was isolated from *Methanobacterium thermoautotrophicum*. The structure of this compound was elucidated by various two-dimensional nuclear magnetic resonance techniques and confirmed by fast atom bombardment mass spectrometry. The structure of 5,10-methenyl-5,6,7,8-tetrahydromethanopterin shows one additional carbon atom as compared to the parent compound methanopterin. This additional carbon atom is rapidly labeled in vivo by $^{13}CO_2$. It is shown that 5,10-methenyl-5,6,7,8-tetrahydromethanopterin is a physiologically active one-carbon carrier at the formyl level of oxidation in the methanogenic pathway from CO_2.

During short-term labeling experiments with cells of *Methanobacterium thermoautotrophicum* it was demonstrated that $^{14}CO_2$ was incorporated in substantial amounts into a so-called yellow fluorescent compound (Daniels & Zeikus, 1978). Cells of *Methanosarcina barkeri*, grown in the absence of hydrogen, incorporated $^{14}CH_3OH$ into a closely related yellow fluorescent compound. Note that in the absence of hydrogen *M. barkeri* performs the reaction $4CH_3OH \rightarrow 3CH_4 + CO_2$, whereas in the presence of hydrogen the reaction $CH_3OH + H_2 \rightarrow CH_4 + H_2O$ is performed. In the presence of hydrogen and $^{14}CH_3OH$ the yellow fluorescent compound was not labeled, which indicated that this compound plays a role in the oxidation of CH_3OH to CO_2 by *M. barkeri* (Kenealy & Zeikus, 1982).

On the basis of spectral properties and degradation studies it was erroneously assumed that the yellow fluorescent compound present in *M. thermoautotrophicum* was a carboxy-

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