Importance of Aliphatic Side-Chain Structure at Positions 2 and 3 of the Insulin A Chain in Insulin–Receptor Interactions†

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Received November 6, 1991; Revised Manuscript Received January 14, 1992

ABSTRACT: In order to evaluate the cause of the greatly decreased receptor-binding potency of the naturally occurring mutant human insulin Insulin Wakayama ([Leu^{A3}]insulin, 0.2% relative potency), we examined (by the semisynthesis of insulin analogues based on N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin) the importance of aliphatic side chain structure at positions A2 and A3 (Ile and Val, respectively) in directing the interaction of insulin with its receptor. Analogues bearing glycine, alanine, α-amino-*n*-butyric acid, norvaline, norleucine, valine, isoleucine, *allo*-isoleucine, threonine, *tert*-leucine, or leucine at positions A2 or A3 were assayed for their potencies in competing for the binding of ^{125}I-labeled insulin to isolated canine hepatocytes, as were analogues bearing deletions from the A-chain amino terminus or the B-chain carboxyl terminus. Selected analogues were also analyzed by far-UV CD and absorption spectroscopy of Co^{2+} complexes. Our results identify that (a) Ile and Val serve well at position A2, whereas residues with other side chains (including those with straight chains, alternatively configured β-branches, or a γ-branch) exhibit relative receptor-binding potencies in the range 1–5%; (b) greater flexibility is allowed side-chain structure at position A3, with Ile, *allo*-Ile, α-amino-*n*-butyric acid, and *tert*-Leu exhibiting relative receptor-binding potencies in the range 11–36%; and (c) simultaneous replacements at positions A2 and A3, and deletions of the COOH-terminal domain of the insulin B chain in related analogues, yield cumulative effects. These findings are discussed with respect to a model for insulin–receptor interactions that involves a structure-orienting role for residue A2, the direct interaction of residue A3 with receptor, and multiple separately defined elements of structure and of conformational adjustment.

The discovery within the past decade of naturally occurring abnormal human insulins [produced as the result of point mutations within the human genome; see Tager (1990) for a review] has led to renewed focus in examining insulin structure–function relationships. Importantly, the sites of amino acid replacement in the abnormal human insulins (Val^{A3}, His^{B10}, Phe^{B24}, and Phe^{B25}) have been highly conserved during animal evolution and are dispersed throughout the three-dimensional structure of the insulin molecule (Blundell et al., 1972; Baker et al., 1988). Although considerable attention has been placed on understanding the importance of the replacement of Phe^{B24} by Ser in Insulin Los Angeles and that of Phe^{B25} by Leu in Insulin Chicago [cf. Tager (1990)], our understanding of the effects of replacing Val^{A3} by Leu in Insulin Wakayama is still limited. In fact, the replacement of the β-branched side chain of Val with the γ-branched side chain of Leu at position A3 results in a natural insulin variant possessing the lowest receptor binding potency (0.2% of that of insulin; Nanjo et al., 1986; Kobayashi et al., 1986) of any abnormal human insulin studied to date. The importance of Insulin Wakayama is heightened by considering (a) the unexpected major consequence of exchanging one branched aliphatic amino acid side chain for another, (b) the prior placement of the NH_2-terminal region of the insulin A chain within the receptor-binding domain of the hormone (Blundell et al., 1972; DeMeyts et al., 1978), (c) the occurrence of Val^{A3} within the evolutionary conserved sequence Gly-Ile/Val-Val-Glu/Asp, and (d) the extension of the causative point mutation to multiple apparently unrelated families in Japan [see Steiner et al. (1990)].

Several aspects of insulin structure provide a framework on which to base consideration of structure–function relationships with respect to both the NH_2-terminal domain of the insulin A chain and the disposition of residue A3. First, the insulin A chain can be considered in terms of a somewhat disordered, NH_2-terminal α-helix (residues A2–A8) oriented in an antiparallel fashion to a COOH-terminal α-helix (residues A13–A20) (Blundell et al., 1972; Chothia et al., 1983). Second, the disposition of the region corresponding to residues A1–A6 differs between the two monomers of the crystallographic 2-Zn insulin hexamer (Chothia et al., 1983); these differences are due in part to close lattice contacts and crystal packing in the hexamer and are related to concomitant changes in residues B28–B30. Third, during molecular dynamical simulations, the domain comprising residues A1–A6 is found to undertake two distinct, but reversibly formed, conformations (Kruger et al., 1987). Fourth, residue Gly^{A1} is found in the crystallographic structure of insulin to occupy a compact surface pocket and to undergo multiple close contacts (involving both hydrogen bonding and a salt bridge between its α-amino group and the γ-carboxyl group of Glu^{A4}) (Blundell et al., 1972; Baker et al., 1988). Fifth, the side chain of residue Ile^{A2} is found to be buried as part of the hydrophobic core of the molecule; it is in van der Waals contact with the side chain of Tyr^{A19} (Blundell et al., 1972; Derewenda et al., 1990). Sixth, the side chain of residue Val^{A3} is at the base of a short surface pocket (Blundell et al., 1972); the degree of exposure of the residue to solvent would increase significantly in despentapeptide-(B26–B30)-insulin, an analogue with full receptor binding potency (Bi et al., 1984; Dai et al., 1987; Derewenda et al., 1990). Very important recent data on the NMR structure of insulin identify (a) the retention of α-helix in the region of residues A2–A8 for insulin in solution, (b) the very similar structures of the region for insulin, despentapeptide-

† These studies were supported by Grants DK18347 and DK20595 from the National Institutes of Health.
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0006-2960/92/0431-3204$03.00/0

insulin, and monomeric insulin analogues, (c) the slow deuterium exchange within the region expected for a stable peptide structure in solution, and (d) interactions both between residues Ile^{A2} and Tyr^{B26} and between residues Val^{A3} and Pro^{B28} (Weiss et al., 1989, 1991; Hua & Weiss, 1990, 1991).

Taken together, the above findings identify the NH_2-terminal α-helical domain of insulin as exhibiting both a fundamentally stable conformation and a conformation with the propensity for structural change. It should be noted that although both crystallographic and NMR data identify a close packing between the NH_2-terminal domain of the insulin A chain and the COOH-terminal domain of the insulin B chain, insulins cross-linked between these two domains (by chemical means or by the introduction of a peptide bond between Lys^{B29} and Gly^{A1} in so-called "miniproinsulin") exhibit significantly decreased potency for receptor interactions, notwithstanding their near native structures (Brandenburg et al., 1973; Cutfield et al., 1981; Markussen et al., 1985; Nakagawa & Tager, 1989; Derewenda et al., 1991). In order to gain a better understanding of the importance of the NH_2-terminal domain of the insulin A chain in insulin–receptor interactions and of the specific importance of residue A3 with respect to the replacement of Val^{A3} by Leu in Insulin Wakayama, we undertook (a) to prepare by semisynthesis a series of insulin analogues containing natural and unnatural aliphatic amino acid replacements at positions 2 and 3 of the insulin A chain and (b) to study the effects of these replacements on the potency of insulin–receptor interactions. Overall, our findings identify separate roles for residues Ile^{A2} and Val^{A3} in determining the fit of insulin for its receptor and the importance of conformational adjustment in the NH_2-terminal domain of the insulin A chain (as in the COOH-terminal domain of the insulin B chain; Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1989; Derewenda et al., 1990; Hua et al., 1991) in achieving a high-affinity state of ligand–receptor interaction.

Materials and Methods

Materials. Porcine insulin and [[^{125}I]iodo-Tyr^{A14}]insulin were obtained from Eli Lilly and Co. (Indianapolis, IN). L-*allo*-Isoleucine (Ail)[1] (Bachem Bioscience, Switzerland) and L-*tert*-leucine (Tle) (Aldrich, Milwaukee, WI) were converted to their corresponding N^{α}-*tert*-butyloxycarbonyl (Boc) derivatives by use of di-*tert*-butyl pyrocarbonate according to Moroder et al. (1976). These protected amino acids, the Boc derivatives of L-α-amino-*n*-butyric acid (Abu), L-norvaline (Nva), and L-norleucine (Nle) (from Sigma, St. Louis, MO), and N^{α}-Boc-*O*-*tert*-butyl-Thr (from Bachem Bioscience) were converted to their corresponding *N*-hydroxysuccinimide esters by standard methods (Anderson et al., 1964).

Preparation of the N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-Bisacetyl Derivatives of Desdipeptide-(A1–A2)-insulin and Destripeptide-(A1–A3)-insulin. N^{α}-Gly^{A1}-Phenylthiocarbamyl- (Ptc-) insulin was prepared by reacting insulin (0.1 mmol) with phenyl isothiocyanate (0.35 mmol) for 2 h at 22 °C in a solution containing methanol (100 mL) and 5% (v/v) aqueous triethylamine brought to pH 9.3 with HCl (25 mL) (Drewers et al., 1981; Nakagawa & Tager, 1989). The crude product was purified by reverse-phase HPLC by use of a C-18 column and the aqueous triethylammonium phosphate/acetonitrile solvent system that has been described before (Shoelson et al., 1983). The product corresponding to the major peak was desalted on a column of Bio-Gel P-4 using 3 M acetic acid and was freeze dried, yielding 45 μmol of material. The material was dissolved in 3 mL of dimethylformamide (DMF) containing 2 mmol of *N*-methylmorpholine and was reacted for 3 h at 22 °C with 1 mmol of *N*-hydroxysuccinimido acetate. The resulting N^{α}-Gly^{A1}-Ptc-N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin was precipitated and washed with diethyl ether. The product was treated with trifluoroacetic acid (3 mL, 1 h, 22 °C) to remove Ptc-Gly and to obtain (subsequent to precipitation by ether) N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1}-insulin. The truncated peptide (0.04 mmol) was subjected to a full cycle of Edman degradation by reaction for 1 h at 22 °C with phenyl isothiocyanate (0.2 mmol) and *N*-methylmorpholine (0.1 mL) dissolved in DMF (2 mL) followed by precipitation and treatment with trifluoroacetic acid as described above. At this point, half of the product was retained for subsequent use, and half was subjected to an additional cyclic of Edman degradation. The crude products were purified by reverse-phase HPLC, desalted by gel filtration, and freeze dried to yield 7.5 and 6.8 μmol of the desdipeptide-(A1–A2) and destripeptide-(A1–A3) derivatives of bisacetyl-insulin, respectively.

Preparation of Analogues of Bisacetyl-insulin Containing Amino Acid Replacements at Positions A2, A3, or A2 plus A3. For the synthesis of analogues with replacements at position A2, N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2}-insulin (1 μmol) was reacted for 3–48 h at 22 °C in 0.2 mL of DMF containing 10–20 μL of *N*-methylmorpholine with the appropriate N^{α}-Boc-amino acid *N*-hydroxysuccinimide ester (Gly, 10 μmol; Tle, 100 μmol; other amino acids, 20 μmol). Reaction progress was monitored by analytical reverse-phase HPLC. When the reaction was nearly complete, the mixture was diluted with 1 mL of 3 M acetic acid and the resulting solution was gel-filtered on a column (1.5 × 50 cm) of Bio-Gel P-4 using 3 M acetic acid as the solvent. Fractions in the major peptide peak were pooled, freeze dried, and treated with trifluoroacetic acid (1 mL) for 45 min at 0 °C to remove the Boc protecting group. The products (freeze dried from water) were subsequently treated with N^{α}-Boc-Gly *N*-hydroxysuccinimide ester (5 μmol) for 2–6 h at 22 °C, as described above, to complete the peptides. The Boc group was removed by treatment with trifluoroacetic acid, and the peptides were freeze dried.

For the synthesis of analogues with amino acid replacements at position A3 or at positions A2 and A3, N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2},Val^{A3}-insulin was used as the starting material. Peptide synthesis proceeded by use of Boc-amino acid *N*-hydroxysuccinimide esters and the procedures described above. All insulin analogues were purified by reverse-phase HPLC by use of C-18 columns and were desalted by gel filtration. Analytical HPLC of the final products identified the purity of the peptides to be >95%. When appropriate, peptide compositions were confirmed by amino acid analysis. The yields of the final products ranged from 16 to 47%, based on the amount of truncated insulin analogue used in the initial step of peptide synthesis.

Preparation of Trisacetyl Derivatives of Insulin and Insulin Analogues. For the preparation of N^{α}-Gly^{A1},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-trisacetyl-insulin, insulin (10 μmol), dissolved in 2 mL of dimethyl sulfoxide, was reacted for 3 h at 22 °C with 150 μmol of *N*-hydroxysuccinimido acetate. The reaction was terminated by the addition of 3 M acetic acid at 0 °C, the mixture was gel-filtered on Bio-Gel P-6, appropriate fractions were pooled, and the product was freeze dried. For the preparation of the trisacetyl derivatives of truncated insulin analogues, the des-Gly^{A1}, des-Gly^{A1},Ile^{A2}, or des-

[1] Abbreviations: Abu, α-amino-*n*-butyric acid; Nva, norvaline; Nle, norleucine; Ail, *allo*-isoleucine; Tle, *tert*-leucine; Boc, *tert*-butyloxycarbonyl; HPLC, high-performance liquid chromatography; Ptc, phenylthiocarbamyl; DMF, dimethylformamide.

Gly^{A1},Ile^{A2},Val^{A3} counterparts of N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin (0.2 μmol), prepared as described above, were reacted for 5 h at 22 °C in a mixture containing DMF (0.1 mL), *N*-methylmorpholine (5 μL), and *N*-hydroxysuccinimido acetate (2 μmol). The products were purified as described above for trisacetyl-insulin.

Preparation of N^{α}-Phe^{B1}-Acetyl-despentapeptide-(B26–B30)-[Leu^{A3},Phe^{B25}-α-carboxamide]insulin. N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Leu^{A3}]insulin (prepared as described above, 0.05 μmol) was dissolved in 0.05 mL of 0.2 M Tris buffer (containing 10 mM $CaCl_2$) brought to pH 8.0 with HCl and was incubated for 1.5 h at 37 °C with tosylphenylalanine chloromethyl ketone-treated trypsin (15 μg). A solution containing the hydrochloride salt of the peptide Gly-Phe-Phe-α-carboxamide (5 μmol), *N*-methylmorpholine (1 μL), DMF (0.06 mL) and 1,4-butanediol (0.06 mL) was then added to the trypsin-cleaved insulin analogue, as was an additional 15 μg of trypsin. The mixture was kept for 18 h at 12 °C to allow for enzyme-catalyzed peptide bond formation between Arg^{B22} of the insulin analogue and Gly^{1} of the tripeptide (see Inouye et al., 1979; Nakagawa & Tager, 1986; and Morihara et al., 1986). The reaction was terminated by the addition of 3 M acetic acid; the product was purified by gel filtration on Bio-Gel P-6 in 3 M acetic acid followed by reverse-phase HPLC. The yield of the purified peptide (after it had been desalted on Bio-Gel P-4) was 26% of theoretical.

Preparation of the Desoctapeptide-(B23–B30) Derivatives of Insulin Analogues Containing Amino Acid Replacements at Position A2 or A3. Desoctapeptide-(B23–B30)-insulin (50 μmol dissolved in 3 mL of dimethyl sulfoxide) was allowed to react for 80 min at 22 °C with 65 μmol of 2-Boc-oximino-2-phenylacetonitrile (to yield predominantly the corresponding N^{α}-Gly^{A1}-Boc peptide). *N*-Hydroxysuccinimido acetate (0.25 mmol) was then added and the reaction was continued for 50 min to acetylate free amino groups. The mixture was diluted with DMF (2 mL), the peptide was precipitated by the addition of diethyl ether (120 mL), and the washed and dried precipitate was dissolved in trifluoroacetic acid (6 mL) to remove the Boc group. The product was freeze dried from 3 M acetic acid and was purified by reverse-phase HPLC and by gel filtration. The yield of N^{α}-Phe^{B1}-acetyl-desoctapeptide-(B23–B30)-insulin was 63% of theoretical. Portions of the product were subjected to 2 or 3 cycles of Edman degradation, as appropriate, and as described above. The HPLC-purified truncated peptides were then used as a scaffold for *N*-hydroxysuccinimide ester-based semisynthesis as described above for full-length analogues of insulin.

Additional Methods. Procedures for the isolation of canine hepatocytes and for their use in receptor binding experiments have been described before (Bonnevie-Nielsen et al., 1982; Nakagawa & Tager, 1986). Cells (1.6×10^6/mL) were incubated with [[^{125}I]iodo-Tyr^{A14}]insulin plus selected concentrations of insulin or insulin analogues (determined by UV absorbance) for 30 min at 30 °C. CD spectra were determined at 22 °C by use of a JASCO Model J-600 spectropolarimeter at peptide concentrations of 90 μg/mL in 10 mM KH_2PO_4 adjusted to pH 7.4 with NaOH or 54 μg/mL in a mixture of this buffer and trifluoroethanol (60:40 v/v). Cylindrical cells of 1 mm optical path length were used. Six spectra (taken at a scan speed of 20 nm/min) were averaged for each peptide studied. The T → R structural transitions of insulin$_6$(Co^{2+})$_2$ complexes (transitions that are induced by phenol and are stabilized by thiocyanate) were examined by methods described previously (Roy et al., 1989; Thomas & Wollmer, 1989; Nakagawa & Tager, 1991). Visible spectra were recorded for peptides (0.17 mM final concentration) dissolved in 0.05 M Tris brought to pH 8.0 by the addition of HCl by use of a Beckman Model DU-40 spectrophotometer. Further details are provided in the legend to Figure 6.

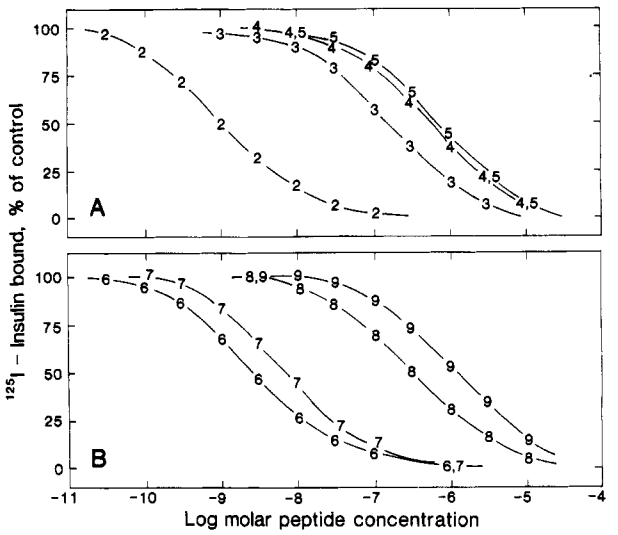

FIGURE 1: Inhibition of binding of ^{125}I-labeled insulin to isolated canine hepatocytes by truncated insulin analogues. Incubations were performed as described under Materials and Methods. Control binding is defined as the amount of radiolabeled ligand which became cell associated in the absence of competitor; all data have been corrected for so-called nonspecific binding that was detected in the presence of 10 μM insulin. Quantitative information is provided in Table I; identifying numbers in Table I, rather than symbols, are used to indicate the peptide under consideration. Panel A: 2, inhibition by N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin; 3, 4, and 5, inhibition by analogues of peptide 2 in which one (Gly^{A1}), two (Gly^{A1} and Ile^{A2}), or three (Gly^{A1}, Ile^{A2}, and Val^{A3}) residues have been deleted from the amino terminus of the A chain, respectively. Panel B: 6, inhibition by N^{α}-Gly^{A1},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-trisacetyl-insulin; 7, 8, and 9, inhibition by analogues of peptide 6 in which one (Gly^{A1}), two (Gly^{A1} and Ile^{A2}), or three (Gly^{A1}, Ile^{A2}, and Val^{A3}) have been deleted from the A chain, respectively, and the A-chain α-amino group has been derivatized by acetylation. See Table I and text for further details.

RESULTS

Receptor Binding Studies. As described under Materials and Methods, our approach to the preparation of insulin analogues containing amino acid deletions from the amino terminus of the insulin A chain or amino acid replacements at position A2 or A3 depended upon (a) the preparation of N^{α}-Gly^{A1}-Ptc-insulin, (b) the acetylation of free amino groups at positions N^{α}-Phe^{B1} and N^{ϵ}-Lys^{B29}), (c) one, two, or three cycles of Edman degradation, (d) as appropriate, the stepwise addition of up to three residues to the A-chain amino terminus by use of Boc-amino acid *N*-hydroxysuccinimide esters, and (e) the use of the shortened bisacetyl-insulin to act as a scaffold for solution-phase peptide synthesis. This semisynthetic scheme was designed with the preparation of numerous insulin analogues in mind and with the knowledge that derivatization of residues Phe^{B1} and Lys^{B29} has little effect on the potency of insulin–receptor interactions (Lindsay & Shall, 1971; Nakagawa & Tager, 1989). Table I identifies that N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin retains 76% of the receptor-binding potency of native insulin and that the hepatocyte insulin receptor exhibits an apparent dissociation constant of 0.84 nM for the bisderivatized analogue.

As illustrated by Figure 1A (see Table I), the deletion of one, two, or three residues from the amino terminus of the A chain in bisacetyl-insulin results in analogues (peptides 3–5) with 0.58, 0.14, and 0.11%, respectively, of the receptor-binding potency of the parent compound. On the one hand, these

Table I: Identification and Receptor-Binding Potencies of Insulin and Insulin Analogues[a]

identifying no.	peptide	potency relative to insulin	potency relative to $N^{\alpha B1}$,$N^{\epsilon B29}$-bis-acetyl-insulin
1	insulin	100	
2	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin	76 ± 2 (12)	100
3	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1}-insulin	0.44 ± 0.05 (4)	0.58
4	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2}-insulin	0.11 ± 0.02 (4)	0.14
5	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2},Val^{A3}-insulin	0.083 ± 0.011 (4)	0.11
6	N^{α}-Gly^{A1},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-trisacetyl-insulin	22 ± 3 (3)	29
7	N^{α}-Ile^{A2},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-trisacetyl-des-Gly^{A1}-insulin	12 ± 2 (5)	16
8	N^{α}-Val^{A3},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-trisacetyl-des-Gly^{A1},Ile^{A2}-insulin	0.23 ± 0.00 (3)	0.30
9	N^{α}-Glu^{A4},N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-trisacetyl-des-Gly^{A1},Ile^{A2},Val^{A3}-insulin	0.050 ± 0.010 (3)	0.066
10	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Gly^{A2}]insulin	0.90 ± 0.09 (3)	1.18
11	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ala^{A2}]insulin	0.46 ± 0.07 (4)	0.61
12	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Abu^{A2}]insulin	2.7 ± 0.5 (4)	3.6
13	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Nva^{A2}]insulin	2.8 ± 0.5 (3)	3.7
14	N$^{\alpha}$-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Nle^{A2}]insulin	0.75 ± 0.11 (3)	0.99
15	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Gly^{A3}]insulin	0.065 ± 0.014 (4)	0.086
16	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ala^{A3}]insulin	1.4 ± 0.2 (4)	1.8
17	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Abu^{A3}]insulin	9.8 ± 0.9 (3)	13
18	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Nva^{A3}]insulin	1.1 ± 0.1 (3)	1.4
19	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Nle^{A3}]insulin	0.29 ± 0.04 (4)	0.38
20	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Val^{A2}]insulin	29 ± 2 (3)	38
21	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ail^{A2}]insulin	2.6 ± 0.1 (3)	3.4
22	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Tle^{A2}]insulin	1.6 ± 0.2 (3)	2.1
23	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Thr^{A2}]insulin	0.073 ± 0.004 (2)	0.096
24	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ile^{A3}]insulin	8.6 ± 1.4 (4)	11
25	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ail^{A3}]insulin	14 ± 1 (4)	18
26	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Tle^{A3}]insulin	27 ± 3 (4)	36
27	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Thr^{A3}]insulin	10 ± 1 (2)	13
28	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Val^{A2},Ile^{A3}]insulin	1.6 ± 0.1 (2)	2.1
29	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Abu^{A2},Abu^{A3}]insulin	0.31 ± 0.09 (3)	0.41
30	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Gly^{A2},Gly^{A3}]insulin	0.011 ± 0.002 (2)	0.014
31	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Leu^{A2}]insulin	3.4 ± 0.7 (4)	4.5
32	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Leu^{A3}]insulin	0.14 ± 0.01 (4)	0.18
33	N^{α}-Phe^{B1}-acetyl-despentapeptide-(B26–B30)-[Leu^{A3},Phe^{B25}-α-carboxamide]insulin	0.24 ± 0.00 (2)	0.32
34	N^{α}-Phe^{B1}-acetyl-desoctapeptide-(B23–B30)-[Leu^{A3}]insulin	0.0054 ± 0.0006 (3)	0.0071
35	N^{α}-Phe^{B1}-acetyl-desoctapeptide-(B23–B30)-[Ala^{A2}]insulin	0.0026 ± 0.0003 (2)	0.0034
36	N^{α}-Phe^{B1}-acetyl-desoctapeptide-(B23–B30)-[Ala^{A3}]insulin	0.0038 ± 0.0001 (2)	0.0050

[a] The analogues prepared for this study and their respective receptor-binding potencies (relative both to the potency of porcine insulin and to the potency of N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-modified porcine insulin) are identified above. Details of the semisynthetic methods and cell incubation conditions are provided under Materials and Methods. Relative receptor-binding potency is defined as ((concentration of porcine insulin causing half-maximal inhibition of binding of [[^{125}I]iodo-Tyr^{A14}]insulin to receptor)/(concentration of analogue causing half-maximal inhibition of binding of [[^{125}I]iodo-Tyr^{A14}]insulin to receptor)) × 100. Each value represents the mean ± SD of multiple determinations; the number of separate determinations is shown in parentheses. The concentration of insulin causing half-maximal inhibition of radiolabeled insulin binding was 0.64 ± 0.06 nM (n = 16). Since ≤10% of the 20 pM radiolabeled hormone became cell associated in the experiments reported (even in the absence of competitor), the data were not significantly affected by variations in ligand concentrations caused by receptor binding. The relative binding potencies reported in the table can therefore be considered under most circumstances to reflect relative binding affinities.

findings parallel previous work identifying the importance of Gly^{A1} in directing the affinity of insulin–receptor interactions (Brandenburg et al., 1975). On the other, they delineate the cumulative effect of deletions within the region. A greater understanding of the importance of these deletions arises from consideration of the corresponding acetylated derivatives, as documented in Figure 1B and Table I. That is, whereas acetylation of Gly^{A1} in bisacetyl-insulin (to achieve tris-acetyl-insulin, peptide 6) causes a small decrement in receptor-binding potency, acetylation of Ile^{A2} in des-Gly^{A1}-bisacetyl insulin (to achieve peptide 7) actually causes a 34-fold increase in the potency of the truncated analogue. Both of these findings have been reported before (Zahn et al., 1972; Katsoyannis & Zalut, 1972). The acetylation of the desdipeptide and destripeptide insulins (peptides 4 and 5) to give peptides 8 and 9, however, has little effect on the decreased receptor-binding potency that attends amino acid deletions from the amino terminus of the insulin A chain. Taken together, the results of Figure 1 identify (a) the small, but significant, importance of the α-amino group of Gly^{A1} per se in achieving a high affinity state of insulin–receptor interactions, (b) the very detrimental effects that result from removing structural elements (either Gly or the acetyl group) that engage the α-amino group of Ile^{A2} in amide bond, and (c) the additional negative effects that arise from further deletions involving the removal of Ile^{A2} or of Ile^{A2} plus Val^{A3} (whether or not the newly formed amino group is masked by acetylation). The separate importance of the Xxx–Ile^{A2} amide bond and of the Ile^{A2} side chain itself are consistent with the stabilizing hydrogen bonds and van der Waals interactions that have been described for this region of the molecule as the result of crystallographic analysis (Blundell et al., 1972; Baker et al., 1988). The very low receptor-binding potencies of the des-Gly^{A1},Ile^{A2},Val^{A3} insulin analogue and its acetylated counterpart (peptides 5 and 9, respectively; about 0.1% of the potency of insulin) are little different from the receptor-binding potency of desoctapeptide-(B23–B30)-insulin (Kikuchi et al., 1980; Riemen et al., 1983; Nakagawa & Tager, 1986). They identify the region A1–A3 [like the region B23–B25; cf. Riemen et al. (1983) and Nakagawa and Tager (1986)] as a tripeptide domain of particular importance in insulin–receptor interactions.

Our first attempts to investigate the importance of side-chain structure at positions A2 and A3 of insulin relied on the

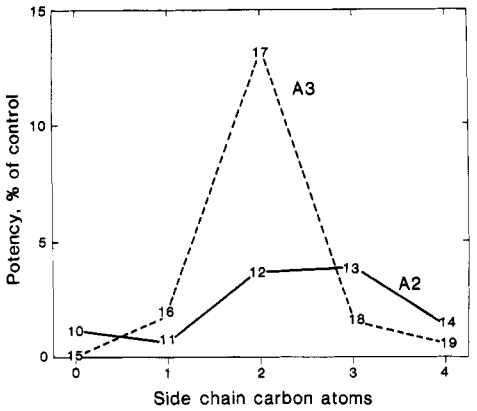

FIGURE 2: Receptor binding potencies of insulin analogues with unbranched amino acid replacements at position A2 or A3 as a function of side-chain length. Data were collected from experiments taking the course described in the legend to Figure 1 and under Materials and Methods. Receptor binding potencies relative to the potency of N^{α}-Gly^{A1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin are plotted against the number of carbon atoms distal to C^{α} for analogues bearing replacements at position A2 (—) or position A3 (---). Quantitative information is provided in Table I; identifying numbers in Table I, rather than symbols, are used to indicate the peptide under consideration. 10, 11, 12, 13, and 14, potency of analogues of N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin in which Ile^{A2} has been replaced by Gly, Ala, Abu, Nva, and Nle, respectively. 15, 16, 17, 18, and 19, potency of analogues of N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin in which Val^{A3} has been replaced by Gly, Ala, Abu, Nva, and Nle, respectively. See Table I and text for further details.

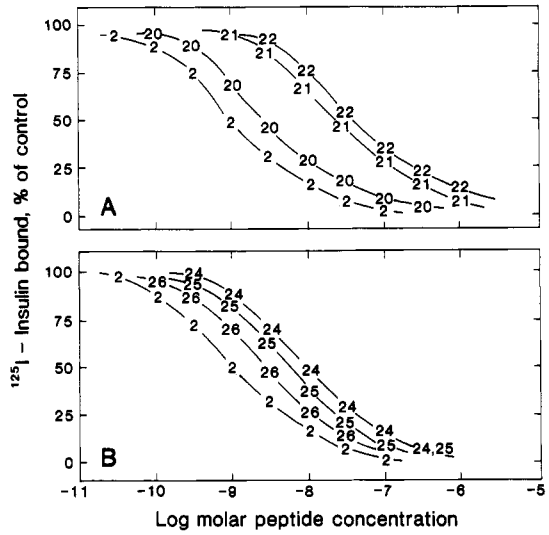

FIGURE 3: Inhibition of binding of ^{125}I-labeled insulin to isolated canine hepatocytes by insulin analogues bearing β-branched amino acid replacements at position A2 or A3. Incubations were performed as described under Materials and Methods. Control binding is defined as the amount of radiolabeled ligand which became cell associated in the absence of competitor; all data have been corrected for so-called nonspecific binding that was detected in the presence of 10 μM insulin. Quantitative information is provided in Table I; identifying numbers in Table I, rather than symbols, are used to indicate the peptide under consideration. Panel A: 2, inhibition of binding by N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin; 20, 21, and 22, inhibition by analogues of peptide 2 in which Ile^{A2} has been replaced by Val, Ail, and Tle, respectively. Panel B: 2, inhibition of binding by N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin; 24, 25, and 26, inhibition by analogues of peptide 2 in which Val^{A3} has been replaced by Ile, Ail, and Tle, respectively. See Table I and text for further details.

semisynthesis of analogues containing Gly or unbranched aliphatic residues (Ala, Abu, Nva, and Nle) at each of these sites [cf. Okada et al., (1981), Kitagawa et al., (1984), and Kobayashi et al. (1986) for previous related studies]. The results of corresponding receptor-binding studies are presented in Figure 2 and Table I (peptides 10–19). The receptor-binding potencies of analogues containing replacements of Ile^{A2} fall into a group and range from 0.6 to 3.7% of the potency of the parent bisacetyl-insulin. Notwithstanding a slight preference for a side chain with two or three carbon atoms (corresponding to Abu and Nva, respectively), the receptor-binding potencies of analogues in this series cannot be easily correlated with respect to the existence or length of side chain in a defined way. In contrast, Figure 2 and Table I identify for analogues containing the same replacements at position A3 a defined preference for Abu, a residue containing a side chain with two carbon atoms. The results of Figure 2 suggest separate mechanisms for the decreased receptor-binding potency attending replacement of Ile^{A2} or Val^{A3}. For position A2, it seems that the loss per se of the β-branched amino acid precludes the formation of a high-affinity state of ligand–receptor interaction. For position A3, it seems that the necessary structural locus can be filled in significant part by a residue (Abu) with appropriate length.

Further studies addressed the specific importance of a β-branch in the side chains of residues placed at position A2 or A3. Figure 3A and Table I identify that, whereas Val (a residue that can be considered to be isosteric with Ile) is well tolerated at position A2 (the resulting analogue exhibiting a receptor-binding potency of 38% relative to the parent compound), other β-branched residues (including Ail, Tle, and Thr) are not. While the very low receptor-binding potency of the Thr-containing analogue (about 0.1% of that of the parent, Table I) might be expected (since Ile^{A2} normally occurs in a hydrophobic region of the insulin monomer, see introduction), the low potencies of the Ail- and Tle-containing analogues (2–3% relative to the parent compound) identify the profound importance of the disposition of β-branched mass, or of its implications in overall secondary structure, in determining the receptor-binding potencies of relevant analogues. These results contrast sharply with those obtained for analogues having identical amino acid replacements at position A3, as identified in Figure 3B and Table I. Three particular findings should be noted. First, notwithstanding that Val appears to serve best at position A3 (as in natural insulin), the bisacetyl derivatives of [Ile^{A3}]insulin and [Ail^{A3}]insulin (peptides 24 and 25, respectively) exhibit 11–18% of the receptor-binding potency of the parent compound. Second, Tle is surprisingly well tolerated at position A3, with the corresponding analogue (peptide 26) exhibiting 36% of the receptor-binding potency of the derivatized insulin bearing Val at this position and 17-fold higher potency than the analogue containing Tle at position A2 (peptide 22). Third, the analogue with Thr at position A3 (peptide 27) exhibits a relative potency (13%) slightly higher than that of the analogue with Ile at the same site and a full 130-fold higher than the analogue containing Thr at position A2 (peptide 23). These results identify important differences that apply to the effects of replacing Ile^{A2} and Val^{A3} by other β-branched amino acid residues. Most notably, the site represented by residue A2 seems to require a hydrophobic, β-branched side chain with well-defined geometry, whereas the site represented by residue A3 exhibits significant flexibility in tolerating to similar degrees residues with β-branched side chains having altered geometries, the *tert*-butyl group, or even polar constituents.

Experiments addressing the independence or interdependence of amino acid replacements at positions A2 and A3 were undertaken by constructing analogues in which both residues were replaced within a single insulin analogue. As documented in Table I, the analogue containing an inversion of the sequence

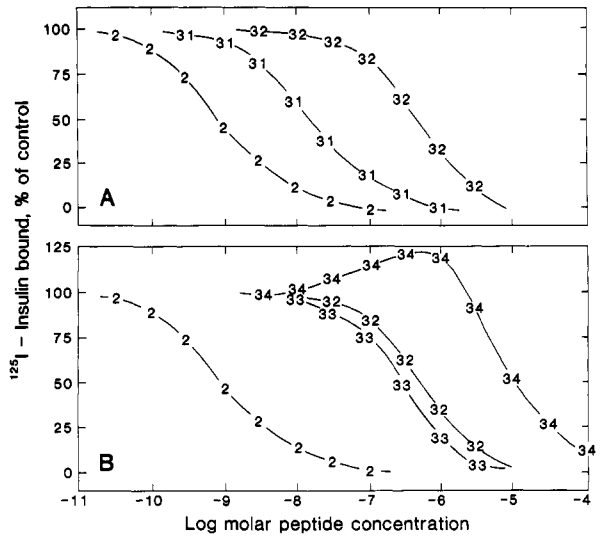

FIGURE 4: Inhibition of binding of ^{125}I-labeled insulin to isolated canine hepatocytes by insulin analogues bearing replacements by Leu at position A2 or A3. Incubations were performed as described under Materials and Methods. Control binding is defined as the amount of radiolabeled ligand which became cell associated in the absence of competitor; all data have been corrected for so-called nonspecific binding that was detected in the presence of 10 μM insulin. Quantitative information is provided in Table I; identifying numbers in Table I, rather than symbols, are used to indicate the peptide under consideration. Panel A: 2, inhibition by N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin; 31, inhibition by the analogue of peptide 2 in which Ile^{A2} has been replaced by Leu; 32, inhibition by the analogue of peptide 2 in which Val^{A3} has been replaced by Leu. Panel B: 2, inhibition by N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin; 32, inhibition by the analogue of peptide 2 in which Val^{A3} has been replaced by Leu; 33, inhibition by the analogue of peptide 2 in which Val^{A3} has been replaced by Leu, Phe^{B25} has been replaced by Phe-α-carboxamide, and residues B26–B30 have been deleted; 34, inhibition by the analogue of peptide 2 in which Val^{A3} has been replaced by Leu and residues B23–B30 have been deleted. See Table I and text for further details.

Ile^{A2}-Val^{A3} to Val^{A2}-Ile^{A3} (peptide 28) exhibits about 2% of the receptor-binding potency of its bisacetyl counterpart having the natural sequence. Notably, this value is close to the product of the effect of replacing Ile^{A2} by Val (peptide 20, 38% of control binding) and of replacing Val^{A3} by Ile (peptide 24, 11% of control binding). Other related examples include the simultaneous replacement of residues A2 and A3 by Abu (peptide 29) or by Gly (peptide 30). In all cases, replacement of both residues results in markedly, and cumulatively, decreased potency. These findings suggest that residues A2 and A3 act independently (or nearly so) in the attainment of a high-affinity state of insulin–receptor interactions. The exceeding low receptor binding potency of the Gly^{A2},Gly^{A3} bisacetyl-insulin analogue (a potency nearly 10 times lower than that found for desoctapeptide-(B23–B30)-insulin; Kikuchi et al., 1980; Riemen et al., 1983; Nakagawa & Tager, 1986) further indicates the combined importance of both side-chain and main-chain conformation in the acquisition of structure compatible with high-affinity ligand interactions with the insulin receptor.

As a final test of the importance of aliphatic side-chain structure at positions A2 and A3 in the affinity of the insulin receptor for hormone, we constructed analogues bearing Leu in place of Ile^{A2} or Val^{A3} (the latter yielding an analogue corresponding to the natural insulin variant Insulin Wakayama; see introduction). Figure 4A and Table I identify that replacement of Ile^{A2} by Leu (to yield peptide 31, an analogue with 4.5% of control receptor-binding potency) is as easily (or as poorly) tolerated as replacement of the same residue by Abu and Nva. At the same time, Figure 4A and Table I identify that replacement of Val^{A3} by Leu (to yield peptide 32, an analogue corresponding to Insulin Wakayama and one exhibiting 0.18% of control receptor binding) results in an analogue with a relative receptor-binding potency well below the value obtained for any analogue containing a straight-chain or β-branched residue at the same position (peptides 16–19 and 24–27 in Table I). This finding identifies the replacement of Val^{A3} by Leu as being of special significance and suggests that the γ-branch of Leu (as opposed to the β-branch of Ile or Ail or even the straight chain of Nle) results among these amino acid isomers (a) in an analogue with ineffectively distributed side-chain mass at position A3 and (b) in an analogue with a correspondingly reduced receptor-binding potency.

Since, as noted in the introduction, the NH_2-terminal residues of the insulin A chain are closely disposed to the COOH-terminal residues of the insulin B chain [a region that has already been suggested to participate in insulin–receptor interactions through conformational adjustments in the peptide backbone; cf. Nakagawa and Tager (1986, 1987) and Mirmira and Tager (1989)], we questioned whether the effects of replacing Val^{A3} by Leu might have their origin in altered structural relationships between the NH_2-terminal domain of the insulin A chain and the COOH-terminal domain of the insulin B chain. Figure 4B and Table I show, however, that this is not the case. That is, (a) the relative receptor-binding potency of the Leu^{A3}-containing full insulin analogue (peptide 32) is little different from that of the corresponding analogue in which the COOH-terminal five residues of the B chain have been deleted (peptide 33), (b) the effect of deleting the COOH-terminal octapeptide of the insulin B chain combines with the effect of replacing Val^{A3} by Leu to yield an analogue with exceedingly low receptor-binding potency (peptide 34), and (c) the effect of deleting the COOH-terminal octapeptide of the insulin B chain combines with the effect of replacing Ile^{A2} or Val^{A3} by Ala (peptide 35 or 36, respectively) to yield analogues with receptor-binding potencies lower than those applicable to either modification alone by more than an order of magnitude. Taken together, the results of Figures 1–4 identify that residues A2, residue A3, and residues associated with the COOH-terminal domain of the insulin B chain play separate roles in determining the affinity of the hepatocyte insulin receptor for ligand.

Spectroscopic Studies of Insulin Structure. We next applied far-UV CD spectroscopy to selected analogues to consider how the amino acid replacements described above might have affected secondary structure in the important NH_2-terminal domain of the insulin A chain. Panels a and b of Figure 5 show, as a control, results obtained for desoctapeptide-(B23–B30)-insulin. Whereas this analogue exhibits a significantly perturbed CD spectrum in phosphate buffer, in the presence of trifluoroethanol (a solvent that has been proposed to enhance intramolecular hydrogen bonding and α-helical structure; Nelson & Kallenbach, 1986, 1989) the CD spectra of desoctapeptide-insulin and insulin are indistinguishable. These data suggest that the truncated analogue, notwithstanding its low biological potency, retains the full ability of insulin to undertake solvent-enhanced helical conformations applicable to the A and B chain domains of the hormone. In contrast, panels c and d of Figure 5 show much smaller changes between the CD spectra of destripeptide-(A1–A3)-bisacetyl-insulin and bisacetyl-insulin in buffer but a signficant divergence between the two spectra in the presence of trifluoroethanol. We interpret these results as suggesting that (a) deletion of the first three residues of the insulin A chain (residues that can be

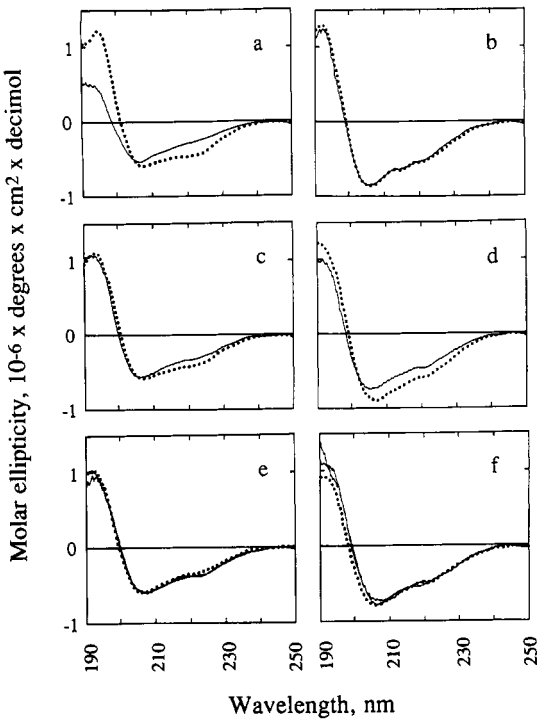

FIGURE 5: Far-UV CD spectra of insulin and selected insulin analogues in aqueous solution and in the presence of trifluoroethanol. CD spectra were recorded for analogues dissolved in 10 mM KH_2PO_4 brought to pH 7.4 with NaOH (90 μg/mL peptide) or in a mixture of this buffer and trifluoroethanol in the ratio 60:40 (54 μg/mL peptide). Further details are provided under Materials and Methods. Quantitative data on these and other analogues at 208 and 222 nm are provided in Table II. Panels a and b: insulin (peptide 1, ···) and desoctapeptide-(B23–B30)-insulin (—) in the absence and presence of trifluoroethanol, respectively. Panels c and d: N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin (peptide 2, ···) and peptide 2 lacking the NH_2-terminal three residues of the A chain (Gly^{A1}, Ile^{A2}, and Val^{A3}; peptide 5, —) in the absence and presence of trifluoroethanol, respectively. Panels e and f: N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2},Val^{A3}-insulin (peptide 5, ···)and N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin (peptide 2) substituted with Gly at position A2 or A3 (peptides 10 and 15, both —) in the absence and presence of trifluoroethanol, respectively.

expected to participate in α-helix formation; see introduction) limits the overall extent to which helical structure can be induced in the insulin molecule, (b) the lesser molar ellipticity recorded for the shortened analogue in the presence of trifluoroethanol, relative to that recorded for the control peptide (see Table II), arises from the loss of a potential helix-forming region near the NH_2 terminus of the A chain, and (c) as judged by the smaller differences in molar ellipticity observed for these two peptides in buffer alone relative to those observed in buffer plus trifluoroethanol (Figure 5, panels c and d, and Table II), the applicable region undertakes an identifiable, but still not fully helical, conformation in simple aqueous solution. These conclusions are augmented by considering corresponding data obtained for analogues containing replacements of Ile^{A2} or Val^{A3} by Gly (peptide 10 or 15, respectively). Figure 5, panels e and f, and Table II identify for both of these analogues (in buffer alone or in buffer plus trifluoroethanol) CD spectra nearly identical to the respective spectra observed for the destripeptide-(A1–A3)-insulin derivative described above. Thus, the introduction of the helix-destabilizing residue in full-length insulin analogues has essentially the same effect as deleting the entire region in question.

The application of methods similar to those described above to analogues containing amino acid replacements at position 2 or 3 of the insulin A chain are addressed by reference to the

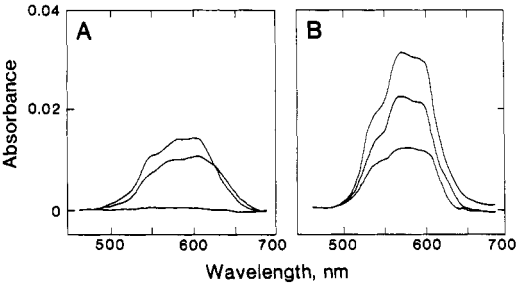

FIGURE 6: Spectroscopic studies of hexamer function by selected insulin analogues. Panel A: visible spectra obtained as the result of incubating 0.17 mM peptides with 0.06 mM $CoCl_2$ and 50 mM phenol (all final concentrations) in 50 mM Tris buffer at pH 8.0. Panel B: visible spectra obtained as a result of adding NaSCN (800 mM final concentration) to the solutions analyzed in panel A. For both panels, the uppermost curve corresponds to peptide 32 (N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Leu^{A3}]insulin), the middle curve corresponds to peptide 15 (N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Gly^{A3}]insulin), and the lowermost curve corresponds to peptide 5 (N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2},Val^{A3}-insulin). It should be noted that the spectra corresponding to the Leu^{A3}-substituted analogue do not differ from those obtained for the parent compound (peptide 2, N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin). See Materials and Methods and text for further details.

quantitative data provided in Table II. Four findings should be noted. First, amino acid replacements at position A2 tend to have considerably less effect on the molar ellipticities of the corresponding analogues at 208 and 222 nm (regions of CD spectra considered to reflect α-helical content; Johnson, 1988) than do equivalent substitutions at position A3. Second, the degree to which the CD spectra of these analogues are perturbed relative to the control peptide cannot be correlated in any simple way with the relative receptor-binding potencies of the corresponding insulin derivatives. Third, the analogue containing Leu in place of Val^{A3} exhibits CD spectral parameters close to those of the control, notwithstanding that it possesses only 0.2% of the receptor-binding potency of bisacetyl-insulin. Fourth, the analogue containing Tle in place of Val^{A3} exhibits CD spectral parameters more similar to those applicable to the Gly^{A3} derivative than to the control, notwithstanding that it possesses a 400-fold greater receptor-binding potency than its glycine-substituted counterpart. On the one hand, these data suggest a complex interplay between secondary and local structure and the fit of insulin with its receptor. On the other, they identify that the inability of an insulin analogue containing an amino acid replacement in the NH_2-terminal domain of the A chain to undertake a nativelike structure in simple aqueous solution, or in solutions containing the structure-modifying solvent trifluoroethanol, does not preclude effective insulin–receptor interactions.

Additional studies addressed the importance of the NH_2-terminal domain of the insulin A chain with respect to the propensity of insulin to form metal ion coordinated hexamers in solution and to undergo the so-called T → R structural transition. As has been well documented, octahedrally coordinated insulin$_6$(Co^{2+})$_2$ complexes in the T state undergo a conformational adjustment in the presence of phenol in which (a) the ligand field changes to tetrahedral geometry (resulting in an intense absorbance between 500 and 650 nm) and (b) the NH_2-terminal domain of the insulin B chain undertakes an α-helical rather than an extended conformation (the R state) (Roy et al., 1989; Thomas & Wollmer 1989). The subsequent addition of thiocyanate or other lyotropic anions stabilizes the tetrahedrally coordinated R state and results in increased optical absorbance in the same region. Figure 6 demonstrates that bisacetyl-insulin (like insulin itself) readily undergoes the T → R transition, yielding the spectroscopic

Table II: CD Spectral Data of Insulin and Selected Insulin Analogues[a]

identifying no.	peptide	molar ellipticity (10^{-6} deg cm^2 dmol^{-1}) buffer 208 nm	buffer 222 nm	buffer + trifluoroethanol 208 nm	buffer + trifluoroethanol 222 nm
1	insulin	−0.639	−0.511	−0.916	−0.587
	desoctapeptide-(B23–B30)-insulin	−0.569	−0.313	−0.905	−0.573
2	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-insulin	−0.623	−0.441	−0.905	−0.580
5	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-des-Gly^{A1},Ile^{A2},Val^{A3}-insulin	−0.617	−0.366	−0.776	−0.485
10	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Gly^{A2}]insulin	−0.596	−0.387	−0.763	−0.482
11	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ala^{A2}]insulin	−0.653	−0.399	−0.876	−0.545
12	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Abu^{A2}]insulin	−0.621	−0.396	−0.852	−0.536
14	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Nle^{A2}]insulin	−0.637	−0.390	−0.833	−0.535
22	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Tle^{A2}]insulin	−0.601	−0.375	−0.828	−0.544
31	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Leu^{A2}]insulin	−0.640	−0.409	−0.830	−0.529
15	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Gly^{A3}]insulin	−0.587	−0.371	−0.736	−0.484
16	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Ala^{A3}]insulin	−0.572	−0.375	−0.788	−0.519
17	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Abu^{A3}]insulin	−0.604	−0.390	−0.759	−0.501
19	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Nle^{A3}]insulin	−0.565	−0.352	−0.769	−0.513
26	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Tle^{A3}]insulin	−0.587	−0.383	−0.777	−0.513
32	N^{α}-Phe^{B1},N^{ϵ}-Lys^{B29}-bisacetyl-[Leu^{A3}]insulin	−0.629	−0.401	−0.855	−0.565

[a] CD spectra were recorded for insulin and selected analogues dissolved in (a) 10 mM KH_2PO_4 brought to pH 7.4 by the addition of NaOH (90 μg/mL peptide) or (b) a mixture of this buffer and trifluoroethanol in the ratio 60:40 (54 μg/mL peptide). Further analytical details are provided under Materials and Methods. The table provides quantitative information obtained at both 208 and 222 nm. The full far-UV CD spectra of some of these analogues are shown in Figure 5. Data are reported in terms of molar ellipticity (rather than mean residue ellipticity) to facilitate the comparison of both full-length and truncated analogues.

signature typical of the ligand field adjustment. [Gly^{A3}]bisacetyl-insulin (an analogue with a significantly perturbed CD spectrum, see earlier) also undergoes the transition but yields a hexameric complex with optical absorbance slightly less than that applicable to the control peptide; related findings have been interpreted in terms of an altered equilibrium for complex formation and the formation of a less stable metal-liganded hexamer (Nakagawa & Tager, 1991). An extreme example is reached in destripeptide-(A1–A3)-insulin. This truncated analogue apparently exhibits no propensity to undergo the T → R transition in the presence of phenol alone but exhibits the characteristic visible spectrum (at low absorbance) when the tetrahedrally liganded hexamer is stabilized by the addition of thiocyanate. These results identify that (a) the phenol binding cavity (a cavity which is formed from the packing of residues B1–B8 against the A chain of another molecule within the insulin hexamer and which is stabilized by hydrogen bonds between the phenolic hydroxyl group and both the A6 carbonyl oxygen and the A11 amide nitrogen; Derewenda et al., 1989) is only marginally perturbed in a low-potency full-length analogue and (b) related conformational adjustments are not prevented by replacements or even deletions at the relevant site. Importantly, the analogue resulting from the replacement of Val^{A3} by Leu (one related to Insulin Wakayama) retains an ability to form tetrahedrally coordinated insulin$_6$(Co^{2+})$_2$ complexes no different from that found for insulin itself (data not shown).

Discussion

Our use of semisynthetic insulin analogues with deletions or replacements in the NH_2-terminal domain of the A chain has permitted a detailed analysis of the importance of local side-chain structure in contributing to the affinity of insulin–receptor interactions. Importantly, previous work (involving chemical modifications or the synthesis of selected analogues) has already identified the NH_2-terminal A-chain domain as lying within the receptor-binding region of the hormone (Blundell et al., 1972; DeMeyts et al., 1978; Baker et al., 1988). As noted in the introduction, (a) the NH_2-terminal domain of the insulin A chain undertakes an α-helical conformation as determined by both crystallography and NMR, (b) this α-helical segment can be seen to move somewhat among different crystallographic forms of the hormone, (c) the peptide carbonyl of Gly^{A1} is engaged in multiple hydrogen-bonding interactions, (d) the side chain of Ile^{A2} is found to make van der Waals contacts with the side chain of Tyr^{A19} within the hydrophobic core of insulin monomer, and (e) the side chain of Val^{A3}, while it is not in contact with solvent, is directed toward the surface of the molecule (Blundell et al., 1972; Baker et al., 1988). Our analysis of analogues bearing deletions or substitutions within this region identifies the amino group of Gly^{A1}, the Gly^{A1}–Ile^{A2} peptide bond, and the side chains of both Ile^{A2} and Val^{A3} as contributing structural elements important in conferring affinity to insulin–receptor interactions. Although the very great loss in potency attending deletion of Gly^{A1} from Phe^{B1},Lys^{B29}-bisacetyl-insulin (to yield an analogue with only 0.6% of the receptor-binding potency of the parent peptide) might suggest that Gly^{A1} or the Gly^{A1}–Ile^{A2} peptide bond is of overriding importance in this respect, additional results suggest otherwise. That is, given the low receptor-binding potencies of analogues bearing replacements of Ile^{A2}, Val^{A3}, or both Ile^{A2} and Val^{A3} by Gly (peptides 10, 15, and 30; about 1, 0.1, and 0.01% relative potency, respectively), it would appear that perturbations of the Gly^{A1}–Ile^{A2} peptide bond might destabilize insulin structure in the truncated analogue to the extent that the usual roles of the Ile^{A2} and Val^{A3} side chains cannot be fulfilled. Accordingly, the NH_2-terminal domain of the insulin A chain might best be viewed, in part, as comprising a network of structural elements that cooperatively determine the potential for each to have effect.

Our findings with respect to the importance of side-chain structure at positions 2 and 3 of the insulin A chain are summarized in Figure 7, a figure which considers structural change in terms of the progressive addition of methylene groups to glycine to achieve residues with straight, β-branched, or γ-branched side chains. The scheme illustrates that replacement of Ile^{A2} by Val (a residue occasionally found at position A2 in nature) causes only a small decline in receptor-binding potency, whereas other replacements having alternatively configured side chains yield analogues with only 1–5% of the potency of insulin. It thus appears that high-affinity insu-

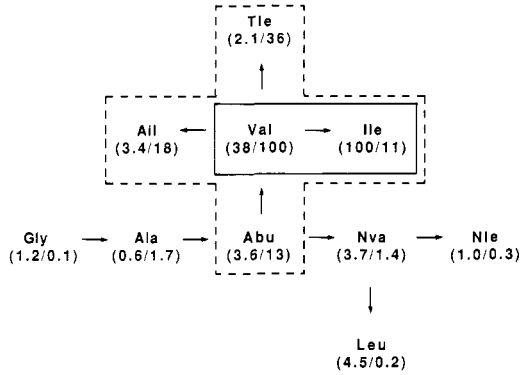

FIGURE 7: Scheme for considering the relative receptor-binding potencies of insulin analogues bearing replacements at position A2 or A3 with respect to side-chain structure. The scheme considers the progressive addition of methylene groups (a) to the structure of Gly to achieve a series of stepwise elongated, straight-chain aliphatic residues, (b) to the structure of Abu to achieve a series of β-branched, aliphatic residues with stepwise increases in bulk, and (c) to the structure of Nva to achieve Leu. Numbers appearing in parentheses under each residue indicate the potencies of analogues bearing the residue at position A2 (left) or position A3 (right), both relative to N^{α}-Phe^{B1}-N^{ϵ}-Lys^{B29}-bisacetyl-insulin. The residues boxed in a solid line indicate analogues bearing acceptable (relative potency $\geq$ 10%) amino acid replacements at position A2. Those boxed in a dashed line indicate analogues bearing acceptable (relative potency $\geq$ 10%) amino acid replacements at position A3. See Table I and text for further details.

lin–receptor interactions preclude significant variation in side-chain structure at position A2. The low receptor-binding potency of the analogue containing Ail at position A2 was unexpected and emphasizes the critical importance of the placement of side-chain mass in maintaining effective ligand structure. Given the relatively high potency of the Val^{A2}-containing analogue (38% relative to the parent peptide having Ile at that position), this result suggests that the elongation of the Val side chain in the wrong direction (by even a single methylene group) to achieve Ail, rather than the shortening of the ethyl group of the Ile side chain in the usual direction, induces an unacceptable local peptide conformation. The close packing described for the side chains of Ile^{A2} and Tyr^{A19} both in the Zn insulin hexamer by use of crystallographic methods (Blundell et al., 1972; Baker et al., 1988) and in the insulin monomer by use of NMR methods (Weiss et al., 1989, 1991; Hua & Weiss, 1990, 1991) thus appears to apply as well to insulin when it is bound to its receptor. The narrow grouping of the receptor-binding potencies of analogues exhibiting inappropriate distribution of side-chain mass at position A2 (see above) further suggests that the importance of the side chain of Ile^{A2} arises from its role in orienting the NH_2-terminal domain of the insulin A chain relative to the rest of the molecule. The extent to which the Ile^{A2} side chain might actually make contact with the receptor remains unclear, however.

As identified in Figure 7, the importance of side-chain structure at residue Val^{A3} seems to differ in several ways from that at residue Ile^{A2}. First, the exchange of Val^{A3} by Ile is not as well accommodated as that of Ile^{A2} by Val. Second, the side chain of Ail is as easily accepted at position A3 as that of Ile, whereas it is not at position A2. Third, a discrete preference is observed within the series of straight-chain aliphatic amino acids for replacement of Val^{A3} by Abu (a residue which could easily fit the space taken by Val), whereas none is seen for replacement of Ile^{A2}. Fourth, Tle is well accepted at position A3, whereas it is not at position A2. Taken together, these results identify significantly greater flexibility in the acceptance of alternatively configured side-chain mass at position A3 relative to that which applies to position A2. Given (a) the very different propensities of acceptable residues at position A3 to direct the formation of α-helix [Abu > Ile > Val $\gg$ Tle; cf. Lyu et al. (1991) and Padmanabhan and Baldwin (1991)], (b) the much higher receptor-binding potencies of analogues with replacements of Val^{A3} by Abu, Ile, Ail, or Tle relative to those with replacements of Val^{A3}, for example, by Nva or Gly [residues with the propensity for forming or for disordering α-helix, respectively; cf. Lyu et al. (1991) and Padmanabhan and Baldwin (1991)], and (c) the orientation of the Val^{A3} side chain toward the surface of the insulin monomer, it appears that the position A3 side chain participates in conferring affinity to insulin–receptor interactions by means of direct receptor contact rather than by means of main-chain orientation. In the same way that Nva or Nle could not possibly fit a receptor site designed for Val, Leu at position A3 [the residue applicable to the natural mutant insulin Insulin Wakayama; cf. Shoelson et al. (1983) and Nanjo et al. (1986)] would find great difficulty (due to its γ-branch) in fitting a site designed for a residue with a branch one carbon atom closer to the peptide backbone.

The cause of the greatly decreased potency of Insulin Wakayama thus appears to arise in large part simply from a bad fit of residue Leu^{A3} with receptor. Whereas a mutant human insulin with replacement of Ile^{A2} by Leu would undoubtedly result in a phenotype little different from that which applies to subjects expressing Insulin Wakayama (since endogenously produced insulin could not compensate for the diminished potency of the mutant hormone), replacement of Ile^{A2} by Leu would result in an analogue with more than 20-fold higher affinity than that which results by replacement of Val^{A3} by the same residue. The specific importance of Leu thus differs for its effects at position A2 (where it apparently disrupts main-chain orientation), at position A3 (where it apparently affects side-chain contact with receptor), and at position B6 (where, in normal insulin, it apparently participates by way of inducing general hydrophobic interaction; Nakagawa & Tager, 1991).

The separate roles of aliphatic side-chain structure at positions A2 and A3 are emphasized by results obtained through simultaneous replacements of both residues. That is, (a) reversal of the usual Ile^{A2}-Val^{A3} sequence yields an analogue with receptor-binding potency very much lower than that which applies to the singly substituted analogues and (b) simultaneous replacement of Ile^{A2} and Val^{A3} by Abu or by Gly yields analogues for which receptor-binding potency is greatly reduced relative to corresponding analogues in which only one of these residues is replaced. An additional cumulative effect occurs in analogues substituted at position A2 or A3 upon deletion of the COOH-terminal octapeptide of the insulin B chain (a region that has also been placed within the receptor-binding domain of the insulin molecule and that lies in apposition to the NH_2-terminal domain of the insulin A chain and to the hydrophobic core of insulin in most structures of the hormone; Blundell et al., 1972; DeMeyts et al., 1978; Baker et al., 1988; Derewenda et al., 1990). Notwithstanding these cumulative effects (see Table I), changes in the free energies of interaction of the doubly substituted analogues (peptides 28–30) and the truncated, substituted analogues (peptides 34–36), relative to the sum of free energies of the singly modified analogues within each pair, range from 0.1 to −2.2 kcal/mol (for [Abu^{A2},Abu^{A3}]insulin and the desoctapeptide counterpart of [Leu^{A3}]insulin, respectively). Thus, our results suggest that these three domains of the insulin molecule function in quite different but perhaps not totally independent

ways in determining the affinity of insulin–receptor interactions.

The importance of conformational change has recently become a focus for considering the mechanisms by which insulin and its receptor combine to achieve an effective ligand–receptor complex. That is, (a) conformational adjustments in the COOH-terminal domain of the insulin B chain have been proposed to occur during high-affinity interactions of insulin with receptor (Nakagawa & Tager, 1986, 1987; Mirmira & Tager, 1989; Derewenda et al., 1990; Hua et al., 1991), (b) such adjustments would alter the structural relationships between the COOH-terminal B-chain domain and the NH_2-terminal A-chain domain of the hormone, (c) residues A2 and A3 would become significantly more solvent exposed in the full-potency insulin analogue despentapeptide-(B26–B30)-[Phe^{B25}-α-carboxamide]insulin (Fisher et al., 1985; Nakagawa & Tager, 1986; Derewenda et al., 1990), (d) the solution structure of [Gly^{B24}]insulin, a high-potency analogue (Mirmira & Tager, 1989), has been shown by NMR methods to represent an unfolded molecule in which the COOH-terminal B-chain domain is displaced to expose both the hydrophobic core of the insulin molecule and the side chains of residues A2 and A3 (Hua et al., 1991), and (e) an insulin analogue in which the carboxyl group of Lys^{B29} is linked to the amino group of Gly^{A1} by peptide bond (an analogue with structure isomorphic to that of insulin itself and with little possibility for conformational change) exhibits an unmeasurably low potency for interaction with the insulin receptor (Markussen et al., 1985), notwithstanding that additional insulin analogues cross-linked between residues Gly^{A1} and Lys^{B29} exhibit receptor-binding potencies about 10% of those exhibited by their un-cross-linked counterparts (Brandenburg et al., 1972, 1973; Nakagawa & Tager, 1989). One of these analogues (containing the diaminosuberoyl group) has also been demonstrated to be crystallographically isomorphous with insulin (Cutfield et al., 1981).

Two aspects of our current studies directly apply to considerations of conformational adjustment in the insulin molecule. First, whereas insulin substituted with Thr at position A3 exhibits 13% of the receptor-binding potency of the parent peptide (in keeping with the potencies of related β-branched residues placed at the same position), the substitution of Ile by Thr at position A2 results in an analogue with only about 0.1% of the receptor-binding potency of insulin. These results indicate that residue A3 readily tolerates solvent exposure and ensuing hydration, whereas residue A2 does not. Second, the helix-breaking amino acid Tle (Lyu et al., 1991) is readily accommodated at position A3 (whereas, apparently for reasons of side-chain fit within the insulin monomer, it is not at position A2). Since CD spectroscopy identifies that the Tle^{A3}-containing analogue is structurally more similar to the analogue from which residues A1–A3 have been deleted (or to analogues containing Gly in place of residues A2 or A3) than it is to insulin itself, it appears that exact helical structure within the NH_2-terminal domain of the insulin A chain is not necessary for high-affinity ligand interactions with the insulin receptor. As a corollary, these results suggest that (a) residue 3 of the insulin A chain exists in a perturbed conformation when the hormone is bound to the insulin receptor and (b) a structural adjustment of the NH_2-terminal domain of the insulin A chain away from an α-helical conformation plays an important role in determining the affinity of insulin–receptor interactions.

Overall, our results identify the very different importance of side-chain structure at positions 2 and 3 of the insulin A chain. On the one hand, it seems that the side chain of residue A2 by its interaction with the side chain of residue A19 forms a tether to which the NH_2-terminal domain of the insulin A chain is tied through intramolecular contacts. On the other, it seems that the side chain of residue A3 might make contact with receptor to provide direct binding energy applicable to ligand–receptor interactions. The contact of the residue A3 side chain with receptor would require an adjustment in main-chain structure, however, since Val^{A3} is normally directed outwardly but is positioned in a solvent-inaccessible pocket in the usual structure of the insulin molecule. Movement of the COOH-terminal B-chain domain away from the hydrophobic insulin core would enhance the accessibility of the position A3 side chain to the receptor (Baker et al., 1988; Mirmira & Tager, 1989; Derewenda et al., 1990; Hua et al., 1991). The perturbed conformation expected to result from replacement of Val^{A3} by Tle suggests, however, that the adjustment necessary to bring the Val^{A3} side chain in contact with receptor might well require movement within the NH_2-terminal domain of the insulin A chain as much as it requires movement within the COOH-terminal domain of the insulin B chain. In both cases, conformational adjustments in the insulin main chain appear to play a critical role in allowing the Val^{A3} side chain to express its potential in governing the quality of insulin's fit with its receptor.

Acknowledgments

We thank Paul Rubinstein for preparing isolated hepatocytes and Crystal Sherman-Jones for assistance in the preparation of the manuscript.

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