Mutational Analysis of Invariant Valine B12 in Insulin: Implications for Receptor Binding†

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ABSTRACT: An invariant residue, valine B12, is part of the insulin B-chain central α-helix (B9−B19), and its aliphatic side chain lies at the surface of the hydrophobic core of the insulin monomer in close contact with the neighboring aromatic side chains of phenylalanines (B24 and B25) and tyrosines (B26 and B16). This surface contributes to the dimerization of insulin, maintains the active conformation of the insulin monomer, and has been suspected to be directly involved in receptor recognition. To investigate in detail the role of the B12 residue in insulin−receptor interactions, we have synthesized nine analogues bearing natural or unnatural amino acid replacements for valine B12 by chemical synthesis of modified insulin B-chains and the subsequent combination of each synthetic B-chain with natural insulin A-chain. The receptor binding potencies of the synthetic B12 analogues relative to porcine insulin were determined by use of isolated canine hepatocytes, and the following results were obtained: isoleucine, 13%; *allo*-isoleucine, 77%; *tert*-leucine, 107%; cyclopropylglycine, 43%; threonine, 5.4%; D-valine, 3.4%; α-amino-*n*-butyric acid, 14%; alanine, 1.0%; and glycine, 0.32%. Selected analogues were also analyzed by far-UV circular dichroic spectroscopy and by absorption spectroscopy of their complexes with Co^{2+}. Our results indicate that *β*-branched aliphatic amino acids are generally tolerated at the B12 position with specific steric preferences and that the receptor binding potencies of these analogues correlate with their abilities to form dimers. Furthermore, the structure−activity relationships of valine B12 are quite similar to those of valine A3, suggesting that valine residues at both A3 and B12 contribute to the insulin−receptor interactions in a similar manner.

The structure of insulin has long been a subject of investigation as an important model of a small protein hormone of great biological importance. Over the last 30 years, crystal structures of insulins of various species and various modified forms derived by chemical synthesis or by bioengineering have been analyzed (*1*−*10*). The natural insulins crystallize as zinc-coordinated hexamers in various forms (*1*−*4*, *11*−*13*) and in the absence of metal ions crystallize as dimers (*14*). In nature, insulins are stored as 2Zn hexamers in the pancreas and are dissociated to monomers during the circulation in blood (*2*). In recent years, progress in NMR spectroscopy has enabled the structural analysis of monomeric insulins in solution, yielding valuable results, which are rapidly accumulating (*15*−*25*). The hydrophobic cluster on the surface of the monomer that is also a dimer-forming interface has long been suspected to contribute to the receptor binding of insulin (*26*). However, this hypothesis had to be reconsidered when the crystal structures of A1−B29 cross-linked insulin and "miniproinsulin" (the α-carboxyl group of Lys^{B29} is peptide-bonded to the α-amino group of Gly^{A1}) were analyzed (*27*, *28*). Meanwhile, the discoveries of mutant insulins often associated with mild diabetes (see review *29*) stimulated investigations on amino acid residues important for biological activity including residues Phe^{B24}, Phe^{B25}, and Val^{A3}. These residues are invariant in vertebrates throughout evolution, and detailed investigations have confirmed their importance, along with other invariant residues, for the biological function of the hormone.

Insulin consists of two peptide chains, A and B, which are linked covalently by two disulfide bonds. The 21 residue A-chain contains an additional intrachain disulfide bond and is normally organized with N-terminal and C-terminal α-helices connected by a loop. The 30 residue B-chain begins with an extended N-terminus (B1−8), followed by a central α-helix (B9−19), a *β*-turn (B20−23), and an extended C-terminal region (B24−30) which is closely packed with the central α-helix. The side chain of the invariant residue, Val^{B12}, is part of a hydrophobic patch on the surface of the insulin monomer, and its side chain is in defined contact within the monomer to Phe^{B24} and Tyr^{B26} (Figure 1). Two Val^{B12} side chains also make contacts and are buried by dimer formation. In the 2Zn insulin hexamer, the metal coordination is octahedral (T_6-state) (*1*−*3*), while in the presence of phenol the metal coordination becomes tetrahedral (R_6-state) which involves a secondary structural change of residues B1−B8 (from extended to α-helical) (*13*, *30*). The intermediate state

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‡ In memory of Dr. Howard S. Tager, Ph.D. (1945−1994), with whom these studies were initially started.

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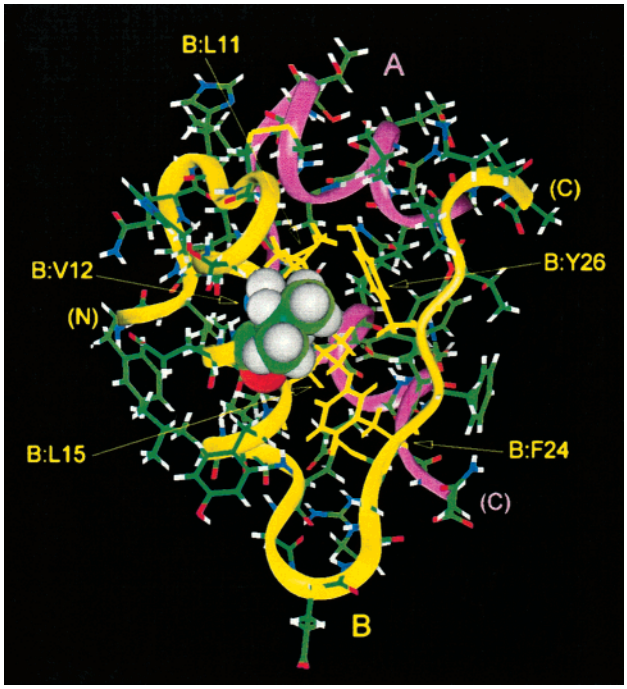

FIGURE 1: View of the insulin monomer (cubic crystal) (*14*). The ribbon diagram shows the main chains of A (pink) and B (yellow), and N- and C-termini of the chains are indicated as (N) and (C). The residue Val B12 is shown in van der Waals image, and the side chains of contact residues (B11, B15, B24, and B26) are shown as yellow skeletons. The figure was prepared using the program Insight (Biosym Technologies, Inc., San Diego).

(T_3R_3 or $T_3R^f_3$) is also known in the presence of anions or phenolic compounds (*7*, *11*, *12*). This structural transformation (T → R) has also been studied extensively in solution (*31*−*34*).

Some B12 analogues having substitutions with hydrophobic amino acids, such as Ile, Phe, Leu, D-Ala, and α-aminoisobutyric acid, or with hydrophilic amino acids, such as Asn, Glu, and Thr, have been reported (*35*−*38*). We undertook a further detailed investigation of this critical residue using a series of systematic replacements at this position. The resulting analogues were carefully analyzed on the potencies of insulin−receptor interactions, on the conformational changes observed by far-UV[1] CD spectra, and on the abilities to undergo T → R conformational transitions. Our results underscore the important role of Val^{B12} in insulin−receptor interactions.

EXPERIMENTAL PROCEDURES

Materials. Porcine [[^{125}I]iodo-Tyr^{A14}]insulin (receptor grade) was purchased from Du Pont (Wilmington, DE). *N-tert*-Butyloxycarbonyl (Boc)[1] derivatives of L-α-amino-*n*-butyric acid (Abu), D-valine, and carboxypeptidase A (type I-Dfp) were obtained from Sigma (St. Louis, MO), and L-*tert*-leucine (Tle) was from Aldrich (Milwaukee, WI). *Staphylococcus aureus* protease (strain V8) and trypsin (TPCK-treated) were purchased from Pierce (Rockford, IL) and Worthington (Freehold, NJ), respectively. Bio-Gels are from Bio-Rad Laboratories (Hercules, CA).

Synthesis of DL-*Cyclopropylglycine (H-*DL-*Cpg-OH).* The amino acid was synthesized by the Bucherer modification of the Strecker procedure (*39*, *40*) by use of cyclopropanecarboxyaldehyde (10 g) as a starting material. The product was recrystallized from water (yield 9.4 g, 54%).

*Preparation of H-*L-*Cpg-OH.* H-DL-Cpg-OH (9.2 g) was converted to the *N*-trifluoroacetyl derivative according to the general procedure (*41*). The product was recrystallized from a mixture of ethyl acetate and petroleum ether (yield 10.6 g, 63%; mp 124−125 °C). The derivative (10.1 g) was treated with carboxypeptidase A (19.2 mg, 1000 units) at 37 °C for 1 h, and the mixture was acidified to pH 3 with 5 N HCl and heated for 30 min at 100 °C. The small amount of precipitates (denatured enzyme) were removed by filtration, and the filtrate was concentrated to about 30 mL under reduced pressure. Trifluoroacetyl derivative (mostly D-isomer) was extracted with ethyl acetate (3 times), and the aqueous solution was neutralized and concentrated further until the L-amino acid began to crystallize. After cooling to 4 °C, the crystals were collected by filtration; a second crop of the product was also obtained as crystals after addition of ethanol to the filtrate. The combined yield of L-amino acid was 1.48 g (54%).

Synthesis of Boc-Cpg-OH. H-L-Cpg-OH (0.92 g) was converted to its Boc-derivative by a standard method (*42*). The compound was crystallized from a mixture of ethyl acetate and petroleum ether (yield 1.44 g, 83%; mp 111−113 °C).

Synthesis of Boc-Leu-Tle-OH. H-Tle-OH (262 mg) was dissolved in water (4.8 mL) together with sodium bicarbonate (336 mg). To this was added a solution of Boc-Leu-*N*-hydroxysuccinimide ester (788 mg) in dioxane (6 mL). The mixture was stirred for 20 h at room temperature. The progress of the condensation reaction was monitored by thin-layer chromatography on silica gel using a solvent system of chloroform−methanol−acetic acid (95:5:3, v/v/v) and a detection of peptide spots by spraying ninhydrin reagent-HCl and heating. The reaction mixture was concentrated under reduced pressure to an oily residue, which was crystallized upon acidification with 0.5 N HCl (8 mL) and a slight dilution with water. After cooling to 4 °C, the crystals were collected by filtration, washed with cold water, and dried to give 620 mg (90% yield) of the product.

Synthesis of B-Chain Analogue Di-S-sulfonates. Peptides were synthesized by standard solid-phase methods by use of an Applied Biosystems model 430A peptide synthesizer as described before (*43*). Typically, the Boc-Ala-phenylacetamidomethyl resin (0.5 mmol) was elongated to residue B13, the resulting peptidyl resin was divided into four parts, and the synthesis was continued separately for each part to complete the B-chain sequence with various amino acid residues substituted for Val at B12. As an exception, bulky Tle was incorporated into the peptidyl resin as dipeptide, Boc-Leu-Tle. Preliminary experiments showed that the removal of the Boc-group from the N-terminus of the Tle-peptide on the solid support was extremely difficult even under the condition of prolonged treatment with trifluoroacetic acid. To overcome this problem, we synthesized the Boc-dipeptide which was incorporated manually into the peptidyl resin by use of dicyclohexylcarbodiimide and

[1] Abbreviations: Abu, α-amino-*n*-butyric acid; Ail, *allo*-isoleucine; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; Cpg, C^{α}-cyclopropylglycine; DOI, des-octapeptide(B23−B30)-insulin; DPI, des-pentapeptide(B26−B30)-insulin; rp-HPLC, reversed-phase high-performance liquid chromatography; Tle, *tert*-leucine; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

1-hydroxybenzotriazole in dimethylformamide. Quantitative ninhydrin test (*44*), after the condensation reaction for 20 h, showed a coupling yield of 73%. Repeating the procedure increased the yield up to 88%. The remaining free amino groups were acetylated, and automated synthesis by the standard procedure was continued. The peptidyl resins were subjected to cleavage by liquid hydrogen fluoride containing 10% anisole (v/v) for 60 min at 0 °C. The peptide was isolated as di-*S*-sulfonates by oxidative sulfitolysis as described previously (*45*, *46*) and purified by a combination of gel-filtration on Bio-Gel P-6 in 0.1 M ammonium bicarbonate and rp-HPLC on a C-4 column using a solvent system of 0.1 M phosphoric acid/0.05 M triethylamine (adjusted to pH 3.0 with sodium hydroxide). After desalting by dialysis, purified B-chain analogue di-*S*-sulfonates were obtained as a powder upon lyophilization. The purity of each analogue was confirmed by analytical rp-HPLC (>90%).

Preparation of Insulin Analogues with Replacements at Position B12. Chain combination between natural A-chain and synthetic B-chain with various amino acid substitutions at position 12 followed the published methods (*47*). Typically, dithiothreitol (10.5 μmol) was added at 4 °C to a solution of porcine A-chain tetra-*S*-sulfonate (3 μmol) and a synthetic B-chain di-*S*-sulfonate (1 μmol) in 0.1 M glycine adjusted to pH 10.5 with sodium hydroxide (total volume of 2 mL). The mixture was stirred for 1−2 days at 4 °C. The progress of a chain combination reaction was monitored occasionally by analytical rp-HPLC. The reaction mixture was acidified with acetic acid (0.5 mL) and then gel-filtered on a column of Bio-Gel P-4 using 3 M acetic acid as the solvent. Materials in fractions corresponding to the molecular weight of insulin were pooled and freeze-dried. The crude peptides thus obtained were further purified by rp-HPLC on C-4. The purity of each analogue was confirmed by analytical rp-HPLC (both on C-4 and on C-18) and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (PerSeptive Biosystems, Farmingham, MA). The formation of the correct disulfide bridges in each insulin analogue was confirmed by peptide mapping after digestion with *Staphylococus aureus* V8 protease as described (*43*, *48*). The yield for each chain combination reaction after purification, based on the amount of each B-chain employed, was as follows: Ile^{B12}, 15%; Ail^{B12}, 22%; Tle^{B12}, 13%; Thr^{B12}, 13%; Cpg^{B12}, 18%; D-Val^{B12}, 1.2%; Abu^{B12}, 25%; Ala^{B12}, 11%; and Gly^{B12}, 1.0%.

Preparation of Des-pentapeptide(B26−B30)-[Phe^{B25}-α-carboxamide]insulin (DPI-amide) Analogues. Selected insulin analogue with a replacement at B12 (0.05 μmol) was dissolved in 0.2 M Tris−acetate (pH 8.0 and containing 10 mM calcium acetate) (0.05 mL). The mixture was incubated for 1.5 h at 37 °C with tosylphenylalanine chloromethyl ketone-treated trypsin (15 μg). A solution containing the trifluoroacetate salt of the tripeptide, Gly-Phe-Phe-amide (10 μmol), in *N*-methylmorpholine (2.2 μL), dimethylformamide (0.06 mL), and 1,4-butanediol (0.06 mL) was then added to the trypsin-cleaved insulin analogue together with an additional trypsin (30 μg). The mixture was kept for 1 day at 12 °C to allow for enzyme-catalyzed peptide bond formation between Arg^{B22} of the des-octapeptide(B23−B30)-insulin (DOI) analogue and the tripeptide (*49*, *50*). The reaction was terminated by the addition of 3 M acetic acid, and the product was purified by gel-filtration and rp-HPLC.

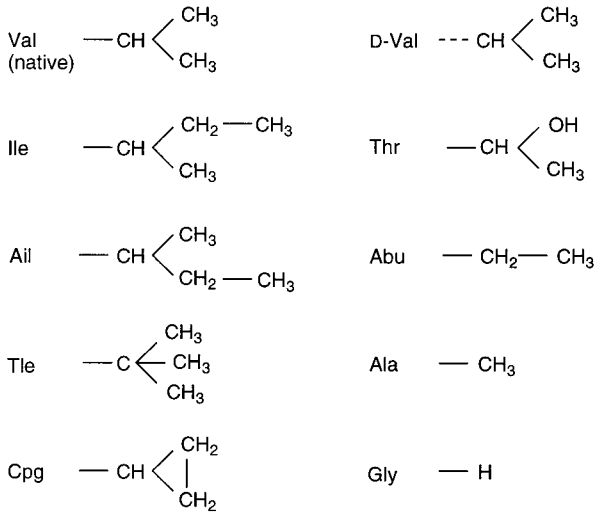

FIGURE 2: Side chain structures of the amino acids incorporated into position B12 of insulin in this study.

Receptor Binding Assay. Procedures for the isolation of canine hepatocytes and for their use in receptor binding experiments have been described elsewhere (*51*, *52*). Cells (1.6×10^6/mL) were incubated with [(^{125}I)iodo-Tyr^{A14}]insulin and selected concentrations of insulin or insulin analogues (determined by UV absorbance) for 30 min at 30 °C.

Spectroscopic Procedures. CD spectra were determined at 22 °C by use of a Jasco J-600 spectropolarimeter (Tokyo, Japan) at a peptide concentration of 90 μg/mL in 0.02 M Tris·HCl at pH 7.4. Cylindrical cells of 1 mm optical path length were used. Six spectra were averaged for each peptide studied. The T → R structural transition of insulin$_6$(Co^{2+})$_2$ complexes induced by phenol and stabilized by thiocyanate was examined by methods described (*33*, *34*, *43*). The concentration of insulin and insulin analogues was approximately 1 mg/mL, and the buffer was 0.05 M Tris·HCl at pH 8.0.

RESULTS

Chain Combination. Insulin analogues bearing various replacements at position B12 (see Figure 2) were prepared by chain combination between S-sulfonated insulin A-chains and S-sulfonated synthetic B-chain analogues in the presence of dithiothreitol at pH 10.5 (*37*, *43*, *47*). Each analogue was purified by a combination of size-exclusion chromatography and preparative rp-HPLC. The final yields for most analogues were relatively good (between 11% and 25%) except for the Gly^{B12} and D-Val^{B12} analogues (approximately 1%). The results suggest that the overall secondary structures, particularly the B-chain α-helix and β-turn, are well maintained by substituting Val^{B12} with Ile, Ail, Tle, Cpg, Thr, Abu, or Ala, and these derivatives all fold relatively well. Substitution of Val^{B12} by Gly or D-Val probably leads to an unfavorable packing of the hydrophobic core, either due to the lack of the side chain or due to an improper accommodation of the side chain; as a result, these analogues seem to fold poorly.

Receptor Binding Studies. The relative receptor binding potencies of synthetic analogues determined with insulin receptors isolated from canine hepatocytes are shown in Figure 3A−C and Table 1. The first group (Figure 3A) presents the effects of replacing Val^{B12} by other hydrophobic β-branched amino acid residues with an extra methylene

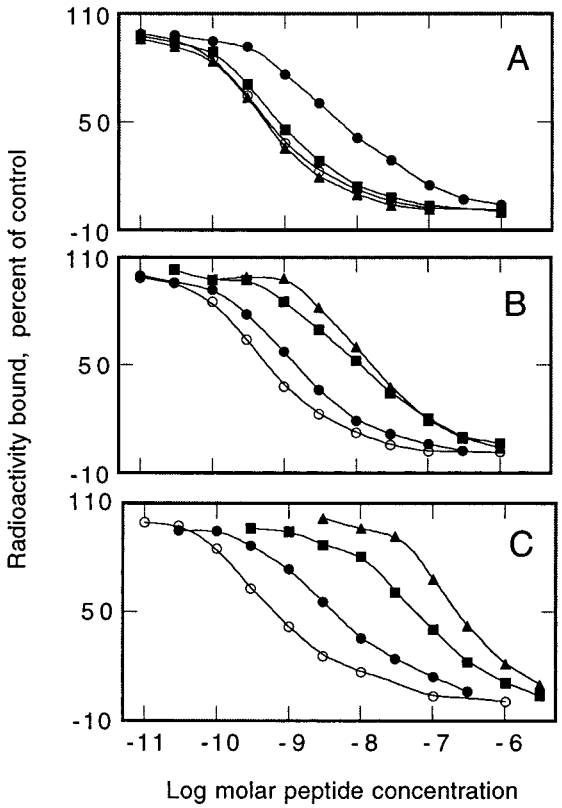

FIGURE 3: Inhibition of binding of ^{125}I-labeled insulin to isolated canine hepatocytes by insulin and insulin analogues. Incubations were performed as described under Experimental Procedures. Control binding is defined as the amount of radiolabeled ligand which became cell-associated in the absence 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 1. Panel A: insulin (○); [Ile^{B12}]insulin (●); [Ail^{B12}]insulin (■); and [Tle^{B12}]insulin (▲). Panel B: insulin (○); [Cpg^{B12}]insulin (●); [Thr^{B12}]insulin (■); and [D-Val^{B12}]insulin (▲). Panel C: insulin (○); [Abu^{B12}]insulin (●); [Ala^{B12}]insulin (■); and [Gly^{B12}]insulin (▲).

group. An extension to either γ-carbon of the side chain yields Ile^{B12} or Ail^{B12} analogues. Binding affinities of these two analogues relative to that of natural insulin are significantly different; 13% for [Ile^{B12}]insulin and 77% for [Ail^{B12}]-insulin, respectively, indicating that the environments of these newly added δ-methyl groups are different. One is well accommodated on the hydrophobic surface, but the other significantly impairs the structure necessary to induce proper receptor binding. An additional analogue that has an extra methyl group attached to the β-carbon of the Val, [Tle^{B12}]-insulin, exhibits a surprisingly high binding potency (107%) compared to natural insulin. The side chain of Tle does not protrude from the hydrophobic surface like the Ile or Ail side chain but is more rigid. This feature is somehow favorable for interaction with the receptor. The second group (Figure 3B) presents the effects of replacing the side chain of Val^{B12} with β-branched amino acid residues having an unnatural or hydrophilic side chain. An analogue in which two γ-carbons of Val are covalently bonded, [Cpg^{B12}]insulin, shows reduced but still adequate receptor binding affinity (43%). The loss of two hydrogens reduces the size of the side chain, which may be reflected in the reduced binding affinity of this analogue. The analogue, which has a hydroxyl group in place of one of the γ-methyl groups of valine,

Table 1: Receptor Binding Potencies of Insulin and Insulin Analogues[a]

peptide	potency relative to insulin %	±SD	(*n*)
porcine insulin	100		
[Ile^{B12}]insulin	13	±1	(8)
[Ail^{B12}]insulin	77	±2	(8)
[Tle^{B12}]insulin	107	±1	(3)
[Cpg^{B12}]insulin	43	±2	(3)
[Thr^{B12}]insulin	5.4	±0.5	(5)
[D-Val^{B12}]insulin	3.4	±1	(3)
[Abu^{B12}]insulin	14	±1	(8)
[Ala^{B12}]insulin	1.0	±0.06	(4)
[Gly^{B12}]insulin	0.32	±0.05	(3)
[Ile^{B12}]DPI-amide	15	±3	(3)
[Ail^{B12}]DPI-amide	102	±20	(3)
[Thr^{B12}]DPI-amide	13	±2	(3)
[Abu^{B12}]DPI-amide	27	±1	(5)

[a] 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 0.69 ± 0.03 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.

[Thr^{B12}]insulin, shows a relatively low binding affinity (5.4%). [This result is inconsistent with the reported value of 56% in receptor binding assays on human placental membranes by Wang et al. (*38*).] The analogue in which the L-configuration of the α-carbon was changed to the D-configuration, [D-Val^{B12}]insulin, shows markedly reduced receptor binding affinity (3.4%) relative to that of the natural hormone. The change is probably due to an unfavorable accommodation of the side chain as it was reflected in the chain combination yield. The third group (Figure 3C) presents the effects of the consecutive removal of carbon atoms from the side chain of Val^{B12}, yielding [Abu^{B12}]-, [Ala^{B12}]-, and [Gly^{B12}]insulin. The relative receptor binding potencies of these analogues are reduced to 14%, 1%, and 0.32%, respectively. Of these, [Abu^{B12}]insulin is still tolerated, and it folds as well as natural insulin, as reflected in its high yield on chain combination. [Ala^{B12}]Insulin also folds relatively well but its receptor binding potency is severely diminished, and [Gly^{B12}]insulin folds very poorly and shows even further reduced receptor binding potency. These results indicate that for both parameters at least up to a γ-carbon is required in the side chain of B12 and the removal of both γ-carbons is detrimental.

Since the side chain of Val^{B12} lies in close proximity to the B-chain β-strand and is in contact with the side chains of Phe^{B24} and Tyr^{B26}, it was of interest to test how these substitutions in the side chain of Val^{B12} would affect the conformational changes postulated to take place at the C-terminus of the B-chain during receptor binding (*19, 28, 53, 54*). We therefore prepared four truncated derivatives of [Ile^{B12}]-, [Ail^{B12}]-, [Thr^{B12}]-, and [Abu^{B12}]insulin for this purpose. The resulting DPI-amide derivatives of the B12

analogues show higher receptor binding potencies than their full-length analogues (see Table 1). Of these, [Ile^{B12}]DPI-amide and [Ail^{B12}]DPI-amide exhibit increased receptor binding potencies (about 1.2-fold) than their full-length analogues, corresponding well with the known increase in potency of the DPI-amide form vs full-length natural insulin. However, [Thr^{B12}]DPI-amide and [Abu^{B12}]DPI-amide exhibit further increased receptor binding potencies (about 2-fold) than their full-length analogues. In the former two analogues, regardless of their significantly different receptor binding potencies, deletion of the B-chain C-terminal five residues induces the same degree of enhancement in receptor binding potencies, suggesting that in these analogues the analogous conformational changes as in the case of natural insulin take place upon receptor binding. However, in the latter two analogues, the C-terminal truncation relieves additional unfavorable negative constraints caused either by the introduction of a polar hydroxyl group in the otherwise hydrophobic surface (in the case of Thr^{B12}) or by the introduction of a side chain that is too small to fill the surface (in the case of Abu^{B12}). Thus, these intramolecular conformational perturbations appear to interfere with appropriate conformational changes necessary for receptor recognition. These truncation studies suggest that replacement of residue B12 by amino acids with hydrophobic or β-branched side chains does not affect the mode of interaction with the receptor, whereas replacement with nonhydrophobic or non-β-branched amino acids causes more unfavorable structural changes within the insulin molecule and these negative effects are partially reversed by removal of the B-chain C-terminal five residues.

Circular Dichroic Studies. CD spectra of insulin and B12 analogues at 190−250 nm are presented in Figure 4A−D except for [D-Val^{B12}]insulin which has not been obtained in a quantity to access CD studies. The apparent monomer−dimer association constant at pH 8 for natural insulin has been estimated to be 2.22×10^5 M^{-1} by Goldman and Carpenter (*55*); the peptide concentration used in our experiments, 1.5×10^5 M^{-1}, is thus in the range of the dimer−monomer equilibrium. When insulin dimers dissociate to monomers, ellipticity minima at 222 nm predominantly decrease, but the minima at 209 nm do not change much; as a consequence, the ellipticity ratio at 209 nm vs 222 nm increases with dilution (*56*). Assuming that global secondary structural changes such as apparent distortions of the B-chain central α-helix do not occur by replacing the Val^{B12} in insulin by other amino acids, the values obtained for the ellipticity ratio 208 nm/222 nm for each analogue at the same peptide concentration with the natural insulin should provide information on the relative ability of the analogues to dimerize. Due to the limited amount of each peptide available, near-UV CD spectra were not measured.

All panels in Figure 4 include metal-free porcine insulin as a standard. The CD spectra of [Ile^{B12}]insulin and [Ail^{B12}]-insulin are shown in panel A; an almost similar spectrum to that of natural insulin is observed for [Ail^{B12}]insulin, but a slightly diminished secondary structure is observed for the steric isomer [Ile^{B12}]insulin. In panel B, the CD spectra of [Tle^{B12}]insulin and [Thr^{B12}]insulin are compared. The CD spectrum of [Tle^{B12}]insulin is very similar to the natural insulin, while the spectrum of [Thr^{B12}]insulin is more perturbed; again, these results correspond to their relative

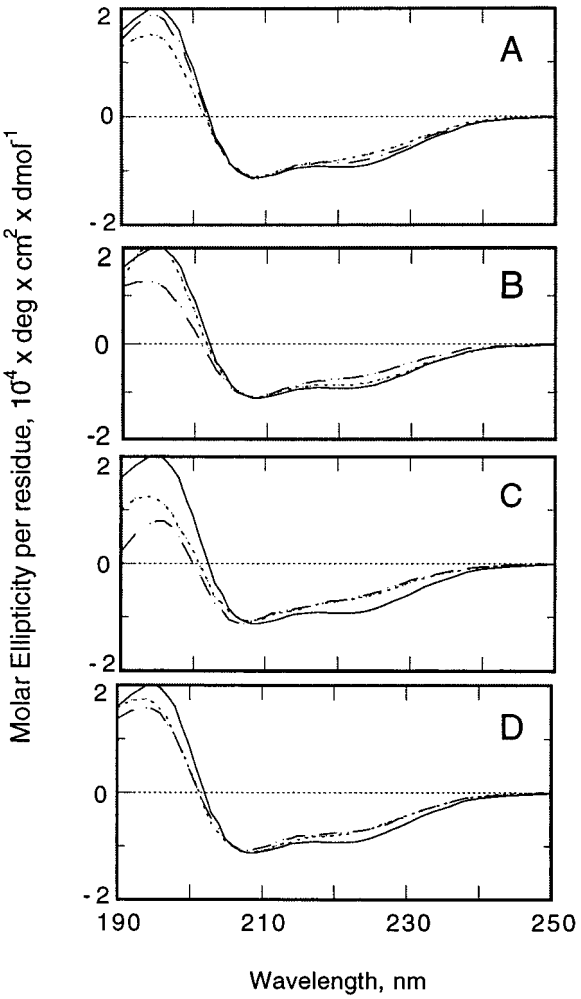

FIGURE 4: CD spectra of insulin and insulin analogues in the far-UV. Spectra were recorded by use of a 1 mm cylindrical cell for all peptides at a concentration of about 90 μg/mL dissolved in 10 mM Tris·HCl (pH 7.4). Quantitative data on these analogues at 208 and 222 nm are provided in Table 2. CD spectra of zinc-free insulin (—) are included in all panels. Panel A: [Ile^{B12}]insulin (• • •) and [Ail^{B12}]insulin (- - -). Panel B: [Tle^{B12}]insulin (• • •) and [Thr^{B12}]insulin (- - -). Panel C: [Ala^{B12}]insulin (• • •) and [Gly^{B12}]-insulin (- - -). Panel D: [Abu^{B12}]insulin (• • •) and [Cpg^{B12}]insulin (- - -).

receptor binding potencies. The spectra of [Ala^{B12}]insulin and [Gly^{B12}]insulin are shown in panel C. Again, the perturbations in the CD spectra reflect their reduced receptor binding abilities. As shown in panel D, the CD spectra of [Abu^{B12}]-insulin and [Cpg^{B12}]insulin, which have intermediate receptor binding potencies, are similar although their side chain structures are quite different.

Our present and previous (*43*) measurements of CD spectra of porcine insulin at a concentration of 15 μM at pH 8.0 give an ellipticity ratio of approximately 1.25, which is in a good agreement with the ratio reported by Wood et al. (*56*) for bovine insulin at a concentration of 38 μM at pH 7.8. The ratio for des-octapeptide(B23−B30)-insulin (DOI) which has no B-chain C-terminal octapeptide to form an intermolecular antiparallel β-sheet is approximately 1.82. This is also in good agreement with the ratio of 1.79 (209 nm/222 nm) reported for naturally occurring monomeric guinea pig insulin (*56*). The quantitative ellipticity values at 208 and 222 nm and their ratios for all B12 analogues except for [D-Val^{B12}]-insulin are summarized in Table 2. We plotted the above

Table 2: CD Spectral Data of Insulin and Selected Insulin Analogues[a]

peptide	mean residual ellipticity (deg cm^2 dmol^{-1})		
	208 nm	222 nm	208 nm/222 nm
porcine insulin	−12648	−10259	1.23
[Ile^{B12}]insulin	−12350	−8254	1.50
[Ail^{B12}]insulin	−12508	−9157	1.37
[Tle^{B12}]insulin	−12263	−8992	1.36
[Cpg^{B12}]insulin	−11878	−8249	1.44
[Thr^{B12}]insulin	−12409	−7725	1.61
[Abu^{B12}]insulin	−12669	−8333	1.52
[Ala^{B12}]insulin	−11790	−7416	1.59
[Gly^{B12}]insulin	−12372	−7130	1.73

[a] CD spectra were recorded for insulin and selected analogues dissolved in 10 mM Tris·HCl (pH 7.4) (90 μg/mL peptide). Further analytical details are provided under Experimental Procedures. The table provides quantitative information obtained at both 208 and 222 nm.

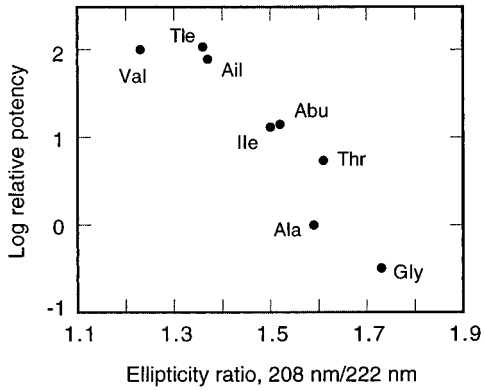

FIGURE 5: Relationship between the receptor binding potencies and the dimer-forming capabilities of insulin and the B12 analogues. The logarithms of the receptor binding potencies of B12-substituted analogues relative to insulin (defined as 100%) are plotted against the ratios of the observed molar ellipticities (208 nm/222 nm) of insulin and the B12 analogues obtained by measurements of CD spectra (see Table 2 for the quantitative data).

values against logarithmic receptor binding potencies of B12 analogues. As shown in Figure 5, structural perturbations at the monomer−monomer interface greatly affect interactions with insulin receptors.

Visible Spectroscopic Studies. Insulin hexamers octahedrally liganded with Co^{2+} in solution transform to tetrahedrally liganded complexes in the presence of phenol or phenol-like compounds. The complexes are further stabilized by addition of sodium thiocyanate, which enhances absorption in the region 500−650 nm. This $T_6 \rightarrow R_6$ structural transition (*32*) is typical of insulin as studied in detail in solution (*33, 34*). Figure 6 shows the spectra obtained for insulin and selected B12 analogues. The native $(insulin)_6$-$(Co^{2+})_2$ complex undergoes the transition in the presence of phenol alone (Figure 6A, dashed line), and the addition of sodium thiocyanate further enhances the effect (solid line). Analysis of the spectra for [Abu^{B12}]insulin (Figure 6B) indicates a somewhat reduced but similar structural transition as in the case of natural insulin. The analysis of [Tle^{B12}]-insulin (Figure 6C) shows that the structural transition is retained but is largely reduced in degree despite its high receptor binding potency. The spectra for [Ala^{B12}]insulin which has only 1% of the receptor binding potency of normal insulin show substantial capability to undergo the transitions (Figure 6D). The measurements of [Thr^{B12}]insulin result in

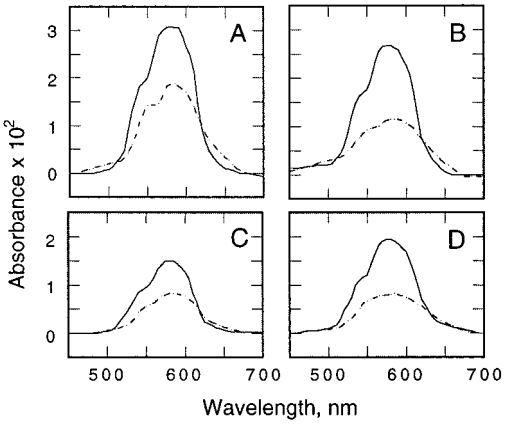

FIGURE 6: Spectroscopic studies of Co^{2+}-coordinated hexamer function by insulin and selected insulin B12 analogues. For each panel, curves represent spectra obtained as the result of incubating 0.17 mM insulin or a B12 analogue with 0.06 mM $CoCl_2$ and 50 mM phenol in 50 mM Tris·HCl buffer at pH 8 both before (· · ·) and after (—) the addition of 800 mM NaSCN to stabilize potential metal ion-coordinated complexes: (A) insulin; (B) [Abu^{B12}]insulin; (C) [Tle^{B12}]insulin; and (D) [Ala^{B12}]insulin.

a similar degree of structural change to that shown by [Tle^{B12}]-insulin (data not shown) despite its substantially lower binding potency in comparison to that of [Tle^{B12}]insulin. These measurements have not been performed for the other analogues. Overall, these results clearly indicate a lack of correlation between the analogues' abilities to undergo T → R structural transitions and their insulin receptor binding potencies.

DISCUSSION

Our results presented here demonstrate the important role of Val^{B12} in insulin−receptor interactions, as earlier postulated from the crystal structure of insulin (*26*). The Val^{B12} resides in the B-chain central α-helix, and its side chain forms part of the nonpolar surface of the monomer which is buried by the formation of the antiparallel β-sheet dimer.

Any alteration of the side chain at B12 is expected to affect the packing of the B-chain C-terminal β-strand, in particular the packing of the side chains of Phe^{B24} and Tyr^{B26} which are in van der Waals contacts with the side chain of Val^{B12} in the hexameric or dimeric insulin crystal (*1−4, 11−14, 28*) or in monomeric insulins in solution (*15, 17, 57−61*). The correct insulin fold within the monomer would be impaired, which in turn would affect monomer−monomer interactions involving residues B24−B28. Although the dimer is not an active species, it is worthwhile to investigate how this perturbation at the monomer−monomer interface also modulates hormone−receptor interactions.

We have observed that there is an apparent correlation between the receptor binding potencies of B12 analogues and their dimer-forming abilities, expressed as the ellipticity ratio (208 nm/222 nm), as shown in Figure 5. Native insulin forms dimers readily, but a potent analogue, [Tle^{B12}] insulin, which shows a slightly higher receptor binding potency than native hormone is somewhat impaired in dimerization. This indicates that the Val at B12 is structurally a best fit for dimer formation and also for hexamer formation, but this structure is not necessarily a best fit for receptor binding; rather, a slight perturbation of the monomer surface caused by the substitution of Val by Tle may be preferred for

receptor binding. Since Tle is a more bulky amino acid than Val, an increased hydrophobicity in a limited space may be advantageous for receptor recognition. The rigidity of residue Tle probably causes a reduced structural transformation of the hexameric metal complex of the analogue from the T-state (extended at B1−B8) to the R-state (continuous α-helix at B1−B19) by the presence of phenol, even in the presence of thiocyanate ion (*33, 34*). As the main-chain amide hydrogen of Val^{B12} in molecule I of 2Zn insulin forms a good hydrogen bond with the carbonyl oxygen of Gly^{B8} (*3*), the increased bulk at B12 is likely to affect the structural change at Gly^{B8}. Cornish et al. (*62*) reported, in their studies on T4 lysozyme, that incorporation of Tle into an α-helix is destabilizing because the β-branched methyl groups can cause distortions in the local helix backbone. But in some contexts the conformational rigidity of β-branched amino acids may be stabilizing because it lowers the entropic cost of forming favorable side chain van der Waals contacts. The latter may be the case for [Tle^{B12}]insulin, and this feature is favorable for interaction with the receptor. In our previous work with Val^{A3} substitutions, we have observed a similar character in the Tle^{A3} analogue, which has shown a higher receptor binding affinity than the Ile^{A3} or Ail^{A3} analogues (*63*). A remarkable difference in the receptor binding potencies of [Ile^{B12}]insulin and [Ail^{B12}]insulin is also reflected in their monomer−monomer interactions. [Ail^{B12}]Insulin has almost the same capability as [Tle^{B12}]insulin in dimer formation but has a slightly decreased receptor binding potency relative to natural insulin, while [Ile^{B12}]insulin has further reduced receptor binding potency as well as reduced dimer-forming ability. In this regard, [Gly^{B12}]insulin has the lowest dimer-forming capability, while the other B12 analogues are somewhat better, but less than insulin. The fact that the structural perturbations at the monomer−monomer interface due to the replacement of Val^{B12} by other amino acids nearly parallel the receptor binding potencies of the analogues suggests that the dimer-forming surface partly overlaps a major receptor binding site.

Substitution of Val^{B12} by Ala has a profound effect on the relative receptor binding potency (1%). It is due to a lack of a γ-carbon in the side chain but may also in part due to an increased α-helical stability of the backbone. Since [α-aminoisobutyric $acid^{B12}$]insulin has been reported to have 9.3% of the binding potency of native insulin (*37*) and [D-Val^{B12}]insulin in our study also shows higher receptor binding potency (3.4%) than [Ala^{B12}]insulin, a slight distortion of the backbone in addition to an improper disposition of the side chain may affect the receptor binding potencies of these analogues, although all show markedly reduced receptor binding potencies. The above-mentioned [α-aminoisobutyric $acid^{B12}$]insulin has only a slightly lower biological potency than that of [Abu^{B12}]insulin, and both [Ala^{B12}]-insulin and [D-Ala^{B12}]insulin (0.7%) (*37*) have low but similar binding potencies. These results indicate that when residue B12 is replaced with amino acids having small side chains, hydrophobicity is a more dominant factor than is the steric configuration of the α-carbon, whereas when residue B12 is replaced with an amino acid having added bulk to the β- or γ-carbon of the valine, the precise tertiary structure of the monomer surface determines the ability to form dimers and to bind the receptor.

It is interesting that Val^{B12} is invariant throughout vertebrate insulin and insulin-like growth factors. The corresponding position is occupied by Ala in some other insulin superfamilies such as the bombyxins (*64*) or the amphioxus insulin-like peptide (*65)*. The bombyxin II does not have the typical B-chain C-terminal insulin fold (β-turn and β-strand) but has an extended B-chain α-helix (B9−B22) (*66*).

It is now generally considered that insulin binding to its receptor is accompanied by conformational changes that involve a separation of the B-chain C-terminus from the rest of the molecule (*3, 19, 28, 53, 54*). A truncated insulin with a B-chain C-terminal deletion of five residues has reduced but substantial biological activity (*67, 68*) and maintains the insulin fold in the crystalline state (*5*) or in solution (*16, 18, 21, 69*). Amidation of the C-terminal carboxyl group in DPI fully restores (even slightly increases) the biological potency of the native insulin (*70*). All four C-terminally truncated B12 analogues prepared in this study show increased receptor binding potencies in comparison with their full sequence counterparts; an approximately 1.2-fold increase for the Ile^{B12} and Ail^{B12} analogues and a 2-fold increase for the Thr^{B12} and Abu^{B12} analogues, respectively. In the former two analogues, the altered side chains of residue B12 do not directly affect the overall conformational changes associated with insulin−receptor binding. In the latter two analogues, replacement of a γ-methyl group by a hydroxyl group or removal of a γ-methyl group partially interferes with the proper conformational changes for receptor binding, but these negative effects are neutralized by removal of the C-terminal five residues. As a result, the truncated analogues of both Ile^{B12} and Thr^{B12} show almost the same receptor binding potencies while the truncated analogue of Abu^{B12} shows about 2-fold higher receptor binding potency as compared to the truncated analogues of Ile^{B12} and Thr^{B12}. Clearly, Abu^{B12} is a better analogue than Ile^{B12} or Thr^{B12} if the B-chain C-terminal five residues are removed. These findings suggest that in B12 analogues having smaller side chains than Val, the B-chain β-strand (B24−B28) tends to lie too close to the core and interferes with those conformational rearrangements associated with receptor binding, while this negative effect is partially reversed by the truncation of the C-terminal five residues.

Previous studies on a fully active monomeric insulin, [Asp^{B10}, Lys^{B28}, Pro^{B29}]insulin, in solution at pH 8 (*20*) showed that the side chain of Phe^{B25} adapts an outward configuration (corresponding to molecule 2 of 2Zn insulin). However, the recently solved solution structure of a monomeric insulin mutant, des(B30)-[Glu^{B16}, Gly^{B24}]insulin (relative biological potency, 15%), at pH 8 (*25*) revealed a rearrangement of the B-chain C-terminal decapeptide, involving a perturbation of the B20−B23 turn, which allows Phe^{B25} to occupy the position normally taken by Phe^{B24} in native insulin. Their work (*25*) emphasized that upon changing Phe^{B24} to Gly, the underlying hydrophobic core directs a reorientation of exposed side chains to form a novel functional binding surface. Another solution structure of a monomeric mutant, [B24−B25 CO−O]insulin (depsi-insulin), which has only 3−4% of the receptor binding potency of that of native insulin, was also analyzed (*24*). It was found that the B20−B23 β-turn was distorted and the side chain of Phe^{B25} was inserted into the hydrophobic core

as a result of the increased flexibility of the B-chain C-terminus. These results seem to be in agreement with our previous observations on the effects of insertions of extra glycines between Gly^{B23} and Phe^{B24} (*71*). In our present findings involving substitutions of Val^{B12}, the side chain of B12 together with the neighboring side chains of Leu^{B11} and/or Leu^{B15} may reorient the aromatic residues at the B-chain C-terminus during receptor binding. Slight perturbations of the B12 side chain affect this reorientation and influence receptor binding affinity. Of these, the neighboring side chain of Leu^{B15} is probably not involved directly in receptor contacts since [Ala^{B15}]insulin still retains 12% of the receptor binding potency of insulin regardless of its perturbed structure in solution as determined by UV CD spectra (unpublished results). Both Val^{B12} and Phe^{B24} are critical residues for receptor binding; their side chain−side chain packing within the monomer is essential to maintain the correct insulin fold. This contact is maintained even in DPI, regardless of the significant changes in conformation involving both the N- and C-termini of the B-chain (*5*, *16*). The dimer-forming abilities of B12 analogues are almost parallel to their receptor binding affinities, and DPI-amide derivatives of these B12 analogues show further enhanced receptor binding affinities. Together, these findings suggest that the specific hydrophobic interactions between the B-chain α-helix and the aromatic residues in the B-chain C-terminal *β*-strand, in particular the side chain of Phe^{B24}, are likely maintained even during receptor interactions.

We have not observed any correlation between the receptor binding potencies of B12 analogues and their abilities to undergo the T → R transition. In our past studies on B6 analogues (*43*), A3 analogues (*63*), and reduced peptide bond analogues in the positions B24 and B25 or B25 and B26 (*72*), all gave inconsistent results as well as the results obtained in the present studies. However, in the studies on the B-chain C-terminal truncated analogues having one to five glycine insertions between Gly^{B23} and Phe^{B24}, a certain link between the interaction with the receptor and the T → R transition was recognized (*71*). The Phe^{B24}−Phe^{B25}-amide dipeptide plays roles both in interacting with the receptor and in undergoing the T → R conformational change. When both Phe are replaced by Ala, the receptor binding potency of the analogue was severely diminished, and the ability to undergo the T → R transition was lost. An alteration of the side chain of Val^{B12} would affect the positioning of the B-chain C-terminal aromatic residues and, as a consequence, affect monomer−monomer interactions and the insulin−receptor interactions. However, the factors affecting the structural changes of insulin in the presence of receptors and in the presence of metal ion, phenol, plus anion are apparently different; it seems that the insulin−receptor interactions are more dynamic, cooperative, and comprehensive.

Previously, we have investigated the roles of residues Ile^{A2} and Val^{A3} by preparing various substitutions in studies similar to those on Val^{B12} described here. It appears that analogues of Val both at the A3 and at the B12 sites behave similarly in their relative receptor binding potencies, as indicated by log plots of the relative potencies of B12 analogues vs A3 analogues having identical amino acid substitutions (Figure 7). Since all Val^{A3} analogues were prepared as N^{α}-Phe^{B1}, N^{ϵ}-Lys^{B29}-bisacetyl derivatives for the convenience of semi-

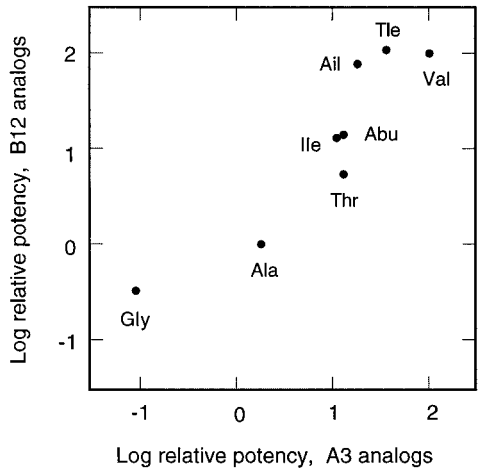

FIGURE 7: Comparison of the relative receptor binding potencies of B12 analogues and A3 analogues. The logarithms of the receptor binding potencies relative to native insulin are plotted against each amino acid substituted. The receptor binding potencies of the A3-substituted analogues relative to insulin are from previous work (*63*).

synthesis, the receptor binding potencies are corrected for the parent insulin derivative, which has 76% of the receptor binding potency of that of natural insulin (*63*). As shown in Figure 7, there is a correlation between the relative receptor binding potencies of both Val mutants at A3 and B12, although a preference for hydrophobicity is more prominent for the B12 side chain than for A3. Some B12 analogues (Tle and Ail) have higher binding potencies than the corresponding A3 analogues, but most analogues with the same amino acid replacement have rather similar receptor binding potencies. This correlation leads to the suggestion that residues Val^{A3} and Val^{B12} in insulin play similar critical roles in insulin−receptor interactions.

In conclusion, our results demonstrate the very important role of the invariant residue Val^{B12} in insulin−receptor interactions. The side chain structure of Val^{B12} is very sensitive to minor modifications both in terms of receptor binding affinity and in terms of dimer-forming ability, which are closely related parameters in the present studies. Deletion of five residues from the B-chain C-terminus of some B12 analogues increased their receptor binding affinities, suggesting a critical role of Val^{B12} for a concerted conformational adjustment during association with the receptor.

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REFERENCES

1. Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S. (1969) *Nature 224*, 491−495.
2. Blundell, T., Dodson, G., Hodgkin, D., and Mercola, D. (1972) *Adv. Protein Chem. 26*, 279−402.
3. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cuttfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M. C., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe,

N., and Vijayan, N. M. (1988) *Philos. Trans. R. Soc. London B319*, 369−456.
4. Derewenda, U., Derewenda, Z. S., Dodson, G. G., and Hubbard, R. E. (1990) in *Handbook of Experimental Biology* (Cuatrecasas, P., and Jacobs, S., Eds.) Vol. 92, pp 23−39, Springer-Verlarg, Berlin.
5. Bi, R. C., Dauter, Z., Dodson, E., Dodson, G., Giordano, F., and Reynolds, C. (1984) *Biopolymers 23*, 391−395.
6. Bentley, G. A., Brange, J., Derewenda, Z., Dodson, E. J., Dodson, G. G., Markussen, J., Wilkinson, A. J., Wollmer, A., and Xiao, B. (1992) *J. Mol. Biol. 228*, 1163−1176.
7. Ciszak, E., Beals, J. M., Frank, B. H., Baker, J. C., Carter, N. D., and Smith, G. D. (1995) *Structure 3*, 615−622.
8. Bao, S.-J., Xie, D.-L., Zhang, J.-P., Chang, W.-R., and Liang, D.-C. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2975−2980.
9. Whittingham, J. L., Havelund, S., and Jonassen, I. (1997) *Biochemistry 36*, 2826−2831.
10. Zeng, Z.-H., Liu, Y.-S., Jin, L., Zhang, Y., Havelund, S., Markussen, J., and Wang, D.-C. (2000) *Biochim. Biophys. Acta 1479*, 225−236.
11. Bentley, G., Dodson, E., Dodson, G., Hodgkin, D., and Mercola, D. (1976) *Nature 261*, 166−168.
12. Smith, G. D., Swenson, D. C., Dodson, E. J., Dodson, G. G., and Reynolds, C. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7093−7097.
13. Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Reynolds, C. D., Smith, G. D., Sparks, C., and Swenson, D. (1989) *Nature 338*, 594−596.
14. Badger, J., Harris, M. R., Reynolds, C. D., Evans, A. C., Dodson, E. J., Dodson, G. G., and North, A. C. T. (1991) *Acta Crystallogr. B47*, 127−136.
15. Weiss, M. A., Nguyen, D. T., Khait, I., Inouye, K., Frank, B. H., Beckage, M., O'Shea, E., Shoelson, S. E., Karplus, M., and Neuringer, L. J. (1989) *Biochemistry 28*, 9855−9873.
16. Hua, Q., and Weiss, M. A. (1990) *Biochemistry 29*, 10545−10555.
17. Kline, A. D., and Justice, R. M. (1990) *Biochemistry 29*, 2906−2913.
18. Boelens, R., Ganadu, M. L., Verheyden, P., and Kaptein, R. (1990) *Eur. J. Biochem. 191*, 147−153
19. Hua, Q. H., Shoelson, S. E., Kochoyan, M., and Weiss, M. A. (1991) *Nature 354*, 238−241.
20. Weiss, M. A., Hua, Q.-X., Lynch, C. S., Frank, B. H., and Shoelson, S. E. (1991) *Biochemistry 30*, 7373−7389.
21. Knegtel, R. M. A., Boelens, R., Ganadu, M. L., and Kaptein, R. (1991) *Eur. J. Biochem. 202*, 447−458.
22. Jorgensen, A. M. M., Kristensen, S. M., Led, J. J., and Balschmidt, P. (1992) *J. Mol. Biol. 227*, 1146−1163.
23. Jorgensen, A. M. M., Olsen, H. B., Balschmidt, P., and Led, J. J. (1996) *J. Mol. Biol. 257*, 684−699.
24. Kurapkat, G., De Wolf, E., Grotzinger, J., and Wollmer, A. (1997) *Protein Sci. 6*, 580−587.
25. Ludvigsen, S., Olsen, H. B., and Kaarsholm, N. C. (1998) *J. Mol. Biol. 279*, 1−7.
26. Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J., and Gammeltoft, S. (1976) *Nature 259*, 369−373.
27. Dodson, E. J., Dodson, G. G., Hubbard, R. E., and Reynolds, C. D. (1983) *Biopolymers 22*, 281−291.
28. Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Bing, X., and Markussen, J. (1991) *J. Mol. Biol. 220*, 425−433.
29. Tager, H. S. (1990) in *Handbook of Experimental Pharmacology* (Cuatrecasas, P., and Jacobs, S., Eds.) Vol. 92, pp 41−64, Springer-Verlag, Berlin.
30. Smith, G. D., and Dodson, G. G. (1992) *Proteins: Struct., Funct., Genet. 14*, 401−408.
31. Renscheidt, H., Strassburger, W., Glatter, U., Wollmer, A., Dodson, G. G., and Mercola, D. A. (1984) *Eur. J. Biochem. 142*, 7−14.
32. Kaarsholm, N. C., Ko, H.-C., and Dunn, M. F. (1989) *Biochemistry 28*, 4427−4435.
33. Roy, M., Brader, M. L., Lee, R. W.-K., Kaarsholm, N. C., Hansen, J. F., and Dunn, M. F. (1989) *J. Biol. Chem. 264*, 19081−19085.
34. Thomas, B., and Wollmer, A. (1989) *Biol. Chem. Hoppe-Seyler 370*, 1235−1244.
35. Brange, J., Ribel, U., Hansen, J. F., Dodson, G., Hansen, M. T., Havelund, S., Melberg, S. G., Norris, F., Norris, K., Snel, L., Sorensen, A. R., and Voigt, H. O. (1988) *Nature 333*, 679−682.
36. Schwartz, G. P., Burke, G. T., and Katsoyannis, P. G. (1981) *Int. J. Pept. Protein Res. 17*, 243−255.
37. Hu, S.-Q., Burke, G. T., Schwartz, G. P., Federigos, N., Ross, J. B. A., and Katsoyannis, P. G. (1993) *Biochemistry 32*, 2631−2635.
38. Wang, Q.-G., Feng, Y.-M., and Zhang, Y.-S. (1996) *Biochem., Mol. Biol. Int. 39*, 1245−1254.
39. Bucherer, H. T., and Lieb, V. A. (1934) *J. Prakt. Chem. (Leipzig) 141*, 5.
40. Bucherer, H. T., and Steiner, W. (1934) *J. Prakt. Chem. (Leipzig) 140*, 291.
41. Weygand, F., and Geiger, R. (1956) *Chem. Ber. 89*, 647.
42. Moroder, L., Hallett, S., Wunsch, E., Keller, O., and Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651−1653.
43. Nakagawa, S. H., and Tager, H. S. (1991) *J. Biol. Chem. 266*, 11502−11509.
44. Sarin, V. K., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147−157.
45. Bailey, J. L., and Cole, R. D. (1959) *J. Biol. Chem. 234*, 1733−1739.
46. Katsoyannis, P. G., Tometsko, A., Zalut, C., Johnson, S., and Trakatellis, A. C. (1967) *Biochemistry 6*, 2635−2642.
47. Chance, R. E., Hoffman, J. A., Kroef, E. P., Johnson, M. G., Schirmer, E. W., Bromer, W. W., Ross, M. J., and Wetzel, R. (1981) in *Peptides: Synthesis, Structure and Function; Proceedings of the Seventh American Peptide Symposium* (Rich, D. H., and Gross, E., Eds.) pp 721−728, Pierce Chemical Co., Rockford, IL.
48. Grau, U. (1985) *Diabetes 34*, 1174−1180.
49. Morihara, K., Ueno, Y., and Sakina, K. (1986) *Biochem. J. 240*, 803−810.
50. Kubiak, T., and Cowburn, D. (1986) *Int. J. Pept. Protein Res. 27*, 514−521.
51. Bonnevie-Nielsen, V., Polonsky, K. S., Jaspan, J. J., Rubenstein, A. H., Schwartz, T., and Tager, H. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2167−2171.
52. Nakagawa, S. H., and Tager, H. S. (1986) *J. Biol. Chem. 261*, 7332−7341.
53. Nakagawa, S. H., and Tager, H. S. (1987) *J. Biol. Chem. 262*, 12054−12058.
54. Mirmira, R. G., and Tager, H. S. (1989) *J. Biol. Chem. 264*, 6349−6354.
55. Goldman, J., and Carpenter, F. H. (1974) *Biochemistry 13*, 4566−4574.
56. Wood, S. P., Blundell, T. L., Wollmer, A., Lazarus, N. R., and Neville, W. J. (1975) *Eur. J. Biochem. 55*, 531−542.
57. Roy, M., Lee, R. W. K., Brange, J., and Dunn, M. F. (1990) *J. Biol. Chem. 265*, 5448−5452.
58. Hua, Q. X., Jia, W., Frank, B. H., and Weiss, M. A. (1993) *J. Mol. Biol. 230*, 387−394.
59. Hua, Q. X., Shoelson, S. E., Inouye, K., and Weiss, M. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 582−586.
60. Ludvigsen, S., Roy, M., Thogersen, H., and Kaarsholm, N. C. (1994) *Biochemistry 33*, 7998−8006.
61. Olsen, H. B., Ludvigsen, S., and Kaarsholm, N. C. (1996) *Biochemistry 35*, 8836−8845.
62. Cornish, V. W., Kaplan, M. I., Veenstra, D. L., Kollman, P. A., and Schultz, P. G. (1994) *Biochemistry 33*, 12022−12031.
63. Nakagawa, S. H., and Tager, H. S. (1992) *Biochemistry 31*, 3204−3214.
64. Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Mizoguchi, A., Fujiwara, Y., Suzuki, A., Takahashi, S.

Y., and Ishizaki, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5840−5843.
65. Chan, S. J., Cao, Q.-P., and Steiner, D. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9319−9323.
66. Nagata, K., Hatanaka, H., Kohda, D., Kataoka, H., Nagasawa, H., Isogai, A., Ishizaki, H., Suzuki, A., and Inagaki, F. (1995) *J. Mol. Biol. 253*, 749−758.
67. Katsoyannis, P. G., Ginos, J., Schwartz, G. P., and Cosmatos, A. (1974) *J. Chem. Soc., Perkin Trans. I*, 1311−1317.
68. Insulin Research Groups (1974) *Sci. Sin. 17*, 779−792.
69. Hua, Q., and Weiss, M. A. (1991) *Biochemistry 30*, 5505−5515.
70. Fischer, W. H., Saunders, D., Brandenburg, D., Wollmer, A., and Zahn, H. (1985) *Biol. Chem. Hoppe-Seyler 366*, 521−525.
71. Nakagawa, S. H., and Tager, H. S. (1993) *Biochemistry 32*, 7237−7243.
72. Nakagawa, S. H., Johansen, N. L., Madsen, K., Schwartz, T. W., and Tager, H. S. (1993) *Int. J. Pept. Protein Res. 42*, 578−584.

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