The A-chain of Insulin Contacts the Insert Domain of the Insulin Receptor

PHOTO-CROSS-LINKING AND MUTAGENESIS OF A DIABETES-RELATED CREVICE

The contribution of the insulin A-chain to receptor binding is investigated by photo-cross-linking and nonstandard mutagenesis. Studies focus on the role of ValA3, which projects within a crevice between the A- and B-chains. Engineered receptor α-subunits containing specific protease sites (“midi-receptors”) are employed to map the site of photo-cross-linking by an analog containing a photoactivatable A3 side chain (para-azido-Phe (Pap)). The probe cross-links to a C-terminal peptide (residues 703–719 of the receptor A isoform, KTFEDYLHVNFVFPRPS) containing side chains critical for hormone binding (underlined); the corresponding sequence of the holoreceptor was shown previously to cross-link to a PapB25-insulin analog. Because Pap is larger than Val and so may protrude beyond the A3-associated crevice, we investigated analogs containing A3 substitutions comparable in size to Val as follows: Thr, allo-Thr, and α-aminobutyric acid (Abα). Substitutions were introduced within an engineered monomer. Whereas previous studies of smaller substitutions (GlyA3 and SerA3) encountered nonlocal distortions, NMR structures of the present analogs are similar to wild-type insulin; the variant side chains are accommodated within a native-like crevice with minimal distortion. Receptor binding activities of AbαA3 and allo-ThrA3 analogs are reduced at least 10-fold; the activity of ThrA3-DKP-insulin is reduced 5-fold. The hormone-receptor interface is presumably destabilized either by a packing defect (AbαA3) or by altered polarity (allo-ThrA3 and ThrA3). Our results provide evidence that ValA3, a site of mutation causing diabetes mellitus, contacts the insert domain-derived tail of the α-subunit in a hormone-receptor complex.

The A-chain of insulin contacts the insert domain of its receptor. This contact is mediated by ValA3, which projects within a crevice between the A- and B-chains. Engineered receptor α-subunits containing specific protease sites (“midi-receptors”) are employed to map the site of photo-cross-linking by a PAP-containing analog. Because PAP is larger than Val and so may protrude beyond the A3-associated crevice, we investigated analogs containing A3 substitutions comparable in size to Val as follows: Thr, allo-Thr, and α-aminobutyric acid (Abα). Substitutions were introduced within an engineered monomer. Whereas previous studies of smaller substitutions (GlyA3 and SerA3) encountered nonlocal distortions, NMR structures of the present analogs are similar to wild-type insulin; the variant side chains are accommodated within a native-like crevice with minimal distortion. Receptor binding activities of AbαA3 and allo-ThrA3 analogs are reduced at least 10-fold; the activity of ThrA3-DKP-insulin is reduced 5-fold. The hormone-receptor interface is presumably destabilized either by a packing defect (AbαA3) or by altered polarity (allo-ThrA3 and ThrA3). Our results provide evidence that ValA3, a site of mutation causing diabetes mellitus, contacts the insert domain-derived tail of the α-subunit in a hormone-receptor complex.

The atomic coordinates and structure factors (code 2jum, 2juu, 2juv) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/). Chemical shifts have been deposited in the BioMagResBank with accession numbers BMRB-15450, BMRB-15454, and BMRB-15455.

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Binding of insulin to its receptor is central to the hormonal control of metabolism. Although recent crystallographic studies have provided insights into the structure of the free receptor ectodomain (1), mechanisms underlying hormone-receptor recognition remain elusive. Insulin is a small globular protein containing two chains, A (21 residues) and B (30 residues), derived from proinsulin (2). In the pancreatic β-cell insulin is stored as Zn2+-stabilized hexamers within secretory granules (2). The hexamers dissociate on secretion into the portal circulation, enabling the hormone to function as a Zn2+-free monomer. In this study we investigate structure-function relationships at position ValA3, a site of mutation associated with diabetes mellitus (3) located within a crevice between A- and B-chains (Fig. 1A).

Diabetes-associated mutations have been identified at three invariant sites as follows: ValA3 → LeuA3 (insulin Wakayama), PheB24 → Ser (insulin Los Angeles), and PheB25 → Leu (insulin Chicago). These variants exhibit impaired receptor binding but to different extents (4). The most severely affected is LeuA3-insulin, whose receptor binding is reduced by 500-fold. Such a marked impairment, unusual among mutant insulins (5–7), suggests either nonlocal structural perturbations distorting multiple contacts or steric clash at a rigid facet of the hormone-receptor interface. Activities of LeuB24-insulin and SerB24-insulin are reduced by 100- and 6-fold, respectively. Whereas the low affinity of LeuB25-insulin is because of the nonplanarity of the variant side chain at the receptor interface (8–11), structure-activity relationships at B24 are not readily rationalized (8, 12, 13). Substitution of residues A3, B24, or B25 by a photoac-
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FIGURE 1. Structure of insulin and the A3-related crevice. A, left, ribbon representation of a T-state insulin molecule (PDB code 4INS); the methyl groups at ValA3 are shown as orange or yellow balls. The A-chain is light blue, and the B-chain dark blue. Right, surface representation of A3-related crevice in native insulin (stereo) highlighting the positions of the ValA3 methyl groups in a crevice between chains. B, left, corresponding molecular model in which ValA3 has been substituted by para-azido-Phe (magenta with the para-azido group represented as a red ball). Center and right, protein surfaces indicating how Pap would protrude from the A3-related crevice as viewed in the same orientation (center) or following a 40° rotation about an axis indicated by the arrow (right). C, schematic models of wild-type and variant side chains in the A3-related crevice: left to right, Val, Aba, Thr, and allo-Thr. The A- and B-chains are indicated by light and dark blue; methyl groups are likewise color-coded in accord with A. The variant side chains are similar in volume and configuration to ValA3 without predicted protrusion from the crevice.

activable amino acid derivative in each case enables efficient and specific photo-cross-linking to the IR (11, 14, 15), providing support for a potential role in receptor binding.

Introduction of photoactivable amino acids at specific sites in a protein by chemical synthesis provides a powerful method to map interaction surfaces (16). A limitation can arise, however, if the photo-probe is significantly larger than the native side chain it replaces, as in such cases the probe may extend beyond the native contact surface (17). Thus, although para-azido-Phe (Pap) is similar in size to Phe or Tyr and so provides a commensurate probe at aromatic sites in insulin (such as TyrB16, PheB24, and PheB25) (14, 18, 19), extension of this strategy to sites containing smaller side chains may be confounded by probe-dependent non-native interactions. Substitution of ValA3 by Pap, for example, is predicted to alter the surface topography of insulin; modeling suggests that the photo-probe protrudes beyond the A3-related crevice (Fig. 1B). Whereas the side chain of ValA3 is contained within this crevice (Fig. 1A), the azido group of PapA3 (red ball in Fig. 1B) is predicted to project 3–5 Å beyond the hormone surface. An analogous limitation can arise in structure-activity studies of analogs containing small-to-large substitutions. Because LeuA3 protrudes beyond the molecular dimensions of ValA3, for example, the very low activity of insulin could reflect steric clash at the hormone-receptor interface even if ValA3 itself were not directly engaged (Supplemental Material). This possibility is not excluded by the native-like crystal structure of LeuA3-insulin as a zinc-stabilized hexamer (20). Conversely, nonlocal structural perturbations may confound use of large-to-small substitutions as exemplified by segmental destabilization of the A1–A8 α-helix on substitution of ValA3 by Gly or Ser (21, 22).

We describe here the contribution of ValA3 to the structure, stability, and function of insulin. The two methyl groups of ValA3 line the floor of an inter-chain crevice (red and yellow balls in Fig. 1A). Structure-activity relationships are first investigated through studies of insulin analogs containing A3 side chains similar in size to Val as follows: α-aminobutyric acid (Aba), Thr, and allo-Thr (Fig. 2). Although none of these side chains would be predicted (in the absence of nonlocal distortions) to interfere with binding, the data are consistent with a significant role for ValA3 in insulin-receptor interactions.

The abbreviations used are: Pap, para-azido-phenylalanine; Aba, α-aminobutyric acid; DG, distance geometry; DRK-insulin, insulin analog containing three substitutions in the B-chain (Asp10, Lys28, and Pro29); DTT, dithiothreitol; 125I-TyrA14-labeled insulin, insulin labeled with 125I at TyrA14; IR, insulin receptor; MS, mass spectrometry; NOE, nuclear Overhauser enhancement; Pmp, para-aminophenylalanine; NOESY, NOE spectroscopy; RMD, restrained molecular dynamics; TEV, tobacco etch virus; PDB, Protein Data Bank; ID, insert domain; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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changes (as in GlyA3 or SerA3 analogs; see Refs. 21, 22). Despite side chains (as in LeuA3-insulin) or transmitted conformational change to protrude from the crevice (Fig. 1C), on engagement with the IR the variant side chains might perturb the packing or polarity of the interface. To address these issues, the substitutions were introduced within an engineered monomer of high activity (DKP-insulin; see Ref. 23) to facilitate spectroscopic studies without confounding effects because of self-assembly. Although none of these substitutions perturbed the efficiency of disulfide pairing in insulin chain combination, each analog exhibits lower thermodynamic stability, presumably because of altered packing or solvation of the variant side chains.1H NMR studies nonetheless demonstrate retention of a native-like A3 crevice without protrusion of the substituted side chains (as in LeuA3-insulin) or transmitted conformational changes (as in GlyA3 or SerA3 analogs; see Refs. 21, 22). Despite such structural similarities, receptor binding activities are lower than that of the parent monomer (DKP-insulin) but higher than that of LeuA3-insulin (24). These studies suggest that ValA3 functions directly in receptor binding.

The second part of this study focuses on photo-cross-linking. Because our initial studies of isosteric analogs suggested that ValA3 directly contacts the IR, we sought to map the site of photo-cross-linking between PapA3-DKP-insulin and the IR α-subunit. Through the use of engineered midi-receptors (25) containing specific protease sites (26), evidence is presented that PapA3 contacts a 17-residue segment at the extreme C-terminus of the α-subunit. Derived from the insert domain (ID), the same segment (residues 704–718 in the IR B-isoform; designated αCT) was shown previously to be a site of photo-cross-linking between a truncated PapB25 analog of insulin and the holoreceptor (14). This segment contains several conserved aromatic and aliphatic residues and so may provide a nonpolar docking site for ValA3 and PheB25. The importance of αCT in hormone binding has been demonstrated by Ala-scanning mutagenesis of the holoreceptor (27, 28).

This study thus brings together total chemical synthesis with receptor engineering to define a key feature of the hormone-receptor complex. Nonstandard mutagenesis of insulin extends the repertoire of conventional site-directed mutagenesis, whereas construction of model midi-receptors facilitates biochemical characterization of photo-cross-linked complexes. These complementary approaches highlight the contribution of the A-chain to the function of insulin at a site of clinical mutation causing diabetes mellitus. To our knowledge, these findings represent the first characterization of a contact between the insulin A-chain and a cognate binding element of the insulin receptor.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Insulin Analogs**—The general protocol for solid-phase synthesis is as described previously (29). In brief, 4-methylbenzhydrylamine resin (0.6 mmol amine/g; Bachem, Inc.) was used as solid support for synthesis of A-chain analogs; N-tert-butoxycarbonyl-O-benzyl-threonine-PAM resin (0.56 mmol/g; Bachem, Inc.) was used for synthesis of a B-chain analog containing substitutions HisB10→Asp, ProB28→Lys, and LysB29→Pro (designated the DKP B-chain). A manual double-coupling protocol was followed (30). The p-NH2 moiety of para-amino-Phe (Pmp) was protected by a 2-chlorobenzyloxy-carbonyl group, which is stable during peptide synthesis. The PmpA3-A-chain also contained a biotin group attached to the ε-amino group of d-LysA1, a well tolerated substitution for GlyA1 (20, 31). Chain recombination employed an S-sulfonated DKP-B-chain and S-sulfonated variant A-chains (~2:1 by weight) in 0.1 M glycine (pH 10.6) in the presence of dithiothreitol (30). Analogs were isolated from the combination mixture (32) and purified as described (33) with essentially native yields. Electrospray mass spectrometry (MS) in each case gave expected values and in the photoactivable derivative verified conversion of Pmp to Pap (14). As a control, a photoactivable analog was similarly prepared containing PapB16, which contacts the N-terminal L1 domain of the IR α-subunit (19); this analog contains biotin linked to the B1 α-amino group. Purities were in each case >98% as evaluated by analytical reverse-phase high performance liquid chromatography. MS revealed no anomalous molecular masses.

**CD Spectroscopy**—CD spectra were obtained using an Aviv spectropolarimeter (34). Samples contained 25–50 μM DKP-insulin or A3 analogs in 50 mM potassium phosphate (pH 7.4); samples were diluted to 5 μM for denaturation studies.

**Thermodynamic Modeling**—Guanidine denaturation data were fitted by nonlinear least squares to a two-state model as described (35). In brief, CD data θ(x), where x indicates the
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centration of denaturant, were fitted by a nonlinear least squares program according to Equation 1,

\[ \theta(x) = \frac{\theta_A + \theta_B e^{-(\Delta G_A - m_B - m_x)/RT}}{1 + e^{-(\Delta G_B - m_A - m_x)/RT}} \]  

(Eq. 1)

where \( x \) is the concentration of guanidine and where \( \theta_A \) and \( \theta_B \) are base-line values in the native and unfolded states. Base lines were approximated by pre- and post-transition lines \( \theta_A(x) = \theta_A^{12O} + m_Ax \) and \( \theta_B(x) = \theta_B^{12O} + m_Bx \) (36). The \( m \) values obtained in fitting the variant unfolding transitions are lower than the \( m \) value obtained in fitting the wild-type unfolding curve (Supplemental Material). To test whether this difference and apparent change in curve (Supplemental Material) might be a consequence of an inability to measure the CD signal from the fully unfolded state, simulations were performed in which the data were extrapolated to plateau CD values at higher concentrations of guanidine; essentially identical estimates of \( \Delta G_A \) and \( m \) were obtained.

NMR Spectroscopy—\(^1\)H NMR spectra were obtained at 600 and 800 MHz under the following three conditions: (i) in 50 mM potassium phosphate (pH 7.0), (ii) in 20% deuterated acid (pH 1.9) at 25 °C, and (iii) in D$_2$O at pH 8.0 and 32 °C as described (37, 38). This range of pH and temperature conditions enables resonances that overlap in one spectrum to be resolved in another. In addition, use of acidic pH in a cosolvent facilitates analysis of amide resonances, some of which are resolved in another. In addition, use of acidic pH in a cosolvent enables resonances that overlap in one spectrum to be resolved in another. In addition, use of acidic pH in a cosolvent facilitates analysis of amide resonances, some of which are reso

Molecular Modeling—Structures were calculated by distance geometry and restrained molecular dynamics (DG/RMD) using X-PLOR (39) as described (19). Geometries were monitored with PROCHECK (40); solvent-accessible areas were obtained by using X-PLOR, and molecular cavities were calculated by using SURFNET (41). Rigid-body models of Pap^A^3^, and Leu^A^3^ insulin analogs were built using InsightII (Accelrys Inc., San Diego) and X-PLOR based on the structure of a T-state crystallographic protomer (2-Zn molecule 1; Protein Data Bank code 4INS).

Biological Assays—Two types of receptor-binding assays were performed. Determinations were performed with 3–6 replicates; values are reported as mean ± S.D. (Table 1). The percentage of tracer bound in the absence of competing ligand was less than 15% to avoid ligand-depletion artifacts. (i) Activities of Thr^A^3^–DKP-insulin and DKP-insulin were first measured relative to 125I-Tyr^A^14^–labeled human insulin using a human placental membrane preparation containing the IR (column 2 in Table 1) (33, 42, 43). This assay is limited by underestimation of the affinities of super-active analogs (such as DKP-insulin (33, 44)) but is included to allow comparison with previous studies of related analogs using this assay (45–47). Relative activity is defined as the ratio of analog to human insulin required to displace 50% of specifically bound 125I-insulin. (ii) To evaluate Aβ^A^3^–, Thr^A^3^, and allo-Thr^A^3^–DKP-insulin (column 3 in Table 1), a cell culture-based assay was employed relative to 125I-Tyr^A^14^–labeled DKP-insulin as described (44, 48). Use of the parent monomeric analog as tracer circumvents uncertainties because of the base-line activity of DKP-insulin. Relative activity is defined as ratio of dissociation constants obtained by nonlinear regression curve fitting. IM-9 human lymphocytes were grown in suspension as described (49) in RPMI media supplemented with 2 mM L-glutamine, 10% fetal calf serum, and 100 units/ml of penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO$_2$ humidified atmosphere. Procedures are described elsewhere (44). In both types of assays data were corrected for nonspecific binding (amount of radioactivity remaining membrane-associated in the presence of 1 μM human insulin). To evaluate the negative cooperativity, a cell culture-based assay was employed (44, 50). A dose-response curve for negative cooperativity was established by measuring extent of dissociation of prebound labeled insulin by increasing concentrations of analogs, after allowing 30 min for dissociation in a 40-fold dilution (50).

Engineered Midi-receptors—An IR-derived midi-receptor (25, 51) was constructed by PCR amplification and ligation of DNA fragments derived from human IR cDNA (isoform B). This α-subunit construct encodes the N-terminal IR signal peptide, domains L1-CR-L2-FnIII-1 (residues 1–601), and the C-terminal insert domain-derived segment (650–719) (25). The C-terminus was extended to contain a Myc epitope (EQKLI-SEEDL), permitting Western blotting by anti-Myc antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To facilitate mapping of photoproducts, two such constructs were designed, each containing specific internal cleavage sites for a protease derived from the tobacco etch virus (TEV; consensus recognition site ENLYFQ ↓ G with cleavage after Q); the wild-type IR α-subunit lacks consensus TEV cleavage sites. (i) Midi-receptor T1 contains a single TEV site inserted following residue 601; cleavage thus liberates a C-terminal polypeptide of 12 kDa (81 residues (IR-(650–719) plus 10-residue c-Myc tag) plus the mass of the photo-cross-linked insulin A- or B-chain. (ii) Midi-receptor T2 contains two consecutive TEV sites inserted after residue 702; complete cleavage in this case liberates a C-terminal peptide of mass 3 kDa (28 residues; IR-(703–719) plus c-Myc tag) plus the mass of the photo-cross-linked insulin chain. cDNA inserts were verified by DNA sequencing.

Expression of Midi-receptors—The above cDNAs were inserted into mammalian expression vector pcDNA 3.1 (Invitrogen). Constructs were transiently expressed by transfection into 293H cells using Lipofectamine 2000 reagent (Invitrogen). Media were collected 72 and 144 h post-transfection and concentrated 7-fold by ultrafiltration (Amicon Ultra 15; Millipore). The concentrated protein solution was adjusted to contain 50 mM Heps (pH 7.4). After addition of sodium azide to a final concentration of 0.02%, the recombinant receptor fragments were stored at 4 °C. Binding of 125I-Tyr^A^14^-insu-

DNA sequencing confirmed the plasmids contained the canonical human IR sequence (89) except for coding mutations H144Y and K224R due to allelic variations present in our cloned IR cDNA (sequences available upon request).
lin by midi-receptors was assayed in duplicate by polyethylene glycol precipitation as described (52).

Photo-cross-linking—Purified IR ectodomain or midi-receptors were incubated overnight with either 125I-labeled or biotinylated photodegradable insulin derivatives at a hormone concentration of 100–200 nM at 4 °C with gentle shaking. Solutions containing the insulin analogs were transferred to a Costar assay plate (Corning Glass) for UV irradiation (20 s at 254 nm) using a Mineralight lamp (model UVG-54, UVP, Upland, CA); the distance from the source was 1 cm. For analysis of ectodomain photoproducts after UV irradiation, covalent complexes were reduced with 2% mercaptoethanol or 100 mM dithiothreitol (DTT) and analyzed by SDS-PAGE.

Mapping of Hormone-Receptor Photoproducts—To characterize midi-receptor complexes, products were immunoprecipitated with anti-Myc antibody (c-Myc (A-14); Santa Cruz Biotechnology) and eluted with a free Myc peptide. 1 unit of TEV protease (Invitrogen) was then added to the eluate, and the digestion allowed to proceed overnight at 30 °C; TEV-digested products were resolved by SDS-PAGE. To detect protease-digested 125I-labeled photoadducts, gels were fixed for 20 min in 10% acetic acid and 25% isopropyl alcohol (v/v), dried onto Whatman 3MM paper, and exposed to x-ray film (Kodak Biomax MS) or a phosphor screen (Packard Cyclone). For detection of protease-digested biotinylated photoadducts, proteins were blotted onto a nitrocellulose membrane and probed with NeutrAvidin (Pierce) or a polyclonal antiserum directed against the N-terminal 20 residues of the α-subunit (N-20, Cruz Biotechnology; herein designated IRαN).

RESULTS

Our study has two parts. Structure-activity relationships are first described among three analogs of DKP-insulin containing isosteric (Thr and allo-Thr) or smaller (Aba) substitutions at A3. Photo-cross-linking studies of PapA3-DKP-insulin to engineered midi-receptor fragments of the IR α-subunit are then described. The midi-receptors, whose hormone-binding properties are similar to those of the IR ectodomain (25, 51), contain specific TEV protease sites to facilitate mapping of photo-cross-linking sites.

Structure-Activity Relationships at Position A3—Receptor-binding affinities of ThrA3-DKP-insulin, allo-ThrA3-insulin, and AbaA3-DKP-insulin are each lower than that of DKP-insulin (Table 1). Effects of AbaA3 and allo-ThrA3 are consistent with previous studies of these substitutions in a native B-chain context (53, 54), validating use of the monomeric DKP template. The activity of AbaA3-DKP-insulin (7 ± 1% relative to DKP-insulin) is significantly less than that of the ThrA3-DKP-insulin (22 ± 3%) but is similar to that of allo-ThrA3-DKP-insulin (5 ± 1%). No disproportionate changes in negative cooperativity were observed among the A3 analogs.

DKP-insulin and its A3 analogs exhibit similar overall secondary structures as inferred from far-UV CD spectra (Supplemental Material). Perturbations in α-helix content, characteristic of GlyA3 and SerA3 analogs (Supplemental Material (21, 22), are thus not observed. Studies of thermodynamic stability by guanidine denaturation, monitored by CD, indicate that each analog exhibits a cooperative and apparent two-state transition but with leftward shift, leading to lower values of the concentration of guanidine at which the protein is half-unfolded (C_mic) (Table 1). Inferred values of the free energy of unfolding (ΔG_u) are lower than that of DKP-insulin (4.9 ± 0.1 kcal/mol) as follows: allo-ThrA3-insulin (4.1 ± 0.1 kcal/mol), AbaA3-DKP-insulin (4.0 ± 0.1 kcal/mol), and ThrA3-DKP-insulin (3.6 ± 0.1 kcal/mol). Decrements in stability (ΔG_u) are thus 0.8 ± 0.2 (allo-ThrA3), 0.9 ± 0.2 (AbaA3), and 1.3 ± 0.2 kcal/mol (ThrA3). These decrements presumably reflect, at least in part, less favorable interactions of the variant side chains in the A3-related crevice (see “Discussion”).

To probe possible conformational changes, structures were determined by two-dimensional 1H NMR spectroscopy in relation to the parent monomer (23). 1H NMR spectra in each case exhibit chemical shift dispersion similar to that of DKP-insulin (Supplemental Material). Although patterns of chemical shifts are generally similar (data not shown), small changes are in each
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(Fig. 3A) and to the ortho resonance of TyrB26 (cross-peak b). Attenuation of related NOEs from these aromatic protons to the γ1-methyl group of ValA3 indicates that conformation of the wild-type side chain is well defined, constrained (as in crystal structures) by its packing within the inter-chain crevice. Similarly, comparison of ThrA3- and allo-ThrA3 analogs demonstrates that inversion of β-carbon chirality is associated with corresponding “inversion” of specific long range NOEs between the single remaining A3 γ-methyl resonance and the aromatic resonances of TyrA19 and TyrB26 (corresponding resonances a, b, and c in Fig. 3, B and C). These NOEs, which are in accord with contacts made in wild-type insulin by the side chain of ValA3, aid in determining the respective side chain conformations of ThrA3- and allo-ThrA3. Analogous A3-related contacts are observed in the NOESY spectrum of AbaA3-DKP-insulin (cross-peaks a and b in Fig. 3D). A summary of NOEs involving the wild-type or variant A3 side chains is given in supplemental Table S2; reference distances are provided based on the crystal structure of a variant zinc KP-insulin hexamer (i.e. bearing substitutions ProB28 → Lys, and

Patterns of inter-residue NOEs among the analogs closely resemble the pattern of NOEs in DKP-insulin, including framework contacts between conserved side chains (PheB24/LeuB15, TyrB26/ValB12, and TyrA19/IleA2). In two-dimensional NMR spectra, the γ1- and γ2-methyl resonances of ValA3 are resolved between 0.8 and 0.9 ppm; NOEs are observed selectively from the γ1-methyl group to the meta ring resonance of TyrA19 (cross-peak a in Fig. 3A) and to the ortho resonance of TyrB26 (cross-peak b). Attenuation of related NOEs from these aromatic protons to the γ1-methyl group of ValA3 indicates that conformation of the wild-type side chain is well defined, constrained (as in crystal structures) by its packing within the inter-chain crevice. Similarly, comparison of ThrA3- and allo-ThrA3 analogs demonstrates that inversion of β-carbon chirality is associated with corresponding “inversion” of specific long range NOEs between the single remaining A3 γ-methyl resonance and the aromatic resonances of TyrA19 and TyrB26 (corresponding resonances a, b, and c in Fig. 3, B and C). These NOEs, which are in accord with contacts made in wild-type insulin by the side chain of ValA3, aid in determining the respective side chain conformations of ThrA3- and allo-ThrA3. Analogous A3-related contacts are observed in the NOESY spectrum of AbaA3-DKP-insulin (cross-peaks a and b in Fig. 3D). A summary of NOEs involving the wild-type or variant A3 side chains is given in supplemental Table S2; reference distances are provided based on the crystal structure of a variant zinc KP-insulin hexamer (i.e. bearing substitutions ProB28 → Lys, and

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packing schemes of Thr$^{A3}$ and allo-Thr$^{A3}$ are in accord with stereospecific differences in their thermodynamic stabilities (Table 1); although each is less stable than DKP-insulin, the greater stability of the allo-Thr$^{A3}$ analog relative to Thr$^{A3}$-DKP-insulin may reflect an unfavorable electrostatic interaction between the $\beta$-oxygen of Thr$^{A3}$ and the $\pi$ electron cloud of Tyr$^{B26}$, which appears to be relieved on chiral inversion.

The absence of significant non-native A3-related features is consistent with (a) maintenance of native-like overall structures and (b) the similar shapes and volumes of Val, Thr, allo-Thr, and Aba. Side chain-specific solvent accessibilities were calculated and compared with those of native insulin (Supplemental Material). As expected based on maintenance of native-like overall folds, the A3 main chain remains inaccessible in each analog, whereas side chain accessibilities are low as in DKP-insulin. Thr$^{A3}$ and Aba$^{A3}$ are associated with a decrease in the precision of the adjoining side chain of GluA4 (Fig. 5, C and D); this is because of attenuation of inter-residue NOEs between H$_9$ of GluA4 and H$_8$ of ThrA8 in the two analogs. Because GluA4 itself does not make an important contribution to receptor binding (5, 57), decreased receptor binding by the present analogs is likely to reflect a direct A3-related perturbation of the hormone-receptor interface (see “Discussion”).

Although the structure of insulin is proposed to change on receptor binding (12, 58, 59), the proximity of the A3-related crevice to the classical receptor-binding surface (A8, B16, and B24–B26) and a nonclassical second site in a T-state protomer (A13 and B17 (60)) is shown in Fig. 6C.

Photo-cross-linking Studies—The IR is a modular protein containing two $\alpha$- and two $\beta$-subunits linked by disulfide bonds (Fig. 6, A and B). The $\alpha$-subunits bind insulin, whereas the $\beta$-subunits contain the cytoplasmic tyrosine kinase domain (59). The isolated ectodomain ($\alpha\beta_2$) retains specific insulin binding activity (although with 10-fold lower affinity (61, 62)).

We have shown previously that insulin derivatives containing Pap at positions A3 or B16 exhibit efficient photo-cross-linking to the IR (19, 31). Although the affinity of the reduced affinity of the isolated ectodomain for insulin is presumably because of enhanced flexibility between the two $\alpha\beta_2$ subunits in the absence of tethering by the transmembrane $\alpha$-helices; native affinity is restored on fusion of the ectodomain $\beta_4$ fragment to either a coiled coil (1) or dimeric Fc domains (90).
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FIGURE 6. Modular structure of the IR and putative receptor-binding surface of insulin. A, schematic representation of αβ2 subunits of IR. Domains are distinguished by color: N- and O-glycosylation (gly) sites are indicated by green and blue balls, and residues critical to insulin binding (as inferred from mutagenesis (28, 93)) are indicated by red arrowheads, predominantly in L1 and αCT. B, modular organization of (αβ)2 holoreceptor and truncated receptor ectodomain ((αβ)Δ2). C, molecular CPK surface of insulin (T-state structure; PDB code 4INS). Classical receptor binding residues span the A-chain (A2, A3, A8, and A21; light red arrowheads), predominantly in L1 and αCT (14), and residues critical to insulin binding (as inferred from mutagenesis (28, 93)) are indicated by red arrowheads. D, photo-cross-linking studies of unlabeled insulin (negative control (con)), PapA3-DKP-insulin, and PapB16-DKP-insulin with ectodomain as visualized by NeutrAvidin (NAV) (upper panel) or ILuN antiserum (lower panel). Sites of photo-cross-linking in the ectodomain and holoreceptor have been mapped to a C-terminal 14-kDa deglycosylated fragment (A3 adduct (20)) or the L1 domain (B16 adduct (19)).

para-amo-PheA3-DKP-insulin is reduced by 30-fold relative to insulin (and so the corresponding azido analog would also be expected to exhibit reduced binding), under our experimental conditions the concentrations of the hormone and receptor are >103-fold higher than the wild-type dissociation constant, thus permitting study of even low-affinity insulin analogs.

To characterize further the photo-cross-linking properties of PapA3- and PapB16-insulin derivatives, we employed the isolated ectodomain and engineered midi-receptors. Photo-cross-linking of PapA3 and PapB16 to the isolated ectodomain was first investigated. Following reduction of the covalent insulin-IR complexes by DTT, a photoadduct between the α-subunit and biotin-tagged insulin derivatives can readily be detected by NeutrAvidin (NAV, upper panel of Fig. 6D); the mobility of this band is essentially identical to that of the free α-subunit (molecular mass 110 kDa) as detected by Western blot (designated IRαN, lower panel of Fig. 6D). Extension of our previous domain mapping strategy by tryptic and chymotryptic diges-

9 Independent mapping of the PapA3 photoproduct was limited by the absence of a corresponding antiserum directed against the C-terminal portion of the α-subunit. Preliminary assignment to the ID was suggested by the similarity between the proteolytic digestion patterns of PapA3 and PapB25 photo-cross-linked complexes, yielding a C-terminal 14-kDa polypeptide containing part of FnIII-2 and the α-specific portion of the ID (31). This assignment depended on the assumption that the predominant site of photo-cross-linking by PapB25-DKP-insulin is within αCT (14).

10 The IC50 of mid-receptor T1 was estimated to be 0.11 and 0.15 nM in two independent assays; the IC50 of T2 was 1.8 and 1.9 nM. Decreased insulin binding by T2 may reflect interference by the inserted pair of TEV sites adjacent to αCT. The observed affinities are nonetheless higher than that reported by Kristensen et al. (31) in studies of a mid-receptor lacking engineered TEV sites (K, 4.4 nM).
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Recent progress in the crystallographic analysis of the IR has revealed that the ectodomain forms an inverted V structure (1). Although approximate insulin-binding sites are suggested by the positions of the dimer-related L1 domains, the structure does not contain bound insulin. Modeling of the insulin-bind-

ing site has been limited by the absence of interpretable electron density for the insert domain, presumably because of its disorder in the free receptor (1). Despite considerable efforts, independent crystals of a hormone-receptor complex have not been obtained. In the absence of direct crystallographic information, structure-function relationships in insulin have been inferred by mutagenesis and chemical modification (5, 6). In this study we have focused on the role of Val\(^{A3}\), an invariant residue in the A-chain and site of clinical mutation associated with human diabetes mellitus (63). A related mutation has been identified in human insulin-like growth factor-I causing developmental abnormalities (64).

**Contribution of Val\(^{A3}\) to Structure and Stability**—Val\(^{A3}\) belongs to the N-terminal A-chain \(\alpha\)-helix (residues A1–A8). Among crystal structures of insulin this helix is more variable than the other \(\alpha\)-helices (A12–A18 and B9–B19; T state) both in overall orientation and internal geometry (5). In solution the A1–A8 segment, unlike the other \(\alpha\)-helices, lacks protected amide resonances in \(D_2O\) solution, providing evidence for conformational fluctuations leading to the breakage of main chain hydrogen bonds (65). The A1–A8 sequence (GIVEQCCT) is notable for \(\beta\)-branched amino acids of low intrinsic helical propensity at positions 2 and 3 (Ile\(^{A2}\) and Val\(^{A3}\)) and an unfavorable C-cap residue at position 8 (Thr\(^{A8}\)) (66–68). In accord with the low intrinsic helical propensity of the A1–A8 sequence, this \(\alpha\)-helix undergoes segmental unfolding on substitution of Ile\(^{A2}\) by Ala (69) or on pairwise substitution of cystine A6–A11 by Ala or Ser (23, 34). Its helical conformation is stabilized by packing of Ile\(^{A2}\) and Val\(^{A3}\) and by the intra-A-chain disulfide bridge.

The A1–A8 \(\alpha\)-helix orients the side chain of Val\(^{A3}\) to pack within a crevice between A- and B-chains. This crevice is in part nonpolar, but its physicochemical properties are made complex by the aromatic rings of Tyr\(^{A19}\) and Tyr\(^{B26}\) (whose \(\pi\) electron clouds are associated with an asymmetric distribution of partial charges (70)) and by solvation at the mouth of the crevice. The present set of analogs permits the contribution of the A3-related crevice to the stability of insulin to be assessed. Although these analogs each retain native-like overall structures, they exhibit reduced stability (Table 1). The decreased stability of Aba\(^{A3}\)-DKP-insulin is remarkable in view of the increased intrinsic helical propensity of Aba relative to Thr; substitution of a \(\beta\)-branched side chain by an unbranched near-isostere would be expected to enhance stability by \(-0.5\) kcal/mol (71–73), and yet the substitution impairs stability by \(0.9 \pm 0.2\) kcal/mol.\(^{11}\) This decrement highlights the distributed importance of packing efficiency within the A3-related crevice; removal of a \(\gamma\)-methyl group presumably leaves a destabilizing gap in the floor of the crevice, in turn altering its dynamics and solvation.

\(^{11}\) The net instability of the Aba\(^{A3}\) analog (0.9 kcal/mol plus a helical propensity term of \(-0.5\) kcal/mol) is thus more than double that expected based on the change in side chain volume (\(-21–29\) \(\AA^3\), corresponding to a decrease in stability of 0.45–0.64 kcal/mol according to an empirical correlation developed by Matthews and co-workers (22 cal/mol/\(\AA^3\) (91)). The volume of Aba was estimated based on the known volumes (\(V\)) of 20 standard amino acids (92): calculations \(V_{\text{Aba}} = V_{\text{Ala}} + (V_{\text{Ala}} - V_{\text{Val}})\) or \(V_{\text{Aba}} = V_{\text{Gly}} + (V_{\text{Gly}} - V_{\text{Val}})\) yields respective estimates of 118.6 and 140 \(\AA^3\). Given the \(V_{\text{Val}} = 140\) \(\AA^3\), a cavity of size 21–29 \(\AA^3\) is thus predicted by rigid-body modeling on substitution of Val by Aba.

**DISCUSSION**

FIGURE 7. Photo-cross-linking studies of engineered midi-receptors. A and B, midi-receptor constructs used in present study; L1, CR, L2, and FnIII-1 are first four domains of IR \(\alpha\)-subunit, whereas IR segment 703–719 (isoform B) is part of insert domain. TEV protease site was inserted at residue 601 in midi-receptor T1 (A); two consecutive TEV sites were inserted after residue 702 in T2 (B). C, photo-cross-linking studies of Pap\(^{A3}\)-DKP-insulin bound to T1 or T2. Photoproducts were analyzed by SDS-PAGE following immunoprecipitation with anti-Myc antiserum and reduction with DTT. Red asterisks indicate photoadducts (18 and \(-5\) kDa) characteristic of Pap\(^{A3}\) cross-linking to C-terminal TEV fragment; the 650–719 segment in T1 and 703–719 segment in T2. The insulin derivative was labeled with both \(125I\) and biotin; gel at left (10\% Tricine) was visualized by \(125I\)-autoradiography, and gel at right (10–20\% Tris-glycine) was probed by NeutrAvidin (NAv). Minus and plus signs at bottom of gels indicate before (\(-\)) or after (\(+\)) digestion by TEV protease. D, control study of Pap\(^{A3}\)-DKP-insulin relative to A3 derivative. Products of photo-cross-linking to T2 were visualized by autoradiography following SDS-PAGE (8–16\% Tris-glycine) before (\(-\)) and after (\(+\)) TEV digestion. Whereas A3 probe cross-links to C-terminal TEV fragment (red asterisk), Pap\(^{B16}\)-DKP-insulin cross-links to the large N-terminal fragment containing L1-CR-L2-FnIII, domains (\(-110\) kDa).
Evidence for such local dynamic changes in Aba\(^{A3}\)-DKP-insulin is provided by a reduction in the density of the inter-residue NOE network near A3.

Because the intrinsic helical propensities of Thr and allo-Thr are similar to that of Val (66–68), the decreased stabilities of Thr\(^{A3}\)- and allo-Thr\(^{A3}\)-DKP-insulin are also likely to reflect the importance of nonpolar packing in the A3-related crevice as in each analog a penalty would be incurred for complete or partial burial of a polar moiety. The decrements differ in severity, 0.8 ± 0.2 (allo-Thr\(^{A3}\)) versus 1.3 ± 0.2 kcal/mol (Thr\(^{A3}\)). The difference in stability on chiral inversion (\(\Delta\DeltaG_m\) 0.5 ± 0.2) may reflect two factors. First, whereas the β-OH moiety of Thr\(^{A3}\) is completely buried, that of allo-Thr\(^{A3}\) is partially exposed. Thus, Thr\(^{A3}\)-DKP-insulin would be expected to incur a greater hydrophobic transfer free-energy penalty than allo-Thr\(^{A3}\)-DKP-insulin (74). Second, respective orientations of the β-OH group differ relative to the aromatic rings of Tyr\(^{A19}\) and Tyr\(^{B26}\), the internal β-OH moiety of Thr\(^{A3}\) abuts the π cloud of Tyr\(^{B26}\) and is near that of Tyr\(^{A19}\). We speculate that this is an example of how protein stability can be modulated by weakly polar interactions (70). In particular, insulin contains conserved aromatic rings (including tyrosines at A19 and B26) whose position and orientation, maintained in the analogs, provide a geometric framework for packing of neighboring aliphatic side chains. These rings exhibit partial positive charges at their edges and partial negative charges at each face (70, 75, 76).

The relative stabilities of A3 analogs may be influenced by our choice of DKP-insulin as a template. Although this template enables effects of substitutions on a monomer to be distinguished from confounding effects on self-association, one of the DKP substitutions (Pro\(^{B28}\) → Lys) may in principle alter the structure or dynamics of the A3-related crevice. In wild-type insulin the B28 pyrrolidine ring contributes to the nonpolar crevice lining and in turn to the environment of Val\(^{A3}\). Although the methylene chain of Lys\(^{B28}\) in part recapitulates this surface, the uniquely constrained shape of a proline is absent. It would be of future interest to investigate the thermodynamic consequences of A3 substitutions in wild-type insulin, including assessment of any changes in dimerization and higher order assembly. It would be of further interest to test whether crevice packing contributes not only to the stability of insulin once folded but also to the efficiency of folding of proinsulin, a process that might be influenced by the relative stabilities of partially folded conformations (77). Whereas the biosynthesis of insulin Wakayama in the pancreatic β-cell is apparently unperturbed (4), mutations elsewhere in proinsulin that block its folding in the endoplasmic reticulum are associated with permanent neonatal-onset diabetes mellitus (78).

**Contribution of Val\(^{A3}\) to Receptor Binding**—The biological importance of Val\(^{A3}\) is suggested by the clinical association between the Leu\(^{A3}\) variant and diabetes mellitus (insulin Wakayama (24)). Yet the extremely low activity of this variant is anomalous among mutant insulins (5). Whereas substitutions at the classical receptor-binding surface ordinarily impair affinity by 5–100-fold, the activity of Leu\(^{A3}\)-insulin is reduced by 500-fold. Leu\(^{A3}\)-insulin nonetheless retains a native-like crystal structure as an \(R_e\) zinc insulin hexamer (20). Such a marked reduction in binding unaccompanied by structural distortions suggests that protrusion of the larger A3 side chain beyond the A3-related crevice may incur steric clash at the hormone-receptor interface, possibly leading to transmitted perturbations at neighboring receptor contacts. This model left open the possibility that the side chain of Val\(^{A3}\) itself is near the receptor but not directly engaged.

To address this possibility, we have chosen to study A3 substitutions of similar size to Val and hence unlikely to protrude from the crevice. Use of a monomeric template circumvents a structural issue inherent in crystallographic studies of zinc insulin hexamers: that self-assembly may obscure possible conformational perturbations through imposition of a shared framework of subunit interfaces. This issue is pertinent to A3 substitutions as the A3-related crevice adjoins the classical dimer interface. That A3 substitutions may result in nonlocal structural perturbations in a zinc-free monomer is suggested by previous spectroscopic studies of Gly\(^{A3}\) and Ser\(^{A3}\) analogs (21, 22). In each case evidence has been obtained that the A1–A8 helix is distorted. The low activities of these analogs (0.1 and <2%, respectively) may thus reflect disruption of multiple hormone-receptor contacts.

Our results demonstrate that substitution of Val\(^{A3}\) by Aba, Thr, or allo-Thr (unlike substitution by Ser or Gly) is structurally well tolerated, and yet (in accord with past studies (6, 53, 54)) impairs receptor binding. The extent of impairment is 4–20-fold, values typical of mutations at the surface of insulin (5). We envisage that such decreased binding reflects perturbed side chain interactions between A3 and a cognate binding pocket of the IR. Of particular interest is the stereospecific difference in activity between Thr\(^{A3}\)-DKP-insulin (affinity ~20% in the IM-9 lymphocyte assay relative to DKP-insulin) and allo-Thr\(^{A3}\)-DKP-insulin (5%). Although the stability of the allo-Thr\(^{A3}\) analog and the local dynamics of its A3-related crevice are less perturbed than these features of Thr\(^{A3}\)-DKP-insulin (see above), the allo-Thr\(^{A3}\) analog is 4-fold less active than the Thr\(^{A3}\) analog. These results suggest that the pro-S γ-methyl group of Val\(^{A3}\) docks against the nonpolar surface intolerant of a hydroxyl substituent, whereas the pro-R γ-methyl group may reside at a less selective microenvironment. Removal of the offending β-OH moiety from allo-Thr\(^{A3}\)-DKP-insulin (yielding the nonstandard Aba\(^{A3}\) analog) results in only a small improvement in binding (this small difference may in fact be within experimental error); the absence of the polar perturbation may largely be offset by introduction of a destabilizing packing defect analogous to that observed in the free hormone.

Direct engagement of Val\(^{A3}\) at the receptor interface may require a change in conformation of the B-chain to expose the A3-related crevice. Such exposure could be associated with detachment on receptor binding of the overlying C-terminal B-chain β-strand (residues B24–B28 (12, 58)). The detachment model is supported by the anomalous properties of several insulin analogs, including the very low activity of mini-proinsulin, a single-chain analog containing a peptide bond between Lys\(^{B28}\) and Ile\(^{A3}\).
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and Gly\textsuperscript{A1} (58, 79), and the enhanced activities of analogs containing D-amino acids at position B24 (80, 81). Detachment of the C-terminal B-chain \(\beta\)-strand would also expose the side chain of Ile\textsuperscript{A2}, rationalizing the 50-fold loss of activity exhibited by allo-Ile\textsuperscript{A2} analogs (6) despite maintenance of native-like structures (45, 82). Packing of the \(\beta\)-branched side chains of Ile\textsuperscript{A2} and Val\textsuperscript{A3} at the receptor interface may underlie their otherwise puzzling conservation within an \(\alpha\)-helix.

Evidence that Val\textsuperscript{A3} directly contacts the IR motivated efforts to map the site of cross-linking between a photo-reactive probe at A3 and the receptor \(\alpha\)-subunit. Previous studies of a holoreceptor complex established that Pap\textsuperscript{A3}-DKP-insulin photo-cross-links to a putative C-terminal fragment whose molecular mass, estimated by SDS-PAGE following deglycosylation, is 14 kDa (\(\sim110–130\) amino acids). Assignment as a C-terminal fragment was based on a correspondence between proteolytic footprints of Pap\textsuperscript{A3}-DKP-insulin and Pap\textsuperscript{B25}-DKP-insulin (19, 20). Proteolytic footprints of the holoreceptor-derived photo-products are essentially identical to those derived from the isolated ectodomain.

Here, we have exploited “midi-receptors” (active dimeric fragments of the \(\alpha\)-subunit in the absence of the \(\beta\)-subunit; see Ref 51) to obtain a rapid and simple mapping protocol. To this end, we have introduced engineered protease TEV site(s) within the midi-receptor without loss of hormone binding activity. This assay provides evidence that Pap\textsuperscript{A3}-DKP-insulin forms a photoproduct with the 650–719 segment (construct T1) and the 703–719 segment (construct T2). The A3 probe thus contacts the same C-terminal peptide (\(\alpha\)CT) as does a Pap\textsuperscript{B25} probe (14). Interestingly, B25 is also a site of clinical mutation causing diabetes mellitus (insulin Chicago (4)). The sequence of \(\alpha\)CT (KTFEDYLHNVVPRPS) contains a mixture of hydrophobic and hydrophilic amino acids; residues critical to hormone binding (boldface in sequence above) have been identified by Ala-scanning mutagenesis (28, 83). These residues may either contact insulin or mediate interactions between \(\alpha\)CT and other domains of the receptor \(\alpha\)-subunit. The residues in \(\alpha\)CT that contribute to the binding sites for residues A3 and B25 are presumably aromatic or nonpolar.

The structure of the IR ectodomain (Fig. 8, A and B; see Ref. 1, 84), provides a framework for understanding insulin binding. Unfortunately, interpretable continuous electron density was lacking for portions of FnIII-2 and the insert domain (dashed red lines in Fig. 8A). Intriguingly, an unassigned region of poor electron density (comprising \(\sim20\) residues) was observed adjoining the hormone-binding surface of the L1 domain (green in Fig. 8C) (1). The present results support assignment of this density to \(\alpha\)CT. A dual structural role of \(\alpha\)CT, simultaneously docking against L1 and the bound insulin, would rationalize the large number of critical residues identified by Ala-scanning mutagenesis and the severity of their impact on hormone binding (28, 83).

The putative proximity of L1 and \(\alpha\)CT would also rationalize how consecutive photoreactive probes in insulin (at positions B24 and B25) can cross-link to these respective domains of the IR (18). These L1 and \(\alpha\)CT domains could belong to the same \(\alpha\)-subunit (a cis-model) or to dimer-related \(\alpha\)-subunits (a trans-model). Recent mutagenesis results support a trans-model (52). In this regard it is noteworthy that the present substitutions at

FIGURE 8. Crystal structure of receptor ectodomain and possible position of \(\alpha\)CT segment. A, ribbon model of component protomer (PDB code, 2DTG). Individual domains L1, CR, L2, FnIII-1, FnIII-2, and FnIII-3 are shown in gray, black, blue, red, purple, and dark blue, respectively. Missing or discontinuous electron density (residues 655–755; IR isoform A) is depicted in schematic form by red dashed lines (not intended to represent actual conformation). B, crystal structure of inverted V ectodomain dimer. C, L1 domain (gray ribbon) in relation to unassigned and discontinuous electron density (green), potentially from \(\alpha\)CT; density may represent \(\sim20\) residues of insert domain. Residues in L1 critical to hormone binding (as inferred from Ala-scanning mutagenesis (93) are shown as red stick. C was kindly provided by C. W. Ward.
A3 cause no disproportionate perturbations in negative cooperativity, whereas this phenomenon is impaired by substitutions at B25 (85). Because the mechanism of negative cooperativity presumably involves binding of one or more additional insulin molecules to already occupied receptors, this difference between A3 and B25 analogs suggests structural differences in how insulin binds to such additional sites. Whether this in turn implies corresponding differences in a singly occupied high affinity complex (as in a trans model) is unclear. In either cis- or trans-models, the absence of interpretable electron density for the C-terminal portion of the α-subunit suggests that this region is less well ordered in the absence of insulin binding. We therefore imagine that the insert domain undergoes a disorder-to-order transition on insulin binding, i.e. both insulin and its receptor exhibit induced fit. Hormone-dependent folding of the IR may contribute to the mechanism of signal transduction, leading to activation of the cytoplasmic tyrosine kinase domains.

Concluding Remarks—This study has demonstrated the importance of a conserved side chain (ValA3) to the structure, stability, and function of insulin. Our strategy has combined structural studies with receptor-based mapping of photo-cross-linked adducts. Use of isosteric analogs as probes of structure-activity relationships circumvents possible confounding effects of larger side chains (protrusion from the A3-related crevice) or smaller side chains (nonlocal structural perturbations). The properties of these analogs demonstrate that ValA3, despite its low intrinsic helical propensity, contributes to the stability of the free hormone through its packing in an inter-chain crevice. Photo-cross-linking studies suggest that analogous packing occurs at the receptor interface, mediated by the C-terminal tail of the α-subunit. Receptor binding is impaired by the present isosteric substitutions and is incompatible with LeuA3 (insulin Wakayama), leading to diabetes mellitus. Because ValA3 is not accessible in the free hormone, a conformational change in the overlying B-chain may be required for its engagement. Extension of the present strategy to other sites in insulin may enable construction of a model of the hormone-receptor complex. Such a model may enable design of novel insulin agonists for the treatment of diabetes mellitus.

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