How insulin binds to the insulin receptor has long been a subject of speculation. Although the structure of the free hormone has been extensively characterized, a variety of evidence suggests that a conformational change occurs upon receptor binding. Here, we employ chiral mutagenesis, comparison of corresponding D and L amino acid substitutions, to investigate a possible switch in the B-chain. To investigate the interrelation of structure, function, and stability, isomeric analogs have been synthesized in which an invariant glycine in a β-turn (GlyB8) is replaced by D- or L-Ser. The D substitution enhances stability (ΔΔGₐ 0.9 kcal/mol) but impairs receptor binding by 100-fold; by contrast, the L substitution markedly impairs stability (ΔΔGₐ −3.0 kcal/mol) with only 2-fold reduction in receptor binding. Although the isomeric structures each retain a native-like overall fold, the L-SerB8 analog exhibits fewer helix-related and long range nuclear Overhauser effects than does the D-SerB8 analog or native monomer. Evidence for enhanced conformational fluctuations in the unstable analog is provided by its attenuated CD spectrum. The inverse relationship between stereospecific stabilization and receptor binding strongly suggests that the B7–B10 β-turn changes conformation on receptor binding.

Insulin is a small globular protein containing two chains, A (21 residues) and B (30 residues, Fig. 1A). Stored in the β cell as a Zn²⁺-stabilized hexamer, the hormone functions as a Zn²⁺-free monomer. Although the structure of the free hormone has been well characterized by X-ray crystallography and NMR spectroscopy (Fig. 1B, Refs. 1–3), it is not known how insulin binds to its receptor. Indeed, studies of single chain analogs have suggested that classical structures represent inextensible conformations (4, 5). What sites in the insulin molecule might function as a structural switch? Here, we employ chiral mutagenesis, comparison of corresponding D and L amino acid substitutions (6), to uncover non-standard structure-function relationships. Our studies focus on the B7–B10 β-turn, the hinge between the N-terminal β-strand and central α-helix of the B-chain. An inverse relationship between protein stability and activity provides a stereospecific signature of induced fit.

Our strategy exploits the right hand side of the Ramachandran plane: substitution of a glycine with positive φ angle (and hence residing in a region ordinarily “forbidden” to L amino acids) by a D amino acid (7, 8). The site of substitution (GlyB8; arrow in Fig. 1A and red balls in Fig. 1B) is invariant among mammalian insulins and insulin-like growth factors (9). GlyB8 lies on the surface of a type II β-turn comprising residues B7–B10 (Fig. 1, C and D). This turn contains CysB7 (part of the canonical A7–B7 disulfide bridge; highlighted in gold in Fig. 1, C and D) and so provides a link between the central B-chain α-helix and the A-chain. Substitution of GlyB8 by L amino acids impairs the folding of a single chain insulin precursor (10) and impedes disulfide pairing in insulin chain combination (6). Substitution of a turn-specific D-glycine by a D amino acid would by contrast be expected to enhance the stability of the native fold (7, 8). Respective sites of L and D substituents are indicated in blue (the pro-L Hα of GlyB8; Fig. 1D) or magenta (pro-D Hα). Whereas previous studies of D- and L-AlaB8 insulin analogs established such reciprocal effects on stability, the L analog was observed to misfold; aberrant aggregation prevented structural studies and confounded interpretation of its biological activity (6). Fortuitously, these limitations may be circumvented through the use of D- and L-Ser.

In this article we describe the solution structures of D- and L-SerB8 analogs of an engineered insulin monomer. Such engineering is required to avoid dimerization and higher order protein assembly (11), which would otherwise limit the feasibility of NMR analysis (12). A monomeric template is provided by DKP-insulin, which contains three amino acid substitutions in the B-chain (AspB10, LysB28, and ProB29, magenta in Fig. 1A and Ref. 13). The structure of DKP-insulin and its use as a template for study of mutations of interest have previously been described (14, 15). Our results demonstrate that whereas both D- and L-SerB8 DKP-insulin retain native-like overall folds, the L analog exhibits a profound decrease in thermodynamic stability associated with attenuated...
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EXPERIMENTAL PROCEDURES

Materials—Human insulin was kindly provided by Eli Lilly and Co. (Indianapolis, IN). All other chemicals were of analytical grade (Fisher Chemicals).

Synthesis of Insulin Analogs—The tetra-S-sulfonate derivative of the human A-chain was obtained by oxidative sulfitolysis (17). B-chain analogs were prepared by solid phase chemical synthesis as described (17). In brief (N-tert-butoxycarbonyl, O-benzyl)-Thr-PAM resin (0.56 mmol/g; Bachem, Inc.) was used as solid support for synthesis of B-chain analogs. The d-Ser\textsuperscript{B8}-DKP B-chain was prepared by automated solid-phase synthesis using F-moc chemistry (18). For synthesis of the L-Ser\textsuperscript{B8}-DKP B-chain, a manual double-coupling t-BOC protocol was followed (17, 19). d- and L-Ser\textsuperscript{B8}-DKP-insulin were prepared by chain combination (17, 20). Analogs were purified by reverse-phase high performance liquid chromatography (HPLC) as described (17). Whereas the yield of the d analog was at least as high as that of DKP-insulin, the yield of the L analog was reduced by \(10\)-fold. Predicted molecular masses were confirmed by matrix-assisted laser desorption ionization (MALDI-TOF) mass spectrometry (MS).

Receptor Binding Assays—Relative activity is defined as the ratio of analog to wild-type human insulin required to displace 50% of specifically bound \(^{125}\text{I}\)-human insulin. A human placental membrane preparation containing the insulin receptor (IR) was employed as described (21). In all assays the percentage of tracer bound in the absence of competing ligand was \(<15\%\) to avoid ligand-depletion artifacts.

Circular Dichroism—Far-ultraviolet (UV) CD spectra were obtained as described (22). Spectra, acquired with an Aviv spectropolarimeter (Aviv Biomedical, Inc., Lakewood, NJ), were normalized by mean residue ellipticity. Estimates of secondary structure were obtained by deconvolution using an iterative singular value decomposition algorithm as described (23). Samples were dissolved in 10 mM phosphate and 50 mM KCl (pH 7.4) at a protein concentration of \(\sim 25 \mu\text{M}\). For equilibrium denaturation studies, samples were diluted to 5 \(\mu\text{M}\); guanidine-HCl was employed as denaturant (24). Data were obtained at
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### TABLE 1
Activities and stabilities of insulin analogs

| Analog            | Binding | ΔG_u | C_mol | m^2 |
|-------------------|---------|------|-------|-----|
|                   | kcal/mol M | kcal/mol/M |
| Insulin (In)      | 100     | 4.4 ± 0.1 | 5.3 ± 0.1 | 0.84 ± 0.01 |
| DKP-insulin       | 161 ± 19 (6) | 4.9 ± 0.1 | 5.8 ± 0.1 | 0.84 ± 0.01 |
| l-Ser^B8^-DKP-In  | 90 ± 6 (3) | 1.9 ± 0.1 | 3.7 ± 0.2 | 0.89 ± 0.02 |
| d-Ser^B8^-DKP-In  | 1.1 ± 0.1 (3) | 5.8 ± 0.1 | 6.5 ± 0.1 | 0.51 ± 0.01 |

- Binding activity is defined by affinity for the human placental insulin receptor relative to native insulin (100%).
- Under these conditions the K_d for native insulin is 0.48 ± 0.06 nM. The number of replicates is given in parentheses.
- ΔG_u indicates apparent change in free energy on denaturation in guanidine-HCl as extrapolated to zero denaturant concentration by a two-state model (25).
- C_mol is defined as that concentration of guanidine-HCl at which 50% of the protein is unfolded.
- m^2 provides slope in plotting unfolding free energy ΔG_u versus molar concentration of denaturant; this slope is proportional to the protein surface area exposed on unfolding.

### RESULTS

D- and l-Ser^B8^-DKP-insulin exhibit receptor-binding affinities of 1.1 ± 0.1 and 90 ± 6% relative to native human insulin (column 2 in Table 1). Because the relative affinity of DKP-insulin is 161 ± 19 under these conditions (enhanced binding is caused by the Asp^B10 substitution (29)), specific effects of the D and l B8 substitutions on affinity relative to the parent monomer are revealed. The far-UV CD spectrum of d-Ser^B8^-DKP-insulin exhibits a slight accentuation of helix-sensitive wavelengths (Fig. 2A). Conversely, the CD spectrum of l-Ser^B8^-DKP-insulin is attenuated at helix-sensitive wavelengths (open circles in Fig. 2A).

Because the precision of the measured ellipticities in the range 200–250 nm is ±1%, these stereospecific differences are significant. Respective values of mean residue ellipticity at 196, 208, and 222 nm are given in Table 2, A. Deconvolution suggests d-specific enhancement, and complementary l-specific attenuation, of mean α-helix content (Table 2, B). Such changes may in principle reflect either static structural changes or stereospecific modulation of conformational fluctuations within helical elements (see below).

D- and l-Ser^B8^-DKP-insulin exhibit marked differences in thermodynamic stability. Whereas the d-Ser^B8^- analog is more resistant to denaturation in concentrated solutions of guanidine hydrochloride than is DKP-insulin, the l analog is more sensitive (Fig. 2B). Fitting of these CD-detected denaturation curves by a two-state model yields ΔΔG_u values of 0.9 ± 0.2 kcal/mol (d analog) and −3.0 ± 0.2 kcal/mol (l) relative to DKP-insulin (Table 1). The difference in stability between stereoisomers is thus 3.9 ± 0.3 kcal/mol. This difference is significantly greater than would be expected based on chiral inversion within an isolated β-strand and so implies non-local effects of the substitution in one or both analogs.
based on guanidine denaturation, the rate of amide proton exchange in 20% deuterioacetic acid and 80% D$_2$O is markedly accelerated in the l-Ser$_{88}$ analog and retarded in the D-Ser$_{88}$ analog (see supplemental material). Quantitative interpretation of protection factors is limited by the absence of baseline exchange rates in this co-solvent.

NMR Studies of the d Isomer—Chemical shifts are essentially identical to those observed in DKP-insulin; significant changes (magnitude >0.1 ppm) are observed only at neighboring residues Cys$_{B7}$ and Ser$_{89}$ (supplemental material). The novel d-Ser$_{88}$ spin system is well resolved (Fig. 3A). Analysis of secondary structure, based on diagnostic strings of $d_{NN}$, $d_{ON}$, $d_{(i,i+3)}$, and $d_{(i,i+4)}$ NOEs, is identical to that of DKP-insulin (supplemental material). The pattern of long range inter-residue NOEs is likewise similar, in each case consistent with structures of T-state crystallographic protomers. Key native-like long range NOEs are observed, for example, between aromatic and aliphatic side chains (Fig. 4, A and B). These include contacts between the side chains of Phe$_{B7}$/Leu$_{B15}$ and Tyr$_{B26}$/Val$_{B12}$, indicative of native-like B-chain supersecondary structure; between Tyr$_{A19}$/Ile$_{A1}$, indicative of native-like A-chain supersecondary structure; and between Phe$_{B1}$/Leu$_{A13}$, His$_{B24}$/Ile$_{A10}$, Tyr$_{A19}$/Leu$_{B15}$, and Tyr$_{B26}$/Val$_{A3}$, indicative of a native-like orientation between chains. The upfield chemical shifts of the Leu$_{B15}$ methyl resonances, sensitive to the ring current of Phe$_{B24}$, are essentially identical in the spectrum of the d-Ser$_{88}$ analog (0.13 and 0.54 ppm) and in the spectrum of DKP-insulin (0.15 and 0.54 ppm). The upfield chemical shift of the Val$_{B12}$ H$_{B}$ resonance, sensitive to the ring currents of Phe$_{B24}$ and Tyr$_{B26}$, is also not significantly perturbed (3.12 ppm in the d-Ser$_{88}$ analog versus 3.18 ppm in DKP-insulin; random-coil value 4.18 ppm). Such correspondence of secondary shifts provides evidence of structural similarity given the steep dependence of upfield ring currents on the relative distance and orientation between these side chains.

A summary of inter-residue NOEs is given as a diagonal plot (Fig. 3C). Maintenance of T-state specific long range interactions by the N-terminal arm of the B-chain is demonstrated by retention of native-like inter-chain NOEs between the B1-A13 and B5-A10

**FIGURE 3.** Two-dimensional NMR identification of d- and l-Ser$_{88}$ spin systems and diagonal plot of inter-residue NOEs. TOCSY spectra of d-Ser$_{88}$-DKP-insulin (A) and l-Ser$_{88}$-DKP-insulin (B) in the region containing the AMX spin system of respective B8 side chains. Spectra (mixing times 55 ms) were observed in D$_2$O at 32 °C and pD 7.6 (direct meter reading). C and D, diagonal plot of d-Ser$_{88}$-DKP-insulin (C) and l-Ser$_{88}$-DKP-insulin (D) shown inter-residue NOEs, respectively. NOEs between side chains are shown at lower right (open boxes); NOEs between main chain protons or between main chain and side chains are shown at upper left (filled boxes). Red squares in C indicate NOEs consistent with T-state crystal structures but unobserved in the spectrum of l-Ser$_{88}$, DKP-insulin. In C, NOEs a–c indicate (a) a set of contacts between the Ile$_{A10}$ side chain and main chain atoms of Asn$_{B3}$, Gln$_{B4}$, and His$_{B5}$; (b) contact between the side chains of His$_{B5}$ and Thr$_{A8}$; and (c) the set of contacts between the Leu$_{B8}$ side chain and H$_{B}$ of Leu$_{B11}$ across the β-turn. In D, red boxes indicate NOEs present in the l analog but not in the d analog. Respectively green and red boxes d and e indicate (d) Cys$_{A11}$ side chain to H$_{B}$ of Cys$_{B8}$; and (e) contact between H$_{B}$ of Tyr$_{A19}$ and meta resonance of Phe$_{B24}$.
side chains; examples are provided by contacts between the side chain of IleA10 and main chain atoms of AsnB3, GlnB4, and HisB5 (labeled a in Fig. 3C) and between the side chains of HisB5 and ThrA8 (labeled b). These contacts are extended by a network of interchain NOEs involving neighboring main chain and side chain protons in segments A6–A11 and B3–B7. The orientation of the B9–B19 α-helix relative to the A-chain, as probed by a network of long range NOEs in the hydrophobic core (supplemental material), is also unaffected. NOEs are observed from B8 HN to the methyl resonances of LeuB11 and from B8 HN to the 1-CH3 resonance of ValB12; these contacts are in accord with corresponding NOEs in DKP-insulin. A novel side chain-specific NOE is observed from the H11002-CH2 group of D-SerB8 to the aromatic meta resonance of TyrB26 (but not to the methyl groups of ValA3, ValB12, and LeuB11, which might also be plausible based on wild-type crystal structures).

NMR Studies of the L Isomer—The L-SerB8 spin system is likewise well resolved (Fig. 3B), and essentially complete resonance assignment was obtained (supplemental material). Inversion of B8 Cα chirality introduces non-local perturbations in NOEs and chemical shifts. Although the overall pattern of inter-residue NOEs is native-like, helix-related strings of dNN,dN, d(i, i+3), and d(i, i+4) contacts are less complete than those observed in spectra of DKP-insulin or D-SerB8-DKP-insulin. In total 51 fewer helix-related NOEs are observed; their attenuation seems consistent with the attenuated CD spectrum of the L analog. Chemical shifts are similar to but distinct from those in D-SerB8-DKP-insulin; significant changes (magnitude >0.1 ppm) are summarized in Table 3. Stereospecific perturbations are observed at adjoining residues CysB7 and SerB9, within contiguous structural elements (Δδ 0.22 ppm at GlnB4 Hα and 0.16 ppm at AspB7 Hβ), and at transmitted sites (0.19 ppm at CysB7 Hβ and 0.15 ppm at the meta resonances of PheB26). Significant chemical-shift differences between D and L analogs occur at residues A7, A10, B4, B5, B9, and B11 at pH 8; a corresponding set of perturbations is seen in 20% deuteroacetic acid (residues A2, A7, B4–B6, and B10–B13).

Despite perturbations in chemical shifts and partial attenuation of helix-related NOEs, L-SerB8-DKP-insulin retains key native-like long range contacts (Fig. 4C). As in the D-SerB8 analog, native-like contacts between the side chains of PheB24/
LeuB115 and TyrB26/ValB12 are maintained. However, the most upfield methyl chemical shift of LeuB115 (0.22 ppm) is less shifted relative to the random coil value (0.90 ppm) than the corresponding methyl resonance in DPK-insulin (0.15 ppm) or D-SerB8, DPK-insulin (0.13 ppm). Likewise, the upfield chemical shift of the ValB12 Hα resonance (3.28 ppm) is less shifted relative to the random coil value (4.18 ppm) than in DPK-insulin (3.18 ppm) or D-SerB8, DPK-insulin (3.12 ppm). This subtle trend in secondary shifts correlates with the order of thermodynamic stability (ΔGj; Table 1) and CD-derived helix contents (Table 2, B). We speculate that attenuation of 1H NMR secondary shifts in the l-SerB8 analog and attenuation of helix-specific mean residue ellipticities have a common physical origin: conformational fluctuations leading to averaging of ring current shifts in one case and optical chirality in the other.

Despite such subtle 1H NMR features, a subset of native-like long range NOEs are retained between A- and B-chains. As in DPK-insulin and D-SerB8, DPK-insulin, strong contacts are observed between the side chains of TyrA19 and LeuB115. Although NOEs involving the N-terminal arm of the B-chain are less prominent than in the spectrum of the D analog (see below), T-like positioning of the N-terminal segment is retained as indicated by long range NOEs between the side chains of PheB25/LeuA11 and HisB7/IleA10. The orientation of the canonical B9–B19 α-helix relative to the A-chain is likewise defined by an analogous network of long range NOEs (Fig. 3D and supplemental material). Nevertheless, the number of long range contacts in the spectrum of the l analog (89 NOEs) is significantly smaller than in the spectrum of the D analog (122 NOEs). NOEs present in the D analog but not in the l analog are highlighted in red in Fig. 3C. Also as in DPK-insulin and D-SerB8, DPK-insulin, native-like long range NOEs are maintained within the A-chain between the side chains of IleA2 and TyrA19 (Fig. 4C). Similarity of the conformation of the A6–A11 disulfide bridge is indicated by maintenance of an NOE between CysA11 Hα and CysA6 Hα (labeled d in Fig. 3D). Contacts are also retained between side chains PheB24/LeuB15, TyrB26/ValB12, and TyrA19/LeuB115. These and related NOEs define native-like supersecondary structures within each chain and constrain the orientation between chains. Unlike in DPK-insulin and D-SerB8, DPK-insulin, native-like long range NOEs between ValA3 and LeuB11 are not observed.

Highlighted in red in the diagonal plots (Fig. 3, C and D) are NOEs observed in one analog but not the other. The total number of inter-residue contacts identified in l-SerB8, DPK-insulin (299 NOEs) is substantially less than the number observed in the D analog (414 NOEs); more red boxes are thus seen in Fig. 3C than in Fig. 3D. The missing NOEs include local, medium range, and long range contacts. The following contacts are remarkable for their absence in the spectrum of l-SerB8, DPK-insulin. (i) Whereas an NOE is observed between the β protons of D-SerB8 and the aromatic ring of TyrB26, no such NOE is observed in l-SerB8, DPK-insulin, presumably because of the altered orientation of the l side chain. (ii) Instability of the B7–B10 β-turn is suggested by the absence (relative to DPK-insulin and the D-SerB8 analog) of the following turn-related NOEs: LeuB6 Hα/LeuB11 Hα (boxes c in Fig. 3C), CysB7 Hβ/AspB10 Hμ, CysB7 Hα/LeuB11 Hα, CysB7 Hβ/LeuB11 δ1,2 CH3, CysB7 Hα/LeuB11 Hμ, CysB7 Hβ/LeuB11 δ1,2 CH3, and SerB8 Hα/ValB12 γ1 CH3. (iii) Propagation of such instability into the B9–B19 α-helix is suggested by the absence of native-like NOEs SerB9 Hα/ValB12 γ1 CH3, SerB9 Hμ/ValB12 γ1,2 CH3, SerB9 Hμ/ValB12 γ1 CH3, SerB9 Hμ/ValB12 γ1 CH3, AspB10 Hα/AlaB14 Hα, and LeuB6 δ1,2 CH3/AlaB14 H4. The l-SerB8-specific perturbation extends into the N-terminal arm of the B-chain. Absent or markedly attenuated are native-like inter-chain NOEs between side chains AsnB3/IleA10 and HisB7/ThrA8. Also absent are NOEs from the side chain of IleA10 to AsnB3 Hβ and GinB4 Hα. Whereas NOEs from the side chain of CysB7 to CysA6 Hα are also absent, the spectrum of the l analog contains cystine-related NOEs CysB7 Hμ/CysA7 Hα and CysB7 Hβ/CysA7 Hα such contacts, not observed in DPK-insulin or the D-SerB8 analog, indicate an altered or more flexible disulfide linkage. l-SerB8, specific NOEs (red in Fig. 3D) also affect contacts between the aromatic ring of PheB25 and the Hα protons of CysA20 and AsnA21. Subtle differences in side chain packing are further indicated by a novel NOE between the Hβ of TyrA19 and meta resonance of PheB24 (labeled e in Fig. 3D). Although in each case consistent with crystal structures, such NOEs are not observed in DPK-insulin and indicate an altered orientation of the B24 and B25 side chains. Overall T-like positioning of the N- and C-terminal segments is nonetheless retained in each case as indicated by maintenance of canonical long range NOEs. Attenuation of long range NOEs not directly involving l-or D-SerB8 provides evidence that stereospecific frustration (or stabilization) of the B7–B10 β-turn is coupled to conformational fluctuations elsewhere in the protein. 1H NMR analysis, when considered in appropriate detail, thus corroborates the apparent attenuation of organized structure implied by CD.

Solution Structures—DG/RMD structures were calculated according to NOE, J-coupling, and hydrogen-bond-related restraints (supplemental material). The total number of restraints employed in calculating the d- and l-SerB8 ensembles are 631 and 537, respectively (∼11–12 restraints per residue). Root-mean-square deviations (RMSDs) are provided as supplemental material. The ensembles verify that the solution structures of d- and l-SerB8, DPK-insulin (Fig. 5, A and B, respectively) are similar to the T-like conformation of DPK-insulin (black ribbon, Fig. 5). The l-SerB8 ensemble exhibits a small shift in the position of the N-terminal A-chain α-helix relative to the B-chain. Although analogous structural variation is observed in comparison of crystal structures (supplemental material), it is possible that an l-SerB8-specific perturbation is transmitted to the A-chain via the adjoining A7–B7 disulfide bridge. The precisions of the ensemble do not allow atomic scale definition of a discrete pathway of conformational change. Although the precisions of the two ensembles are similar (main chain RMSDs 0.50 Å (α) and 0.61 Å (l) for residues B3–B27 and A2–A20), the d structure is better defined in the neighborhood of B8 and within the B1–B7 arm. B8 Ramachandran angles in the d ensemble are φ = 60.1° ± 8.1° and ψ = −150.0° ± 8.2°. These values cluster as expected on the right side of the Ramachandran plot, near the ψ angle of GlyB8 in crystallographic T-state protomers (55.6° ± 2.8°). The ψ angle in the DG/RMD ensemble is also similar to that of GlyB8 in T-state crystallo-
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FIGURE 5. DG/RMD ensembles. Front and back views of d-SerB8-DKP-insulin (A) and l-SerB8-DKP-insulin (B). In each case the A-chain is shown in gray and B-chain in blue. Left and right images are related by a rotation of 180° about the vertical axis. The d- and l-SerB8 side chains are shown in green and purple, respectively (arrowheads). Ribbons indicate the mean structure of DKP-insulin. Arrow indicates the region of decreased precision in the d analog; asterisk indicates the region of decreased precision in the l analog. Structures were aligned according to the main chain atoms of residues A2–A7, A13–A19, and B9–B23.

graphic protomers (−131.2° ± 4.2°). The local precision of the l ensemble is not sufficient to determine the B8 conformation.³ It would be of future interest to define these angles at high resolution through x-ray crystallography.

DISCUSSION

The present study exploits chiral stabilization or destabilization of the B7–B10 β-turn by respective d- or l-SerB8 substitutions to probe structure-function relationships. The substitutions were incorporated into an engineered insulin monomer (13) to circumvent otherwise confounding effects of self-association. Substitution of GlyB8 by d-Ser enhances stability but markedly impairs receptor binding; its substitution by l-Ser markedly impairs stability but is compatible with substantial activity. Such an inverse correlation between stability and activity suggests that the native B7–B10 β-turn undergoes a change in conformation in receptor binding.

Use of a d amino acid substitution highlights the power of the total chemical synthesis of proteins to elucidate non-standard structure-activity relationships. Despite the general robustness of insulin chain combination to diverse substitutions, however, low yields were encountered in the synthesis of l-SerB8-DKP-insulin; the yield of the d-SerB8 analog was by contrast at least as high as that obtained in corresponding syntheses of DKP-insulin. The origin of this stereospecific impairment of chain combination is not well understood. Because efficient syntheses of unstable insulin analogs have previously been reported (15, 24, 32), we imagine that l-SerB8 imposes a kinetic barrier to disulfide pairing. It is possible that in a reaction intermediate the main chain conformation of residue B8 affects the orientation of CysB7 and in turn its alignment with CysA7. Anomalously low yields have likewise been encountered by Katsoyannis and co-workers (17) on interchange of residues LeuB11 and ValB12 (also near cystine A7–B7) and on substitution of LeuA16 (near cystine A20–B19; Ref. 33). It would be of interest should this pattern of yields generalize to the efficiency or fidelity of folding of corresponding mutant proinsulins in the endoplasmic reticulum of the pancreatic β cell.

³ Two families of structures are consistent with the restraints: one with B8 dihedral angles (ϕ = 60.1 ± 8.1, ψ = −149.7 ± 8.2) and the other with angles (ϕ = −157.63 ± 37.2, ψ = −161.7 ± 8.2).

Sterespecific Effects on Stability—CD and NMR spectra of d-SerB8-DKP-insulin closely resemble those of the parent DKP-insulin monomer; (14). The pattern of inter-residue NOEs is similar to that of DKP-insulin; differences in chemical shift are confined to the immediate neighborhood of the d side chain. The enhanced thermodynamic stability evident by resistance to denaturation in concentrated solutions of guanidine-HCl is likely to reflect in part stabilization of a nascent native-like B7–B10 turn in the unfolded state, which would reduce the entropic penalty of folding. The increased stability of d-SerB8-DKP-insulin (∆∆G0, 0.9 ± 0.2 kcal/mol) is similar to that observed by Raleigh and co-workers (8) in studies of analogous d-Ala substitutions in unrelated globular domains.

The magnitude of chiral stabilization of d-SerB8-DKP-insulin (∆∆G0, 0.9 ± 0.2 kcal/mol) is less than that of d-AlaB8-DKP-insulin (∆∆G0, 1.5 ± 0.1 kcal/mol; 6). This difference may arise in part from residue-specific effects in their respective unfolded states and in part from structural perturbations in the folded state. Among crystal structures in general Ser is more likely than Ala to exhibit positive ϕ angles, suggesting that in an unfolded polypeptide d-Ser would be less effective than d-Ala in constraining the ϕ angle to the right hand side of the Ramachandran plot. Nonetheless, such subtle residue-specific effects in the unfolded state would seem insufficient to account for the 0.6 kcal/mol difference between the stabilities of d-AlaB8 and d-SerB8 analogs. Indeed, because complete exclusion of the right side of the Ramachandran plane would be associated with an entropic contribution of only RT ln (2) (i.e. 0.4 kcal/mol at
Waals interactions between the D-methyl group and surrounding aliphatic and aromatic side chains: D-AlaB8 partially inserts within a local non-polar pocket formed by ValA3, LeuB11, ValB12, and TyrB26 (6). Such favorable packing is partially disrupted by the polar D-Ser side chain. Effects of local structure may be further modulated by differences in solution free energy near the B8 D side chain. These considerations indicate that the net effect of D amino acid substitutions in a globular protein is likely to reflect both general chiral restriction of the main chain $\phi$ angle and the residue-specific side chain environment.

**Stereospecific Effects of Protein Dynamics—**CD and NMR spectra of L-SerB8-DKP-insulin seem at first glance to have contradictory implications. On the one hand, the CD spectrum of the L analog is attenuated relative to that of DKP-insulin, suggesting a decrease in helix content. On the other hand, the endpoints of its three helical segments, as defined by strings of local and medium range NOEs, are unchanged. This seeming paradox is resolved by consideration of the physical origins of these respective spectra and by detailed analysis of the network of helix-related NOEs. CD provides an ensemble average of the mean helix content. Such features can be attenuated by either segmental changes in conformation or dynamic perturbations. An example of the former is the segmental unfolding of insulin in response to a cavity-forming mutation IleA2→Ala: this substitution in DKP-insulin leads to disorder of the N-terminal segment of the A-chain (15, 22). In this case the attenuated CD helix content is in accord with discrete loss of helix-related NOEs.

A contrasting example of dynamic CD attenuation is provided by the apparent increase in helix content on insulin assembly (23). Although NMR-derived helical endpoints in an isolated monomer are consistent with crystal structures of dimers and hexamers (1), the attenuated helical CD signature of the monomer is likely to reflect enhanced conformational fluctuations. Physical evidence for such fluctuations and their damping on assembly has been provided by analysis of helix-specific Raman vibrational band widths (34). Such damping of conformational fluctuations is likely to underlie the anomalous accentuation of helical CD features observed on substitution of GlyB8 by D-Ser. Although the D analog likewise exhibits identical NMR-defined helical segments, such accentuation implies a non-local coupling between fluctuations in the B8-related $\beta$-turn and fluctuations in (at least one) $\alpha$-helix. We speculate that D-SerB8 constrains local unfolding of the adjoining B9–B19 $\alpha$-helix; transmitted effects to the A-chain cannot be excluded.

Although L-SerB8-DKP-insulin retains a native-like overall fold and helical substructure, its NOESY spectrum contains fewer helix-related and long range contacts than are observed in DKP-insulin or the D-SerB8 analog. In addition, chemical-shift perturbations are observed throughout the molecule. These findings strongly suggest that its attenuated CD spectrum, perturbations essentially mirror image to those of the D analog, reflects conformational excursions leading to transient distortions in helical main chain geometry. Because helical elements in insulin function in receptor recognition (1, 21, 35–37), we imagine that helical destabilization would in itself be expected to impair binding. The substantial receptor binding activity of D-SerB8-DKP-insulin may therefore be retained despite such transmitted perturbations.

We suggest that the high affinity of L-SerB8-DKP-insulin, a seeming paradox in light of its instability and perturbed CD spectrum, reflects the net result of favorable and unfavorable factors. In this model destabilization of the native B7–B10 $\beta$-turn would enhance receptor binding whereas transmitted destabilization effects of helices would decrease binding. Although local effects of the D side chain at the hormone-receptor interface cannot be excluded, the 10-fold better binding of D-SerB8-DKP-insulin relative to D-AlaB8-insulin (6) suggests that D-SerB8 is locally well tolerated. Although it is formally possible that the side chains of D substitutions incur steric clash at the receptor interface, this possibility seems unlikely in light of the 5-fold better binding of a bulky D amino acid side chain (d-para-aminophenylalkane) than D-AlaB8 (6).

Conversion of this side chain to the photoactivatable analog d-para-azido-phenyl results in negligible photo-cross-linking to the insulin receptor, in striking contrast to the efficient photo-cross-linking of such insulin derivatives in the classical receptor-binding surface (36–38). The respective 100-fold and 1000-fold decrements in receptor binding exhibited by D-SerB8-DKP-insulin and D-AlaB8-DKP-insulin are larger than those usually incurred by mutations associated with modest changes in side chain volume (supplemental material).

**B8 Chirality and the TR Transition—**Although the present study has focused on chiral mutagenesis of an engineered insulin monomer, our results are pertinent to crystallographic studies of zinc-insulin hexamers. Such assemblies exhibit a ligand-dependent equilibrium among $T_{o},T_{t}R_{3}^{f}$, and $R_{6}$ hexamers (1, 39). In the T-state as in an engineered monomer in solution (3, 14), the B-chain contains an extended N-terminal arm (residues B1–B6) and type II $\beta$-turn (B7–B10) followed by the central $\alpha$-helix (B9–B19). In the alternative R-state the B-chain contains an extended $\alpha$-helix (residues B1–B19; Refs. 39 and 40). These structural differences are illustrated in the cylinder models shown in Fig. 1B. Despite the extensive crystallographic characterization of zinc insulin hexamers, the relationship between these structures and the active conformation of insulin has long been the subject of speculation (1, 6, 31).

The TR transition is accompanied by a change in sign of the $\phi$ angle of GlyB8 from positive (in the T-state-specific $\beta$-turn) to negative (in the R-state-specific $\alpha$-helix). D amino acids at B8 would thus favor the T-state whereas L substitutions would favor the R-state. Within zinc hexamers chiral substitutions would thus be expected to shift the equilibrium among $T_{o},T_{t}R_{3}^{f}$, and $R_{6}$ hexamers in one direction or the other. Within the DKP-insulin monomer D-SerB8 stabilizes a T-like confor-

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4. K. Huang, B. Xu, P. K. Katsymannis, and M. A. Weiss, unpublished results.
5. The TR transition is also characterized by a change in the handedness of cystine $A_{7}$–$B_{7}$. The sulfur atoms of the latter are exposed in the T-state but buried in a nonpolar crevice in the R-state. We speculate that coupling between the B8 $\phi$ angle and handedness of cystine $A_{7}$–$B_{7}$ may account for the low yield of chain combination in synthesis of L-SerB8-DKP-insulin.
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nformation. Although L-Ser\textsuperscript{B8} destabilizes this conformation, no evidence of an R-like monomeric state is observed. In particular, minor helix-related NOEs in the B1-B8 segment diagnostic of a subpopulation of R-state conformers have not been detected. Further, the attenuated CD spectrum of L-Ser\textsuperscript{B8}.DKP-insulin is opposite to the increased helix content observed in CD studies of T\textsubscript{3}R\textsubscript{3}, and R\textsubscript{9} hexamers (41). That similar attenuation occurs in the CD spectrum of L-Ala\textsuperscript{B8}.DKP-insulin (6) indicates that this effect is unrelated to the relative intrinsic helical propensities of alanine or serine (42).

The very low activities of d-Ser\textsuperscript{B8} and d-Ala\textsuperscript{B8} analogs, despite maintenance of a native T-like conformation of enhanced stability, strongly suggest that the T-state must undergo a conformational change on receptor binding. This does not imply, however, that the receptor-bound state of insulin resembles the R-state. It is possible, for example, that the receptor-bound state of insulin undergoes a conformational change on receptor binding. This approach, designated the TR transition. We and others have proposed that detachment of the C-terminal B-chain residues B1–B4 may be deleted without significant change in receptor binding (43). We imagine that the TR transition exploits the intrinsic flexibility of Gly\textsuperscript{B8}. The structural reorganization of the zinc insulin hexamer may otherwise be unrelated to the induced fit of the active monomer. Resolving this issue will require a co-crystal structure of a hormone-receptor complex.

The incomplete correspondence between the TR transition and the mechanism of receptor binding is further illuminated by studies of the single chain analog mini-proinsulin (4). This inactive analog exhibits native assembly and interconversion among T\textsubscript{o}, T\textsubscript{3}R\textsubscript{3}, and R\textsubscript{6} hexamers (41). Further, its crystal structure reveals that the B29-A1 tether does not constrain or perturb either T or R protomers (4). The inactivity of mini-proinsulin thus indicates that aspects of induced fit in the hormone-receptor complex must exceed or be unrelated to the TR transition. We and others have proposed that detachment of the C-terminal B-chain \beta-strand, locked into place within the dimer interface of zinc insulin hexamers, releases a receptor binding arm that exposes an otherwise hidden A-chain surface (31, 37, 44–46).6 We thus envisage that both the N- and C-terminal segments of the B-chain reorganize on receptor binding.

In summary, our results strongly suggest that the classical insulin T-state represents an inactive conformation of the hormone. By identifying a critical hinge point at Gly\textsuperscript{B8}, studies of mirror-image D and L substitutions provide evidence for induced fit on receptor binding. This approach, designated chiral mutagenesis, exemplifies a general strategy for the analysis of protein function through total chemical synthesis. The receptor-bound structure of insulin promises to enable design of novel agonists for the treatment of diabetes mellitus.

\textsuperscript{6} In classical structures, the C-terminal B-chain \beta-strand covers Ile\textsuperscript{A2} and Val\textsuperscript{A3}. Analogs containing allo-Ile\textsuperscript{A2} (in which the chirality of the \beta-carbon is inverted; 18) or Leu\textsuperscript{A2} exhibit native structure but low activity (26, 37, 47). Destabilization of the B-chain \beta-strand by substitution of Phe\textsuperscript{A24} by \alpha-Phe enhances activity (13, 48).

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