Insulin contains a β-turn (residues B20–B23) interposed between two receptor-binding elements, the central α-helix of the B chain (B9–B19) and its C-terminal β-strand (B24–B28). The turn contains conserved glycines at B20 and B23. Although insulin exhibits marked conformational variability among crystal forms, these glycines consistently maintain positive ϕ dihedral angles within a classic type-I β-turn. Because the Ramachandran conformations of GlyB20 and GlyB23 are ordinarily forbidden to L-amino acids, turn architecture may contribute to structure or function. Here, we employ “chiral mutagenesis,” comparison of corresponding D- and L-Ala substitutions, to investigate this turn. Control substitutions are introduced at GluB21, a neighboring residue exhibiting a conventional (negative) ϕ angle. The D- and L-Ala substitutions at B23 are associated with a marked stereospecific difference in activity. Whereas the D-AlaB23 analog retains native activity, the L analog exhibits a 20-fold decrease in receptor binding. By contrast, D- and L-AlaB20 analogs each exhibit high activity. Stereospecific differences between the thermodynamic stabilities of the analogs are nonetheless more pronounced at B20 (ΔΔG°u 2.0 kcal/mole) than at B23 (ΔΔG°u 0.7 kcal/mole). Control substitutions at B21 are well tolerated without significant stereospecificity. Chiral mutagenesis thus defines the complementary contributions of these conserved glycines to protein stability (GlyB20) or receptor recognition (GlyB23).

Insulin, a small globular protein secreted by pancreatic β cells, plays a central role in the control of vertebrate metabolism (1). The hormone is stored in glucose-regulated secretory granules as microcrystalline arrays of Zn2+-stabilized hexamers (2) and functions as a Zn2+-free monomer (3). The structure of insulin is well characterized (3). In the T state,1 the predominant conformation of the monomer in solution (4–6), the A chain contains N- and C-terminal α-helices; the B chain contains a central α-helix flanked by N- and C-terminal extended segments (Fig. 1A). The product of a single-chain precursor, designated proinsulin (7), insulin contains three disulfide bridges required for its folding, stability, and function (5, 8–10). Structure-function relationships have been extensively investigated (for review, see Ref. 11).

The B chain of insulin contains two β-turns (boxed in Fig. 1A, see below). The first is a type-II β-turn comprising residues B7–B10 (black box); the second is a type-I β-turn comprising residues B20–B23 (red box; ball-and-stick model in Fig. 1B). Each contains one or more conserved glycines: GlyB8, GlyB20, and GlyB23 (Fig. 1C). These residues exhibit positive ϕ angles (“d-glycines”) and, therefore, reside on the right side of the Ramachandran plane in regions unfavorable for L-amino acids. GlyB8, which adjoins the A7–B7 inter-chain disulfide bridge, is invariant among vertebrate insulins and highly conserved among insulin-related growth factors (black box in Fig. 1C). Mutations at B8 markedly impair the stability of insulin and the yield of chain combination (12, 13); the efficiency of recombinant expression of a single-chain precursor (mini-proinsulin) in Saccharomyces cerevisiae is likewise reduced (14, 15). Whereas such unstable insulin analogs can be highly active, D-amino acid substitutions at B8 augment stability but markedly impair activity (12, 13). Despite these profound stereospecific differences, the overall structures of D- and L B8 analogs closely resemble native insulin (13, 16).

In this report we employ “chiral mutagenesis” to investigate the B20–B23 β-turn. The turn sequence is notable for conserved negative and positive charges (GluB21 and ArgB22 in eutherian mammals) flanked by glycines (Fig. 1C). Experimental design is motivated by the pattern of main-chain dihedral angles (Table 1). To investigate the interrelation of structure, activity, and stability, isomeric analogs have been synthesized in which GlyB20 or GlyB23 is substituted by D- or L-Ala; control

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2 The on-line version of this article (available at http://www.jbc.org) contains Figs. S1–S3, providing additional molecular graphics.

1 Supported in part by the Diabetes Research and Training Center at the University of Chicago.  
2 To whom correspondence should be addressed: Dept. of Biochemistry, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4935. Tel: 216-368-5991; Fax: 216-368-3419; E-mail: maw21@case.edu.

3 In zinc-insulin hexamers, insulin folds as T or R state protomers. These conformations differ in the secondary structure of B1–B8, extended in the T state and α-helical in the R state. The TR transition is also characterized by a change in the handedness of cystine A7–B7; 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 ϕ angle and handedness of cystine A7–B7 may account for the low yield of chain combination in synthesis of analogs containing L-amino acid substitutions at B8. The conformation of the B20–B23 β-turn is similar in the T and R states.
substitutions have likewise been introduced at B21. Characterization of corresponding D- and l-Ala analogs demonstrates a wide spectrum of stereospecific perturbations, enabling residue-specific decomposition of turn function. Whereas the main-chain conformation and negative charge of GluB21 are of marginal importance, consistent with previous studies (17, 18), GlyB20 and GlyB23 make key yet complementary contributions: a D-conformation at B20 enhances stability, whereas that at B23 is essential for receptor recognition. l-Ala substitutions at either position impede disulfide pairing. Together, these results illuminate the critical contributions of a conserved β-turn to the folding, stability, and function of insulin.

**EXPERIMENTAL PROCEDURES**

*Materials*—Human insulin and des-octapeptide(B23–B30)-AspB10-insulin were provided by Eli Lilly and Co. (Indianapolis, IN). Isolated A-chain tetra-S-sulfonate derivatives were obtained by oxidative sulfitolysis of insulin followed by separation of S-sulfonated A and B chains (19). Trypsin (t-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was purchased from Worthington (Freehold, NJ). Bio-Gels are from Bio-Rad Laboratories. N-α-tert-Butyloxy carbonyl(t-Boc)-protected D-amino acids are from Bachem (Torrance, CA). All other N-t-Boc-protected amino acids and N-t-Boc-O-benzylthreonine-4-hydroxymethylphenylacetic acid methyl ester resin were from Applied Biosystems (Foster City, CA). Analytical and preparative reverse-phase HPLC were performed with Vydec columns (C4 or C18, Hesperia, CA). The molecular weight of each analog was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Relative yields of HPLC-purified products (purity of >98%, Table 2) were determined by analytical HPLC.

**Chain Combination**—Insulin chain combination (25) was effected by interaction of S-sulfonated derivatives of the A-chain (5 μmol) and B-chain analogs (2 μmol) in 0.1 M glycine buffer (pH 10.6, 4 ml) in the presence of dithiothreitol (18 μmol). Each analog was purified by size-exclusion chromatography (B20 and B23 analogs, Bio-Gel P-4 or P-6) in 0.1 M NH4CO3 and preparative reverse-phase HPLC on C4 or C18 columns. The purity of each B-chain analog was confirmed by analytical HPLC.

**Semi-synthesis of l-AlaB23-DKP-Insulin**—Because of the low yield of AlaB23-DKP-insulin on chain combination (above), additional material was prepared by a trypsin-catalyzed semi-synthetic method that circumvents the inefficiency of disulfide pairing (26, 27). In brief, des-octapeptide(B23–B30)-AspB10-insulin (15 mg), synthetic octapeptide (Ala-Phe-Phe-Thr-Lys-Pro-Thr, 20 mg), and l-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (1.5 mg) were dissolved in a mixture of dimethylacetamide/1,4-butanediol/0.2 M Tris acetate (pH 8) containing 10 mM calcium acetate and 1 mM EDTA (35:35:30, v/v, 0.4 ml). The final pH of the mixture was adjusted to 7.0 with 10 μl of N-methylmorpholine and incubated at 12 °C for 2 days. An additional aliquot of enzyme (1.5 mg) was added after 1 day. The reaction mixture was acidified and purified by preparative reverse-phase HPLC (C4) with a final yield of 5.6 mg.

**TABLE 1**

| Residue | φ | ψ |
|---------|---|---|
| GlyB20  | 55.9 ± 6.9 | -138.7 ± 7.0 |
| GluB21  | -59.2 ± 14.7 | -19.3 ± 13.2 |
| ArgB22  | -67.8 ± 5.6  | -35.7 ± 7.5  |
| GlyB23  | 84.1 ± 8.6  | -178.8 ± 4.6  |

The conformation of the B20–B23 turn was analyzed in 14 T state crystallographic protomers (PDB accession codes 4INS, 2INS, 1APH, 1CPH, 1DPH, 1TRZ, 1TYL, 1TYM, 1ZNI, 1LPH, and 1G7A). Glycines residues with positive φ angles are designated ’d’glycines by analogy to the conformational preferences of d-amino acids.
Receptor Binding Assays—Relative activity is defined as the ratio of analog to wild-type human insulin required to displace 50% of specifically bound $^{125}$I-human insulin. A human placental membrane preparation containing the IR was employed as described (28). In all assays the percentage of tracer bound in the absence of competing ligand was $\leq 15\%$ to avoid ligand-

![Structure of insulin and B-domain sequences](image)

**A**. Cylinder model of an insulin monomer showing positions of the two $\beta$-turns in the B chain (boxed); residues B7–B10 (type II $\beta$-turn, box 1) and B20–B23 (type I $\beta$-turn, box 2). The A chain contains shaded cylinders, and the B chain contains an open cylinder and arrow. C$_y$ positions of invariant GlyB8 and GlyB23 are indicated by red circles; the position of conserved GlyB20 is indicated by a green circle. B. Structure of B20–B23 $\beta$-turn. Residues B20 and B23 are shown in green and red, respectively; residues B21 and B22 are shown in black. Coordinates of T-state protomer were obtained from T$_6$ zinc hexamer (2-Zn molecule 1, Protein Data base entry 4INS). C. Sequence alignment of vertebrate insulin B chains and homologous B domains of insulin-like growth factors I and II. Turns 1 and 2 are boxed. GlyB8 and GlyB23 (invariant) are highlighted in red, and GlyB20 (highly conserved) are green. Cystines are in boldface.

**Figure 1. Structure of insulin and B-domain sequences.** A, cylinder model of an insulin monomer showing positions of the two $\beta$-turns in the B chain (boxed): residues B7–B10 (type II $\beta$-turn, box 1) and B20–B23 (type I $\beta$-turn, box 2). The A chain contains shaded cylinders, and the B chain contains an open cylinder and arrow. C$_y$ positions of invariant GlyB8 and GlyB23 are indicated by red circles; the position of conserved GlyB20 is indicated by a green circle. B, structure of B20–B23 $\beta$-turn. Residues B20 and B23 are shown in green and red, respectively; residues B21 and B22 are shown in black. Coordinates of T-state protomer were obtained from T$_6$ zinc hexamer (2-Zn molecule 1, Protein Data base entry 4INS). C, sequence alignment of vertebrate insulin B chains and homologous B domains of insulin-like growth factors I and II. Turns 1 and 2 are boxed. GlyB8 and GlyB23 (invariant) are highlighted in red, and GlyB20 (highly conserved) are green. Cystines are in boldface.

Receptor Binding Assays—Relative activity is defined as the ratio of analog to wild-type human insulin required to displace 50% of specifically bound $^{125}$I-human insulin. A human placental membrane preparation containing the IR was employed as described (28). In all assays the percentage of tracer bound in the absence of competing ligand was $<15\%$ to avoid ligand-

![Structure of insulin and B-domain sequences](image)
depletion artifacts. Assays were repeated three to five times (legend to Table 2). Changes in relative receptor-binding affinities were converted to changes in free energy of binding ($\Delta \Delta G_b$ in kcal/mole) according to the equation, $\Delta G_b = -RT \ln(K_{eq})$.

**CD Spectroscopy**—Far-UV CD spectra were obtained using a thermistor-controlled Aviv spectropolarimeter equipped with an automated titration unit for guanine denaturation studies. Spectra (repeated twice) were obtained from samples containing 25–50 $\mu$M insulin analog in 10 mM potassium phosphate (pH 7.4) and 50 mM KCl at 4 °C. Equilibrium denaturation studies were conducted at 4 and 25 °C. Estimates of secondary structure were obtained by deconvolution (64). Samples were diluted to 5 $\mu$m; guanidine-HCl was employed as the denaturant (29).

**Thermodynamic Modeling**—Guanidine denaturation data were fitted by non-linear least squares to a two-state model (30). In brief, CD data $\theta(x)$, where $x$ indicates the concentration of denaturant, were fitted by a nonlinear least-squares program according to Equation 1,

$$
\theta(x) = \frac{\theta_0 + \theta_1 e^{-(-\Delta G_{H,b} - m \chi)/RT}}{1 + e^{-(-\Delta G_{H,b} - m \chi)/RT}} \quad \text{(Eq. 1)}$

where $x$ is the concentration of guanidine hydrochloride and where $\theta_0$ and $\theta_1$ are baseline values in the native and unfolded states. These baselines were approximated by pre- and post-transition lines $\theta_0(x) = \theta_0^H + m_0 x$ and $\theta_1(x) = \theta_1^H + m_1 x$. Fitting the original CD data and baselines simultaneously circumvents artificats associated with linear plots of $\Delta G$ as a function of denaturant according to $\Delta G_b(x) = \Delta G_{H,b} + m x$ (30).

**RESULTS**

**Overview of Experimental Design**—The B20–B23 $\beta$-turn adjoins the A20–B19 disulfide bridge and is interposed between two critical receptor-recognition elements (11), the central $\alpha$-helix and its C-terminal $\beta$-strand of the B chain (Fig. 1). The location and broad conservation of this turn suggest important roles in folding or function. Our studies focus on $\alpha$-glycines at positions B20 and B23. Molecular models suggest that $\alpha$- or $\gamma$-Ala substitutions at these sites would be well accommodated on the surface of a native-like structure (see supplemental material).

**Synthesis**—Seven analogs of DKP-insulin containing substitutions at positions B20, B21, or B23 were prepared by total chemical synthesis (Table 2). Incorporation of the three “DKP” substitutions in the B chain enabled study of $\beta$-turn variants in a uniform monomeric context (20). This framework facilitates interpretation of physical studies as otherwise apparent effects of $\beta$-turn substitutions could be influenced by associated perturbations in self-assembly (31). The activity of DKP-insulin is ~2-fold higher than that of human insulin (20). Broad experience with DKP-insulin analogs indicates that effects of additional substitutions on receptor binding mirror those of the same substitutions in wild-type insulin.

Synthetic yields, reflecting the efficiency of disulfide pairing in chain combination (29), varied over a 7-fold range (Table 2). Whereas high yields were obtained in syntheses of $\alpha$-Ala$^{B20}$ and $\gamma$-Ala$^{B23}$ analogs, low yields were obtained in corresponding $\gamma$-Ala syntheses. The yield of $\gamma$-Ala$^{B20}$, DKP-insulin was impaired to an extent similar to that previously observed in the biosynthetic expression of a corresponding single-chain analog (1-$\gamma$-Ala$^{B23}$-mini-proinsulin) in S. cerevisiae (18). By contrast, 2-fold-reduced yields were obtained without stereospecificity in control syntheses of d-$\gamma$-Ala$^{B23}$ and $\gamma$-Ala$^{B23}$-DKP-insulin (Table 2), also consistent (in the case of $\gamma$-Ala$^{B23}$) with biosynthetic experience (18). Chiral inversion of the B20 $\alpha$-carbon (i.e. substitution of the native L-Glu$^{B21}$ by d-Glu$^{B21}$) also resulted in a 2-fold lower yield. The marked stereospecific differences in yield among B20 and B23 analogs were remarkable in relation to prior syntheses of unstable and partially unfolded A-chain variants in which yields were essentially unaffected (5G-DKP-insulin and DKP-des-[A6–A11]) (Table 2) (5, 8, 29). The latter analogs contained substitutions in the N-terminal segment of the A chain (see legend to Table 2) that are remote from the B20–B23 $\beta$-turn. To circumvent the inefficiency of disulfide pairing encountered in the synthesis of 1-$\gamma$-Ala$^{B23}$-DKP-insulin, additional material was prepared by semi-synthesis using a preformed insulin framework (des[B23–

**TABLE 2**

**Properties of insulin analogs**

DKP-insulin contains three substitutions in the B chain that prevent classic self-association (see “Experimental Procedures”). Analogues were synthesized as diastereomeric pairs; DKP-insulin (like human insulin) contains L-Glu$^{B21}$. Percent receptor-binding affinities (relative to human insulin) were determined at 4 °C (13); five replicates of L-insulin, B20 analogs, and B23 analogs) or three replicates (B21 analogs) were obtained. L-Glu$^{B21}$-DKP-insulin in B indicates the parent analog; affinity shown was obtained at time of B21 studies and differs slightly from earlier studies. Assay underestimates relative affinities >150%. The dissociation constant for native human insulin is 0.4–0.5 nM.

| Analog | Activity | Yield$^a$ | $\Delta G_b$ | $C_{\text{null}}$ | m$^f$ |
|--------|----------|-----------|-------------|-----------------|------|
|        | % kcal/mol | µ | kcal/mole/mol |
| A) Thermodynamics at 4 °C | | | | | |
| Human insulin | 100 | 100 | 4.4 | 5.3 | 0.84 ± 0.01 |
| i-L-Glu$^{B21}$-DKP-In | 202 ± 16 | 190 | 3.8 | 5.7 | 0.67 ± 0.02 |
| d-5G-DKP-In | 227 ± 31 | 114 | 5.8 | 6.0 | 0.80 ± 0.01 |
| d-5G-DKP-In | 8 ± 2 | 13 | 4.4 | 6.2 | 0.71 ± 0.01 |
| d-5G-DKP-In | 175 ± 20 | 79 | 5.1 | 6.1 | 0.84 ± 0.01 |
| 5G-DKP-In$^b$ | <0.01 | 102 | 2.7 | 4.2 | 0.63 ± 0.01 |
| d-5G-DKP-In$^b$ | <1 | 90 | <2.2 | 3.5 | 0.65 ± 0.07 |
| B) Thermodynamics at 25 °C | | | | | |
| Human insulin | 100 | 100 | 3.3 | 4.8 | 0.70 ± 0.01 |
| i-L-Glu$^{B21}$-DKP-In | 208 ± 26 | 110 | 4.0 | 6.0 | 0.70 ± 0.01 |
| d-Glu$^{B21}$-DKP-In | 196 ± 11 | 57 | 3.9 | 5.9 | 0.67 ± 0.01 |
| i-L-5G-DKP-In | 115 ± 13 | 55 | 4.1 | 5.7 | 0.71 ± 0.01 |
| d-L-5G-DKP-In | 137 ± 18 | 40 | 3.7 | 5.9 | 0.62 ± 0.01 |

$^a$ Yields are based on HPLC-purified product in a single synthesis. Multiple syntheses of DKP-insulin indicate that yields are reproducible to within ± 10% of the mean value. In a typical reaction final yields of 4.5–5.0 mg of DKP-insulin were obtained following chain combination employing 20 mg of variant B chain and 40 mg of A chain (as S-sulfonate derivatives). Bold values highlight marked perturbations of i- or d-Ala substitutions on activity (L- or d-Ala$^{B21}$), yield (B20 and B21), and stability (B20 and B23). Values in italics indicate enhanced stability of $\alpha$-Ala and $\gamma$-Ala analogs at B20 and B23.

$^b$ $\Delta G_b$ indicates apparent change in free energy on denaturation in guanidine-HCl as extrapolated to zero denaturant concentration by a two-state model (30). Statistical uncertainty of two-state fitting is in each case ± 0.05 kcal/mole or less with the exception of D-5G-DKP-des-[A6–A11] (for which only upper bounds may be extracted (8)).

$C_{\text{null}}$ is defined as that concentration of guanidine-HCl at which 50% of the protein is unfolded. Statistical uncertainty of two-state fitting is in each case ± 0.1 m or less.

$^c$ The m value provides the slope in plotting unfolding free energy $\Delta G_b$ versus molar concentration of denaturant; this slope is proportional to the protein surface area exposed on unfolding.

$^d$ Analog contains four A-chain substitutions: residues A2–A5 were each replaced by glycine (29).

$^e$ Analog contains pairwise substitution of cystine A6–A11 by serine as described previously (5, 8).
This protocol does not apply to B20 analogs.

Structure and Stability—Far-UV CD spectra of the analogs were in each case similar to that of DKP-insulin (Fig. 2). Spectra of the B20 analogs (Fig. 2A), B21 analogs (Fig. 2B), and B23 analogs (Fig. 2C) in each case approximated the parent spectrum (blue line in each panel). Values of mean residue ellipticity at helix-sensitive wavelengths (196, 208, and 222 nm) are given in Table 3A. Whereas stereospecific spectral differences were not significant at B20 and B21 (Fig. 2A and B, respectively), the spectrum of D-AlaB23-insulin (black line in Fig. 2C) exhibited an accentuation of mean residue ellipticity at 208 and 222 nm (Table 3A). This accentuation was not due to a change

FIGURE 2. Far-UV CD spectra of insulin analogs. A, comparison of spectra of DKP-insulin and B20 analogs: DKP-insulin (solid blue line), D-AlaB20 (dashed black line), and L-AlaB20 (filled green diamonds). B, comparison of DKP-insulin and B21 analogs: DKP-insulin (solid blue line), D-GluB21 (dashed black line), L-AlaB21 (filled brown squares), and D-AlaB21 (brown open circles). C, comparison of DKP-insulin and B23 analogs: DKP-insulin (solid blue line), D-AlaB23 (solid black line), and L-AlaB23 (filled red triangles). Mean residue ellipticities and selected deconvolution parameters are given in Table 3.

FIGURE 3. Thermodynamic studies of monomeric insulin analogs. CD-detected unfolding transitions induced by guanidine hydrochloride are plotted as percent change in mean residue ellipticity at 222 nm. A, comparison of DKP-insulin and B20 analogs at 4 °C: DKP-insulin (solid blue line), L-AlaB20 (red open diamonds), D-AlaB20 (filled red triangles), D-AlaB20 (solid black line), and D-AlaB20 (dashed black line). B, DKP-insulin and B21 analogs at 25 °C: solid blue line, DKP-insulin; dashed black line, D-GluB21; filled brown squares, L-AlaB21; and brown open circles, D-AlaB21. Inferred thermodynamic parameters and other two-state model parameters are given in Table 2.

TABLE 3
CD parameters and deconvolution
For the parameters, spectra were obtained at 4 °C in 50 mM KCl and 10 mM potassium phosphate (pH 7.4); [θ] indicates the mean residue ellipticity. Values at 196 nm are of lower precision (±1°) due to higher dynode voltage. Values of mean residue ellipticity at 208 and 222 nm were determined to ±1%; errors at 196 nm were ±5%. Fractional deconvolution parameters fα, fβ, fπ, and fr indicate percent α-helix, β-sheet, β-turn, and random coil, respectively. Deconvolution of CD spectra was obtained using the Selec3 method of Sreerama and Woody (64). The basis set employs stably folded globular proteins and may not pertain to flexible polypeptides or molten domains.

| A) Analog | [θ]α | [θ]β | [θ]π | [θ]rc |
|-----------|------|------|------|------|
| DKP-insulin (DKP-In) | 25 | -18.0 | -10.7 | 0.60 |
| L-AlaB20-DKP-In | 17 | -17.7 | -10.4 | 0.59 |
| D-AlaB20-DKP-In | 23 | -18.0 | -10.7 | 0.58 |
| D-GluB21-DKP-In | 23 | -18.1 | -10.7 | 0.57 |
| L-AlaB21-DKP-In | 22 | -17.1 | -10.3 | 0.60 |
| D-AlaB21-DKP-In | 23 | -16.4 | -9.7 | 0.59 |
| D-GluB21-DKP-In | 23 | -18.2 | -10.5 | 0.58 |
| L-AlaB23-DKP-In | 23 | -18.1 | -10.2 | 0.57 |
| D-AlaB23-DKP-In | 24 | -20.2 | -12.4 | 0.61 |

| B) Analog | fα | fβ | fπ | fr |
|-----------|----|----|----|----|
| DKP-insulin | 43 | 14 | 14 | 29 |
| L-AlaB20-DKP-In | 43 | 15 | 20 | 22 |
| D-AlaB20-DKP-In | 42 | 17 | 18 | 23 |
in protein concentration, because the normalized spectrum, exhibiting a subtle change in overall shape, did not differ from the parent spectrum by a uniform scaling factor. Despite increased ellipticity at 208 and 222 nm, their ratio is similar to that of DKP-insulin (Table 3); further, deconvolution of the spectrum of d-AlaB23-DKP-insulin did not suggest an increase in α-helix content (Table 3B). Because residue B23 is located at the junction of a β-turn and β-strand and so does not lie within an α-helix, we attribute the subtle change in its CD spectrum to transmitted conformational effects (see “Discussion”).

The β-turn variants exhibited differences in overall thermodynamic stability as probed by CD-detected denaturant-induced unfolding (Fig. 3). As expected for substitutions of glycines with positive φ angles (i.e. in the D region of the Ramachandran plot; 32), d-AlaB20 and d-AlaB23 analogs exhibited greater stability than the parent DKP-insulin (column 4 in Table 2). Application of a two-state model permitted thermodynamic parameters to be extracted; the precision of fitting of free energies (ΔGm) was generally ± 0.05 kcal/mole. The gain in free energy (ΔΔGm, 0.9 ± 0.1 and 0.2 ± 0.1 kcal/mole, respectively) was more marked at position B20 than at B23, indicating that site-specific factors influence thermodynamic effects of D substitutions even in a solvent-exposed turn. Conversely, l-Ala substitutions at these sites impaired stability (Table 2A). The loss of free energy was more marked at B20 (open red diamonds in Fig. 3A, ΔΔGm = −1.1 ± 0.1 kcal/mole) than at B23 (filled red triangles, ΔΔGm = −0.5 ± 0.1 kcal/mole). Stereospecific differences in free energy between B20 and B23 diastereomers were thus 2.0 ± 0.1 kcal/mole (B20) and 0.7 ± 0.1 kcal/mole (B23). The m value, a parameter correlated with the extent of change in solvation of non-polar surfaces unfolding, was higher for the d-AlaB20 and d-AlaB23 analogs (0.80 ± 0.01 and 0.84 ± 0.01 kcal/mole/m, respectively) than in DKP-insulin (0.70 ± 0.01) or the corresponding l analogs (0.67 and 0.71, respectively). This stereospecific increase essentially restored the m parameter to its original value in wild-type insulin (0.84 ± 0.01). Because the reduction in the m value in DKP-insulin is likely to represent partial destabilization of the C-terminal B-chain β-strand due to the “KP” substitutions (ProB28 → Lys, and LysB29 → Pro), we speculate that the d-Ala substitutions at B20 and B23 damp fluctuations in this β-strand.

Control analogs at B21 by contrast exhibited less marked differences. Whereas l-AlaB21 and d-AlaB21 analogs exhibited stabilities similar to that of DKP-insulin (containing l-GluB21), that of d-AlaB21-DKP-insulin was somewhat reduced (ΔΔGm = −0.3 ± 0.1 kcal/mole). Although the B21 substitutions in principle affected electrostatic interactions (due to loss or chiral displacement of the side-chain carboxylate of GluB21), their similar stabilities suggest that this charge does not significantly contribute to the stability of insulin. Stereospecific effects on m values were also less marked at B21; values were slightly reduced for the d analogs, opposite to the pattern at B20 and B23 (Table 2). Stereospecific effects of the present substitutions were less marked in general than those of chiral substitutions previously described at position B8 (13, 16).

Biological Activities—The analogs exhibited a wide spectrum of receptor-binding affinities (Table 2). Representative receptor binding assays are shown in Fig. 4 (data points △ and ○) relative to native human insulin and DKP-insulin (filled lines). Substitution of GlyB20 by l-Ala (data points △ in Fig. 4A) conferred native or perhaps enhanced binding in accord with a previous report that l-AlaB20-insulin exhibits native or enhanced bind-
Chiral Mutagenesis of Insulin

ing to the secreted receptor ectodomain (18). Similarly, D-Ala$^{B20}$-DKP-insulin exhibited native or enhanced binding (∇ in Fig. 4A). Although at B23 the more stable D analog was more active than the less stable L analog (Fig. 4C), at B20 D- and L-analogs exhibited similar activities despite their different stabilities (Fig. 4A). Together, these results demonstrate that stability and activity cannot generally be correlated as previously proposed (33). The D- and L-Ala$^{B21}$-DKP-insulin analogs each exhibited similar small reductions in receptor binding relative to DKP-insulin (Δ and ∇, respectively, in Fig. 4B; bottom two lines in Table 2). Thus, despite the conservation of a negatively charged side chain at B21, this charge makes only a modest contribution to receptor binding.

Our receptor binding studies compare with previous reports as follows. The low activity of L-Ala$^{B21}$-DKP-insulin (~20-fold lower than that of DKP-insulin, boldface in column 2 of Table 2A) is in accord with an early report by Inouye and colleagues (34) in which both receptor binding and potency (in stimulating glucose update by adipocytes) were found to be reduced by 25- to 30-fold. That the activity of D-Glu$^{B21}$-DKP-insulin was similar to that of DKP-insulin (Table 2) is also in accord with previous studies of Glu$^{B21}$-insulin (17). The 2-fold lower activity of L-Ala$^{B21}$-DKP-insulin (relative to DKP-insulin) is not in accord with a previous study in which this substitution was reported to enhance binding of insulin to the receptor ectodomain by 2-fold (18). Stereospecific effects of these and B8 substitutions on activity and stability are summarized in the histogram shown in Fig. 5.

DISCUSSION

In this report we have investigated the B20–B23 β-turn in insulin by chiral mutagenesis. The structure of this turn is shown in Fig. 5C in relation to the A20–B19 disulfide bridge and core packing. The B20–B23 turn is integral to canonical B-chain super-secondary structure: the chain reversal enables the C-terminal β-strand to pack against the central α-helix. Although the turn is more flexible than these adjoining structural elements (as indicated by higher crystallographic B factors (3)), its pattern of hydrogen bonds and dihedral angles (characteristic of a type-I β-turn) is essentially identical among multiple crystal forms, including T$_{50}$, T$_{46}R_{50}$, and R$_{6}$ zinc-insulin hexamers (3, 35). The turn conformation is independent of zinc binding and hexamer assembly (36–38). Studies of insulin analogs have suggested that the turn is stabilized by Phe$_{B24}$ (4, 39, 40), because the aromatic ring packs against Leu$_{B15}$ and Cys$_{B19}$ at the edge of the hydrophobic core (Fig. 5C).

The B20–B23 sequence is conserved among vertebrate insulins and insulin-like growth factors. The consensus is Gly-X-Y-Gly in which X is usually an acidic residue and Y is usually Arg (Fig. 1C). The flanking glycines exhibit postive φ angles, whereas intervening residues B21 and B22 exhibit negative φ angles (Table 1). Gly$_{B23}$ is invariant, whereas Gly$_{B20}$ is strongly but not rigorously conserved. In this report, comparison of corresponding D- and L-amino acid substitutions at positions B20 and B23 have enabled the respective contributions of these glycines to structure and function to be resolved. Site-specific inversion of chiral centers provides a powerful approach with which to test structure-function relationships in proteins amenable to total chemical synthesis.

Stereospecific Effects on Synthetic Yield—Substitution of Gly$_{B20}$ or Gly$_{B23}$ by L-Ala impairs chain combination and the thermodynamic stability of the folded state (Table 2). Chain combination is under kinetic control (19, 41); therefore, effects of substitutions on the efficiency of disulfide pairing is not well correlated with effects on stability. Indeed, the yield of L-Ala$^{B23}$-DKP-insulin (13% relative to DKP-insulin) was somewhat lower than that of the L-Ala$^{B20}$ analog (19%), despite their opposite order of stabilities (Table 2A). Such subtle discordance between yields and stabilities is in accord with past experience (29). More marked examples are observed for pairwise substitution of cysteine by Ser (DKP-des-[A6–A11]; Table 2A) or following the simultaneous substitution of residues A2–A5 by Gly (5G-DKP-insulin). These analogs exhibit very low stabilities (ΔΔG$_{o}$ > 2.2 kcal/mole) and yet undergo efficient chain combination (29, 42). Thus, perturbation of chain combination by L-Ala substitutions at B20 and B23 cannot be ascribed to modest decrements in stability; indeed, the L-Ala$^{B23}$ analog is as stable as native insulin (Table 2A). The L-specific perturbations of chain combination are rescued by C$_{α}$-chirality inversion in syntheses of corresponding D-Ala analogs.

We propose (a) that formation of a nascent B20–B23 turn occurs in an on-pathway folding intermediate in the kinetic mechanism of disulfide pairing and (b) that this chain reversal is favored by positive ϕ angles at B20 and B23. A nascent B20–B23-associated chain reversal with packing of Phe$_{B24}$ against the central B-chain α-helix has been observed in engineered models of two-disulfide proinsulin intermediates containing either pairings (A20–B19 and A6–A11) or (A20–B19 and A7–B7) (5, 8, 9). Similar conformational features have been observed in homologous models of insulin-like growth factor-I folding intermediates (43, 44), including a small peptide model of the key one-disulfide intermediate containing cystines 18–61 (canonical cystine A20–B19 in insulin). These models provide evidence for the stepwise stabilization of specific structures on successive disulfide pairing.

Thiol-trapping studies of oxidative protein-folding intermediates of both a single-chain insulin analog and insulin-like growth factor-I suggest that cystine A20–B19 is the first disulfide bridge to be formed (46–48). Pairing of cystine A20–B19 is proposed to be directed by the nascent structure of a specific folding nucleus containing native-like B9–B26 super-secondary structure and the C-terminal A-domain α-helix. Although flexible, side-chain packing within this nucleus may resemble interactions in the native state (Fig. 5C). We imagine that L-Ala substitutions at B20 and B23 destabilize this nucleus and so impair the kinetic efficiency of the conformational search; D-Ala substitutions would have the opposite effect. Although the present chain-combination studies were performed under strongly alkaline conditions, their physiological relevance is suggested by the parallel effects of site-directed Ala mutations on the biosynthetic expression of mini-proinsulin analogs in yeast (18). It would be of interest to test the effects of such Ala

5 Q. X. Hua, J. Mayer, W. Jia, J. Zhang, and M. A. Weiss, submitted for publication.
substitutions on the foldability of proinsulin in transfected mammalian secretory cell lines.

**Stereospecific Effects on Stability**—Whereas \( \alpha \)-Ala substitutions at B20 and B23 impair the stability of insulin, \( \beta \)-Ala and \( \alpha \)-Ala at B21 (horizontal axis), the black bars indicate \( \beta \)-analog, and gray bars indicate \( \alpha \)-analog; \( \alpha \)-Glu\( ^{12} \)DKP-insulin is not shown. Contrast in findings between chiral substitutions at B23 and control substitutions at B21 are highlighted in red and green, respectively. \( \alpha \)_histogram of protein stabilities showing marked stereospecific effects at B8 (observed with either (DL)-Ala or (DL)-Ser (13, 16)) and progressively less marked effects at B20, B23, and B21. \( \beta \)_histogram of receptor-binding free energies exhibits a distinct pattern. Decreased binding of B8 analogs (less marked for \( \alpha \)-Ser\( ^{6} \)DKP-insulin) contrasts with native or modestly increased activities of B20 analogs. The red asterisk indicates decreased activity of \( \alpha \)-Ala\( ^{22} \)DKP-insulin.

**C** Molecular structure (stereo pair) showing B20–B23 \( \beta \)-turn and adjoining portions of the A and B chains. Selected side chains highlight long range contacts between chains within B-chain supersecondary structure. A-chain side chains are labeled at the left, and B-chain side chains at the right. Gly\( ^{20} \) and Gly\( ^{23} \) are shown in green and red, respectively; B21 and B22 main-chain atoms are black; the A20–B19 disulfide bridge is gold; the A chain is purple; and the B chain are blue. Coordinates were obtained from Protein Data base entry 4INS.

**FIGURE 5.** Stereospecific effects of \( \beta \)- and \( \alpha \)-substitutions and structural environments. A and B, histograms showing changes in protein stability (A) and receptor binding (B). Positive bars indicate gain in stability or binding; negative bars indicate decrease. For each residue position (B8 Ala, B8 Ser, B20, B23, and B21; horizontal axis), the black bars indicate \( \beta \)-analog, and gray bars indicate \( \alpha \)-analog; \( \alpha \)-Glu\( ^{12} \)DKP-insulin is not shown. Contrast in findings between chiral substitutions at B23 and control substitutions at B21 are highlighted in red and green, respectively. A, histogram of protein stabilities showing marked stereospecific effects at B8 (observed with either (DL)-Ala or (DL)-Ser (13, 16)) and progressively less marked effects at B20, B23, and B21. B, histogram of receptor-binding free energies exhibits a distinct pattern. Decreased binding of B8 analogs (less marked for \( \alpha \)-Ser\( ^{6} \)DKP-insulin) contrasts with native or modestly increased activities of B20 analogs. The red asterisk indicates decreased activity of \( \alpha \)-Ala\( ^{22} \)DKP-insulin. C, molecular structure (stereo pair) showing B20–B23 \( \beta \)-turn and adjoining portions of the A and B chains. Selected side chains highlight long range contacts between chains within B-chain supersecondary structure. A-chain side chains are labeled at the left, and B-chain side chains at the right. Gly\( ^{20} \) and Gly\( ^{23} \) are shown in green and red, respectively; B21 and B22 main-chain atoms are black; the A20–B19 disulfide bridge is gold; the A chain is purple; and the B chain are blue. Coordinates were obtained from Protein Data base entry 4INS.


**Chiral Mutagenesis of Insulin**

studies of d-Ala<sup>88</sup>-DPK-insulin (ΔΔG<sub>c</sub> 1.5 kcal/mole (13)) and d-Ser<sup>88</sup>-DPK-insulin (ΔΔG<sub>c</sub> 0.9 kcal/mole (16)). Studies of d-Ala substitutions in turns within unrelated globular proteins by Raleigh and coworkers have yielded ΔΔG<sub>c</sub> values in the range of 0.60–1.87 kcal/mole (32).

That the stabilizing effect of d-Ala substitutions in turns varies from case to case is likely to reflect the details of these systems: the extent of native-like structure in respective unfolded state ensembles, favorable or unfavorable interactions by the d-Ala side chains in respective folded states, and possible structure-specific perturbations of protein solvation. We suggest that the marginal increase in stability associated with d-Ala<sup>B23</sup> (the lowest observed to date) is a consequence of transmitted conformational changes. In particular, the favorable entropic effect of the d-Ala<sup>B23</sup> substitution may be offset by perturbed packing of Phe<sup>B24</sup>. Although the altered CD spectrum of this analog is consistent with this view, rigorous interpretation will require the three-dimensional structure of this analog to be determined. Conversely, the very marked stabilization caused by substitution of Gly<sup>B88</sup> by d-Ala is likely to be due to favorable interactions of the d-methyl group near the protein surface: d-Ala<sup>B28</sup> partially inserts within a local non-polar pocket formed by Val<sup>A3</sup>, Leu<sup>B11</sup>, Val<sup>B12</sup>, and Tyr<sup>B26</sup> (13). We thus envisage that d-Ala<sup>B28</sup> not only biases the local conformation of the unfolded state ensemble, but also “repairs” a potential packing defect near the protein surface. These examples illustrate the potential complexity of interpreting in atomic terms how substitutions (whether by d- or L-amino acids) affect protein stability.

It would be of future interest to determine the high resolution crystal structures of the present analogs. A salient question is whether respective L- or d-amino acid substitutions do indeed impose stereospecific preferences for ϕ angles on the left or right sides of the Ramachandran plot; for example, would substitution of Gly<sup>B20</sup> by l-Ala cause miscalibration of the B20 ϕ angle, distorting the conformation of the β-turn, or would the overall structural context override the intrinsic chiral preferences of the l- or d-amino acids, thereby restoring a nature-like local structure? These questions are pertinent to interpretation of both stabilities and activities (below). The lower stability of l-Ala<sup>B20</sup>—DPK-insulin might reflect strain, for example, due to accommodation of an l-amino acid in a “forbidden” Ramachandran region. Alternatively, l-Ala<sup>B20</sup> might exhibit a canonical negative ϕ angle, leading to distortion of turn architecture and so introducing strain at other sites. Analogous considerations apply to the substitutions at B21 and B23. Given the range of ΔG<sub>c</sub> values (Table 2), the cost (in free energy) of such local or non-local accommodation presumably differs from position to position in the turn.

*Stereospecific Effects on Activity*—The d-amino acid substitutions at B20, B21, and B23 are compatible with native or enhanced receptor binding. Such functional compatibility is in striking contrast to the profound inhibition of receptor binding associated with substitution d-Ala<sup>B88</sup> (13). The absence of stereospecific effects at B21 suggests that native or non-native conformations at this site are equally competent to bind to the receptor; i.e. this portion of the protein surface is not engaged at the hormone-receptor interface. Effects of these and other substitutions on the free energy of receptor binding are illustrated in the histogram shown in Fig. 5B. Whereas the l-Ala<sup>B20</sup> analog (the least stable among the present analogs) exhibited high activity; the less destabilized l-Ala<sup>B23</sup> analog exhibited low activity.

Although the present structure-activity relationships are difficult to interpret in the absence of a co-crystal structure of the hormone-receptor complex, our overall results suggest that neither B20–B23 β-turn geometry nor its local stability correlate with activity. We speculate that maintenance of a positive dihedral angle at Gly<sup>B23</sup> facilitates engagement of the neighboring aromatic side chains of Phe<sup>B24</sup>, Phe<sup>B25</sup>, and Tyr<sup>B26</sup> at the hormone-receptor interface. The critical importance of these residues is highlighted by clinical mutations associated with diabetes (49). Anomalies in structure-activity relationships at residues in the C-terminal region of the B chain (50–53) have stimulated interest in possible conformational changes on receptor binding, focusing attention on the role of the B20–B23 β-turn. Whereas Liang and colleagues (39) have proposed that a native turn conformation is required for receptor binding, for example, such structures may in fact represent inactive conformers. This possibility is suggested by studies of an inactive single-chain analog (“mini-proinsulin”) in which a peptide bond tethers the C-terminal β-strand of the B-chain (Lys<sup>B25</sup>) to the N-terminal α-helix of the A-chain (Gly<sup>A1</sup>) (54, 55). Reorganization of the B20–B23 β-turn might enable the B24–B28 β-strand to detach from the core to contact the receptor (4). The low activity of l-Ala<sup>B23</sup> analogs may thus reflect impaired induced fit, due either to miscalibration of the B23 ϕ angle or steric clash of the ϕ-methyl group at the receptor interface.

The B24–B28 detachment hypothesis is supported by the high activities of analogs that exhibit structural perturbations of the B chain. Substitution of Phe<sup>B24</sup> (which anchors the C-terminal β-strand to the core (Fig. 5C and Ref. 3)) by glycine leads to an altered turn structure with near-native activity (4, 40). Further, the activity of insulin is enhanced by chiral substitution of Phe<sup>B24</sup> by D-Phe or d-Ala (20, 50); these substitutions appear to be incompatible with native structure. Whereas the C-terminal segment of the B chain functions to contact the N- and C-terminal domains of the receptor α-subunit (56), its reorganization is proposed to expose an otherwise hidden functional surface in the A chain (4, 54, 57–59).<sup>6</sup> Such models are consistent with photo-cross-linking studies of site-specific *para*-azido-Phe derivatives of insulin (56, 59, 60). In addition to the aromatic residues at B24–B26, key receptor-binding determinants include the side chains of Ile<sup>A2</sup>, Val<sup>A5</sup>, and Val<sup>B12</sup> (22, 61–63). Alanine scanning mutagenesis suggests that the C-terminal A-chain α-helix does not make a significant contribution to binding (18).

*Concluding Remarks*—The total chemical synthesis of proteins enables the efficient introduction of non-standard amino acids, extending the repertoire of site-directed mutagenesis to explore structure-activity relationships. A classic probe in synthetic chemistry is provided by inversion of chiral centers to

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<sup>6</sup> In classic structures of insulin the C-terminal B-chain β-strand covers Ile<sup>A2</sup> and Val<sup>A5</sup>. Analogs containing *allo*-Ile<sup>A2</sup> (in which the chirality of the β carbon is inverted (63)) or Leu<sup>A5</sup> exhibit native structure but low activity (57–59).
permit comparison of diastereomers. Protein diastereomers containing corresponding D- and L-amino acid substitutions at conserved glycines exploit the unique flexibility of this achiral residue to occupy a broad range of conformations in the Ramachandran plane. Comparison of such reciprocal substitutions provides a general strategy to explore determinants of protein stability and conformational change (13, 32, 45).

Application of chiral mutagenesis to the conserved B20–B23 \(\beta\)-turn of insulin distinguishes between determinants of stability and function. The two glycines in this turn exhibit reciprocal stereospecific signatures on substitution by D- or L-alanine. Whereas the D-conformation of one residue (Gly\(^{B23}\)) contributes to stability, the other (Gly\(^{B20}\)) is required for high affinity receptor recognition. We envisage that Gly\(^{B23}\) functions in the mechanism of induced fit to optimize binding of the C-terminal strand of the B chain between domains of the insulin receptor (56, 59). Further, given L-Ala-associated perturbations in efficiency of disulfide pairing, we speculate that a nascent chain reversal in the unfolded polypeptide facilitates the oxidative folding of proinsulin in the endoplasmic reticulum. Together, our results strongly suggest that the broad conservation of Gly\(^{B20}\) and Gly\(^{B23}\) among vertebrate insulins and insulin-like growth factors is enjorced by the complementary contributions of the B20–B23 \(\beta\)-turn to folding, stability, and function.

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REFERENCES

1. Dodson, G., and Steiner, D. (1998) Curr. Opin. Struct. Biol. 8, 189–194
2. Michael, J., Carroll, R., Swift, H. H., and Steiner, D. F. (1987) J. Biol. Chem. 262, 16531–16535
3. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. I., Dodson, G. G., Hodgkin, D. M., Hubbard, R. B., Neals, N. W., and Reynolds, C. D. (1988) Philos. Trans. R. Soc. London Ser. B 319, 369–456
4. Hua, Q. X., Shoelson, S. E., Kochoyan, M., and Weiss, M. A. (1991) Nature 354, 238–241
5. Hua, Q. X., Hu, S. Q., Frank, B. H., Jia, W., Chu, Y. C., Wang, S. H., Burke, G. T., Katsoyannis, P. G., and Weiss, M. A. (1996) J. Mol. Biol. 264, 390–403
6. Olsen, H. B., Ludvigsen, S., and Kaarsholm, N. C. (1996) Biochemistry 35, 8836–8845
7. Steiner, D. F. (1967) Trans. N. Y. Acad. Sci. 30, 60–68
8. Weiss, M. A., Hua, Q.-X., Jia, W., Chu, Y.-C., Wang, R.-Y., and Katsoyannis, P. G. (2000) Biochemistry 39, 15429–15440
9. Hua, Q.-X., Nakagawa, S. H., Jia, W., Hu, S. Q., Chu, Y.-C., Katsoyannis, P. G., and Weiss, M. A. (2001) Biochemistry 40, 12299–13211
10. Guo, Z.-Y., and Feng, Y.-M. (2001) Biochem. Biophys. Res. Commun. 282, 433–448
11. De Meyst, P., and Whittaker, J. (2002) Natl. Acad. Sci. U. S. A. 99, 12978–12983
12. Barany, G., and Merrifield, R. B. (1980) in The Peptides (Gross, E., and Meienhofer, J., eds) pp. 3–284, Academic Press, New York
13. Nakagawa, S. H., Tager, H. S., and Steiner, D. F. (2000) Biochemistry 39, 15826–15835
14. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H. (1967) Bull. Chem. Soc. Jpn. 40, 2164–2167
15. Bailey, J. L., and Cole, R. D. (1959) J. Biol. Chem. 234, 1733–1739
16. Chance, R. E., Hoffman, J. A., Kroepp, E. P., Johnson, M. G., Schirmer, W. E., and Borrer, W. W. (1981) in Peptides: Synthesis, Structure and Function; Proceedings of the Seventh American Peptide Symposium (Rich, D. H., and Gross, E., eds) pp. 721–728, Pierce Chemical Co., Rockford, Ill.
17. Inouye, K., Watanabe, K., Morihira, K., Tochino, Y., Kanaya, T., Emura, J., and Sakakibara, S. (1979) J. Am. Chem. Soc. 101, 751–752
18. Kubik, T., and Cowburn, D. (1986) Int. J. Pept. Protein Res. 27, 514–521
19. Weiss, M. A., Hua, Q.-X., Jia, W., Nakagawa, S. H., Chu, Y.-C., Hu, S.-Q., and Katsoyannis, P. G. (2001) J. Biol. Chem. 276, 40018–40024
20. Hua, Q.-X., Chu, Y.-C., Jia, W., Phillips, N. F. B., Wang, R.-Y., Katsoyannis, P. G., and Weiss, M. A. (2002) J. Biol. Chem. 277, 43443–43453
21. Sondheim, R. F., Fang, X., and Shelton, V. M. (2000) Methods Enzymol. 317, 393–409
22. Huang, K., Xu, B., Hu, S. Q., Chu, Y. C., Hua, Q. X., Qu, Y. L., Bi, W., Wang, S., Wang, R. Y., Nakagawa, S. H., Theede, A. M., Whittaker, J., De Meysts, P., Katsoyannis, P. G., and Weiss, M. A. (2004) J. Biol. Chem. 329, 529–530
23. Anil, B., Song, B., Tang, Y., and Raleigh, D. P. (2004) J. Am. Chem. Soc. 126, 13194–13195
24. Bi, R. C., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Giorgino, F., Holst, P., Miller, J. A., and Weiss, M. A. (1996) Acta Crystallogr. Sect. B Struct. Sci. 42, 127–136
25. Bi, R. C., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Giorgino, F., Reynolds, C. D., and Tolley, S. P. (1983) Acta Crystallogr. Sect. B Struct. Crystallogr. Cryst. Chem. 39, 90–98
26. Dai, J.-B., Lou, M.-Z., You, J.-M., and Liang, D.-C. (1987) Sci. Sin. 30, 55–65
27. Bao, S. X., Jie, D. L., Zhang, J. P., Chang, W. R., and Liang, D. C. (1997) Proc. Nutl. Acad. Sci. U. S. A. 94, 2975–2980
28. Ludvigsen, S., Olsen, H. B., and Kaarsholm, N. C. (1998) J. Biol. Chem. 273, 1–7
29. Katsoyannis, P. G., and Tometzko, A. (1966) Proc. Nutl. Acad. Sci. U. S. A. 55, 1554–1561
30. Weiss, M. A., Nakagawa, S. H., Jia, W., Xu, B., Hua, Q. X., Chu, Y. C., Wang, R. Y., and Katsoyannis, P. G. (2002) Biochemistry 41, 809–819
31. Narhi, L. O., Hua, Q. X., Arakawa, T., Fox, G. M., Tsai, L., Rosenfeld, R., Holst, P., Miller, J. A., and Weiss, M. A. (1993) Biochemistry 32, 5214–5221
32. Hua, Q. X., Narhi, L., Jia, W., Arakawa, T., Rosenfeld, R., Hawkins, N., Miller, J. A., and Weiss, M. A. (1996) J. Biol. Chem. 271, 297–313
33. Zhao, M., Nakagawa, S. H., Hua, Q. X., and Weiss, M. A. (1998) Proc. Am. Peptide Symp. 369–371
34. Hober, S., Forsberg, G., Palm, G., Hartmanis, M., and Nilsson, B. (1992) Biochemistry 31, 1749–1756
35. Miller, J. A., Narhi, L. O., Hua, Q. X., Rosenfeld, R., Arakawa, T., Rohde, M., Prestrelski, S., Lauren, S., Stoney, K. S., Tsai, L., and Weiss, M. A. (1993) Biochemistry 32, 5203–5213
36. Qiao, Z. S., Guo, Y. Y., and Feng, Y. M. (2001) Biochemistry 40, 2662–2668
Chiral Mutagenesis of Insulin

49. Steiner, D. F., Tager, H. S., Chan, S. J., Nanjo, K., Sanke, T., and Rubenstein, A. H. (1990) Diabetes Care 13, 600–609

50. Kobayashi, M., Ohgaku, S., Iwasaki, M., Maegawa, H., Shigeta, Y., and Inouye, K. (1982) Biochem. Biophys. Res. Commun. 107, 329–336

51. Mirmira, R. G., and Tager, H. S. (1989) J. Biol. Chem. 264, 6349–6354

52. Mirmira, R. G., Nakagawa, S. H., and Tager, H. S. (1991) J. Biol. Chem. 266, 1428–1436

53. Nakagawa, S. H., and Tager, H. S. (1987) J. Biol. Chem. 262, 12054–12058

54. Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Bing, X., and Markussen, J. (1991) J. Mol. Biol. 220, 425–433

55. Hua, Q.-X., Hu, S. Q., Jia, W., Chu, Y.-C., Burke, G. T., Wang, S. H., Wang, R. Y., Katsoyannis, P. G., and Weiss, M. A. (1998) J. Mol. Biol. 277, 103–118

56. Xu, B., Hu, S. Q., Chu, Y. C., Huang, K., Nakagawa, S. H., Whittaker, J., Katsoyannis, P. G., and Weiss, M. A. (2004) Biochemistry 43, 8356–8372

57. Xu, B., Hua, Q. X., Nakagawa, S. H., Jia, W., Chu, Y. C., Katsoyannis, P. G., and Weiss, M. A. (2002) J. Mol. Biol. 316, 435–441

58. Wan, Z., Xu, B., Chu, Y. C., Katsoyannis, P. G., and Weiss, M. A. (2003) Biochemistry 42, 12770–12783

59. Wan, Z., Huang, K., Xu, B., Hu, S. Q., Wang, S., Chu, Y. C., Katsoyannis, P. G., and Weiss, M. A. (2005) Biochemistry 44, 5000–5016

60. Kurose, T., Pashmforoush, M., Yoshimasa, Y., Carroll, R., Schwartz, G. P., Burke, G. T., Katsoyannis, P. G., and Steiner, D. F. (1994) J. Biol. Chem. 269, 29190–29197

61. Kobayashi, M., Takata, Y., Ishibashi, O., Sasaoka, T., Iwasaki, T. M., Shigeta, Y., and Inouye, K. (1986) Biochem. Biophys. Res. Commun. 137, 250–257

62. Kitagawa, K., Ogawa, H., Burke, G. T., Chanley, J. D., and Katsoyannis, P. G. (1984) Biochemistry 23, 1405–1413

63. Nakagawa, S. H., and Tager, H. S. (1992) Biochemistry 31, 3204–3214

64. Sreerama, N., and Woody, R. W. (1993) Anal. Biochem. 209, 32–44