A central tenet of molecular biology holds that the function of a protein is mediated by its structure. An inactive ground-state conformation may nonetheless be enjoined by the interplay of competing biological constraints. A model is provided by insulin, well characterized at atomic resolution by x-ray crystallography. Here, we demonstrate that the activity of the hormone is enhanced by stereospecific unfolding of a conserved structural element. A bifunctional strand mediates both self-assembly (within β-cell storage vesicles) and receptor binding (in the bloodstream). This strand is anchored by an invariant side chain (PheB24); its substitution by Ala leads to an unstable but native-like analog of low activity. Substitution by D-Ala is equally destabilizing, and yet the protein diastereomer exhibits enhanced activity with segmental unfolding of the β-strand. Corresponding photoactivable derivatives (containing L- or D-para-azido-Phe) cross-link to the insulin receptor with higher D-specific efficiency. Aβ-cell secretory granules (1). The hexameric core (Fig. 2A) contains an N-terminal β-helix, non-canonical turn, and second helix; the B-chain contains an N-terminal segment, central α-helix, and C-terminal β-strand. The B-chain is maintained in an isolated monomer wherein the side chain of PheB24 (tawny in Fig. 2A), packing against the central α-helix of the B-chain, provides a “plug” to seal a crevice in the hydrophobic core (Fig. 2B). Anomalies encountered in previous studies of insulin analogs suggest that PheB24 functions as a conformational switch (4, 7, 10–14). Whereas L-amino acid substitutions at B24 generally impair activity (even by such similar residues as L-Tyr) (15), a seeming paradox is posed by the enhanced activity of nonstandard analogs containing D-amino acids (Table 1) (10–12).

Why do D-amino acid substitutions at B24 enhance the activity of insulin? In this study, we describe the structure and function of insulin analogs containing L-Ala or D-Ala at B24 (Fig. 2, C and D). Our studies were conducted within an engineered...
monomer (DKP-insulin, an insulin analog containing three substitutions in the B-chain: AspB10, LysB28, and ProB29) to circumvent effects of self-assembly (16). Whereas the inactive L-analog retains a native-like structure, the active D-analog exhibits segmental unfolding of the B-chain. Studies of corresponding analogs containing either L- or D-photoactivable probes (L-para-azido-PheB24 or D-para-azido-PheB24 (L- or D-PapB24), obtained from photostable para-amino-Phe (Pmp)

precursors (17)) demonstrate specific cross-linking to the IR. Although photo-contacts map in each case to the N-terminal domain of the receptor α-subunit (the L1 β-helix), higher cross-linking efficiency is achieved by the D-probe. Together, this and the following study (6) provide evidence that insulin deploys a detachable arm that inserts between domains of the IR.

Induced fit of insulin illuminates by its scope general principles at the intersection of protein structure and cell biology. Protein evolution is enjoined by multiple layers of biological selection. The pathway of insulin biosynthesis, for example, successively requires (a) specific disulfide pairing (in the endoplasmic reticulum), (b) subcellular targeting and prohormone processing (in the trans-Golgi network), (c) zinc-mediated protein assembly and microcrystallization (in secretory granules), and (d) exocytosis and rapid

### TABLE 1

| Analog Affinity | Ref. |
|-----------------|------|
| 140 Lymphocytes 10 | (d-PheB24, insulin) |
| 140 Hepatocytes 68 | (L-AlaB24, insulin) |
| 140 Lysophosphatidylethanolamine 69 | (D-PheB24, insulin) |
| 140 Lysophosphatidylethanolamine 11 | (D-AlaB24, insulin) |
| 140 Lysophosphatidylethanolamine 11 | (GlyB24-DKP-insulin) |
| 200 CHO cells 12 | (DKP-insulin) |
| 180 CHO cells 12 | (D-PheB24, DKP-insulin) |
| 17 CHO cells 12 | (L-AlaB24, DKP-insulin) |
| 50 CHO cells 12 | (GlyB24-DKP-insulin) |

- Affinities are given relative to wild-type insulin (100%).
- Lymphocytes are human, and hepatocytes are rat; CHO designates Chinese hamster ovary.
- Standard deviations are not provided in this reference.
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disassembly of insulin hexamers (in the portal circulation), in turn enabling binding of the monomeric hormone to target tissues (1). Each step imposes structural constraints, which may be at odds. This study demonstrates that stereospecific pre-detachment of a receptor-binding arm enhances biological activity but impairs disulfide pairing and renders the hormone susceptible to aggregation-coupled misfolding (18). Whereas the classical globular structure of insulin and its self-assembly prevent proteotoxicity (3, 19), partial unfolding enables receptor engagement. We envisage that a choreography of conformational change has evolved as an adaptative response to the universal threat of toxic protein misfolding.

EXPERIMENTAL PROCEDURES

Synthesis of Insulin Analogs—Human insulin was obtained from Lilly and Novo Nordisk (Copenhagen, Denmark). Synthesis of variant B-chains, insulin chain combination, and protein purification were performed as described (Refs. 14 and 20; see also supplemental “Experimental Procedures”). B-chain analogs each contained three “DKP” substitutions to prevent self-association of insulin (HisB10 → Asp, ProB28 → Lys, and LysB29 → Pro) (8, 16). Whereas the yield of L-PmpB24-DKP-insulin was similar to that of DKP-insulin, the yield of L-AlaB24-DKP-insulin was reduced by 2-fold; the yields of D-AlaB24-DKP-insulin and D-PmpB24-DKP-insulin were reduced by 4-fold. Precursor Pmp analogs were converted to their azido form as described (17, 20). Pmp/Pap analogs were prepared with a B1-linked biotin tag to enable detection by an avidin-based reagent (NeutrAvidin, Pierce).

Receptor Binding Assays—Activities of insulin analogs were evaluated by a competitive displacement assay using a human placental membrane preparation (21); cross-binding to the type I insulin-like growth factor receptor was evaluated as described (22). The percentage of tracer bound in the absence of competing ligand was in each case <15% to avoid ligand depletion artifacts. Assays were performed in triplicate.

Circular Dichroism—Far-UV CD spectra were obtained as described (23). Spectra were normalized by mean residue ellipticity. Samples were dissolved in 10 mM potassium phosphate (pH 7.4) and 100 mM KCl at a protein concentration of ∼25 μM. For equilibrium denaturation studies, samples were diluted in the same buffer to 5 μM; guanidine HCl was employed as denaturant (23). Data were obtained at 25 °C and fitted by nonlinear least squares to a two-state model (24).

1H NMR Spectroscopy—Spectra were obtained at 600 and 800 MHz in aqueous solution at pH 7–8 at 25 and 32 °C and in 20% deuterioacetic acid at 25 °C as described (4, 25). Distance geometry and restrained molecular dynamics calculations were performed as described (20).

Photo-cross-linking Studies—Wheat germ-aggulatin-purified IR ectodomain and holoreceptor were prepared as described (26, 27); methods are provided under supplemental “Experimental Procedures.” Photocross-linking of biotin-labeled Pap analogs to the isolated ectodomain and solubilized IR (isoform B) was induced by UV irradiation (17); photoproducets were characterized by SDS-PAGE and Western blotting as described (20, 26, 27). Gels were probed with NeutrAvidin to detect the B1-linked biotin tag and with polyclonal antiserum recognizing the N-terminal segment of the IR α-subunit (designated IRα-N; Santa Cruz Biotechnology). Control experiments verifying specificity were performed to demonstrate competition between binding of the Pap analogs and native ligands (human insulin and insulin-like growth factor I) (27).

Insulin Fibrillation—Proteins were made 60 μM in degassed phosphate-buffered saline (10 mM phosphate and 140 mM NaCl at pH 7.4) with 0.1% sodium azide in pre-sterilized glass vials with airtight sealed caps (Allergy Laboratories Inc.). Vials were rocked on a BD Biosciences nutator at ~60 rpm as described (28). At successive times, aliquots were withdrawn with a sterilized single-use syringe. Aliquots were added to a thioflavin T solution for fluorescence assay. Emission spectra were collected in 1-cm quartz cuvettes from 470 to 500 nm following excitation at 450 nm; the integration time was 1 s. Assays were performed in duplicate (D-AlaB24-DKP-insulin) or triplicate (AspB10-des-octapeptide-B23-B30)-insulin, DKP-insulin, and its L-AlaB24 analog).

RESULTS

Analogs of DKP-insulin containing L-amino acid substitutions at B24 (“L-analogs”) exhibit reduced receptor-binding affinities (relative to the parent monomer), whereas D-analogs exhibit enhanced affinities (Fig. 3 and Table 2). No disproportionate changes were observed in the extent of low affinity
TABLE 2
Properties of insulin analogs

| Protein           | Affinity*  | Stability*  |
|-------------------|------------|-------------|
| Insulin           | 100 (12)   | 3.3 ± 0.1   |
| DKP-insulin       | 175 ± 31 (9)| 4.3 ± 0.1*  |
| ϑ-AlaB24-DKP-insulin | 3.6 ± 0.7 (3) | 3.5 ± 0.1  |
| d-AlaB24-DKP-insulin | 199 ± 35 (3) | 3.6 ± 0.1  |
| ϑ-AlaB24, Bi-DKP-insulin | 132 ± 5 (3) | ND"        |
| d-AlaB24, Bi-DKP-insulin | 61 ± 5 (3) | ND"        |
| ϑ-AlaB24, Bi-DKP-insulin | 210 ± 27 (3) | ND        |
| d-AlaB24, Bi-DKP-insulin | 147 ± 3 (3) | ND        |

* Relative affinity is based on studies of binding to a human placental membrane preparation (21). Numbers in parentheses refer to replicates. The hormone-receptor dissociation constant under assay conditions is (5.0 ± 0.4) × 10⁻¹⁰ mol/L.

" Thermodynamic stability is based on CD-detected guanidine denaturation studies at 25 °C (24).

The enhanced stability of DKP-insulin relative to insulin is due to substitution HisB24 → Asp (70). This substitution blocks the trimer interface and provides a more favorable N-Cap [i.e. the terminal residue of an a-helix] for the central a-helix; it is also responsible for the enhanced activity of the analog (16, 71). The two substitutions in the C-terminal b-strand (ProB24 → Lys and LysB29 → Pro), which impair classical dimerization (72), are destabilizing (73).

b Bi indicates a biotin tag at the N terminus of the B-chain.

ND, not determined.

2. Spectra were obtained at 600 MHz, pD 7.7, and 32 °C; the mixing time was 200 ms. The far-UV CD spectra of ϑ-AlaB24-DKP-insulin and d-AlaB24-DKP-insulin are each similar to that of DKP-insulin (supplemental Fig. 1A). Protein denaturation studies (supplemental Fig. S1B) indicated that the ϑ- and d-AlaB24 analogs are less stable than the parent monomer, with similar decrements in unfolding free energy (ΔΔG = 0.8 ± 0.2 and 0.7 ± 0.2 kcal/mol, respectively) (Table 2 and supplemental Table S1).

Solution Structures—The 1H NMR spectra of ϑ- and d-AlaB24 analogs each exhibit resonance line widths (in aqueous solution at neutral pH) similar to those of DKP-insulin (8, 16), suggesting that the analogs are likewise monomeric. The variant spectra exhibit attenuated secondary shifts consistent with the absence of the PheB24 ring current (29); complete resonance assignment was in each case obtained (supplemental Tables S2–S5). ϑ- and d-analogs exhibit native-like patterns of helix-related nuclear Overhauser effects (NOEs) within canonical a-helical segments (residues A2–A7, A13–A19, and B9–B19) and the B7–B10 b-turn (Fig. 4, C and D; see also supplemental Fig. S2). Long-range NOEs within the a-helical subdomains likewise resemble those of DKP-insulin (8). These include diagnostic side chain contacts (i) within the A-chain (IleB12-LeuB16 and IleA12-TyrA19; the latter set of NOEs is observed as cross-peaks g, h, and i in Fig. 4A and as cross-peaks g and h in Fig. 4B) and (ii) between A- and B-chains (IleA10-HisA13, LeuA15-PheB11, and TyrA19-LeuB15); the latter gives rise to cross-peaks g, h, u, and v in Fig. 4A and cross-peak v in Fig. 4B). Interchain NOEs near presumed disulfide bridges are in each case consistent with native pairing.

The NOE spectra of ϑ- and d-AlaB24-DKP-insulin exhibit striking differences with respect to the B24–B28 segment (Fig. 4, A and B). The aromatic resonances of PheB25 and TyrB26 provide probes for the packing of this segment against the a-helical subdomain. Whereas the ϑ-analog exhibits a native-like pattern of long-range NOEs (Fig. 4A), few such contacts are observed in the d-analog (Fig. 4B). Of particular importance is the ϑ-specific maintenance of an NOE between TyrB26 and the methyl resonances of IleB24 and ValA3 in the N-terminal A-chain a-helix (cross-peaks a, k, l, m, n, o, p, q, r, and s; Fig. 4B) as follows: a) A19-H2, 0.1 kcal/mol, respectively). Attenuation of these and related NOEs in d-AlaB24-DKP-insulin provides evidence for ste-
Stereospecific detachment of the C-terminal segment. Whereas intermediate and strong NOEs are observed in the L-AlaB24 analog between TyrB26 and the central B-chain helix (ValB12 and LeuB15; cross-peaks b, c, d, and e in Fig. 4, A and C), at neutral pH, the corresponding NOEs are absent in the D-analog. The NOESY spectrum of D-AlaB24-DKP-insulin in an organic co-solvent (20% deuterioacetic acid), although otherwise native-like, exhibits weak B26-related NOEs (cross-peaks c and d) in Fig. 4D) in association with weak non-native B25-B12 and B25-B16 NOEs (arrow), suggesting transient contacts by either aromatic ring within a disordered C-terminal segment.

Structures were calculated by distance geometry and restrained molecular dynamics on the basis of ~700 restraints (15 restraints/residue); statistical parameters are provided in supplemental Table S7. The structure of DKP-insulin (Fig. 5B, PheB24 in black) recapitulates the conformation of crystallographic protomers and so provides a native base line (Fig. 5A) (8). The analogs exhibit marked differences in the B-chain. Whereas the B-chain of the L-analog recapitulates a native-like (but less well defined) U-shaped supersecondary structure (Fig. 5C), residues B20–B30 in the D-analog are essentially unrestrained (Fig. 5D). In the distance geometry and restrained molecular dynamics ensemble, the latter segment is depicted by a near-random conformational sampling; such modeling may overestimate the extent of conformational excursions.

L- and D-analogs each retain native-like α-helical subdomains. Subtle differences are nonetheless observed between the α-helical orientations (Fig. 5, C and D). Whereas almost all helix-related and interhelical NOEs are shared between analogs, a small number of differences are observed (Fig. 4, C and D); these appear unrelated to the relative extent of resonance overlap. Such differences presumably reflect transmitted effects of the B24 substitutions on segmental structure and dynamics. Because aliphatic side chains in the α-helical subdomain (IleA2, ValA3, IleB11, ValB12, and LeuB15) ordinarily engage the C-terminal B-chain segment, the D-analog exposes associated hydrophobic surfaces. Mutagenesis suggested that these surfaces participate in receptor binding (30).

**Photocross-linking Studies**—B1-biotin-labeled L- and D-photoactivable B24 derivatives of DKP-insulin were prepared by chemical synthesis; the corresponding derivative at B25 was prepared as a control (17, 26). Fidelity of synthesis and conversion of photostable Pmp (amino) precursors to Pap (azido) derivatives were verified by electrospray mass spectrometry. Pap analogs and the IR ectodomain (Fig. 6A) were mixed at 1:1 stoichiometry at protein concentrations (~200 nM) >100-fold higher than the weakest dissociation constant of the photostable precursors; the predominant species is thus expected to be a 1:1 bimolecular complex. A short UV exposure time (20 s at 254 nm) yielded essentially complete photolysis or cross-linking (17, 26). Whereas the L-para-azido-PheB24 analog was found to exhibit a photocross-linking efficiency (Fig. 6B, upper panel, lane 12) similar to that of the B25 control (lane 4) (~10–20% of complexes cross-linked) (26), d-PapB24 photocross-linking...
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FIGURE 6. Photocross-linking studies of the IR ectodomain. A, domain organization of the IR. Left, organization of the disulfide-bridged α- and β-subunits; middle, holoreceptor as a disulfide-bridged dimer of αβ-units; right, isolated holoreceptor containing truncated β-subunits. The α-subunit contains (from the N to C terminus) the following modules: L1 (large domain 1), the CR domain, L2 (large domain 2), Fn1 (the first fibronectin type III homology domain), Fn2a (the N-terminal portion of the second fibronectin type III homology domain), and ID-N (the N-terminal portion of the insert domain). The β-subunit contains (from the N to C terminus) the following modules: ID-C (the C-terminal portion of the insert domain), Fn2b (the C-terminal portion of the second fibronectin type III homology domain), Fn3 (the third fibronectin type III homology domain), TM/JM (the transmembrane α-helix and juxtamembrane domain), TK (the tyrosine kinase domain), and JCT (the C-terminal domain of the β-subunit). Dashed lines outline domains (light gray) not present in the crystal structure of the IR ectodomain (see Fig. 7C). B, Western blots showing wheat germ-agglutinin-purified ectodomain of the IR (αβ)2, 290 kDa; α, 115 kDa; β, 30 kDa) cross-linked with DKP-insulin containing Pap substituted at B25 (lanes 1–4), B24 (α-chirality) (lanes 5–8), or B24 (β-chirality) (lanes 9–12) treated without (odd-numbered lanes) or with (even-numbered lanes) UV irradiation. Analog labels have a biotin tag at B1. After cross-linking, reduction with diethiothreitol (upper and middle panels), SDS-PAGE separation, and blotting onto nitrocellulose membranes, cross-linked adducts were probed with alkaline phosphatase-conjugated NeutrAvidin (NAV; upper panel). Control blots probed with anti-IR α-subunit antibody (IRα-N, middle panel; after diethiothreitol reduction) demonstrate equal amounts of IR. Similarly, control blots probed with NeutrAvidin (lower panel; without diethiothreitol reduction) demonstrate equal amounts of insulin analog. Control lanes, with any of the three Pap derivatives, a photocross-linked band was not detected in the absence of the IR and UV irradiation (lanes 1, 5, and 9), in the absence of the IR and in the presence of UV irradiation (lanes 2, 6, and 10), or in the presence of the IR and in the absence of UV irradiation (lanes 3, 7, and 11).

Stabilizing efficiency was at least 1.5-fold higher (lane 8). Similar results were obtained using the holoreceptor (see below). Control blots demonstrated equal loading of the ectodomain (Fig. 6B, middle panel) and insulin derivative (lower panel). As expected, in the absence of the ectodomain, photocross-linked complexes were not observed either before (lanes 1, 5, and 9) or after (lanes 2, 6, and 10) UV irradiation; covalent complexes were likewise not formed in the presence of the ectodomain but in the absence of irradiation (lanes 3, 7, and 11).

Mapping of photoproducts by limited proteolysis was undertaken in corresponding holoreceptor complexes (26). The structural basis of such mapping within the “inverted V” (αβ)2-dimer is illustrated in Fig. 7 (A–D). The results indicate that L-PapB24 photocross-links to the L1 domain of the IR α-subunit (Fig. 7) as found previously for the L-para-azido-PheH926 derivative (26). The mapping strategy exploited exposed basic side chains near the junction of L1 and the cysteine-rich (CR) domain (magenta arrowhead in Fig. 7A); the positions of these side chains are peripheral to the putative hormone-binding interface of the ectodomain (Fig. 7, C and D). Limited tryptic cleavage of the photocross-linked complexes yielded a predominant 31-kDa glycosylated fragment (Fig. 7E) recognized by antiserum against the N-terminal 20 residues of the α-subunit (IRα-N) (Fig. 7F). Upon deglycosylation, the apparent mass of this fragment is 20 kDa, indicating that it contains the L1 domain (residues 1–158) and at most a small portion of the CR domain.

Insulin Fibrillation—Partial unfolding of an insulin monomer at elevated temperatures (>50 °C) promotes formation of amloid (Fig. 8A) (31–34). Such fibrillation occurs via a nucleation-growth mechanism with a lag phase (leading to an amyloidogenic nucleus) and log phase (leading to formation of mature fibrils) (18). Analogous nucleation-growth mechanics underlie deposition of pathological amyloid (19), suggesting that the susceptibility of insulin to fibrillation provides a model for proteotoxicity. Partial thermal unfolding of insulin leads to destabilization of the C-terminal segment of the B-chain (purple dashed line in Fig. 8B) (35). Because this partial fold is reminiscent of the conformation of d-AlaB24-DKP-insulin, we investigated whether the analog might exhibit a foreshortened lag time. Fibrillation was monitored by thioflavin T fluorescence upon gentle agitation of the protein solution in phosphate-buffered saline at pH 7.4 at 37 °C. Under such conditions, DKP-insulin forms fibrils in 12.4 ± 2.5 days. The lag time of the d-AlaB24 analog is reduced by ~40% (7.0 ± 0.7 days); that of the d-AlaB24 analog is more markedly accelerated (between 2 and 3 days).

These observations demonstrate that L- and d-AlaB24-DKP-insulin are each more susceptible to fibrillation than the parent monomer. Although this in accord with their reduced global stabilities, the difference between L- and d-analogs correlates with the extent to which hydrophobic surfaces (concealed in native insulin by the C-terminal B-chain segment) are abnormally exposed. Accelerated fibrillation of the d-analog is in accord with the enhanced amyloidogenicity of insulin analogs containing C-terminal B-chain deletions (18). Under the present conditions, the truncated analog AspB10-des-octapeptide(B23–B30)-insulin (obtained by tryptic removal of the C-terminal segment) exhibits a lag time of 4.3 ± 1.5 days. Although this fragment (shorn of its receptor-binding arm) is without biological activity, NMR studies indicate maintenance of a native-like α-helical domain similar to that of d-AlaB24-DKP-insulin.

DISCUSSION

Crystal structures of insulin have been determined at atomic resolution, enabling detailed analyses (30, 36, 37). Studies of single-chain insulin analogs nonetheless suggest that such structures depict inactive conformations (supplemental Fig. S3) (38, 39). Single-chain insulin analogs contain short con-
necting segments that tether the C terminus of the B-chain to
the N terminus of the A-chain (38–43). Although structurally
well tolerated, tethers of less than four residues markedly
impair receptor binding (8, 40, 44). Activity is partially restored
with connecting domains of six or more residues (39, 45, 46).

Similar length-dependent effects have been observed on in-
terposing chemical cross-linking reagents between the B-chain
(using the e-amino group of LysB28) and the N terminus of the
A-chain (47). These findings suggest that the C-terminal
B-chain β-strand (residues B24–B30; highlighted in blue in Fig.

**FIGURE 7.** Mapping of photo contacts in the holoreceptor. A, schematic model of the holoreceptor as an (αβ)2-dimer containing an inverted V ectodomain, transmembrane α-helices (TM; cylinders), and intracellular tyrosine kinase domains (TK). The magenta arrowhead indicates tryptic sites at the L1-CR domain junction. B, crystal structure of the αβ3 protomer of the IR ectodomain (β3, truncated β-subunit) (Fig. 6A) with component domains as labeled: front view (left) and side view (right). Domains are color-coded as shown in Fig. 6A. The insert domain (ID) exhibits missing or discontinuous electron density (residues 655–755; IR isoform A); only the respective N- and C-terminal subsegments of ID-N and ID-C are well defined (orange). C, crystal structure of the ectodomain dimer showing the front view (left) and side view (right). Basic side chains near the L1-CR domain junction (Lys149, Lys164, Lys166, and Lys181) are shown. The arrow indicates the proposed binding cavity of insulin. D, space-filling model of L1 (residues 1–158; gray) and the adjoining portion of the CR domain (residues 159–309; black). Basic side chains near the L1-CR domain junction are highlighted in magenta. Coordinates were obtained from Protein Data Bank codes 2DTG and 2HR7. E and F, time course of tryptic digestion of the Pap- and L-para-azido-PheB24-cross-linked holoreceptor complex as detected by NeutrAvidin (E) or Western blotting using antiserum recognizing the N-terminal 20 residues of the α-subunit (IRα-N) (F). Photoproducts were resolved by SDS-PAGE after reduction of disulfide bridges. The α- and l-photoprobes each cross-linked to an N-terminal L1 fragment. In each panel, lanes 1 and 6 contain unirradiated complexes; lanes 2–5 and 7–10 provide respective time points at 0, 1, 2, and 10 min.
1) reorganizes to contact the IR (7, 11, 38). In this study, we have reported that stereospecific detachment of the C-terminal segment of the B-chain enhances receptor binding; D- and L-phosphotropes at the site of substitution cross-link efficiently to the IR. The accompanying article provides evidence for the insertion of the B-chain β-strand between domains of the IR (6).

Conformational Switch—Evidence for a receptor-directed conformational change at B24 has been previously provided by structural studies of GlyB24 analogs (7, 13). This non-conservative substitution leads to only a small decrease in activity (11, 15). That the packing of the B24–B28 zation of the B-chain (7, 13). The extent of perturbation has been previously provided by synthetic photoscanning in the accompanying article (6). We imagine that the activity of D-AlaB24-DKP-insulin, although high, would be even higher but for a trade-off: the benefit provided by destabilization of an inhibitory conformation is offset by the cost of refolding the C-terminal B-chain segment upon receptor binding. Similarly, because truncated analogs lack part of the inhibitory segment, the low affinity of D-PheB24, des-pentapeptide(B26–B30)-insulin-amide (see above) (50) is presumably due to the cost of refolding incurred in the absence of an offsetting benefit. The low activity of L-AlaB24-DKP-insulin (despite partial destabilization of its C-terminal arm) may reflect local perturbation of the B24–B28 segment upon receptor binding. Conversely, binding region (52); these include IleA2, ValA3, ValB12, and TyrB16 in the α-helical domain and Ph(9, 14, 20, 54, 55). Compelling examples are provided by substitution of IleA2 by allo-Ile (i.e. inversion of β-carbon chirality) (14, 47, 56) and substitution of ValA3 by Leu, a clinical mutation associated with diabetes mellitus (56–58). We suggest that upon binding of the wild-type hormone to the IR, the insulin monomer undergoes analogous segmental detachment and receptor-dependent refolding, likewise enabling close engagement of underlying nonpolar surfaces. Direct evidence is provided by synthetic photoscanning in the accompanying article (6). We imagine that the activity of D-AlaB24-DKP-insulin, although high, would be even higher but for a trade-off: the benefit provided by destabilization of an inhibitory conformation is offset by the cost of refolding the C-terminal B-chain segment upon receptor binding. Similarly, because truncated analogs lack part of the inhibitory segment, the low affinity of D-PheB24, des-pentapeptide(B26–B30)-insulin-amide (see above) (50) is presumably due to the cost of refolding incurred in the absence of an offsetting benefit. The low activity of L-AlaB24-DKP-insulin (despite partial destabilization of its C-terminal arm) may reflect local perturbation of the B24 receptor contact, as packing of L-PheB24 against the L1 β-helix ordinarily acts as a trigger for further conformational change (11, 15).

Other Sites of Induced Fit—Evidence that the T-state-specific B7–B10 β-turn undergoes a change in conformation upon receptor binding has also been obtained through chiral mutagenesis. D-Amino acid substitutions within the turn (mimicking the positive φ dihedral angle of GlyB8) stabilize insulin but markedly impair receptor binding (3). L-Amino acid substitutions at B8 are destabilizing but can be compatible with high

3 Whereas reattachment of the C-terminal segment in a GlyB24 analog has been reported via mimicry of the Ph–Leu interaction by the neighboring PheB24 side chain (13), in our hands, the solution structure of GlyB24, DKP-insulin is essentially identical to that of D-AlaB24, DKP-insulin (Q.-X. Hua, B. Xu, K. Huang, S.-Q. Hu, S. Nakagawa, W. Jia, S. Wang, J. Whitaker, P. G. Katsoyannis, and M. A. Weiss, manuscript in preparation). This implies that an open ground-state conformation does not require a D-amino acid substitution and so could in principle be accessed in nature if favorable as a biological adaptation.
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activities (4). The photocross-linking studies presented in the following article suggest that such impairment is unlikely to represent steric clash by the introduced d-side chain and so indicates an additional site of induced fit (6).

Induced fit of the N-terminal segment of the B-chain is in accord with classical allostery among zinc insulin hexamers (the TR transition; see Fig. 8 in the accompanying article (6)) (30). Indeed, in this transition, the conformation of GlyB28 changes from d-like (ϕ > 0) to l-like (ϕ < 0). Furthermore, long-range coupling between sites of conformational change at B8 and B24 would be consistent with a subtle feature of the TR transition: the salient R-state-specific change in conformation of the N-terminal segment of the B-chain (including in the sign of the B8 ϕ angle) is associated with slight separation of the C-terminal B-chain β-strand from the A-chain, in turn breaking a T-state-specific hydrogen bond between PheB25–HN and TyrA19–C=O (30). Thus, although frank detachment of the β-strand is constrained by assembly (i.e. by the dimer-related β-sheet shared among Tα, TβRβ, and Rα structural families) (30), conformational variability among zinc insulin hexamers foreshadows molecular mechanisms of induced fit upon receptor binding at both ends of the B-chain.

Determinants of Disulfide Pairing—Insulin chain combination (air oxidation of isolated A- and B-chains) (59) yields native disulfide pairing and thus provides a peptide model of proinsulin folding (60). The reaction is under kinetic control and limited by off-pathway reactions (formation of cyclic A- and B-chains, B-chain polymers, and B-chain fibrils) (23, 61, 62). Although chain combination is generally robust to amino acid substitutions (23), we have found that substitution of PheB24 by d- or l-Ala caused a 4- or 2-fold reduction in yield, respectively. The reactions exhibited proportionate increases in side products rather than formation of insulin disulfide isomers (61, 62). Such impaired yields are unlikely to reflect decreased end-product stability because d- or l-AlaB24-DKP-insulin (although less stable than DKP-insulin) are as stable as wild-type insulin (Table 2). The absence of correlation between synthetic yield and thermodynamic stabilities among these and other analogs (23) suggests that PheB24 plays a kinetic role in direct disulfide pairing. In accord with this hypothesis, the B24 aromatic ring participates in nascent native-like B-chain secondary structure in partially folded insulin analogs lacking either cystine A6–A11 or A7–B7, constructed as models of oxidative folding intermediates (8, 63, 64). Of the three native disulfide bridges, PheB24 is close to only cystine A20-B19.

We propose that in an initial stage of chain combination, PheB24-related hydrophobic clustering favors the alignment of CysB20 and CysA20 for disulfide pairing. Upon substitution of PheB24 by d- or l-Ala, such clustering is presumably less efficient. It would be of interest to investigate whether B24 substitutions likewise affect the foldability of proinsulin in mammalian cells (25).4 The importance of structural interactions flanking disulfide bridges for the cellular folding of proinsulin is highlighted by an emerging data base of mutations causing diabetes as discussed in the following article (6). As a consequence of induced fit, such interactions may be dispensable in the mature hormone once folding has been achieved (25). Foldability thus imposes an evolutionary constraint that is, in principle, independent of receptor binding.

Self-assembly and Proteotoxicity—The classical "closed" conformation of insulin mediates its hexameric assembly (Fig. 8A, left), which in β-cells provides a stable storage form of the hormone within secretory granules (1). Such assembly is proposed to protect the β-cell from proteotoxicity due to aggregation-coupled misfolding (1, 3) and in turn to formation of fibrils (Fig. 8B, right) (18). Insulin fibrillation is delayed by native self-assembly and promoted by partial unfolding (33). Such fibrillation leads to canonical cross-β-structure (as observed in diverse pathological amyloid deposits) and so may be regarded as an assay for toxic protein misfolding (19).

PheB24 packs within the dimer-related β-sheet and so has a dual function in self-assembly and receptor binding (30). We have found that substitution of PheB24 by d- or l-Ala reduces the lag time prior to fibrillation (relative to DKP-insulin). Although these analogs exhibit similar global stabilities, the onset of fibrillation is more rapid in the d-Ala analog. We envisage that segmental unfolding and detachment of the C-terminal segment of the B-chain expose underlying hydrophobic surfaces that in turn participate in formation of an amyloidogenic nucleus (Fig. 8A) (34, 35). That these or related surfaces participate in receptor binding suggests that the susceptibility of the wild-type hormone to fibrillation, apparently conserved among vertebrate insulins (18), is intrinsic to its mechanism of action (28). The interrelation between conformational distortion of the insulin monomer and its receptor-bound structure is illustrated in schematic form in Fig. 8A (middle).

An inhibitory extension of the B-chain may have evolved to reduce the propensity of an active monomer to undergo aggregation-coupled misfolding with its potential proteotoxicity (65). This hypothesis is supported by the increased susceptibility of truncated insulin analogs to fibrillation (18). Similarly, substitution of flanking GlyB23 by Ala (5), a perturbation of the B20–B23 β-turn that presumably redirects the C-terminal segment, accelerates fibrillation to an extent similar to that of l-AlaB24.5 Conversely, immobilization of the C-terminal segment of the B-chain in its inhibitory conformation within inactive single-chain insulin analogs (40, 41) confers marked protection against fibrillation (18, 28). These principles may provide a foundation for the design of thermal fibrillation-resistant single-chain analogs for clinical use in challenged regions of the developing world (46).

Although of major concern in pharmaceutical chemistry (18), insulin fibrillation rarely occurs in vivo6 and is not ordi-

4 Of particular interest is SerB24-insulin, a classical insulinopathy whose structure in 20% deuterioacetic acid (78) resembles that of l-AlaB24-DKP-insulin. Although SerB24-insulin is partially active, the potential contribution of misfolding of the variant proinsulin to β-cell dysfunction is discussed in the following article (6).

5 K. Huang and M. A. Weiss, unpublished data.

6 The pathogenesis of diabetes mellitus in the octagon deus New World rodent is associated with insulin-specific islet amyloidosis. Degu insulin is highly divergent, containing variations at B10, B28, and B29 that are predicted to impair its dimerization and zinc-mediated hexameric assembly (79).
narily present in human amyloid deposits. As a seeming paradox, the absence of pathological fibrillation may highlight its underlying importance in the evolution of insulin-like sequences. Diverse site-directed mutations in insulin promote fibrillation in vitro, whereas protective substitutions are seldom encountered (33, 34). Like the dog that did not bark in the nighttime,7 the general avoidance of amyloidogenic variants among vertebrate insulin sequences implies a selective advantage to their exclusion. Resistance to fibrillation defines a biological constraint that is in principle independent of receptor binding.

Concluding Remarks—The total chemical synthesis of proteins facilitates incorporation of nonstandard amino acids as probes of structure-activity relationships (66). Of particular interest is the inversion of chirality at potential sites of conformational change (3, 5, 67). Whereas D-amino acid substitutions may be employed to stabilize a β-turn (testing its contribution to folding or function) (4), within an α-helix or β-strand such substitutions are likely to be destabilizing. In this study, we have explored such stereospecific perturbation to illuminate the role of an invariant aromatic side chain (PheB24) in folding, activity, and misfolding. We propose that the structure of the distorted D-analog foreshadows the wild-type mode of receptor recognition and so enables enhancement of biological activity. In the following article, this model is tested by systematic photocross-linking studies (6). The resulting “photoscan” provides evidence that detachment of the C-terminal segment of the B-chain leads to its insertion between receptor domains.

Insulin has long provided a model for analysis of protein structure and function. The results of this study of chiral substitutions at B24 suggest that induced fit upon receptor binding represents the molecular adaptation of a globular protein to multiple competing biological constraints. The invariance of PheB24 among vertebrate insulins is presumably enjoined by its complementary roles in folding, protective self-assembly, and signaling. We envisage that partial unfolding extends the accessible receptor-binding surface but also exacerbates the risk of non-native aggregation. The striking resemblance between the proposed active conformation of insulin and the partial fold of an amyloidogenic intermediate rationalizes the general susceptibility of vertebrate insulins to fibrillation. The complex conformational life cycle of insulin from biosynthesis to receptor binding thus highlights the cryptic role of toxic misfolding as a universal constraint in the evolution of polypeptide sequences.

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This metaphor refers to a “curious incident” in Arthur Conan Doyle’s classic story The Adventure of Silver Blaze. Just as the absence of barking provided Sherlock Holmes with a critical clue, the rarity of insulin fibrillation in vivo points to the evolution of protective mechanisms.
Stereospecific Protein “Un-design”

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