Activities of Monomeric Insulin Analogs at Position A8 Are Uncorrelated with Their Thermodynamic Stabilities*

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Previous studies have demonstrated that the potency and thermodynamic stability of human insulin are enhanced in concert by substitution of ThrA8 by arginine or histidine. These surface substitutions stabilize the N-terminal α-helix of the A chain, a key element of hormone-receptor recognition. Does enhanced stability necessarily imply enhanced activity? Here, we test by structure-based mutagenesis the relationship between the stability and activity of the hormone. To circumvent confounding effects of insulin self-association, A chain analogs were combined with a variant B chain (AspB10, LysB28, and ProB29 (DKP)) to create a monomeric template. Five analogs were obtained by chain combination; disulfide pairing proceeded in each case with native yield. CD and 1H NMR spectra of the DKP analogs are essentially identical to those of DKP-insulin, indicating a correspondence of structures. Receptor binding affinities were determined by competitive displacement of 125I-insulin from human placental membranes. Thermodynamic stabilities were measured by CD titration; unfolding was monitored as a function of guanidine concentration. In this broader collection of analogs receptor binding affinities are uncorrelated with stability. We suggest that receptor binding affinities of A8 analogs reflect local features of the hormone-receptor interface rather than the stability of the free hormone or the intrinsic C-capping propensity of the A8 side chain.

The functional surface of insulin has long been the object of speculation (1–3). Despite many years of investigation by mutagenesis, NMR spectroscopy and x-ray crystallography, structures of insulin and insulin analogs do not consistently predict relative potencies (3–8). These observations suggest that a change in structure occurs on receptor binding (7, 8). This intriguing but controversial hypothesis (9) has motivated examination of the relationship between the thermodynamic stability of insulin analogs and receptor binding (10). Do mutations that stabilize the native insulin T state (Fig. 1) also enhance receptor binding, and if so, how can such effects be compatible with an induced fit mechanism of hormone-receptor recognition? The present study demonstrates that, contrary to a previous proposal (10), the activity and stability of insulin are uncorrelated among a set of A chain analogs. The substitutions affect the C-terminal residue and putative electrostatic capping box of a conserved recognition α-helix.

A subset of insulin analogs with enhanced potency has been found to exhibit enhanced stability (Table I and Ref. 10). This influential study focused on the substitution of ThrA8 in human insulin by alanine, histidine, or arginine. The A8 site defines one edge of the classical receptor-binding surface of insulin (1–3) and delimits the A1–A8 α-helix (asterisk in Fig. 1). The enhanced activities of the HisA8 and ArgA8 analogs (relative to ThrA8) were ascribed to thermodynamic stabilization of this critical helix because of their higher helical propensities, including more favorable C-cap properties (11, 12). Additional stability may derive from an electrostatic interaction between the A8 side chain and the negative charge of GluA4 (a potential C-capping box; Ref. 10). Whether a correlation between stability and activity is general or limited to these particular analogs is not known. Because this issue has functional implications in relation to the active conformation of insulin, we prepared multiple analogs, including substitutions containing negative and polar side chains at A8. In this broader collection we find that activity and stability are uncorrelated. A negatively charged GluA8 substitution, for example, stabilizes the free hormone but impairs receptor binding. The contribution of a putative (GluA4, HisA8) electrostatic capping box to the stability and activity of insulin was tested by the second site substitution GluA4 → Ala and found not to be significant.

Experimental design employs an engineered insulin monomer as a framework for A8 substitutions. The monomer, designated DKP-insulin (13–15), exhibits enhanced activity. Self association is prevented by three substitutions in the B chain (AspB10, LysB28, and ProB29) as described previously (13). The solution structure of DKP-insulin (16) resembles the crystallographic T state (Ref. 3 and Fig. 1). The DKP B chain is readily combined with variant A chains to provide a monomeric template (15). In this article we describe the synthesis and characterization of five analogs of DKP-insulin. Our results demonstrate that receptor binding is uncorrelated with either observed thermodynamic stabilities or tabulated helical capping propensities (11, 12). We propose a model in which the A8 side chain lies at the periphery of the insulin receptor and can thus introduce favorable or unfavorable local interactions at the edge of the receptor. Diverse side chains are readily accommodated at this edge and may be incorporated into novel analogs of therapeutic interest.

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FIG. 1. Stereo ribbon representation of the porcine insulin monomer, as inferred from the crystal structure of 2-Zn (T) porcine insulin (molecule 1; Chinese nomenclature) (3). The B chain is shown in black, and the A chain is shown in gray. The asterisk in the left-hand panel indicates the A8 side chain (threonine). For reference the four tyrosine and two phenylalanine side chains are also shown; these provide informative NMR probes as shown in Fig. 4. The NMR structure of DKP-insulin (16) resembles the crystallographic T state protomer (3).

EXPERIMENTAL PROCEDURES

Materials
4-Methylbenzhydramine resin (0.6 mmol of amine/g; Star Biochemicals, Inc.) was used as solid support for synthesis of the A chain analogs; (N-term-butoxycarbonyl, O-benzyl-threonine-phenylacetamido-methyl resin (0.56 mmol/g; Bachem, Inc.) was used as solid support for synthesis of the DKP B chain analog. N-Term-butoxycarbonyl amino acids and derivatives were obtained from Bachem and Peninsula Laboratories; N,N,N’-dicyclohexylcarbodiimide and N-hydroxybenzotriazole (recrystallized from 95% ethanol) were from Fluka. Amino acid analyses of synthetic chains and insulin analogs were performed after acid hydrolysis; protein determinations were carried out by the Lowry method using native insulin as standard. Chromatography resins were preswollen microgranular carboxymethylcellulose (CM-cellulose; Whatman CM52), DE53 cellulose (Whatman), and Cellex E (Ecteola cellulose); preswollen microgranular carboxymethylcellulose (CM-cellulose; Whatman, Manchurc, Russia) and reverse-phase HPLC on a Vydac 218 TP column (0.46 × 25 cm); the latter used a flow rate of 0.5 ml/min with 20–80% linear gradient of 80% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 80 min. Recrromatography of this material on reverse-phase HPLC under the same conditions gave in each case a single sharp peak. The HisA8 A chain was also purified by the native human B chain to obtain HisA8 human insulin as a positive control. Amino acid analyses and mass spectrometry in each case gave expected values.

Peptide Synthesis

The general protocol for solid phase synthesis is as described (17). A manual double-coupling protocol was followed (18, 19). The C-terminal Asp residue in the synthesis of the A chain was incorporated into solid support by coupling N-term-butoxycarbonyl Asp–N-benzyloxycarbonyl with 4-methylbenzhydramine resin. After the final deprotection the Asp residue was converted to an Asn residue.

Synthetic A chain S-Sulfonate Analogs—From 606 mg of GluA8 peptide resin, after deblocking, sulfitolysis, and chromatographic purification, ~116 mg of purified S-sulfonated A chain were obtained. After analogous processing the following yields of other analogs were obtained: 125 mg of purified S-sulfonated chain from ~602 mg of GluA8 peptide resin, ~235 mg of purified chain from 1.03 g of HisA8 peptide resin, ~165 mg of purified chain from 0.8 g of [AlaA14, HisA16] peptide resin, and 136 mg of purified chain from 0.75 g of AlaA14 peptide resin. AlaA14 A chain was also prepared as a negative control (see text). Synthetic B Chain S-Sulfonate—From 610 mg of [AspB10, LysB28, ProB29] peptide resin after deblocking, sulfitolysis and chromatographic purification, ~125 mg of purified S-sulfonated B chain was obtained.

Peptide Purification

Crude S-sulfonated A chains were purified by chromatography on a Cellex E column (1.5 × 47 cm) as described (18, 19), dialyzed against distilled water, and lyophilized to yield the purified A chain S-sulfonate A8 analogs. Crude S-sulfonated DKP B chain was likewise purified on a cellulose DE53 column (1.5 × 47 cm), dialyzed, and lyophilized to yield the [AspB10, LysB28, ProB29] B chain S-sulfonate.

Chain Recombination

Chain recombination employed S-sulfonated A chain and DKP B chain (~2:1 by weight) in 0.1 M glycine buffer (pH 10.6) in presence of dithiothreitol. Insulin analogs were isolated from the combination mixture as described (18, 19) and purified on a CM-cellulose column (0.9 × 23 cm) and reverse-phase HPLC on a Vydac 218 TP column (0.46 × 25 cm); the latter used a flow rate of 0.5 ml/min with 20–80% linear gradient of 80% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 80 min. Recrromatography of this material on reverse-phase HPLC under the same conditions gave in each case a single sharp peak. The HisA8 A chain was also purified with the native human B chain to obtain HisA8 human insulin as a positive control. Amino acid analyses and mass spectrometry in each case gave expected values.

Receptor Binding Studies

Radiolabeled [125I-TyrA8] human insulin was purchased from American Pharmacia Biotech. Receptor binding assay of insulin analogs were performed as described (20) with minor modifications. Human placental cell membranes were prepared (21), stored at ~80 °C in small aliquots, and thawed prior to use. Membrane fragments (0.025 mg of protein/tube) were incubated with radiolabeled insulin (~30,000 cpm) in the presence of selected concentrations of unlabeled peptide for 18 h at 4 °C in a final volume of 0.25 ml of 0.05 M Tris-HCl and 0.25% (w/v) bovine serum albumin at pH 8. Subsequent to incubation, each mixture was diluted with 1 ml of ice-cold buffer and centrifuged (10,000 × g) for 5 min at 4 °C. The supernatant was then removed by aspiration, and the membrane pellet was counted for radioactivity. The data were corrected for nonspecific binding (amount of radioactivity remaining membrane-associated in the presence of 1 µM human insulin). Each determination was performed with three or four replicates (Table II); the values are reported as the mean and standard deviation of these multiple measurements. Binding studies of control analog HisA8 human insulin were undertaken with both placenta-derived membrane fragments at 4 °C and hepatic insulin receptor preparations at 24 °C (17, 18).

Spectroscopy

1H NMR spectra of DKP-insulin and DKP analogs were obtained at 500 or 600 MHz at 25 °C in aqueous solution (pH 7.0, pD 6.6, direct meter reading); the protein concentration was 1.5 mM. Resonance assignments for DKP-insulin have been described (16) and extended by inspection to analogs. CD spectra of DKP-insulin analogs were obtained using an Aviv spectropolarimeter equipped with thermister temperature control and automated titration unit for guanidine denaturation studies. Denaturation studies were conducted in 10 mM potassium phosphate (pH 7.4) and 50 mM KC1. CD spectra were obtained at a protein concentration of 50 µM; the samples were diluted to 5 µM for equilibrium denaturation studies (Fig. 3, C and F); to allow comparison with the conditions used by Kaarsholm and co-workers (10), guanidine CD studies of the unfolding of human insulin represent measurement in the presence of 1 M guanidine HCl. Each determination was performed with three or four replicates (Table II); the values are reported as the mean and standard deviation of these multiple measurements. Binding studies of control analog HisA8 human insulin were undertaken with both placenta-derived membrane fragments at 4 °C and hepatic insulin receptor preparations at 24 °C (17, 18).

Control Studies

The values obtained herein for activity and stability in part differ from published reports because of conditions of study.

Receptor Binding Studies—Use of placental membrane preparations at 4 °C leads to lower estimates of enhanced activity than use of hepatic membrane preparations at 24 °C (18) or IM-9 lymphocyte membranes.
Table II

| Analog       | Placental receptor binding | $\Delta G_u$   | $\Delta G_a$   | $C_{\text{mid}}^{b}$ | $m^{c}$ |
|--------------|---------------------------|----------------|----------------|----------------------|---------|
| Insulin      | 100                       | 4.4 ± 0.1      | -              | 5.3 ± 0.1            | 0.84 ± 0.01 |
| DKP-insulin  | 161 ± 19 (4)              | 4.8 ± 0.2      | -              | 5.8 ± 0.1            | 0.94 ± 0.01 |
| Ala$^{A4}$ DKP-insulin | 152 ± 24 (3)              | 4.6 ± 0.1      | -0.3 ± 0.1     | 6.0 ± 0.1            | 0.77 ± 0.01 |
| His$^{A8}$ DKP-insulin | 167 ± 21 (6)              | 5.2 ± 0.1      | 0.4 ± 0.3      | 6.3 ± 0.1            | 0.83 ± 0.01 |
| [Ala$^{A4}$, His$^{A8}$] DKP-insulin | 209 ± 40 (3)              | 5.0 ± 0.1      | 0.2 ± 0.2      | 5.7 ± 0.1            | 0.88 ± 0.02 |
| Gln$^{A8}$ DKP-insulin | 122 ± 1 (3)               | 5.0 ± 0.1      | 0.2 ± 0.3      | 6.4 ± 0.1            | 0.78 ± 0.01 |
| Glu$^{A8}$ DKP-insulin | 61 ± 2 (3)                | 5.9 ± 0.1      | 1.1 ± 0.3      | 6.6 ± 0.2            | 0.89 ± 0.03 |

$^{a}$ The activity was measured by relative affinity for the placental insulin receptor; the number of assays is given in parentheses with standard deviations provided. Under these conditions the $K_u$ for native insulin is 0.48 ± 0.06 nM. Control studies were also performed using His$^{A8}$ human insulin (positive control) and Ala$^{A8}$ DKP-insulin (negative control) as described in the text.

$^{b}$ $C_{\text{mid}}$ is defined as that molar concentration of guanidine HCl associated with 50% protein unfolding.

$^{c}$ The $m$ value (kcal/mol/σ) is defined as the slope in plotting the unfolding free energy versus molar concentration of denaturant; this slope is often found to be proportional to the protein surface area exposed on unfolding. Details of the curve fitting are given in Ref. 41.

![Fig. 2. Sequences of insulin A chains.](Image)

The cysteine residues involved in disulfide bridges (A6–A11, A7–B7, and A20–B19) are shown in bold type.

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at 15 °C (23). Positive control studies of His$^{A8}$ human insulin yielded values of 212% ± 34 in the placental membrane assay and higher values in our hands (450–550%) in the hepatic membrane assay in accord with published values (10). Negative control studies of a low affinity substitution (Ala$^{A4}$ DKP-insulin) in the placental membrane assay yielded values of ~1%, which is also in accord with published values (24, 25).

Guanidine Titrations—The His$^{A8}$ substitution in the context of DKP-insulin enhances stability at 4 °C by 0.4 ± 0.1 kcal/mol, which is less than its reported stabilizing effect in the context of native insulin at 25 °C ($\Delta G_a$ = 1.7 kcal/mol; Table I and Ref. 10). To test whether this reflects a difference in experimental conditions or template context (DKP-insulin versus native insulin), we reinvestigated the stability of human insulin and His$^{A8}$ insulin relative to human insulin at 4 and 25 °C. The stabilizing effect of His$^{A8}$ at 4 °C ($\Delta G_a$ = 0.5 ± 0.2 kcal/mol) is similar to that in the DKP context. The higher values reported at 25 °C (10) are reproducible in our hands and reflect the lower relative stability of native human insulin at higher temperatures.

**RESULTS**

Five analogs of DKP-insulin were prepared by total synthesis (Table II and “Experimental Procedures”). Chain combination yielded native disulfide pairing with efficiencies similar to that observed in the total synthesis of native insulin. Non-native disulfide isomers (26) were not observed. Use of the monomer DKP template simplifies biophysical analysis by avoiding possible confounding effects of insulin self-association (15).

**Analogs Exhibit Native Overall Structure—**CD spectra of the analogs exhibit similar helix contents relative to DKP-insulin as illustrated in Fig. 3 (A and D). $^1$H NMR spectra (pH 7 and 25 °C) are similar to those of DKP-insulin; each analog exhibits comparable nonrandom dispersion of chemical shifts characteristic of the T state structure. In addition, each spectrum retains the pattern of resonance line widths and chemical shifts diagnostic of the monomeric state (14). Two-dimensional total correlation $^1$H NMR spectra reveal essentially identical spin systems with corresponding chemical shifts. This correspondence is illustrated for three analogs (Fig. 4); the aromatic resonance of the proteins four tyrosines and three phenylalanines provide intrinsically probes of the major structural elements of the hormone. An example is provided by the chemical shifts of Phe$,B_{24}$ and Tyr$,A_{19}$, whose large secondary shifts reflect their distinct environments in the native hydrophobic core (Fig. 1). Such correspondence of chemical shifts provides evidence of structural similarity.

**Receptor Binding Affinities and Thermodynamic Stability Are Uncorrelated—**Glu$^{A8}$ and Gln$^{A8}$ analogs differ by a single functional group: a side chain carboxylate or carboxamide. The results of activity and stability measurements are given in Table II. Whereas the activity of Gln$^{A8}$ DKP-insulin is ~120% (relative to human insulin), the Glu$^{A8}$ analog is 2-fold less active. Analysis of protein unfolding by contrast demonstrates opposing differences in thermodynamic stability (Fig. 3F). Whereas DKP-insulin exhibits a cooperative and apparent two-state transition with $\Delta G_u$ = 4.8 ± 0.1 kcal/mol, Glu$^{A8}$ DKP-insulin and His$^{A8}$ DKP-insulin each exhibit enhanced stability ($\Delta G_a$ = 5.9 ± 0.1 and 5.2 ± 0.1 kcal/mol, respectively; see Table II). Surprisingly, the extent of stabilization conferred by Glu$^{A8}$ ($\Delta G_a$ = 1.1 ± 0.2 kcal/mol) is greater than that conferred by His$^{A8}$ ($\Delta G_a$ = 0.4 ± 0.2 kcal/mol). Substitution of the negative Glu$^{A8}$ charge by a neutral Glu$^{A8}$ carboxamide attenuates the enhancement in stability. Although qualitative inspection of the unfolding curves strongly suggests that Glu$^{A8}$ DKP-insulin is slightly more stable than DKP-insulin in accord with its greater $C_{\text{mid}}$ guanidine concentration (Table II), the inferred increment in stability is imprecisely determined ($\Delta G_u$ = 0.2 ± 0.3 kcal/mol).

Comparison of Glu$^{A8}$ and Gln$^{A8}$ analogs thus demonstrates...
that a negative charge at A8 can be as stabilizing as was reported for a positive charge (ArgA8; Table I) but less active than a neutral isostere (GlnA8). The native DKP monomer and A8 analogs exhibit similar temperature-dependent changes in mean residue ellipticity at 222 nm in the range 4–50 °C (Fig. 3, B and E). To test whether the enhanced stability of HisA8 DKP-insulin is due to an electrostatic capping box involving GluA4, the latter side chain was substituted by alanine to yield the two analogs AlaA4, HisA8 DKP-insulin and [AlaA4, HisA8] insulin. In either context the alanine substitution is destabilizing by 0.2 kcal/mol. Although this destabilizing effect is consistent with a general contribution of GluA4 to stability, our results exclude a specific role for a putative [GluA4, HisA8] capping box: introduction of a negative charge at A4 is no more stabilizing relative to [AlaA4, HisA8] than it is relative to [AlaA4, ThrA8]. The destabilizing GluA4 → Ala substitution does not impair receptor binding activity in either context.

**DISCUSSION**

The present study addresses two questions: can the stability of insulin be engineered through the use of general principles of peptide chemistry (27, 28), and if so, can such engineering be exploited to enhance its activity? These questions were posed by an influential study by Kaarsholm et al. (10) in which rational optimization of the C-terminal capping box of the A1–A8 α-helix (11, 12) was found to enhance stability and activity in concert (Table I). The present experimental design has tested whether such enhancement is related to the biophysical rationale of the design or is coincidental. Two sets of analogs were synthesized and characterized. First, GluA8 and GlnA8 DKP-insulin enable comparison of two isosteric side chains differing by a single function: the negatively charged carboxylate versus the polar carboxamide. We have found that GluA8 enhances the stability of insulin, which would not be expected based on an electrostatic C-capping mechanism in an isolated α-helix (11, 12). A negative charge at A8 would in fact seem to be unfavorable because of repulsion by the helical dipole and neighboring presence of GluA4. It is possible that the stabilization of the analog is due to the greater helical propensity of Glu (relative to Thr) irrespective of segmental electrostatic interactions. Despite such enhanced stability, the GluA8 analog is ∼3-fold less active than its parent DKP-insulin in receptor binding. The stability of GlnA8 DKP-insulin is similar to that of DKP-insulin. Because Glu and Gln have similar helical propensities (11, 12) and GlnA8 would not incur unfavorable local electrostatic interactions, it is not clear why GluA8 DKP-insulin is substantially more stable than GlnA8 DKP-insulin. Electrostatic contributions to protein stability are complex, including long range interactions, changes in solvation, and distribution of counter ions. The activity of GlnA8 DKP-insulin is between that of GluA8 DKP-insulin and DKP-insulin (Table II).

We next examined the stabilizing mutation HisA8. Our results confirm that this substitution enhances the stability of insulin, although the extent of enhancement is smaller at 4 °C than at 25 °C because of differential thermal destabilization of insulin. GluA4 → Ala substitutions were introduced into DKP-insulin and HisA8 DKP-insulin to test whether such enhanced stability is due to an (i, i + 4) electrostatic capping interaction. The AlaA4 substitution causes the same small decrement in stability in either context. These results suggest that an (i, i + 4) interaction, if present, does not contribute to stability. Thus, whereas physical mechanisms stabilizing insulin must include...
contributions from foundational principles of helical stabilization (24, 25), these factors are not predominant at the A8 position. The GluA4 3 Ala substitution has previously been shown to enhance slightly the activity of native insulin (10, 29). Our results are consistent with this but not sufficiently precise to provide a rigorous assessment. We can conclude, however, that elimination of a putative interaction between the side chains of GluA4 and HisA8 in [AlaA4, HisA8] DKP-insulin does not impair receptor binding activity.

Optimization of stability by protein engineering does not in general provide a consistent criterion for enhancing receptor binding. Mutations that enhance the stability of a DNA-binding domain, for example, do not consistently enhance DNA affinity at permissive temperatures (30). The stability of a protein ($\Delta G$) reflects an overall equilibrium between folded and unfolded states of a protein. A mutational increase in $\Delta G_u$ from 4 to 5.4 kcal/mol, for example, would correspond to an increase in the fraction of folded insulin molecules in an ensemble from 0.9990 to 0.9999%. Should the folded state be the active conformation, such a change would in itself predict only a marginal increase in the availability of active molecules; the associated enhancement in receptor binding would be negligible. Should the folded state be inactive, then stabilizing mutations might even impair activity. In either case the present results, excluding a general correlation between the stability and activity of insulin, are not inconsistent with thermodynamic principles.

Activity of A8 analogs could be enhanced or impaired by three possible residue-specific mechanisms: (i) the mutation could cause a local or nonlocal structural changes in the free hormone; (ii) the mutation could facilitate or hinder a change in the hormone's conformation on receptor binding; or (iii) the
mutation could stabilize or destabilize the hormone-receptor interface irrespective of its effects in the free hormone. We discuss each of these possibilities in turn.

**Structural Changes in the Free Hormone**—The A8 side chain is exposed on the surface of insulin (Fig. 1 and Refs. 1–3). Although crystal structures of A8 analogs have not been described to date, the NMR structure of an engineered insulin monomer containing HisAβ has been reported (31). The structure resembles the crystallographic T state with no non-native interactions involving the mutant side chain. Similarly, qualitative two-dimensional NMR studies of the present analogs demonstrate retention of a native insulin fold (Fig. 4). These data do not exclude small changes in the local structure or dynamics of the A1–A8 α-helix. Because local changes may be uncorrelated with global stabilities, it remains possible that A8 substitutions can affect activity through transmitted effects on classical receptor-binding sites (1–3), e.g. at GlyA1, IleA2, and ValA3. We consider this unlikely in light of the small thermodynamic cost of induced fit observed in receptor-binding studies of unstable two-disulfide analogs of insulin (22) and insulin-like growth factor-1 (32); segmental unfolding of the A1–A8 α-helix (an extreme case of structural and dynamic perturbation) is associated with only modest decrements in affinity. We therefore imagine that any subtle changes in the structure or dynamics of the A1–A8 α-helix in an A8 analog could readily be "repaired" on receptor binding.

**Hindrance or Facilitation of a Conformational Change**—Considerable speculation has focused on conformational changes involving the C-terminal β-strand of the B chain on receptor binding (4, 7, 8, 33–35). Detachment or destabilization of the C-terminal β-strand is suggested, for example, by the enhanced activity of β-amino-acid substitutions at position B24 (3–5). Such a change in conformation would be expected to facilitate contacts between the receptor and the conserved side chains of IleA² and ValA³ as illustrated in Fig. 5. Can an analogous model apply to the A chain? Although this possibility is consistent with the modularity of the structure of insulin (i.e. like that of the C-terminal B chain β-strand, local folding or unfolding of the A1–A8 segment can occur independently of the remainder of the native state; Ref. 16), such a model is unlikely. Unlike the C-terminal B chain β-strand, the N-terminal α-helix of the A chain appears to function as a preformed recognition element (Fig. 5 and Refs. 22 and 31). Further, although we cannot exclude a mechanism by which enhanced segmental stability of the A1–A8 α-helix hinders detachment of the B24–B28 β-strand, the divergent properties of GluAβ-DKP-insulin (more stable but less active) suggests that such transmitted effects are not predominant.

**Local Effects within the Hormone-Receptor Interface**—We posit that the variable activities of A8 insulin analogs reflect primarily the complex chemistry of the hormone-receptor interface. Differences in activity between A8 analogs would then arise from variable local interactions within the complex and not within the free hormone. In accord with classical models of the receptor-binding surface of the hormone (1–3), we imagine that ThrA⁸ is positioned at the edge of the receptor and that this location has a small negative electrostatic potential. Side chains containing a positive charge are slightly favored, whereas side chains containing a negative charge are slightly disfavored. Because of the variety of side chain shapes, sizes, and functionalities that can be accommodated by the receptor at the A8 site, we expect that high affinity complexes can be generated using photoreactive side chains such as para-azido-PheAβ (36) or para-benzoyl-PheAβ (37). It would be of future interest to test whether such derivatized insulins would photo-cross-link to the insulin receptor and, if so, to map sites of attachment.

In summary, the critical importance of the N-terminal α-helix of the A chain (1–3) has focused attention on the structural basis of its stabilization by A8 substitutions and their functional implications. Our results verify that substitutions at A8 can modulate the stability and activity of insulin but exclude a simple electrostatic model of how the A8 side chain contributes to the stability of insulin and do not substantiate a proposed relationship between stability and activity (10). How such diverse substitutions as HisAβ and GluAβ (but not GlnAβ) enhance the stability of insulin remains enigmatic. The complexity of protein structures and interactions implies that changes in physical or functional properties often reflect small differences between large entropic and enthalpic driving forces, including those associated with solvation and counter ion distribution. Deciphering how A8 substitutions influence the activity of insulin will require a crystal structure of the hormone-receptor complex (38). Such a structure would be of both basic and applied interest in relation to diabetes therapy. Positions at the edge of the classical receptor-binding surface of the hormone (1–3), such as A8, B9, B10, B28, and B29, are of particular interest as sites of modification in pharmacologic design of novel analogs (39, 40).

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Activities of Monomeric Insulin Analogs at Position A8 Are Uncorrelated with Their Thermodynamic Stabilities

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