Structure-based stabilization of insulin as a therapeutic protein assembly via enhanced aromatic–aromatic interactions

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Key contributions to protein structure and stability are provided by weakly polar interactions, which arise from asymmetric electronic distributions within amino acids and peptide bonds. Of particular interest are aromatic side chains whose directional π-systems commonly stabilize protein interiors and interfaces. Here, we consider aromatic–aromatic interactions within a model protein assembly: the dimer interface of insulin. Semi-classical simulations of aromatic–aromatic interactions at this interface suggested that substitution of residue TyrB26 by Trp would preserve native structure while enhancing dimerization (and hence hexamer stability). The crystal structure of a [TrpB26]insulin analog (determined as a T3Rf hexamer at a resolution of 2.25 Å) was observed to be essentially identical to that of WT insulin. Remarkably and yet in general accordance with theoretical expectations, spectroscopic studies demonstrated a 150-fold increase in the in vitro lifetime of the variant hexamer, a critical pharmacokinetic parameter influencing design of long-acting formulations. Functional studies in diabetic rats indeed revealed prolonged action following subcutaneous injection. The potency of the TrpB26-modified analog was equal to or greater than an unmodified control. Thus, exploiting a general quantum-chemical feature of protein structure and stability, our results exemplify a mechanism-based approach to the optimization of a therapeutic protein assembly.

Weakly polar interactions are ubiquitous among protein structures (1). Among such interactions, the relative packing of aromatic rings is of particular interest in relation to the organization of protein cores and subunit interfaces (2). Aromatic–aromatic interactions are governed by quantum-chemical properties, which underlie dispersion forces and give rise to asymmetric distribution of partial charges. Whereas aromatic stacking is prominent in nucleic acid structures, pairs of aromatic side chains in proteins more often exhibit edge-to-face (ETF) contacts (3). Can such contacts be exploited in therapeutic protein engineering? Here, we have analyzed aromatic–aromatic interactions in the insulin hexamer (4) as a basis for designing improved long-acting (basal) analogs. This class of analogs is central to the treatment of Type 1 and Type 2 diabetes mellitus (5).

Classical crystal structures of insulin hexamers (6, 7) immediately suggested a pathway of assembly (4). Pertinent to the mechanism of storage in the secretory granules of pancreatic β-cells (8), such assembly is also of pharmacologic importance (9). Insulin assembly both protects the hormone from degradation in pharmaceutical formulations and modulates its pharmacokinetic properties (10). Indeed, the first use of insulin analogs in diabetes therapy reflected efforts to destabilize the insulin hexamer and thereby accelerate absorption of monomers and dimers from the subcutaneous (SQ) depot (summarized in Fig. S1 (11, 12)). Because it is more straightforward to introduce unfavorable substitutions than favorable ones, such engineering (corresponding to rapid-acting analogs) was more successful than complementary efforts to enhance the thermodynamic (and kinetic) stability of the insulin hexamer (13). There are presently three rapid-acting insulin analogs in clinical use (14), but no basal products designed on the basis of enhanced hexamer assembly despite extensive efforts (15).

These difficulties were circumvented by alternative mechanisms of protracted action (acylation and pH-dependent SQ precipitation (14); Fig. S2).

The dimer interface of insulin (repeated three times in the hexamer) contains a cluster of eight conserved aromatic rings

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We dedicate this article to the memory of the late Prof. Colin Ward (Eliza and Walter Hall Institute, Melbourne, Australia).

This article contains Figs. S1–S12 and Tables S1–S13.

The atomic coordinates and structure factors (code 2CK2) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: ETF, edge-to-face; αCT, carboxyl-terminal domain of IR; μIR, domain-minimized insulin micro-receptor; EEC, entropynenthalpy compensation; IR, insulin receptor; L1, leucine-rich domain of IR MD, molecular dynamics; MM, molecular mechanics; PD, pharmacodynamics; PDB, Protein Data Bank; r.m.s. deviation, root mean square deviation; SQ, subcutaneous; QM, quantum-mechanical; SEC, size exclusion chromatography; IGF, insulin-like growth factor; TOCSY, total correlated spectroscopy; i.v., intravenous; Orn, ornithine; WT insulin pertains to the human sequence unless otherwise stated.

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A sequence of insulin with disulfide bridges in black. TyrB26 is highlighted in black; the present TyrB26 → Trp substitution is orange; and modifications in pI-shifted clinical analog glargine in purple. Our semisynthetic pI-shifted analog contained Orn (green) instead of Lys or Arg. B, structure of an insulin monomer; the A chain is shown in black and B chain in green. TyrB26 is red, whereas PheB24 and TyrB16 are blue (PDB code 4INS). C, structure of zinc-coordinated insulin hexamer (T3 state), a trimer of dimers; TyrB26, PheB24, and TyrB16 are color-coded as in B, D, stereo view showing TyrB26 (sticks) in a cavity within insulin dimer (extracted from T3Rf zinc–insulin hexamer, PDB code 1TRZ). E, corresponding stick model with residues labeled.

The present study had three parts. The first employed local modeling, using the standard CHARMM empirical energy function, to probe possible effects of a TyrB26 → Trp substitution on aromatic–aromatic interactions within the wildtype (WT) aromatic cluster. These molecular mechanics (MM) calculations suggested that substitution of TyrB26 by Trp could enhance dimer-related ETF contacts and yet otherwise preserve a native-like interface. We next prepared this analog to examine whether this substitution might indeed stabilize the insulin hexamer and retard its disassembly while preserving the biological activity of the monomeric hormone. The crystal structure of a [TrpB26]insulin analog (as a zinc–insulin hexamer) was essentially identical to that of WT insulin. Finally, we undertook studies in diabetic rats to obtain proof of principle that this approach could extend the duration of insulin action on SQ injection.
To our knowledge, our results represent the first exploitation of aromatic–aromatic interactions to enhance the physical and biological properties of a therapeutic protein. Because standard MM calculations employ a simplified model of aromatic systems (i.e. approximating their quantum-mechanical (QM) properties via partial atomic charges (19, 20)), ab initio QM simulations of the aromatic cluster and their incorporation in QM/MM simulations (21) promise to establish a rigorous foundation for therapeutic protein design, including further optimization through incorporation of modified or nonstandard amino acids (22–24). The present results suggest that insulin’s conserved aromatic cluster can provide a natural laboratory for such foundational analysis and its therapeutic translation.

Results

Molecular mechanics calculations suggested that augmented aromatic–aromatic interactions are possible at the TrpB26 dimer interface

MM simulations were employed to estimate the strength of aromatic–aromatic interactions of TrpB26 at the insulin dimer interface in relationship to those of the native Tyr. These calculations employed the CHARMM empirical energy function in which aromatic rings contain partial atomic charges, parameterized to mimic the electrostatic properties of the π-system (19, 20). Working models of the variant dimer were obtained by local energy minimization (see “Experimental procedures”).

Energies of interaction between the eight aromatic residues at the insulin–dimer interface (TyrB16, PheB24, PheB25, TyrB26, their symmetry-related mates) were calculated using local models in which TrpB26 was substituted within WT T2, R2, and TRf reference dimers (extracted from PDB structures 4INS, 1ZNJ, and 1TRZ) (25). The total interaction energy between B26 and the other aromatic residues at the TrpB26 interface, which was calculated using the full CHARMM potential energy function, was augmented by 2.0 and 0.8 kcal/mol relative to the minimized WT interface in the context of R2 and TRf structures, respectively, and diminished by 1.5 kcal/mol in the context of the T2 structure. Results are summarized in Table S1.

A minimal model was utilized to further evaluate the potential impact of a TrpB26 substitution on aromatic–aromatic interactions at the dimer interface. To this end, a structural model of the dimer interface (extracted from a T6 insulin hexamer; PDB code 4INS) was first built containing residue B26 (TyrB26 or TrpB26) and its nearest aromatic neighbors (PheB24, PheB25, or TyrD16). Simulations predicted the orientation of the B26 ring corresponding to free-energy minima of electrostatic interactions between the aromatic residues (the partial-charge parametrization of aromatic residues in CHARMM is shown in Fig. S3) (23). When substituted at position B26, Trp displayed improved electrostatic interactions with its three aromatic neighbors (relative to the WT Tyr) over a broad range of conformations (Fig. 2). This trend extended to conformations that are sterically permitted in the context of the WT insulin hexamer.

TrpB26 analog exhibited markedly decreased hexamer dissociation rate

The effect of the TrpB26 substitution on the lifetime of insulin hexamers under formulation conditions was assessed in the context of [OrnB29]insulin, a structural equivalent of WT insulin that is amenable to production by trypsin-catalyzed semisynthesis (26). The lifetime of Co2+–substituted, phenol-stabilized (Rg) hexamers of [TrpB26, OrnB29]insulin was assessed at equilibrium in relationship to native [TyrB26, OrnB29]insulin. Optical absorbance spectra of these analogs (characteristic of Co2+ with tetrahedral coordination) were similar to WT (Fig. 3, A and B). Assessment of Rg dissociation rates (summarized in Table 1) revealed a 150-fold increase in hexamer half-life of the TrpB26 analog relative to its parent [OrnB29]insulin (Fig. 3, C and D). This increase is remarkable given that the difference between the half-lives of a rapidly dissociating analog in clinical use (lispro)5 (27–29) and WT is <2-fold (Table 1).

TrpB26 imposed kinetic barriers to the dissociation of hexamers into monomers

Rg dissociation kinetics were further examined by size exclusion chromatography (SEC). The TrpB26 analog (formulated in the presence of phenol and zinc ions) was injected onto an SEC column using a zinc- and phenol-free mobile phase. Subsequent dissociation of the Rg hexamers was monitored in the chromatograms (Fig. 4A, Table 1). Absence of a void volume signal (V0) indicated that none of the proteins formed large nonspecific aggregates. Whereas WT and [OrnB29]insulin eluted as a broad peak representing an association state intermediate between monomer and dimer (9.7 and 8.2 kDa, respectively) and insulin lispro eluted essentially as a monomer (5.1 kDa), [TrpB26, OrnB29]insulin eluted in two distinct peaks. The larger peak corresponded to a trimeric or tetrameric association state (molecular mass 28 kDa), and the smaller corresponded to a monomeric state (4 kDa; Fig. 4, A and B; see Fig. S4)

5 Insulin lispro, containing substitutions ProB28→Lys and LysB29→Pro, exhibited an attenuated 574-nm band (69); this is associated with a greater proportion of spectroscopically attenuated T6 homo/hetero hexamer and invisible T6 hexamer (Fig. 3B).

Figure 2. Molecular simulations of aromatic interactions in the insulin dimer. A, aromatic–aromatic interactions across insulin’s dimer interface involve PheB24, TyrB16, PheB25 (sticks) and either TyrB26 (left) or TrpB26 (right). Residues were extracted from T6 structure 4INS. B, contour maps depicting empirical interaction energies between B26 (Tyr on left and Trp on right) at varying χ1 and χ2 angles and the other three residues shown in A. The orientation of TyrB26 or TrpB26 in the WT or variant crystal structure is indicated by a green ‘x’; orientation of TrpB26 or TrpB26 in the local model is indicated by a green asterisk.
Stabilization of insulin hexamer

Figure 3. Hexamer dissociation of TrpB26 analog. A, tetrahedral Zn\(^{2+}\)-co-ordination site in R\(_6\) insulin hexamer (ball-and-stick model); three His\(^{B10}\) side chains and one chloride ion. B, absorbance spectra of d-d bands in corresponding Co\(^{2+}\) complex; the color code is indicated. C, hexamer dissociation curves as monitored at 574 nm after addition of excess EDTA; the color code is as in B. The lifetime of the [TrpB26, Orn\(^{B29}\)]insulin hexamer was markedly prolonged (asterisk). D, dissociation of [TrpB26, Orn\(^{B29}\)]hexamer from 0 to 8000 s in relationship to that of parent [Orn\(^{B29}\)]insulin (black arrow). Half-lives are given in Table 1.

Table 1

| Analog          | \(t_1/2\) hexamer dissociation | Calculated molecular mass by SEC* |
|-----------------|---------------------------------|----------------------------------|
| Wildtype        | 7.7 (± 1.3)                     | 9.7                             |
| Lispro\(^{B28}\) | 4.6 (± 0.3)                     | 5.1                             |
| Orn\(^{B29}\)   | 8.2 (± 0.8)                     | 8.2                             |
| TrpB26, Orn\(^{B29}\) | 1.2 (± 0.3) \times 10^3        | 28.0, 4.0                       |

*Proteins were made 0.6 mM in a buffer containing ZnCl\(_2\) at a ratio of 2 zinc ions per insulin hexamer and applied to SEC column as described under "Experimental procedures." Masses were calculated from the plot in Fig. 4B.

for reference chromatograms of the monomer and hexamer). These findings suggest that oligomers intermediate between hexamer and dimer may delay dissociation of the TrpB26 analog: in particular, the absence of broad elution tails implies that TrpB26 imposes significant barriers to rapid dissociation.

TrpB26 analog retained native biological activity

The in vitro affinity of the TrpB26 analog for the lectin-purified insulin receptor (IR; isoform B holoreceptor) was determined to be 0.14 (± 0.02) nM, ~50% that of [Orn\(^{B29}\)]insulin (0.07 (± 0.02) nM) and WT insulin (0.08 (± 0.03) nM) (Fig. 5A). The potencies of these analogs, evaluated by intravenous (i.v.) injection in diabetic rats, were nonetheless, indistinguishable from WT insulin (Fig. 5B).

TrpB26 analog displayed a zinc-dependent delay in onset of biological activity

Hexamer assembly delays absorption of insulin from its SQ-injection site (11, 12). To assess the onset and duration of [Trp\(^{B26}\), Orn\(^{B29}\)]insulin relative to [Orn\(^{B29}\)]insulin, the pharmacodynamics (PD) profile of these proteins (made 0.15 mg/ml, corresponding to a monomer concentration of 27 \(\mu\)M and a putative hexamer concentration of 4.5 \(\mu\)M) were evaluated as zinc-free solutions or as pre-assembled phenol-stabilized R\(_6\) hexamers in the presence of excess zinc ions (0.30 mM ZnCl\(_2\); 70 zinc ions per hexamer). A zinc-dependent delay in onset of activity was observed on SQ injection of [Trp\(^{B26}\), Orn\(^{B29}\)]insulin but not on injection of [Orn\(^{B29}\)]insulin or WT insulin (Fig. 5C; see Fig. S5 for WT results). Whereas the latter PD profiles exhibited a nadir at about 120 min irrespective of the zinc ion concentration, the PD profile of [Trp\(^{B26}\), Orn\(^{B29}\)]insulin occurred at (i) 120 min in the absence of zinc ions and (ii) 150 min in the presence of 0.30 mM zinc ions with corresponding delays in the rate of fall over the first 30 min (Fig. 5D). Together with the above in vitro results, these findings suggest that the prolonged lifetime of the TrpB26 R\(_6\) hexamer (as inferred from the above kinetic studies of the Co\(^{2+}\)-substituted hexamer) are responsible for the inferred zinc-dependent delay in SQ absorption.

Trp\(^{B26}\) protracted the PD profile of a model pi-shifted analog

Insulin analogs with isoelectric points (pI) shifted to neutral pH generally exhibit prolonged activity due to precipitation in the SQ depot (30). To determine whether Trp\(^{B26}\) might further prolong the activity of such analogs, this substitution was introduced into a [Gly\(^{A21}\), Orn\(^{B29}\), Orn\(^{B31}\), Orn\(^{B32}\)]insulin. This “glargine-like” framework was designed to recapitulate the pI shift of glargine with greater ease of semisynthesis. The proteins (formulated at 0.6 mM with 0.3 mM ZnCl\(_2\), corresponding to 3 zinc ions per hexamer) were each injected SQ in diabetic rats.

The pi-shifted parent analog displayed peak activity at about 120 min with blood glucose levels returning to baseline after about 360 min. By contrast, its Trp\(^{B26}\) derivative displayed a prolonged PD profile: peak activity occurred 180 min with slow return to baseline after ~800 min (Fig. 5E; see Fig. S6 for i.v. potency). Such a marked delay in peak activity was not observed in the parent glargine-like analog or the Trp\(^{B26}\) derivative when administered in the absence of zinc (Fig. S7). These results suggest that Trp\(^{B26}\) may favorably be incorporated into current basal analogs as a complementary mechanism of prolonged SQ absorption.

Crystal structure of Trp\(^{B26}\) analog demonstrated native-like dimer interface

The crystal structure of [Trp\(^{B26}\), Orn\(^{B29}\)]insulin was determined as a zinc-coordinated hexamer in the presence of phenol to a resolution of 2.25 Å. Diffraction and refinement statistics are provided in Table S2. The asymmetric unit constituted a “TR\(_0\)” dimer (31, 32). The overall structures of the T and R\(_4\) protomers were essentially identical to those of WT insulin (Fig. S8) with respective r.m.s. deviations of 1.11 ± 0.30 and

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6 This analog simulated pi-shifted insulin glargine, which contains two additional arginine residues at the C-terminal B chain (B31 and B32) and a substitution of Asn\(^{A21}\) by Gly (see Fig. 1A) (29, 69). Use of Orn permitted semisynthesis.

7 One protomer in the (dimeric) asymmetric unit adopted the classical “T” conformation (Fig. 18); the other adopted in the frayed R state (31, 32) in which residues B3–B8 extend the B-chain \(\alpha\)-helix.
structures (Tables S5 and S6, respectively) with a slight deviation in the positioning of the peptide backbone to accommodate the larger indole side chain.

Spectroscopic probes revealed native-like structure and thermodynamic stability of TrpB26 analogs in solution

The native-like crystal structure of [TrpB26, OrnB29]insulin is in accordance with its unperturbed circular dichroism (CD) spectrum and thermodynamic stability under monomeric conditions (Fig. 7A). Free energies of unfolding ($\Delta G_u$) $= 3.3 \pm 0.1$ kcal/mol at $25 \degree C$ as inferred from two-state modeling of chemical denaturation (33) were indistinguishable due to small and compensating changes in transition midpoint and slope ($m$ value) (33, 34) (Fig. 7B, Table 2). Further evidence that the crystal structure extends to the monomer in solution was provided by 2D $^1$H NMR studies of TrpB26 substituted within an engineered insulin monomer (lispro (35)). Whereas the spectrum of lispro (at pD 7.6 and 37 °C) exhibits sharp resonances for each aromatic[1H NMR spectrum and thermodynamic stability under monomeric conditions (Fig. 7A). Free energies of unfolding ($\Delta G_u$) $= 3.3 \pm 0.1$ kcal/mol at $25 \degree C$ as inferred from two-state modeling of chemical denaturation (33)) were indistinguishable due to small and compensating changes in transition midpoint and slope ($m$ value) (33, 34) (Fig. 7B, Table 2). Further evidence that the crystal structure extends to the monomer in solution was provided by 2D $^1$H NMR studies of TrpB26 substituted within an engineered insulin monomer (lispro (35)). Whereas the spectrum of lispro (at pD 7.6 and 37 °C) exhibits sharp resonances for each aromatic ring exhibited regiospecific nonlocal nuclear Overhauser enhancements (NOEs) from its six-member moiety to the methyl resonances of ValB12 and IleA2 (Fig. 8A, B). In contrast, the indole ring exhibited regiospecific nonlocal nuclear Overhauser enhancements (NOEs) from its six-member moiety to the methyl resonances of ValB12 and IleA2 (Fig. 8, B–D).

The pattern of secondary shifts in the variant is similar to that in the parent monomer. In particular, the aromatic $^1$H NMR resonances of TrpB26 (red cross-peaks in Fig. 8C) exhibit upfield features (relative to Trp in the isolated B23–B30 octapeptide; dashed lines) similar to those of TyrB26 in the parent spectrum (purple cross-peaks in Fig. 8A versus dotted lines) (36). Dilution of the TrpB26 sample partially mitigated resonance broadening but preserved these trends in dispersion. Indole-specific NOEs indicated that the side chain assumes one predominant and asymmetric conformation within a native-like crevice between A- and B-chain $\alpha$-helices (Table S7). Because TyrB26 undergoes rapid ring rotation about the $\beta_{Tyr}C_{\alpha}$ bond axis (“ring flips”), analogous side chain-specific NOEs (inferred in prior studies from molecular modeling) cannot be observed directly (Table S8).

1.36 ± 0.30. Additional r.m.s. deviations are given in Tables S3 and S4. Side chain packing near the B26 position was largely unperturbed. In both proomers the TrpB26 indole group was oriented with its six-member ring packing against conserved residues IleA2, ValA3, and ValB12 (Fig. 6); the indole NH group is exposed to solvent in the TR dimer and $T_3R$ hexamer. The TrpB26 side chain in both R and T proomers also displayed dihedral angles within the range of the native Tyr in WT crystal
Stabilization of insulin hexamer

Figure 6. Crystal structure of [Trp\textsubscript{B26}, Orn\textsubscript{B29}]insulin. A, electron density of Trp\textsubscript{B26} in T-state protomer showing the surrounding density in TR\textsuperscript{I} asymmetric unit (contour level 2.0 \AA). B, stick model corresponding to map in A; Trp\textsubscript{B26} is orange. C, surfaces of residues surrounding Trp\textsubscript{B26} (sticks) as indicated above. D–F, corresponding map and models of Trp\textsubscript{B26} in the R state protomer.

Figure 7. Thermodynamic stability of [Trp\textsubscript{B26}, Orn\textsubscript{B29}]insulin. A, CD spectra of [Trp\textsubscript{B26}, Orn\textsubscript{B29}]insulin, [Orn\textsubscript{B29}]insulin, and WT insulin. B, guanidine denaturation assays of insulin analogs monitored by ellipticity at 222 nm; color code as in A. Stabilities are given in Table 2.

Table 2
Thermodynamic stabilities of insulin analogs

| Analog                  | \(\Delta G_m^0\) \text{ kcal mol}^{-1}\) | \(C_{\text{mold}}\) \text{ m} | \(m^5\) |
|-------------------------|----------------------------------------|-------------------------------|--------|
| Wildtype                | 3.4 \pm 0.1 \textsuperscript{a}     | 5.0 \pm 0.1                   | 0.68 \pm 0.02 |
| Orn\textsubscript{B29}  | 3.3 \pm 0.1                           | 4.9 \pm 0.1                   | 0.67 \pm 0.01 |
| Trp\textsubscript{B26}, Orn\textsubscript{B29} | 3.3 \pm 0.1                           | 5.1 \pm 0.2                   | 0.64 \pm 0.03 |

\(\Delta G_m^0\): Parameters were inferred from CD-detected guanidine denaturation data by application of a two-state model; uncertainties represent fitting errors for a given data set.

\(m^5\): The m-value (slope \(\Delta (G)/\Delta (M)\)) correlates with surface area exposed on denaturation.

\(C_{\text{mold}}\): Analysis of replicates of [Trp\textsubscript{B26}, Orn\textsubscript{B29}]insulin, parent [Orn\textsubscript{B29}]insulin, and WT samples indicated that experimental standard errors were equal to or less than the above fitting errors: \(\pm 0.1\text{ kcal mol}^{-1}\) \((\Delta G_m^0)\), \(\pm 0.1\text{ m}\) \((C_{\text{mold}})\), and \(\pm 0.01\text{ kcal mol}^{-1}\text{ m}^{-1}\) \((m)\).

Discussion

The physical origins of protein stability and recognition define a foundational problem in biochemistry (38) with central application to molecular pharmacology (39). The zinc–insulin hexamer provides a favorable system for structure-based design due to its long history of crystallographic investigation (40). Indeed, the hexamer’s rigidity, as interrogated by NMR spectroscopy (41), renders the overall structure robust to diverse amino acid substitutions (42, 43), even those that destabilize the dimer interface\(^8\) (32, 44). This rigid framework has often enabled analysis of discrete interactions without complications due to the long-range transmission of conformational change (31, 32).

Our studies, stimulated by the seminal recognition of aromatic–aromatic interactions by Burley and Petsko (2) more than 30 years ago, focused on the classical dimer interface, a basic building block of the hexamer (32). Long appreciated as “a thing of beauty” (4, 45), this interface contains eight aromatic residues, six of which engaged in a successive set of aromatic–aromatic interactions. Quantum-chemical simulations of model systems have suggested that nearest-neighbor interactions predominate even in the presence of multiple rings (46).

MM calculations suggested improved aromatic–aromatic interactions within the variant crystal structure

The contribution of aromatic–aromatic interactions involving Trp\textsubscript{B26} to the stability of the variant dimer interface of the T\textsubscript{9}R\textsuperscript{8} hexamer was evaluated through calculation of nonbonded interaction energies among aromatic residues B16, B24, B25, and B26 in the TR\textsuperscript{I} dimer. These calculations, which employed the variant crystal structure, were in overall accordance with expectations based on our initial local MM-based modeling (above). In particular, based on aromatic–aromatic interactions alone, the Trp\textsubscript{B26}, Orn\textsubscript{B29} dimer displayed an increase in interaction energy of 1.4 kcal/mol relative to WT TR\textsuperscript{I} reference structure PDB 1TRZ; the results of these calculations are given in Table S9, a and b. Although the standard CHARMM empirical energy function, when applied to analyze either crystallographic and MM-minimized models of [Trp\textsubscript{B26}]insulin, suggested that the electrostatic properties of the Trp side chain were the primary contributors to the increased stability of the dimer, this physical interpretation may reflect the limitations of the partial-charge representation (23, 37). Indeed, preliminary \textit{ab initio} QM simulations of a minimal model (consisting of two aromatic rings in vacuo) predicted that enhanced Van der Waals interactions may also make a significant contribution (Fig. S9) (see “Discussion”).
the potential opportunity to enhance its stability through substitution of Tyr_B26 by Trp. Whereas our crystallographic analysis verified that this substitution preserves native architecture, a [TrpB26]insulin analog exhibited a dramatic increase in hexamer lifetime in vitro. Results of animal testing demonstrated native intrinsic potency (i.e., on i.v. bolus injection) but with prolonged activity on SQ injection, presumably due to delayed dissociation of the variant zinc hexamers in the SQ depot.

Protein engineering of insulin analogs is constrained by the complexity of insulin’s “conformational lifecycle”: from oxidative folding intermediates and self-assembly in the pancreatic β-cell (8) to adoption of an active, “open” conformation on receptor binding (Fig. 9, A and B) (16). Specific residues may play distinct roles at each stage. In particular, because interfaces within the insulin hexamer overlap the hormone’s receptor-binding surface, essentially invariant among vertebrates (47), modifications often impaire activity (13). A given WT residue may represent a compromise among competing structural tasks. A recent survey of 18 substitutions at position B26 demonstrated that Tyr is suboptimal with respect to IR-binding affinity but enhances self-assembly relative to more active alternatives (Ala, Ser, or Glu) (18). The latter side chains destabilize the “closed” dimer interface but are favorable at the solvated B26-related edge of the open hormone–receptor interface (Fig. 9C).

**Protective self-assembly is of key pharmacologic importance**

Insulin self-assembly protects the hormone from degradation and toxic misfolding in pancreatic β-cells (49) and in pharmaceutical formulations (49). Because the zinc-insulin hexamer exhibits delayed SQ absorption relative to monomers and dimers, mutational destabilization of the hexamer (10, 12) led to development of rapid-acting insulin analogs (14). Efforts to stabilize the insulin hexamer, as a converse strategy to obtain protracted action, were less successful (13). Current long-acting insulin analogs rely instead on higher-order self-association of hexamers within the SQ depot (30) and binding acylated monomer to albumin as a circulating depot (highlighted in Fig. S2) (50, 51).

The problem of how to improve a protein interface is in general more subtle than its opposite, for destabilizing substitutions abound at conserved interfaces, whereas stabilizing substitutions can be rare (13). Structure-based candidate substitutions may encounter entropy–enthalpy compensation (EEC) (52, 53) or cause unintended biological perturbations (54). The challenge posed by insulin is magnified by the struc-

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9 An experiment of nature provided in vivo evidence for the relationship between zinc coordination and protection from toxic insulin misfolding: in the rodent species Octogon degus, substitution of His B10 by Asn is associated with senile amyloidosis of the islets due to insulin fibrillation (70), resulting in DM (48).
tural elegance of its self-assembly (as emphasized by Hodgkin and colleagues (55) in a classic review). Diverse structure-based strategies were previously undertaken with only limited success. Alternate strategies previously used to create basal insulin analogs are depicted in Fig. S2 and summarized in Table S10.

One approach focused on the general nonpolar character of the dimer interface: additional hydrophobic substitutions were introduced in an effort to enhance this feature (56). Such designs were not successful and also impaired biological activity, although analogs were identified whose sparing solubility slowed SQ absorption (56). A second approach exploited the classical TR transition among insulin hexamers (57). At the pivot point of this allosteric transition (Fig. S10), an invariant glycine (GlyB8) was substituted by Ser in an effort to stabilize the more stable R state hexamer (58). This analog was unstable as a monomer (54) and exhibited reduced activity (58). Yet another approach sought to stabilize the hexamer by relieving electrostatic repulsion created by the internal clustering of six acidic side chains (GluB13): their isosteric substitution by Gln indeed promoted assembly of zinc-free hexamers but impaired biological activity (59). Overlap between the self-assembly surfaces of insulin and its receptor-binding surfaces thus compounded the optimization problem.

The present study revisited the architecture of the insulin hexamer in light of recent insights into the hormone’s receptor-binding surface (18). Prominent roles are played by a quartet of aromatic residues in the C-terminal B-chain strand: TyrB16, PheB24, PheB25, and TyrB26 (33). These four residues, and the clustering of eight dimer-related side chains, have long been the focus of structure-activity relationships (60). Photoactivatable aromatic probes (para-azido-Phe) at any of these sites exhibited efficient cross-linking to the IR (61, 62). The co-crystal structure of an insulin monomer bound to a fragment of the IR ectodomain (the IR model) revealed distinct binding sites at the surface of the L1 domain of the IR (B24, B25, and B26) or its CT element (B24 and B25). PheB24 packs within a nonpolar pocket near aromatic residues in L1 (residues Leu37 and Phe39) and CT (residue Phe714); one wall of this pocket is defined by the aliphatic side chains of LeuB15, CysA20, and CysB19 as in free insulin (16). This environment differs in detail from those in the insulin dimer but exhibits analogous general features. Although aromaticity is not required to fill the B24-binding pocket (33), its specific size and shape constrain potential substitutions. TyrB16 lies at the periphery of L1 (near Phe39 and Lys40). Its substitution by Ala or other aromatic residues preserves activity (63). PheB25 occupies a cleft between αCT...

Figure 9. Binding surface of TyrB26 on an IR fragment. A, model of WT insulin (in classical T-state) overlaid on the structure of insulin bound to an IR fragment (PDB entry 4OGA). The L1 domain and part of the CT domain are shown in powder blue, whereas αCT is shown in purple. PheB24 and TyrB26 are, respectively, shown as gray and red sticks. The B-chain of IR-bound insulin is shown in dark gray (B6–B19) or black (B20–B27); the green tube indicates a classical location within overlay of residues B20–B30 (green arrow), thereby highlighting the steric clash of B26–B30 with αCT. Insertion of the insulin B20–B27 segment between L1 and αCT was associated with a small rotation of the B20–B23 β-turn and changes in main chain dihedral angles flanking B24. B, stick representation of B-chain residues B20–B27 packed between αCT and the L1 β-strand. Color code in insulin segment: carbon atoms (green), nitrogen (blue), and oxygen (red). Residues B6–B19 are shown as a black ribbon, and the A-chain is shown as a yellow ribbon. Key contact surfaces of αCT with B24–B26 are highlighted in magenta and L1 with B24–B26 are highlighted in cyan. C, stereo view of the environment of TyrB26 within its binding site. Neighboring side chains in L1 and αCT are as labeled. This figure was adapted from Ref. 18 with permission of the authors.
residues (Val^15, Pro^176, Arg^177, and Pro^178) that can only accommodate trigonal \(γ\)-carbons (22). B25-related aromatic–aromatic interactions are limited due to the peripheral location of this residue (Fig. S11).

We chose to focus on position B26 because of its broad functional tolerance of diverse substitutions (18). As illustrated above (Fig. 8C), Tyr^B26 binds at the solvated periphery of the \(μ\)1R interface. Indeed, substitution of small, polar, or charged amino acids (such as Ala, Ser, or Glu) enhances receptor affinity, but at the price of impaired self-assembly and decreased thermodynamic stability with heightened susceptibility to physical degradation (16). These findings highlighted the evolutionary importance of the native dimer interface and dual role of Tyr^B26. We thus hypothesized that an aromatic substitution at the B26 position might enhance self-assembly without loss of biological activity.

The classical structure of the insulin dimer motivated study of successive aromatic–aromatic interactions as a physical mechanism of stability (6, 64). The increased stability of ETF aromatic–aromatic interactions involving Trp over those involving Tyr contributes to the increased stability of the Trp^B26 hexamer. The larger size of the delocalized \(\pi\)-orbital of Trp in relationship to that of Tyr causes a stronger negative charge to accumulate on the “face” of the indole ring. For this reason, hydrogen atoms surrounding the aromatic rings of local residues form stronger electrostatic interactions with the face of the indole ring of Trp than with the phenol ring of Tyr (65, 66). Aromatic pairs involving Trp residues are less common than those involving Tyr or Phe (1). However, the strength of aromatic–aromatic interactions involving Trp is evidenced by functional importance of Trp-based interactions; an example is provided by a Trp–Tyr aromatic “lock” that stabilizes the active conformation of the ghrelin receptor (67). Indeed, comparison of electrostatic interactions of Trp^B26 and Tyr^B26 within the minimized model of the B16/B24/B26 aromatic network of insulin revealed that interactions involving Trp were favored over those involving Tyr across a broad variety of orientations of the respective aromatic rings. Extension of the aromatic lock metaphor (introduced by Holst et al. (67) to describe conformational “trapping” in the GPCR structure) to the insulin hexamer highlights the kinetic effect of the B26 substitution on the rate of hexamer disassembly (as probed by the Co\(^{3+}\)-EDTA sequestration assay), which was more dramatic than effects on equilibrium association (as probed by SEC). It would be of future interest to measure activation energies for disassembly. Insight into the structural origins of the prominent Trp^B26-associated kinetic lock may be provided by activated molecular dynamics simulations of hexamer disassembly.

**Trp^B26 side chain exhibited an orientation similar to native Tyr**

The Trp^B26 side chain in the crystal structure of [Trp^B26, Orn^B29]insulin displayed an orientation similar to that of the native Tyr. A slight main chain shift in the B24–B28 \(\beta\)-strand (0.2 (±0.03) Å) was sufficient to enable native-like packing of the larger indole ring against the core of a protomer (Fig. S12). In both T and R\(^*\) subunits, the six-membered component of the indole side chain was oriented toward Ile^A2, Val^A3, and Val^B12. Based on the classical 3.5–6.5 inter-centroid distance, residue Trp^B26 (of the T protomer) showed potential interactions with residues Tyr^D16 and Phe^D24. An interplanar angle of 78 degrees between Trp^B26 and Tyr^D16 was indicative of classical aromatic–aromatic packing within proteins (2), which generally range from 50 to 90 degrees. Trp^B26 and Phe^D24 displayed an interplanar angle of 36 degrees, however, this orientation is less common. Similarly, in the R\(^*\) protomer, Trp^D26 packed near Tyr^B16 and Phe^B24. The interplanar angle between Trp^D26 and Tyr^B16 was 62 degrees, whereas between Trp^D26 and Phe^B24 was 48 degrees. Previous studies have suggested that Trp residues may form aromatic–aromatic interactions over longer distances than those formed by Tyr–Phe pairs (68). Thus, the two intra-chain aromatic pairs, Trp^B26–Phe^B24 and Trp^D26–Phe^D24 (respectively, separated by 7.8 and 7.1 Å) may interact more efficiently than the corresponding Tyr–Phe pairs in WT insulin (ring geometries are summarized in Table S11, a and b).

CHARMM calculations of aromatic–aromatic interactions across the dimer interface of [Trp^B26, Orn^B29]insulin revealed 1.4 kcal/mol increased interaction energy. Residue by residue analysis of each component of the aromatic network indicated that the increased strength of interaction across the dimer interface was the result of the interactions involving Trp^D26 (see Table S9b). This result suggests that Tyr^B26 \(\rightarrow\) Trp may only display stabilizing properties when in an R state protomer. If so, the T\(_{4}\) hexamer formed by [Trp^B26, insulin would be expected to have dissociation kinetics similar to WT insulin, whereas the corresponding R\(_{4}\) hexamer may be expected to have markedly increased stability. The R \(\rightarrow\) T transition, which is rapid in WT insulin on release of phenol, may represent the kinetic barrier responsible for the meta-stable association state observed in SEC experiments.

The packing of Trp^B26/D26 within the respective cores of T- and R\(^*\) crystallographic protomers is similar in each case to the WT Tyr^B26/D26 and oriented such that the indole’s nonpolar six-membered portion projects more deeply into a crevice between A- and B-chains than does its proximal heterocycle. Our \(^1\)H NMR studies of Trp^B26 within an engineered monomer (35) provided evidence that this overall conformation does not require self-assembly. Analogous partial burial of Tyr^B26 and Trp^B26 in the respective protein structures would in itself be expected to augment the variant’s stability due to enhanced solvation free energy (69, i.e. as predicted by water–octanol transfer studies of free Tyr and Trp (69); Table S12). Guanidine denaturation nonetheless, indicated that their stabilities are indistinguishable. (11)

We speculate that the predicted residue-specific differences in solvation free energy are attenuated by differences in protein dynamics leading to EEC (70). Although the two B26 side chains each exhibit upfield secondary \(^1\)H NMR shifts and analogous inter-residue NOEs, it is possible that the substitution is associated with local or nonlocal differences in protein dynam-

**References**

10 The identification of a destabilizing Trp \(\rightarrow\) Tyr mutation in *Escherichia coli* thioesterase-I demonstrated, in breach, the improved cavity-filling properties of Trp relative to Tyr (72).

11 That [Trp^B26, Orn^B29]insulin exhibited a greater exposure of nonpolar surfaces on denaturation in guanidine HCl than did [Orn^B29]insulin was suggested by a change in \(m\) values in two-state modeling (Table 2).
ics. It would be of future interest to investigate dynamic features by amide proton $1^H-2^H$ exchange and heteronuclear NMR relaxation methods (70). Because Trp$_{B26}$ promotes partial dimerization of insulin lispro under NMR conditions (as indicated by concentration-dependent $^1H$ NMR resonance broadening), such studies may require use of an alternative monomeric template.

Given the ubiquity of EEC as a confounding general aspect of protein design (71), the profound effects of Trp$_{B26}$ on the properties of the insulin hexamer seem all the more remarkable. We envision that EEC is circumvented in this case by the rigidity of the insulin hexamer (including the interlocked aromatic residues at its dimer interfaces) with efficient burial of the WT and variant B26 side chains (72). With similar internal structures and external solvation properties, the variant hexamer would gain (relative to WT) two advantages from the Tyr $\rightarrow$ Trp substitution: (i) greater B26-related solvation transfer free energy (73) (Table S12) augmented by (ii) an uncompensated enthalpic advantage arising from more favorable ETF aromatic interactions as next discussed.

Molecular mechanics calculations rationalized physical and pharmacologic properties

The structural rigidity of the R6 insulin hexamer (42, 74) motivated local MM-based modeling to assess the interactions contributing to the stability of the [Trp$_{B26}$]insulin hexamers. Analysis of insulin oligomers by Raman spectroscopy revealed dampened conformational fluctuations of the R6 hexamer in relationship to lower-order oligomers and T$_6$ hexamers (72). Moreover, the thermodynamic stability of the assembly was evidenced by the lack of conformational changes visualized by NMR spectroscopy over a temperature range of 10–80 °C (42). The resistance of the R$_6$ hexamer to structural perturbation has also been shown in the context of mutant insulin analogs: native-like X-ray crystal structures have been reported of R$_6$ containing a broad range of substitutions (Table S13) (17, 75, 76). Even substitutions that were shown to destabilize the dimer interface of insulin, such as the substitution of Phe$_{B24}$ by the nonaromatic cyclohexylalanine, were shown to have little impact on the global structure of the R$_6$ hexamer. For this reason, the Tyr$_{B26}$ $\rightarrow$ Trp substitution was expected to affect only the local structure of the insulin hexamer. Thus, the effects of the mutation were amenable to initial analysis in simplified (eight-ring) models of the dimer interface of insulin.

Energy minimization of a local model of [Trp$_{B26}$]insulin (i.e. as substituted into a WT insulin dimer extracted from a representative crystal structure of a T$_1$R$_4$$_3$ zinc hexamer) yielded a native-like framework with enhanced nearest-neighbor B26-related aromatic–aromatic interactions. The partial-charge (monopole) model of the aromatic rings in the CHARMM empirical energy function, parametrized in accordance with ab initio simulations (77), predicted an increase of 0.8 kcal/mol. Although this calculation could in principle have been confounded by transmitted conformational perturbations and did not consider potential changes in conformational entropy or solvation, its conservative features were verified by X-ray crystallography. That the structure of [Trp$_{B26}$, Orn$_{B29}$]insulin is essentially identical to WT suggests that local properties of Trp$_{B26}$ directly underlie the observed increase in hexamer stability and lifetime.

The general asymmetry of the variant TR of the crystallographic hexamer was associated with differences in the details of corresponding aromatic–aromatic interactions across the dimer interface. Although CHARMM calculations predicted an increase of a 1.4 kcal/mol in interaction energies (0.7 kcal/mol per protomer) in accordance with our initial modeling, residue by residue decomposition ascribed this increase primarily to Trp$_{B26}$ (in the R$'$ protomer) and not to Trp$_{B26}$ (in the T protomer). It is formally possible that Trp$_{B26}$ is only stabilizing in an R state, but additional studies would be required to resolve this issue. Our cobalt-EDTA sequestration studies focused on the R$_6$ state as the preferred storage vehicle in a pharmaceutical formulation. On SQ injection, rapid diffusion of phenolic ligands from the depot leads to a TR transition. That Trp$_{B26}$ was found to delay subsequent absorption into the bloodstream suggests that this substitution also enhances the kinetic stability and lifetime of the T$_6$ hexamer.

A seeming paradox is posed by the evolutionary exclusion of Trp at position B28 of vertebrate insulins despite the evident compatibility of this aromatic side chain with native structure and function. We speculate that this exclusion reflects the biological importance of the rapid disassembly of zinc insulin hexamers on their secretion by pancreatic β-cells. Whereas the enhanced thermodynamic and kinetic stabilities of [Trp$_{B26}$]insulin hexamers would seem favorable for storage within secretory granules (as within pharmaceutical formulations) (8), delayed disassembly of the variant hexamers in the portal circulation would be predicted to reduce the hormone’s bioavailability on first pass through the liver, as insulin dimers and hexamers cannot bind to the IR (78). Such delayed disassembly might also decrease the delivery of free zinc ions to the liver, recently predicted to constitute a regulatory signal in its own right (for review, see Ref. 79).

The resulting impairment in hormonal regulation of hepatic metabolism (and accompanying systemic hyperinsulinemia (80)) could in principle have imposed a selective disadvantage in the course of vertebrate evolution. Although to our knowledge, such a kinetics-based mechanism has not been observed in vivo, the converse, abnormally rapid clearance of insulin, has been found on release from zinc-deficient secretory granules; presumably zinc-free insulin oligomers more rapidly dissociate on dilution in the portal circulation and so are more efficiently cleared than WT zinc insulin hexamers (81). Similarly, the exclusion of Trp at position B26 of vertebrate IGFs may reflect a disadvantageous competition between self-assembly and binding to IGF-binding proteins, which are critical to the integrated physiology of IGF function (for review, see Ref. 82). Such functional complexity may impose hidden constraints on the evolution (and so divergence) of protein sequences. The conserved “aromatic triplet” of vertebrate insulins and IGFs provides a natural laboratory to uncover such evolutionary constraints (45). These considerations further suggest that structural features of a protein pertinent to its endogenous function may in general be distinct from those biophysical properties that may be re-engineered to optimize molecular pharmacology.
Comparison of \([\text{Trp}^{B26}]\text{insulin and a corresponding iodo-Tyr analog}\]

Design of \([\text{Trp}^{B26}]\text{insulin was in part motivated by prior studies of an analog containing 3-iodo-Tyr at the B26 (17, 23, 83). The latter analog ("3I-Y-insulin") exhibited a variety of favorable properties: increased affinity for the IR (83), increased thermodynamic stability, and augmented resistance to fibrillation (17). Moreover, when formulated as an \(R_6\) hexamer, this analog exhibited a decreased dissociation rate (17). Because modified amino acids raise the cost and complexity of protein manufacture, we wondered whether a natural amino acid might mimic, at least in part, the structure of 3I-Y-insulin and so confer favorable pharmacologic properties with conventional manufacturing.

This translational goal motivated our initial MM-based local modeling of \([\text{Trp}^{B26}]\text{insulin. Analysis of the crystal structure of 3I-Y-insulin, determined as an \(R_6\) zinc hexamer (23), revealed that the large, nonpolar iodo-substituent packed within the core of the insulin protomer with preservation of a native-like dimer interface. Molecular dynamics simulations, undertaken with a multipolar electrostatic model of the modified aromatic ring (23), rationalized this conformation: the iodine atom efficiently filled a cryptic packing defect in WT insulin, lined by the conserved side chains of Ile\(^{A2}\), Val\(^{A3}\), and Tyr\(^{A19}\). The enhanced packing efficiency of the modified insulin and novel network of halogen-specific electrostatic interactions ("weakly polar" interactions (11)) appear to underlie the analog's increased thermodynamic stability and resistance to fibrillation. Whereas the standard partial-charge model of aromatic systems failed to account for the conformation of iodo-Tyr observed in the crystal structure, a multipolar electrostatic model rationalized thermodynamic stabilization of the dimer interface by this halogen "anchor" (23). Subtle changes in the geometry of aromatic–aromatic interactions were observed both in the simulations and in the crystal structure. Although activated MD simulations were not undertaken to probe the process of dimer dissociation, we presume that the above mechanisms of ground-state stabilization, enhanced core packing efficiency, and a halogen-specific weakly polar network, also underlie the increased barrier to dissociation as indicated by the prolonged lifetime of the variant \(R_6\) hexamer (23).

Although the profound QM effects of halo-aromatic substitutions, including weakly polar interactions and "halogen bonding" (84), cannot be recapitulated by natural amino acids, it seemed possible that enhanced core packing efficiency might be achieved by analogy to 3I-Y-insulin. Indeed, the steric profile of the asymmetric indole side chain of Trp is similar in size to 3-iodo-Tyr. Accordingly, we imagined that the offset six-membered portion of the bicyclic indole ring might pack within the core of insulin in a manner similar to the iodine atom. In this intuitive picture the extended \(\pi\)-system of Trp\(^{B26}\) was envisioned to interact with neighboring residues to recapitulate, at least in part, the favorable electrostatic properties of iodo-Tyr\(^{B26}\) (23, 37).

The above line of reasoning led to the present set of studies. In accordance with our original intuition and local MM-based modeling, the crystal structures of 3I-Y-insulin and \([\text{Trp}^{B26},\text{Orn}^{B29}]\text{insulin exhibited similar features. Nevertheless, salient differences between the quantum-chemical properties of iodo-Tyr and Trp might make the similar structures of these B26 analogs a fortuitous outcome of distinct mechanisms. Whereas effects of 3-iodo-Tyr on aromatic–aromatic interactions at the insulin dimer interface are subtle, presumably reflecting indirect inductive effects of iodo-substituent (23), substitution of Tyr by Trp introduces a larger aromatic surface at this interface. It is possible that this feature underlies the more marked impact of Trp\(^{B26}\) on insulin oligomerization relative to 3-iodo-Tyr.

Aromatic–aromatic interactions exemplify limitations of classical models

The quantum origins of aromatic–aromatic interactions are complex, and so classical electrostatic models (such as partial-charge model of Tyr in the standard CHARMm empirical energy function) can be incomplete (85). Although QM calculations more fully capture this complexity, a tradeoff is encountered between rigor and computational feasibility, especially in complex systems such as proteins (86), but even in \(ab\ initial\) simulations of benzene–benzene interactions (46). Standard MM and MD methods thus employ parametrized force fields to approximate quantum-chemical interactions (21). Although parameters (e.g. partial charges assigned to an aromatic ring) have been chosen to provide reasonable protein models, such use of "monopolar" electrostatics neglects the polarizability of aromatic systems and so omits dispersion forces (87). Parameterized classical models may thus mischaracterize the strength or directionality of intermolecular interactions, particularly those involving more than one aromatic group or an aromatic group and a charged moiety. Although the pioneering studies of Burley and Petsko (88) suggested that the partial-charge model of aromatic rings in proteins was sufficient for characterizing aromatic–aromatic interactions, more recent work has highlighted its limitations with respect to delineating underlying physical mechanisms (85, 89). The limits of parametrized classical force fields are of particular importance when applications are sought in nonstandard systems (such as unnatural protein mutagenesis (90)) for which the parameters were not intended. Examples are provided by halogen-modified aromatic systems (widely employed in medicinal chemistry) (91), which are associated with marked changes in quantum-chemical properties (92). Formal QM/MM simulations of proteins may nonetheless be circumvented through force fields incorporating multipolar electrostatic models of aromatic–aromatic networks (85).

The correspondence between our initial local MM-based modeling and our experimental findings, however striking, may be coincidental (93): rigorous elucidation of the physics of the variant aromatic cluster at insulin’s dimer interface may require application of free-energy MD-based simulations with explicit inclusion of water molecules ("molecular alchemy" (94)). This approach may also provide insights into whether or how EEC may be circumvented. Nevertheless, standard MM calculations can guide initial biochemical analysis of protein structure as a guide for protein engineering. In the present study such initial modeling reinforced our structural intuition by highlighting the plausibility that substitution of Tyr\(^{B26}\) by Trp might pre-
serve a native-like interface and in fact enhance its weakly polar properties. Because the rigid hexameric framework provided a favorable context for local modeling of amino acid substitutions at subunit interfaces and yet it is the monomer that functions as a hormone in the bloodstream, it would be of future interest to apply more sophisticated computational techniques to simulate the structure and dynamics of [TrpB26]-insulin as a monomer in solution. Such predictions may in principle be tested through biophysical studies of an engineered insulin monomer containing TrpB26. Although this substitution is favorable in the context of the hexamer (and so of potential pharmacologic benefit), design of a monomeric NMR model will need to overcome the confounding effects of TrpB26, as defined in this experimental context, to promote self-association.

Concluding remarks

To our knowledge, this study represents the first exploitation of aromatic–aromatic interactions to enhance the biophysical properties of a therapeutic protein (95). Our approach may be broadly applicable in protein engineering (as ETF interactions are ubiquitous) and generalizable to nonstandard aromatic moieties. The latter would be likely to require QM/MM methods rather than classical force fields parameterized with partial charges (90). Overall effects of such substitutions on protein stability and self-assembly will require an integrated analysis of solvation free energies (86), changes in protein dynamics (96), and potential EEC (97). Three- and four-dimensional heteronuclear NMR experiments would be expected to provide higher-resolution information regarding the local and nonlocal interactions of the optimized aromatic system. In the present application, analyses of 15N relaxation and 1H–2H amide–proton exchange are expected to improve understanding of the molecular dynamics of the TrpB26-modified aromatic cluster and so provide a more rigorous biophysical context for its enhanced self-association properties (70, 96).

The present application to insulin demonstrates a direct relationship between stabilization of the insulin hexamer and prolonged activity of a basal analog. Continuous and flat 24-h insulin activity (“peakless” basal formulations) is of clinical interest in the treatment of Type 1 and Type 2 diabetes mellitus to reduce the risk of hypoglycemia (especially at night) at a given level of glycemic control (98). Because this mechanism is unrelated to present strategies to achieve protracted action, we envision that TrpB26-related enhancement of dimer-specific aromatic–aromatic interactions could favorably be introduced into current basal insulin formulations. In the future a combination of orthogonal molecular strategies might enable development of a once-a-week basal insulin therapy analogous to that of GLP-1 agonists (99). Optimization of weakly polar interactions may thus assume a central place in the toolkit of molecular pharmacology.

Experimental procedures

Materials

Insulin was purchased from BioDel® (Danbury, CT). Insulin glargine was obtained from Lantus® (Sanofi-Aventis, Paris, FR). Reagents for peptide synthesis were as described (100).

Preparation of insulin analogs

Variant insulins were prepared by semisynthesis (101). In brief, synthetic peptides were coupled to a tryptic fragment of insulin (des-octapeptide [B23-B30]insulin) in aqueous/orange solvent using trypsin as a catalyst. Following RP-HPLC purification, predicted molecular masses were confirmed by MS (33).

Hexamer disassembly assays

Disassembly of phenol-stabilized (R6) Co2+-substituted insulin hexamers was monitored as described (102). In brief, WT insulin or variants were made 0.6 mm in buffer containing 50 mm Tris-HCl (pH 7.4), 50 mm phenol, and 0.2 mm CoCl2 (18) and incubated overnight at room temperature to attain conformational equilibrium. Spectra (400–750 nm) were obtained to monitor tetrahedral Co2+ coordination (27) through its signature absorption band at 574 nm (27). Co2+ sequestration was initiated by addition of EDTA to a concentration of 2 mM. Dissociation was probed via attenuation of a 574-nm band (27); data were fit to a monoexponential decay equation (29).

Protein crystallography

Crystals were obtained by hanging drop vapor diffusion at room temperature in the presence of a 1:1.7 ratio of Zn2+ to protein monomer and a 3.5:1 ratio of phenol to protein monomer in Tris-HCl. Diffraction was observed using synchrotron radiation at a wavelength of 0.9795 Å at the Stanford Synchrotron Radiation Light Source (beamline BL7-1); crystals were flash frozen to 100 K. Structure determination was carried out using molecular replacement using CCP4 (103) and Phenix structure-determination suites (104). The resulting structure was validated using a PDB Redo server (105). The lattice contained one TRdimer per asymmetric unit. The main chain conformations of the 97 residues in the refined model of the TRdimer in the asymmetric unit (excluding 2 Thr, 1 Orn, and 1 Phe residues) each resided in a most favored Ramachandran region.

Receptor-binding assays

Analog affinities for detergent-solubilized IR-B holoreceptor were measured by a competitive-displacement assay (18). Successive dilutions of WT insulin or analogs were incubated overnight with wheat germ agglutinin-SPA beads (PerkinElmer Life Sciences), receptor, and radiolabeled tracer before counting (18). To obtain dissociation constants, competitive binding data were analyzed by nonlinear regression by the method of Wang (106).

Rat studies

Male Lewis rats (mean mass ~300 g) were rendered diabetic by streptozotocin. Effects of insulin analogs formulated in Lilly® buffer (18) on blood glucose concentration following SQ injection were assessed in relationship to WT or [OrnB29]insulin (18). [OrnB29]Insulin and [TrpB26, OrnB29] insulin were made in the above buffer. GlyA21, OrnB29, OrnB31, OrnB32, TrpB26 and GlyA21, OrnB29, OrnB31, and OrnB32 were dissolved in diluted HCl (pH 4) containing meta-cresol, glycerol, and a 1:2 ratio of ZnCl2-insulin monomer. Rats
were injected SQ with 3.44 nmol of insulin or insulin analogs (≈12–13.7 nmol).

Animals used in this study were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities of Case Western Reserve University (CWRU) School of Medicine. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) Office at CWRU, which provided Standard Operating Procedures and reference materials for animal use (in accordance with the NIH Guide for the Care and Use of Laboratory Animals). The animal health program for all laboratory animals was directed by the CWRU Animal Resource Center. Animal care and use was further monitored for Training and Compliance issues by Veterinary Services.

Size exclusion chromatography

Analogs were made 0.6 mm in 10 mm Tris-HCl (pH 7.4), 1.6% glycerol (v/v), 0.3 mm ZnCl₂, and 7 mm phenol (33). Insulin samples (20 μl) were loaded on an Enrich® SEC70 column (10 × 300 mm with fractionation range 3–70 kDa); the mobile phase consisted of 10 mm Tris-HCl (pH 7.4), 140 mm NaCl, and 0.02% sodium azide. Elution times were monitored by absorbance at 280 nm. Molecular masses and void volume (V₀) were inferred in reference to standard proteins (18).

Spectroscopy

CD spectra were acquired in 10 mm potassium phosphate (pH 7.4) and 50 mm KCl (33). Free energies of unfolding (∆Gₒ) were inferred at 25 °C from two-state modeling of protein denaturation by guanidine HCl (33, 34). 1H NMR spectra were acquired at 700 MHz at pH 8.0 or pD 7.6 (direct meter reading) at 37 °C (36). Chemical shifts of aromatic protons in residues B24–B24 (Phe-Phe-Tyr or Phe-Phe-Trp) were evaluated in relationship to corresponding chemical shifts in respective octapeptides B23–B30, presumed to represent random-coil shifts.

Molecular mechanics calculations

Calculations were performed using CHARMM (kindly provided by Prof. M. Karplus). Its standard empirical energy function was employed (in whose development aromatic rings were parametrized by partial atomic charges in accordance with ab initio QM simulations (21)). Representative WT insulin dimers were obtained from PDB entries 4INS, 1ZNJ, and 1TRZ. These structures and corresponding TrpB26 homology models were subjected to local energy minimization (100 steps of steepest descent followed by Adopted Basis Newton-Raphson with gradient tolerance tolg 0.0008/10,000 steps). Minimizations were halted either at 1,000 steps or when the above tolerance was reached. Changes in conformation were allowed only to eight side chains (TyrB₁₆, PheB₂₄, PheB₂₅, TyrB₂₆ (or TrpB₂₆), and their dimer-related mates; the remaining atoms in the respective dimers were fixed. Total interaction energies and respective electrostatic components were obtained between the side chain of residue B26 and the neighboring three aromatic side chains (PheB₂₄ and dimer-related TyrD₁₆ and PheD₂₄). Following this survey of crystallographic dimers, such energies were further evaluated in a simplified molecular model that contained only the side chains of residues TyrB₁₆ (or TrpB₁₆) and the same neighboring three aromatic residues as extracted from PDB entry 4INS; this yielded the electrostatic interaction energy map shown in Fig. 2, in which B26 X₁ and X₂ dihedral angles were systematically varied without energy minimization.

Quantum mechanics calculations

Electron density and molecular electrostatic potential of Tyr and Trp side chains were calculated with B3LYP and 6-31G(d) basis sets using Gaussian utility Cubegen in Gaussian09 (77). The isosurface map was generated using Jmol (107). Ab initio energies of interaction between pairs of isolated aromatic rings were determined by calculating interaction energies using the MP2 method with the aug-cc-pVDZ basis set in Gaussian 09 (77).

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