Biophysical Optimization of a Therapeutic Protein by Nonstandard Mutagenesis

STUDIES OF AN IODO-INSULIN DERIVATIVE*

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Background: Therapeutic engineering of insulin analogs is ordinarily limited by a trade-off between pharmacokinetics and stability.

Results: Substitution of TyrB26 in a rapid-acting insulin analog by 3-iodo-TyrB26 enhances its biophysical and pharmaceutical properties.

Conclusion: An unnatural amino acid substitution circumvents insulin pharmacokinetic/stability trade-off.

Significance: Nonstandard mutagenesis can optimize the molecular properties of therapeutic proteins.

Insulin provides a model for the therapeutic application of protein engineering. A paradigm in molecular pharmacology was defined by design of rapid-acting insulin analogs for the prandial control of glycemia. Such analogs, a cornerstone of current diabetes regimens, exhibit accelerated subcutaneous absorption due to more rapid disassembly of oligomeric species relative to wild-type insulin. This strategy is limited by a molecular trade-off between accelerated disassembly and enhanced susceptibility to degradation. Here, we demonstrate that this trade-off may be circumvented by nonstandard mutagenesis. Our studies employed LysB28, ProB29-insulin (“lispro”) as a model prandial analog that is less thermodynamically stable and more susceptible to fibrillation than is wild-type insulin. We have discovered that substitution of an invariant tyrosine adjoining the engineered sites in lispro (TyrB26) by 3-iodo-TyrB26 enhances its thermodynamic stability (ΔΔGfO, 0.5 ± 0.2 kcal/mol), delays onset of fibrillation (lag time on gentle agitation at 37 °C was prolonged by 4-fold), enhances affinity for the insulin receptor (1.5 ± 0.1-fold), and preserves biological activity in a rat model of diabetes mellitus. 1H NMR studies suggest that the bulky iodo-substituent packs within a nonpolar interchain crevice. Remarkably, the 3-iodo-TyrB26 modification stabilizes an oligomeric form of insulin pertinent to pharmaceutical formulation (the R6 zinc hexamer) but preserves rapid disassembly of the oligomeric form pertinent to subcutaneous absorption (T6 hexamer). By exploiting this allosteric switch, 3-iodo-TyrB26-lispro thus illustrates how a nonstandard amino acid substitution can mitigate the unfavorable biophysical properties of an engineered protein while retaining its advantages.

Insulin is a small globular protein containing two chains, A (21 residues) and B (30 residues) (see Fig. 1A) (1). Although the hormone functions as a Zn2+-free monomer in the bloodstream (2), it is stored in pancreatic β-cells as a Zn2+-stabilized hexamer (3). Such self-assembly is of overarching importance to stable pharmaceutical formulation. Indeed, in the absence of self-assembly solutions of insulin would have a limited shelf life. Protective self-assembly thus delays the hormone’s chemical degradation (rearrangement of atoms or chemical bonds in the molecule) and physical degradation (aggregation-coupled misfolding leading to amyloid) (4). Pharmaceutical formulations, recapitulating the storage strategy of the β-cell (3), have enabled the broad distribution, storage, and clinical use of insulin for more than 80 years in the treatment of diabetes mellitus. Despite its biophysical advantages in relation to protein stability, insulin self-assembly delays its absorption from a subcutaneous (SQ) depot and so undermines the therapeutic efficacy of mealtime injections. Because wild-type insulin hexamers are absorbed more slowly than the smaller constituent dimers and monomers, onset of insulin action was traditionally limited by the rate of hexamer disassembly (5). To circumvent this pharmacokinetic barrier, insulin analogs were designed containing amino acid substitutions at or near the classical dimerization surfaces of the zinc insulin hexamer (6). These substitutions led to accelerated hexamer disassembly while preserving native receptor binding affinity and biological activity (7). The clinical success of such rapid-acting insulin...
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analogs represents a pioneering triumph of rational protein design in molecular pharmacology (5).

The molecular elegance of rapid-acting insulin analogs has come at a biophysical cost; such formulations are more susceptible to physical and chemical degradation (especially on dilution and above room temperature) than is wild-type insulin (8, 9). Such degradation reflects a trade-off intrinsic to the logic of their design as the same amino acid substitutions that enable accelerated hexamer disassembly in the subcutaneous depot also undermine the protective effects of these assemblies against degradation in the vial or delivery device. This “molecular Catch-22” has motivated exploration of ancillary technologies to provide an improved combination of formulation stability and pharmacokinetic performance; examples include novel coatings for vials and catheters to reduce surface denaturation of insulin (10) (i.e. enhancing stability in a vial or device without delaying subcutaneous absorption) or local heating of the catheter injection site to enhance local blood flow (11) (i.e. promoting subcutaneous absorption without degrading formulation stability).

In this study we have sought to investigate whether nonstandard protein mutagenesis may be exploited to circumvent the stability-pharmacokinetic trade-off among prandial insulin analogs. Based on classical structure-function relationships as described by D. C. Hodgkin and co-workers (12–14) and Derevenska et al. (15) more than 40 years ago, we hypothesized that the structural opportunities provided by nonstandard functional groups at insulin’s dimer interface might confer simultaneous improvements in stability and pharmacokinetic properties. We thus undertook to synthesize and characterize a derivative of a rapid-acting insulin analog in clinical use (LysB28; ProB29-insulin; lispro or KP-insulin, the active component of Humalog® (Eli Lilly and Co.)) (16) in which an invariant tyrosine (TyrB26) was substituted by 3-iodotyrosine (3-I-Tyr). Choice of this modification was motivated by the unique physicochemical properties of iodo-aromatic groups (17) as exploited in the evolution of thyroid hormones (18). Substitution of TyrB26 by 3-I-Tyr was previously shown to preserve the function of insulin (19, 20).

Our results demonstrate that the 3-I-TyrB26 derivative of KP-insulin exhibits a remarkable combination of biophysical properties pertinent to general principles of protein engineering. In particular, differential effects of this modification within the R6 hexamer (a traditional vehicle for pharmaceutical formulation) versus the T6 hexamer (formed in the SQ depot) promise to provide a molecular strategy to augment protein stability while preserving rapid action.

EXPERIMENTAL PROCEDURES

Preparation of Insulin Analogs—Analogs were generated by trypsin-catalyzed semi-synthesis using des-octapeptide[23-B30]-insulin and a modified octapeptide as described (21). The insulin fragment was generated by tryptic cleavage of human insulin and purified by reverse-phase high performance liquid chromatography (HPLC), whereas the 3-I-Tyr-containing octapeptide (sequence GFF(3-I-T)TKPT) was prepared by solid-phase synthesis (22). After trypsin-mediated formation of a peptide bond between ArgB22 and the octapeptide (a reaction favored by the reduced water activity of a mixed solvent system containing 1,4-butanediol and dimethylacetamide (23)), the resulting insulin analog was purified using preparative reverse-phase HPLC (C4 10 μm 250 × 20 mm; Higgins Analytical Inc., Proto 300). Purity was assessed by analytical HPLC (C4 5 μm, 250 × 4.6 mm; Higgins Analytical Inc., Proto 300). The predicted molecular mass of the insulin analog was verified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an Applied Biosystems 4700 proteomics analyzer (determined mass, 5933 Da; predicted mass, 5933 Da).

Circular Dichroism—Far-ultraviolet (UV) spectra were obtained on an AVIV spectropolarimeter equipped with an automated syringe-driven titration unit. Insulin or insulin analogs were made 50 μM in 10 mM potassium phosphate (pH 7.4) and 50 mM KCl. Spectra were obtained from 200–250 nm as described (24). Thermodynamic stabilities were probed by guanidine hydrochloride-induced denaturation monitored by CD at helix sensitive wavelength 222 nm. Data were fit by nonlinear least squares to a two-state model (25),

\[ \theta(x) = A + \theta_0 e^{\left(\Delta G_{\text{m}}^0 - mx\right)/RT} \]

where \( x \) is the concentration of guanidine hydrochloride and \( \theta_A \) and \( \theta_0 \) represent respective estimates of the base-line ellipticities of the protein in its native and unfolded states as extrapolated to a guanidine concentration of 0. Base-line values were approximated via pre- and post-transition lines represented by equations \( \theta_A(x) = \theta_A^{\text{apo}} + m_A x \) and \( \theta_0(x) = \theta_0^{\text{apo}} + m_0 x \). Such simultaneous fitting avoids artifacts of linear plots of \( \Delta G \) versus concentration of denaturant (26).

Size-exclusion Chromatography and Multilangle Light Scattering (SEC-MALS)—Self-association was monitored by SEC-MALS as described (27). Insulin analogs were made 0.6 mM in a buffer consisting of 25 mM Tris-HCl (pH 7.4), 0.65 mg/ml phenol, 1.6 mg/ml meta-cresol, 16 mg/ml glycerol, and ZnCl₂ at a ratio of 2 zinc ions per insulin hexamer. A protein solution (volume 100 μl) was injected onto a Superdex75 column (nominal fractionation range of 3–70 kDa) at a flow rate of 0.5 ml/min. The mobile phase consisted of 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 0.02% sodium azide with or without 50 mM cyclohexanol (employed as a replacement for phenolic compounds often contained in pharmaceutical formulations).

Light scattering was monitored with a Wyatt Technology miniDAWN TREOS® 3-angle detector. Protein concentrations were determined using an inline Shimadzu SPD-6AV UV-visible detector. Data were analyzed using the ASTRA® software obtained from Wyatt Technology (Santa Barbara, CA).

4 Dilution of rapid-acting insulin analogs by 5-fold shifts the self-association equilibrium; although a predominance of hexamers is maintained, partial disassembly leads to larger fractions of zinc-free dimers and monomers. Whereas such dilutional disassembly in the SQ depot facilitates absorption (5), in a vial or pump reservoir it is associated with enhanced rates of degradation at or above room temperature.

5 Cyclic alcohols such as phenol and meta-cresol are widely used as antiimicrobial excipients in pharmaceutical formulations and in the case of insulin also function as protective allosteric effectors, decreasing rates of protein degradation (47).
Receptor Binding Assays—Affinities of insulin or insulin analogs to the B isof orm of the insulin receptor were measured in a competitive-displacement assay. In brief, microtiter strip plates (Nunc Maxisorb) were incubated at 4 °C overnight with a stock solution (100 μl/well) consisting of 40 μg/ml anti-FLAG immunoglobulin G. Detergent-solubilized lysates of 293 PEAK cells transfected with cDNAs encoding the insulin receptor with C-terminal FLAG tags were purified using wheat germ agglutinin chromatography (28). Partially purified receptors were then immobilized in the coated plates. Plates were extensively washed, and competitive binding assays using labeled tracer 125I-TyrB26-insulin and unlabeled insulin analogs were performed as described (29). Data for homologous and heterologous receptor binding were analyzed as described (30).

Assessment of Fibril Formation—Insulin fibrillation was monitored under two conditions, (i) at 60 μM insulin concentration in phosphate-buffered saline (pH 7.4) containing 0.1% sodium azide with gentle rocking at 37 °C and (ii) at 0.15 mM concentration in a buffer consisting of 7 mM Tris-HCl (pH 7.4), 75 μM ZnCl₂, 16 mg/ml glycerin, 1.6 mg/ml meta cresol, 0.65 mg/ml phenol with gentle rocking at 45 °C. In each case the protein solutions were incubated in glass vials containing a liquid/air interface. Aliquots were taken at regular intervals and frozen to enable analysis of thioflavin T fluorescence at the end of the assay, terminated on visual appearance of cloudiness in the solution (31).

Visible Absorption Spectroscopy—Formation and disassembly of the phenol-stabilized R₆ Co²⁺-substituted insulin hexamer were probed by visible absorption spectroscopy (450–700 nm). Proteins were made 0.6 mM in a buffer consisting of 50 mM Tris-HCl (pH 7.4), 50 mM phenol, 0.2 mM CoCl₂, and 1 mM NaSCN (32). Before the studies, samples were incubated overnight at room temperature. Spectra were then acquired to monitor tetrahedral cobalt coordination with its signature d-d absorption band at 574 nm (32). Kinetics studies to determine the rate of Co²⁺ release from the hexamers exploited metal-ion sequestration by a 10-fold molar excess of EDTA as described (33). The assay was initiated at 25 °C by the addition of an aliquot of EDTA (made 50 mM at pH 7.4) to a final concentration of 2 mM. The intensity of the 574-nm absorption band was monitored on a time scale of seconds-hours. Kinetics data were consistent with a mono-exponential decay due to attenuation of this band in an octahedral Co²⁺-EDTA complex.

NMR Spectroscopy—Spectra were acquired in aqueous D₂O solution (pD 7.7, direct meter reading) at 700 MHz at 32 °C; protein concentrations were ~0.5 mM. Homonuclear two-dimensional nuclear Overhauser effect (NOE) spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) spectra were obtained with mixing times 100 and 52 ms as described (34). 1H NMR chemical shifts were calibrated in parts per million (ppm) relative to trimethylsilyl propionate as an internal standard, assumed to be a 0.00 ppm.

Kinetics of Zinc Extraction—Insulin or insulin analogs were made 0.17 mM in a solution containing 13.2 mM Tris-HCl (pH 7.4) and 30 mM phenol with 1.2 zinc ions per hexamer. A probe of the free concentration of Zn²⁺ was provided by the indicator 2,2',6,6'-terpyridine (35); the reagent was obtained from Sigma. Release of Zn²⁺ from insulin hexamers was thus monitored by UV absorbance at 334 nm upon the addition of a final dye concentration of 500 μM (in marked excess relative to total Zn²⁺ concentration). Data were fit to a mono-exponential equation.

Rodent Assay—Male Lewis rats (mean body mass ~300 grams) were rendered diabetic by treatment with streptozotocin (36). To test in vivo potency, protein solutions containing KP-insulin or analog were constituted in a buffer containing 3.8 mg/ml sodium phosphate (pH 7.4), 8.6 μM ZnCl₂ (3 Zn²⁺:1 insulin hexamer) with the addition of 16 mg/ml glycerin, 1.6 mg/ml meta cresol, and 0.65 mg/ml phenol. To ensure uniformity of the formulations, the insulin analogs were each purified by reverse-phase HPLC, dried to powder, dissolved in the above buffer in the absence of zinc ions at the same maximum protein concentration, and re-quantitated by analytical C4 reverse-phase HPLC; an aliquot of 100 mM ZnCl₂ (pH 4 in 0.1 mM HCl) was added to obtain the targeted Zn²⁺:insulin ratio.

Rats were injected SQ at time t = 0 with 10 μg or 20 μg of KP-insulin or analog in 100 μl of buffer per 300-g mass of rat with the dose being adjusted to each rat’s body mass. Dose-response studies of KP-insulin indicated that at the 20-μg dose, a near-maximal rate of glucose disposal during the first hour after injection was achieved with an EC₅₀ of ~5 μg. Blood was obtained from the clipped tip of the tail at time 0 and every 10 min up to 60 min for the 1st h, every 20 min for the 2nd h, every 30 min for the 3rd h and every hour thereafter. Serial measurements were made using a clinical glucometer (Hypoguard Advance Micro-Draw meter). The efficacy of insulin action was calculated using (a) the change in blood glucose concentration over the first hour and (b) the integrated area between the glucose time dependence and a horizontal line at the starting blood glucose concentration (area over the curve (AOC)). The AOC was calculated for each individual rat (and not based on the mean curve) to avoid artifacts due to rat-to-rat variation in initial values of the blood-glucose concentration; in our rat colony such base-line values exhibited considerable variation, even in the same rat on different days. This variation can have a marked effect on the averaged data obtained at each time point. Assessment of statistical significance was performed using Student’s t test. Intravenous injections into tail veins with the same insulin analogs were carried out at a dose of 10 μg per 100 μl of buffer per 300-g mass of rat; data were analyzed as above.

RESULTS

Synthesis of 3-Iodo-TyrB26-LysB28, ProB29-insulin—Trypsin-catalyzed semi-synthesis enabled efficient preparation of 3-I-TyrB26-substituted KP-insulin (Fig. 1, A and B). The KP substitutions (ProB29 → Lys and LysB28 → Pro) provided both a monomeric template (in the absence of Zn²⁺; Fig. 1C) and a hexameric template wherein TyrB26 packs at an aromatic-rich dimer interface (in the presence of Zn²⁺ and phenol, meta cresol, or cyclohexanol; Fig. 1, D and E) (9). The availability of these alternative templates facilitated comparative studies of biophysical properties pertinent to formulation and biological activity, including the TR allosteric transition (Fig. 1E).

6 Conformational equilibria among zinc-stabilized insulin hexamer types T₆R, T₆R₆, and R₆ (R₆, frayed R state).
Receptor Binding and Activity—The affinity of 3-I-TyrB26-KP-insulin for the lectin-purified insulin receptor (isomor B) is almost 2-fold greater than that of KP-insulin or wild-type insulin (Table 1) in accordance with past studies (20, 37); dissociation constants ($K_d$) are summarized in Table 1. The potency and duration of action of 3-I-TyrB26-KP-insulin in a rat model of diabetes mellitus were nonetheless similar (but not identical) to those of KP-insulin on SQ injection at 100 μg (Fig. 2). Because of variation in the initial blood glucose level between rats, data are shown both in mean initial rates of fall in blood-glucose concentration with identical rates of fall in blood glucose concentration at time 0 and 20–70% of normal in relation to the actual blood glucose concentrations at time 0 and 20–70% of normal in relation to the actual blood glucose concentrations at time 0.

At the lower SQ dose (10 μg; n = 10 per group) the $p$ value was 0.35; at the higher SQ dose (20 μg; n = 10 for the lispro group and n = 11 for the iodo-analog group) the $p$ value was 0.43. If the small apparent differences in mean initial rates of fall in blood-glucose concentration should represent a true difference in initial potency, a power calculation suggests that a trial of >40 rats per group would be required to demonstrate statistical significance.

8 At the lower SQ dose the $p$ value was 0.54; at the higher SQ dose (20 μg) the $p$ value was 0.17. The latter $p$ value was reduced due to the limited glycemic response of 2 of the 11 rats to the higher dose of the iodo-analog; these outliers may have represented injection site errors or physiologic insulin resistance as occasionally observed among outbred male Lewis rats. Exclusion of these outliers would yield a $p$ value of 0.57 in accordance with the lower-dose study. If the small apparent differences in mean AOC in the present data should represent a true difference in potency, a power calculation suggests that a trial of >100 rats per group would be required to demonstrate statistical significance. In standard pharmaceutical formulations any such differences in intrinsic potency would be compensated by redefinition of the formulation strength in relation to international units per mg of protein such that equivalent biological activity per ml is obtained.
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of 3-I-TyrB_{26}-KP-insulin in D_{2}O (asterisk in the bottom trace in Fig. 5C) (42).

An overall similarity between KP-insulin and 3-I-TyrB_{26}-KP-insulin was observed with respect to the overall pattern of chemical shifts of corresponding aromatic resonances and downfield H_{9} resonances (assigned to CysA_{6}, CysA_{7}, CysA_{20}, and CysB_{7}). This correspondence was further delineated in two-dimensional TOCSY spectra as illustrated in the respective aromatic spin systems of KP-insulin (black cross-peaks in the bottom panel of Fig. 5D) and 3-I-TyrB_{26}-KP-insulin (red cross-peaks). Whereas, as expected, no TOCSY cross-peaks involve the isolated C_{2}H proton of 3-I-TyrB_{26} (asterisk in Fig. S5D), J coupling was observed between the C_{3}H and C_{4}H protons opposite to the site of modification. A corresponding set of long range NOEs was observed from these aromatic protons to aliphatic resonances and most prominently involved upfield-shifted methyl groups (Fig. 5D, top). NOESY spectra were obtained with a mixing time sufficiently short (100 ms) such that at this temperature (32 °C) spin diffusion was negligible, thus enabling direct contacts to be observed between specific aromatic ring protons and individual methyl groups.

The most up-field aliphatic ¹H NMR resonances (δ_{1}-CH_{3} resonance at 0.15 ppm in KP-insulin and 0.10 ppm in 3-I-TyrB_{26}-KP-insulin) were assigned to the side chain of LeuB_{15}, which packs beneath the aromatic ring of PheB_{24} and so experiences a ring-current shift (43). Of particular interest are native-like contacts between the side chains of PheB_{24} and LeuB_{15} (cross-peaks n, o, and p in Fig. 5D), TyrA_{19} and IleA_{2} (cross-peaks q and s). Maintenance of these framework inter-residue contacts and ring-current effects provides evidence that iodination of TyrB_{26} does not perturb the overall tertiary structure of the protein. The specific NOEs involving the indi-
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In Fig. 5D, reflecting insertion of the ring into an interchain crevice) in the case of \( C_{\alpha}H \) (opposite side of the ring) such NOEs are markedly attenuated or absent (red arrow and dashed line in Fig. 5D). Such asymmetry implies that the iodo-substituent occupies a favored binding pocket in (or adjoining) the hydrophobic core; this environment hinders rotation of the ring about the \( C_{\gamma}C_{\beta} \) bond axis. These qualitative features do not exclude local reorganization of the B26-related substructure, determining the details of this structure will require more complete \(^1\)H NMR analysis and molecular modeling.

**Insulin Self-assembly**—Evidence that 3-I-Tyr\(^{26}\)-KP-insulin and KP-insulin are each competent to form \( R_6 \) phenol-stabilized Co\(^{3+}\)-insulin hexamers\(^9\) was provided by observation of a characteristic d-d visible absorption band in each case. This blue band, which provides an optical signature of a tetrahedral binding site (Fig. 6A), is prominently observed in the spectrum of 3-I-Tyr\(^{26}\)-KP-insulin.

\(^9\) Insulin is capable of coordinating a variety of divalent metal ions to form hexamers in which the three His\(^{40}\) side chains in each component trimer provide a coordination site (octahedral in \( T_4 \) sites and tetrahedral in \( R_6 \) or \( R_3 \) sites) (1, 61).

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**FIGURE 5. Biophysical assays of structure and stability.** A, far-UV circular dichroism spectra of human insulin (HI, green line), KP-insulin (KP, black line) and 3-I-Tyr\(^{26}\)-KP-insulin (iodo-KP, red dashed line) at pH 7.4 and 25 °C. Ellipticity was normalized per residue. B, guanidine (Gu)-unfolding transitions as monitored by ellipticity at 222 nm. Labeling is as in panel A. Thermodynamic stabilities were derived using a two-state model (Table 1). C, one-dimensional spectra in \( D_2O \); aromatic and downfield \( H_\alpha \) resonances of insulin and insulin analogs. Top, wild-type insulin; middle, KP-insulin; bottom, 3-I-Tyr\(^{26}\)-KP-insulin. Asterisk in the bottom trace indicates the downfield \( C_{\alpha}H \) ring resonance of 3-I-Tyr\(^{26}\). Brackets indicate groupings of histidine residues (left) and \( \alpha \) protons (right). Selected resonances assignments are as labeled: a, His\(^{10}\) C\(_\gamma\)H; b, His\(^{19}\) C\(_\delta\)H; c, Cys\(^{15}\) C\(_\gamma\)H; d, Tyr\(^{19}\) C\(_\gamma\)H; e, Phe\(^{24}\) C\(_\gamma\)H; f, His\(^{25}\) C\(_\gamma\)H; g, Cys\(^{26}\) C\(_\gamma\)H; h, Cys\(^{47}\) C\(_\gamma\)H; i, Cys\(^{48}\) C\(_\gamma\)H. Line broadening and minor peaks in the wild-type spectrum reflect coupled self-association equilibria among dimers, trimers, tetramers, hexamers, and higher-order oligomers. D, two-dimensional spectra of KP-insulin (black) and 3-I-Tyr\(^{26}\)-KP-insulin (red) in \( D_2O \). Bottom, overlaid TOCSY spectra of aromatic spin systems; top, are overlaid NOESY spectra in regions containing cross-peaks between aromatic protons (horizontal axis; \( \omega_z \)) and aliphatic protons, including upfield-shifted methylene groups (vertical axis; \( \omega_x \)). The asterisk indicates the downfield \( C_{\alpha}H \) ring resonance of 3-I-Tyr\(^{26}\). Brackets in the top panel denote aromatic and methyl group resonances. Long range NOE assignments: j, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Tyr\(^{19}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); k, Tyr\(^{19}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); l, Tyr\(^{116}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); m, Tyr\(^{116}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); n, Phe\(^{24}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); o, Phe\(^{24}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); p, Phe\(^{24}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Leu\(^{115}\); q, Tyr\(^{19}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); r, Tyr\(^{119}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); s, Tyr\(^{119}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); t, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); u, Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); v, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); w, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); x, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); y, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); z, 3-I-Tyr\(^{26}\) C\(_\gamma\)H/\( \delta\)-CH\(_2\); Ile\(^{42}\); Ile\(^{42}\).
of 3-I-TyrB26-KP-insulin (open circles in Fig. 6B); its retention suggests that the bulky iodo-substituent is readily accommodated at or near the classical dimer interface (1). The slight attenuation of this feature in the spectrum of the KP-insulin hexamer (thin black line in Fig. 6B) has previously been described (9). The visible absorption spectrum of 3-I-TyrB26-KP-insulin as a metal-ion-stabilized complex (open circles in Fig. 6B) more closely resembles the spectrum of the KP-insulin R6 hexamer than it does the spectrum of the wild-type insulin hexamer, a pattern similar to that seen in CD studies of the metal ion-free proteins (above).

To extend these studies from cobalt hexamers to zinc hexamers (as in a pharmaceutical formulation), we employed SEC-MALS, a technique that combines size-exclusion chromatography with multiangle static light scattering. Because phenol and meta-cresol (classical pharmaceutical excipients and allosteric effectors of the TR transition6 present in the samples) interfere with UV detection of the eluting proteins, the running buffer contained the alternative UV-transparent ligand cyclohexanol10 (44) (left-hand panels of Fig. 7). Although there were no zinc ions in the running buffer, past studies have indicated that its release from hexameric complexes is dependent on their disassembly (45, 46).

Marked differences were observed in this assay between wild-type insulin, KP-insulin, and 3-I-TyrB26-KP-insulin. Whereas wild-type insulin eluted predominantly as a hexamer (peak a in Fig. 7A) with a minor component due to an equilibrium between monomers and dimers (intermediate peak b), KP-insulin eluted predominantly as a broad distribution of species representing progressive disassembly of hexamers into smaller species; the late-eluting peak represents an equilibrium between dimers and monomers (peak b in Fig. 7B). 3-I-TyrB26-KP-insulin exhibited an elution profile intermediate between the latter two patterns. Its greater fraction of intact hexamers (relative to KP-insulin; peak a in Fig. 7C) suggests that the iodo-modification either (or both) enhances the stability of the variant R6 hexamer or (and) introduces a kinetic barrier to its disassembly. Such stabilization or kinetic barrier would be expected to forestall degradation of 3-I-TyrB26-KP-insulin hexamers in a vial or delivery device (47).

SEC-MALS may readily be adapted to probe the transition of a concentrated R6-based insulin formulation (as stabilized by Zn2+ coordination and an allosteric phenolic ligand) to lower molecular weight species as a model of these molecular events in the SQ depot. To this end we sought to mimic the rapid loss of phenol and meta-cresol from the loaded R6 sample (which

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10 Cyclohexanol is the non-aromatic isostere of phenol and is UV-transparent. It is capable of binding to insulin hexamers albeit with lower affinity (44).
occurs on the millisecond time scale as measured by $^1$H NMR (43)) on diffusion of these ligands into cell membranes. This step is followed by slow hexamer disassembly with release of zinc ions and absorption of insulin molecules into capillaries. The initial steps of this process may be modeled in the SEC-MALS assay by use of a running buffer that contains neither zinc ions nor cyclohexanol (27). Under these conditions wild-type insulin eluted with a peak mass of $\approx 110.11$ kDa, corresponding to a dimer-trimer equilibrium; a trailing edge was also observed representing a dimer-monomer equilibrium (Fig. 7 D). KP-insulin was observed in this assay to elute primarily as a single peak with a monomeric mass of $\approx 5.8$ kDa (Fig. 7 E). This chromatographic behavior is in accordance with the design of Humalog® as a rapid-acting insulin analog formulation (16). Despite the stabilizing effect of 3-I-TyrB26 in the first set of SEC-MALS studies (above), the elution profile of 3-I-TyrB26-KP-insulin (Fig. 7 F) in the second chromatographic assay resembles that of KP-insulin (Fig. 7 E). A slight initial shoulder in its elution profile (arrow in Fig. 7 F) corresponds to a trace dimer component not detectable in KP-insulin. We note in passing that 3-I-TyrB26-KP-insulin also exhibited a small delay in its peak elution time (relative to KP-insulin), which is a likely consequence of an increased interaction with the column matrix due to the added hydrophobicity of 3-I-Tyr. These findings suggest that under formulation conditions thermodynamic and kinetic features relevant to the rapid action of KP-insulin on SQ injection would be retained in 3-I-TyrB26-KP-insulin.

Kinetics of Disassembly—Because SEC-MALS suggested kinetic stabilization of the cyclohexanol-induced $R_n$ hexamer by the iodo-modification, we returned to the cobalt assay as an explicit probe for the lifetime of the tetrahedral metal binding site (Fig. 6 A). The d-d absorption band of the wild-type and variant insulin $R_n$ hexamers (stabilized by 50 mM phenol) was, therefore, exploited as a kinetic probe (Fig. 6 B). At time $t = 0$ s, an aliquot of a concentrated EDTA (adjusted to pH 7.4) was added that enabled progressive sequestration of Co$^{2+}$ ions on their release from protein complexes in the course of a self-association equilibrium (Fig. 6 C). The rate of release is slow (minutes to hours) relative to rapid EDTA chelation (whose rate is diffusion limited). Hexamer lifetimes may thus be measured via the progressive disappearance of the d-d optical transitions characteristic of the tetrahedral Co$^{2+}$-coordination site in $R_n$ hexamers. Fitting of the time-dependent attenuation of the 574-nm Co$^{2+}$ band intensity indicated that the

\[\text{Tetrahedrally coordinated Co}^{2+}\text{ provides an absorption spectrum in the visible range due to d-d transitions in its incomplete d-shell; this band is negligible in an octahedral complex (32).}\]
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| Table 2 | Metal ion sequestration studies |
|---------|-------------------------------|
| Protein | Co<sup>2+</sup> extraction<sup>a</sup> | Zn<sup>2+</sup> extraction<sup>b</sup> |
|         | half-life | half-life |
| Wild-type insulin | 13.8 ± 0.4 | 3.6 ± 0.1 |
| KP-insulin | 3.1 ± 0.1 | 1.0 ± 0.1 |
| Iodo-KP-insulin | 25 ± 0.8 | 8.9 ± 0.1 |

<sup>a</sup> Kinetic features of Co<sup>2+</sup> release from protein hexamers were monitored by following the attenuation in absorbance at 574 nm after the addition of excess EDTA. Protein solutions were made 0.6 mM (nominal concentration of monomers) in 50 mM Tris-HCl (pH 7.4), 50 mM phenol, 1 mM NaSCN, and 0.2 mM CoCl<sub>2</sub>.

<sup>b</sup> Sequestration of Zn<sup>2+</sup> was monitored by following the change in absorbance at 334 nm after the addition of excess 2,2'-6',2'-terpyridine to a solution wherein the proteins were made 0.17 mM (nominal concentration of monomers) in a buffer containing 13.2 mM Tris-HCl (pH 7.4) and 30 mM phenol with 1.2 zinc ions per hexamer.

respective lifetimes of R<sub>6</sub> assemblies containing wild-type insulin and KP-insulin are 13.8 ± 0.4 and 3.1 ± 0.1 min at 25 °C (Table 2, Fig. 6C). Remarkably, the lifetime of R<sub>6</sub> hexamers containing 3-I-Tyr<sup>B26</sup>-KP-insulin is 25 ± 1 min under these conditions is even slower than that of wild-type insulin.

To extend these kinetic studies to Zn<sup>2+</sup>-containing R<sub>6</sub> hexamers, we employed a molecular probe whose molar absorbance at 334 nm is markedly enhanced on binding Zn<sup>2+</sup> ions (35). The same trend was observed among the three proteins as obtained in the Co<sup>2+</sup>-based EDTA sequestration assay (Table 2, Fig. 6D). Thus, accommodation of the bulky iodo-aromatic substituent within the dimer interface of the R<sub>6</sub> hexamer imposes a kinetic barrier to its disassembly. The added barrier height (ΔΔF<sub>a</sub>) is estimated as RT ln(k'/k); relative to the rate of disassembly of KP-insulin, this estimate yields a value of 1.2 ± 0.1 kcal/mole at 25 °C.

Insulin Fibrillation—Clinical formulations of insulin and insulin analogs are ordinarily protected from fibrillation through their self-assembly as R<sub>6</sub> zinc hexamers. The above findings, therefore, motivated us to test whether the iodo-Tyr modification might augment such protection. Protein fibrillation is characterized by a lag phase of stochastic onset (before detectable increases in thioflavin T fluorescence or optical scattering) and an elongation phase (48). Although variability in the lag time is characteristic of nucleation-propagation reactions (49), a significant difference was observed between the susceptibility of wild-type insulin to fibrillation under diluted formulation conditions<sup>12</sup> (22 ± 9 days in <i>n</i> = 9 trials; as assessed at a protein concentration of 0.15 mM on gentle agitation at 45 °C in the presence of an air-liquid interface) and that of the less stable KP-insulin analog (14 ± 5 days in <i>n</i> = 8 trials; Fig. 8A). The arrow in the KP-insulin sample refers to an outlier that was off scale (44 days) and was excluded from analysis. Although these are overlapping distributions, Student’s t test yielded a p value of 0.027. Our findings are in accordance with clinical guidelines regarding differences in product shelf lives at room temperature.

Strikingly, the enhanced susceptibility of KP-insulin to fibrillation on temperature stress was mitigated by the iodo modification (22 ± 7 days in <i>n</i> = 6 trials), leading to a set of lag times indistinguishable from those of wild-type insulin. Interestingly, fibrillation studies of the metal ion-free proteins made 60 μM in phosphate-buffered saline (pH 7.4) demonstrated that, as a monomer, 3-I-Tyr<sup>B26</sup>-KP-insulin is less susceptible to fibrillation at 37 °C than either KP-insulin or wild-type insulin (Fig. 8B).

DISCUSSION

In this study a nonstandard amino acid substitution has been exploited to alter the biophysical properties of a protein. The vast chemical space that can in principle be explored with nonstandard functional groups offers an expanded opportunity in protein design (50). The present work focused on a halogenated derivative of an aromatic amino acid. We chose 3-iodo-Tyr because of its unique physicochemical properties (18), evolutionary history (51), and fortuitous tolerance as a modification of insulin (20).

Iodo derivatives of insulin have long been used as a radioactive tracer (e.g. 3-<sup>125</sup>I-Tyr or 3-<sup>127</sup>I-Tyr (52), ordinarily located at position A14 extrinsic to the receptor binding surface of the hormone (19, 37). This A14 modification has no effect on affinity for the insulin receptor. In this context nonradioactive mono-iodo derivatives of insulin were first prepared as reverse-phase HPLC standards to guide purification of a mono-component radioactive derivative (20). Of the three remaining sites of modification in human insulin (i.e. Tyr<sup>A19</sup>, Tyr<sup>B116</sup>, and Tyr<sup>B26</sup>), only the B26 derivative did not exhibit a reduction in receptor binding (20, 37). Indeed, 3-I-Tyr<sup>B26</sup> and (3,5)-I<sup>2</sup>-Tyr<sup>B26</sup> enhance binding (20, 37, 53), motivating the present study. Given recent progress in the crystallographic characterization of model insulin–receptor complexes (54), it would be of future interest to investigate the structural basis of such enhanced affinity.

Protein Allostery—Allosteric regulation of proteins resulting from ligand binding at a site distant from the active site is mediated by transmitted changes in structure (55). A classical example is provided by hemoglobin whose conformational equilibria among T (tense) and R (relaxed) states is modulated by bisphosphoglycerate bound within the central axis of an αβ heterotetramer (56). Insulin hexamers likewise participate in coupled conformational equilibria among T<sub>0</sub>-<sub>T</sub><sub>3</sub>R<sub>3+</sub> and R<sub>6</sub> families of structures (Fig. 1E), so named by analogy to hemoglobin. Although the functional significance of the T → R transition is not well understood (46, 57), studies of insulin analogs have suggested that such allostery exploits sites of conformational change relevant to induced fit of the hormone on receptor binding (54, 58).

<sup>12</sup>Diluted insulin formulations are frequently used in the pediatric population to enhance the precision of dosing (67).
minal binding site does not preexist in the structure of the $T_6$ zinc hexamer but is well defined within $T_6R_3^f$ (with three ligands) and $R_6$ (with six or seven ligands) hexamers (62). Despite the precise spatial organization of this internal binding site as visualized in crystal structures, $^1$H NMR studies have established that exchange between bound and free phenol in solution occurs on the millisecond time scale (43). These concepts are pertinent to the present study, as aromatic alcohols (phenol and meta-cresol) have traditionally been employed in pharmaceutical formulations for their antimicrobial properties (63). Although such insulin formulations date to the 1930s, the additional allosteric role of these ligands was first appreciated only in 1989 (61). Fortuitously, the ligand-dependent T→R transition markedly prolongs the lifetime of the hexamer (35) and augments its resistance to chemical and physical degradation (47). Because such degradation occurs primarily via conformational fluctuations in an insulin monomer (4), protein allostery enhances the effectiveness with which self-assembly enables sequestration of the native monomer (35).

The allosteric role of phenol and meta-cresol gained central importance in the course of efforts to formulate “meal-time” insulin analogs (including KP-insulin) within labile zinc hexamers as a strategy to achieve rapid absorption (9). The two leading such analog formulations (Humalog® and Novolog®) thus contain $R_6$ zinc hexamers (33, 64), which presumably convert transiently to the less stable $T_6$ form in the SQ depot on the rapid dissociation and diffusion of the allosteric ligand (5). Such applications highlight the translational importance of fundamental biophysical concepts.

Although current prandial insulin analog formulations are sufficiently stable to meet the regulatory guidelines of the United States Food and Drug Administration, above room temperature they degrade at rates exceeding that of wild-type human insulin (65). Even at room temperature this differential stability has impact on dilution of the formulation as commonly utilized in the treatment of children (the prescribing information for Humalog can be found on the Eli Lilly website; see also Ref. 67). Whereas vials of wild-type insulin, once opened at room temperature, must be discarded after 28 days at any strength, corresponding vials of Humalog®, (Lys$^{B28}$, Pro$^{B29}$-insulin; insulin lispro) must be discarded on dilution after 14 days. Furthermore, the diluted prandial formulations are not recommended for use in insulin pumps due to the risk of degradation and catheter occlusion given the exposure of the protein solution to fluctuations in temperature and agitation.13 This restriction is inherent in the biophysical properties of the mutant proteins irrespective of the potential clinical advantages of pump therapy in such children (68). Such accelerated degradation could in principle be mitigated through further modification of the protein, but only if the modification does not also hinder absorption, which would sacrifice the therapeutic goal of its design (Fig. 9). This would appear to pose a molecular dilemma, as the original design strategies of current insulin analog formulations work at cross-purposes to stability.

The present study suggests that the 3-I-Tyr$^{B26}$ modification of Lys$^{B28}$, Pro$^{B29}$-insulin augments the kinetic stability of the $R_6$ zinc hexamer (Fig. 9, upper panel) without trapping the $T_6$ hexamer in the SQ depot (Fig. 9, lower panel). Furthermore, this modification enhances the intrinsic resistance of the isolated monomer as well as that of the formulated zinc hexamer to fibrillation above room temperature, the principal mode of insulin degradation pertinent to patient care. Moreover, the augmented thermodynamic stability of the modified monomer (as evaluated in guanidine denaturation studies) predicts enhanced resistance to chemical degradation (8). We envisage that 3-I-Tyr$^{B26}$ mediates such protective effects either through direct interactions of the halogen atom with neighboring side chains or through indirect modulation of “weakly polar” electrostatic interactions (69). Such an indirect mechanism may involve transmitted changes through successive edge-face interactions among the eight aromatic rings at the classical dimer interface (Tyr$^{B16}$, Phe$^{B24}$, Phe$^{B25}$, 3-I-Tyr$^{B26}$, and their symmetry-related partners). Dissecting such mechanisms will

13 Exposure of insulin or insulin analog formulations to hydrophobic interfaces, elevated temperature, and agitation are factors that accelerate the formulation of insoluble precipitates in pump lines and catheters (4). The recommended time of use for diluted Humalog® formulations is 14 days after opening of the vial at room temperature; use in pumps is not approved, but off-label applications have been reported (66).
require future crystallographic, quantum-chemical simulations, and NMR analyses (17).

Properties of Iodo-tyrosine—Iodine lies in column 17 of the periodic table with atomic number 53 and mass 126 atomic mass units; as such, it is the largest element that is essential for vertebrate life. Its atomic radius relative to other atoms in a polypeptide (oxygen, nitrogen, carbon, and hydrogen), and indeed relative to smaller halogen atoms, confers unique properties derived from the average distance of its electrons from the nucleus. Unlike smaller halogens, the electronegativity of iodine is approximately equivalent to that of carbon (2.66 versus 2.55) because its electron density is spread over a larger volume; this spreading also imparts a higher polarization potential relative to smaller halogens (18). Together, these factors render 3-I-Tyr hydrophobic despite the inductive effect of the halogen leading to an electrostatic dipole moment (17). It is possible that the greater size and hydrophobicity of 3-I-Tyr relative to Tyr might account for its biophysical effects on LysB28, ProB29-insulin, such as an augmented kinetic barrier to dissociation of the nonpolar dimer interface. The hydrophobicity of the iodine atom in an iodo-aromatic group is in accordance with crystal structures of protein complexes containing thyroid hormone (see below) (70–72). In this context the asymmetric long-range NOEs selectively observed from the C₆H proton of 3-I-TyrB₂₆ (relative to the opposite C₄H proton) suggest that the iodo-substituent packs within a nonpolar pocket between the A and B chains.

Halogen substitution within an aromatic system (such as in the phenolic ring of Tyr) alters the π orbitals. The electron withdrawing effect of fluorine, for example, diminishes the partial positive charges around the edge of the ring as dramatically observed in perfluorinated benzene with its inverted quadrupole moment (73). By contrast, in the case of iodo-aromatic substituents (and to a lesser extent bromo- and chloro-aromatic substituents), the conjugated π system can, as a seeming paradox, pull electron density away from the halogen, giving rise to a σ-hole on its opposing face (74). This electropositive cap can mediate “halogen bonding,” an interaction analogous to hydrogen bonding wherein the electron-deficient σ-face of the larger halogens can interact with a carbonyl oxygen or other nucleophiles (74). It would be of interest to test whether halogen bonding by 3-I-TyrB₂₆ might contribute to the marked effects of this modification on the kinetic or thermodynamic stability of the R₆ hexamer or to its stabilization of the KP-insulin as a metal ion-free monomer. Resolving this issue will require high resolution structures of each conformational state.

Evolutionary Antecedents—Our use of an iodo-Tyr modification to enhance the biophysical properties of a protein may...
recapitulate an ancestral evolutionary innovation. The relative ease with which iodide can be oxidized (the element is found primarily in seawater as soluble iodide (I−) and iodate groups) (75) has allowed its exploitation by enzymes that direct its incorporation into organic molecules (76). Such an evolutionary innovation lies deep in the history of the Metazoan kingdom as indicated by its detection in certain sponges and corals (77).

The most familiar examples of iodo-aromatic compounds in vertebrate biology are provided by thyroid hormones and their biosynthetic precursors, including 3-I-Tyr and (3,5)-I2-Tyr as modified residues in thyroglobulin (78). Critical to the growth, development, and metabolic homeostasis of vertebrates is the incorporation into organic molecules (76). Such an evolution (75) has allowed its exploitation by enzymes that direct its incorporation into organic molecules (76). Such an evolution (75) has allowed its exploitation by enzymes that direct its incorporation into organic molecules (76).

Concluding Remarks—The present study of the 3-I-TyrB26 derivative of a prandial insulin analog has shown that a single halogen modification can markedly alter the biophysical properties of an allosteric protein, including its stability and self-assembly, while preserving biological activity and rapid action in an animal model of diabetes mellitus. We envisage that the structural principles underlying such iodo-aromatic modifications will be found to recapitulate an ancestral evolutionary innovation in biophysical chemistry lying near the root of the Metazoan kingdom.

The general question of how an iodo-aromatic substitution in a protein can modulate its structure, dynamics, and interactions will require high resolution structures and computational investigation at the level of quantum chemistry (17). Because of the small size of insulin and its potential tractability by multidimensional NMR, crystallographic, and multiscale simulation methods (82), iodo-insulin derivatives promise to provide a general model for the integrated analysis of physical and chemical principles pertinent to the complex biology of thyroid hormone and its evolutionary history. In themselves, the surprising properties of 3-I-TyrB26-LysB28, ProB29-insulin uncovered in the present study illustrate the translational promise of non-standard protein engineering in molecular pharmacology.

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