

An ultra-stable single-chain insulin analog resists thermal inactivation and exhibits biological signaling duration equivalent to the native protein

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Thermal degradation of insulin complicates its delivery and use. Previous efforts to engineer ultra-stable analogs were confounded by prolonged cellular signaling *in vivo*, of unclear safety and complicating mealtime therapy. We therefore sought an ultra-stable analog whose potency and duration of action on intravenous bolus injection in diabetic rats are indistinguishable from wild-type (WT) insulin. Here, we describe the structure, function, and stability of such an analog, a 57-residue single-chain insulin (SCI) with multiple acidic substitutions. Cell-based studies revealed native-like signaling properties with negligible mitogenic activity. Its crystal structure, determined as a novel zinc-free hexamer at 2.8 Å, revealed a native insulin fold with incomplete or absent electron density in the C domain; complementary NMR studies are described in the accompanying article. The stability of the analog (ΔG_U 5.0(±0.1) kcal/mol at 25 °C) was greater than that of WT insulin (3.3(±0.1) kcal/mol). On gentle agitation, the SCI retained full activity for >140

days at 45 °C and >48 h at 75 °C. These findings indicate that marked resistance to thermal inactivation *in vitro* is compatible with native duration of activity *in vivo*. Further, whereas WT insulin forms large and heterogeneous aggregates above the standard 0.6 mM pharmaceutical strength, perturbing the pharmacokinetic properties of concentrated formulations, dynamic light scattering, and size-exclusion chromatography revealed only limited SCI self-assembly and aggregation in the concentration range 1–7 mM. Such a combination of favorable biophysical and biological properties suggests that SCIs could provide a global therapeutic platform without a cold chain.

Insulin has long provided a model for biochemical and biophysical studies of globular proteins with application to human health (1). The first protein to be sequenced and the first recombinant therapeutic protein, insulin is central to the hormonal control of vertebrate metabolism. Since its discovery in 1921, the hormone has provided a mainstay of treatment for diabetes mellitus (2). Whereas insulin itself contains two polypeptide chains (A and B) as the proteolytic product of a single-chain precursor (proinsulin (3)), the hormone belongs to an ancestral metazoan superfamily of single-chain homologs (4), including vertebrate insulin-like growth factors (IGFs (5, 6)) and invertebrate neuroendocrine factors (7). This study and accompanying article (8) drew inspiration from these evolutionary relationships to investigate the structure, stability, and function of a single-chain insulin analog (SCI)⁶ with high biological activity and enhanced thermodynamic stability.

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This article contains Figs. S1–S5, Table S1–S3, and supporting Refs. 1–7.

The atomic coordinates and structure factors (code 5WDM) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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⁶ The abbreviations used are: SCI, single-chain insulin; αCT, C-terminal domain of IR; μIR, domain-minimized insulin micro-receptor; Akt, protein kinase B; ER, endoplasmic reticulum; IEF, isoelectric focusing; IR, insulin receptor; L1, first leucine-rich repeat domain of IR; PD, pharmacodynamics; PDB, Protein Data Bank; r.m.s.d., root-mean-square deviation; rp-HPLC, reverse-phase high-performance liquid chromatography; SQ, subcutaneous; T₆, insulin hexamer form wherein all protomers exist in a T state; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; rt-qPCR, rt-quantitative PCR; AOC, area over the curve; PK, pharmacokinetic; SEC, size-exclusion chromatography; DLS, dynamic light scattering; AUC, area under the curve; MPI, mini-proinsulin.

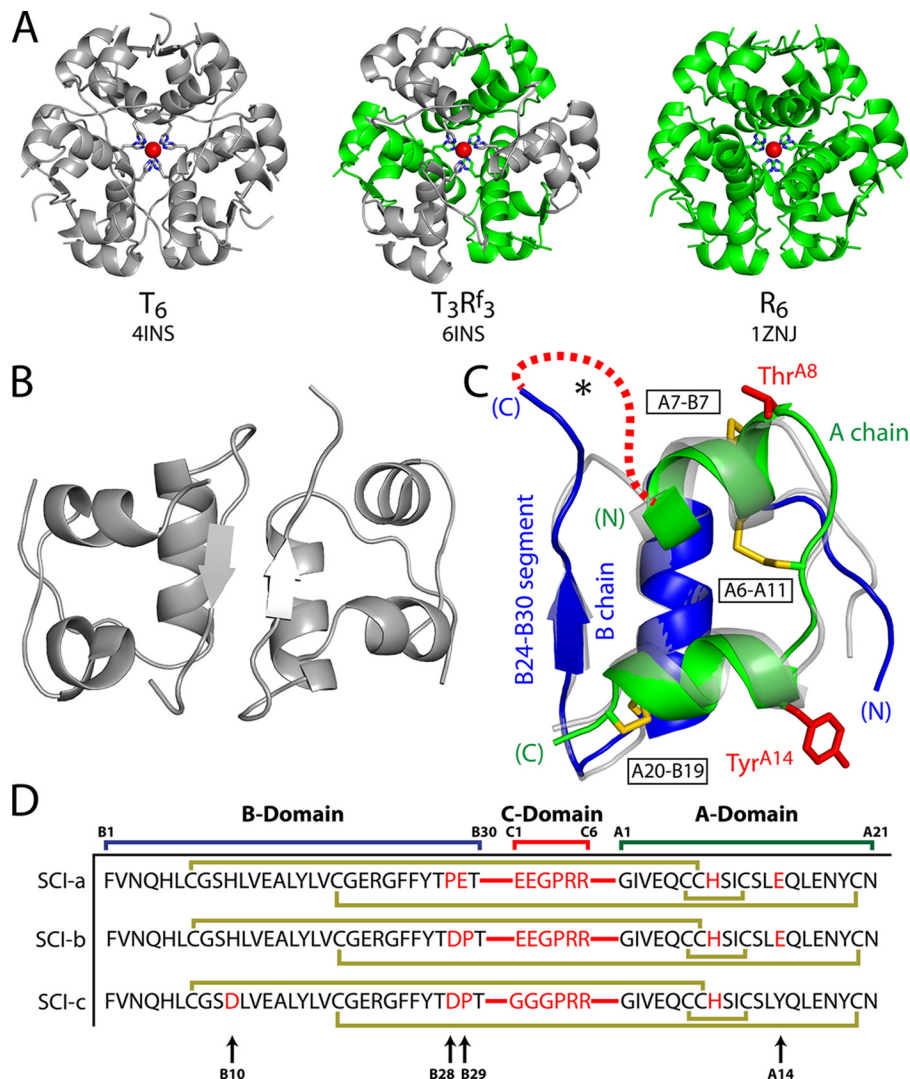


Figure 1. Structural overview and insulin analog sequences. *A*, crystal structures of T_6 (porcine insulin; PDB code 4INS (1)), $T_3R_f^3$ (B29–A1 single chain insulin; PDB code 6INS (19)), and R_6 (human insulin; PDB code 1ZNI) hexamers, as labeled. In each structure, the T state protomers are gray and R state protomers are green. His^{B10} side chains are displayed as sticks with coordinated Zn²⁺ ions as red spheres. The second axial Zn²⁺ ion lies directly behind the first. *B*, anti-parallel β -sheet dimer interface of porcine insulin (PDB code 4INS). *C*, overlay of a T state protomer of B29–A1 single-chain insulin (transparent gray; PDB code 6INS) and porcine insulin (PDB code 4INS). The latter displays the A (green) and B (blue) chains and three native disulfide bridges (yellow sticks): A20–B19, A7–B7, and A6–A11 (black boxed labels). Side chains of WT residues Thr^{A8} and Tyr^{A14} are highlighted as red sticks. The C-terminal B chain segment (important in both dimerization and IR binding) is labeled. Red dashed line and asterisk represent C domains of proinsulin and SCIs. *D*, protein sequences of SCI-a and SCI-b as well as progenitor SCI-c (29). Peptide bonds connecting the A and B domains to the C domain are illustrated with red lines. Substitutions relative to WT insulin are identified by red letters. Gold lines identify the three native disulfide bonds. Black arrows at bottom of *D* highlight residues B10, B28, B29, and A14, key sites of substitution.

The structure of insulin (Fig. 1, *A–C*) has been extensively investigated by X-ray crystallography (1, 9) and NMR spectroscopy (10–13). Although stored within pancreatic β -cells as zinc-stabilized hexamers (14), the hormone functions in the bloodstream as a zinc-free monomer (6). The native conformation contains three α -helices, two in the A chain (21 residues; green in Fig. 1*C*) and one in the B chain (30 residues; blue). Globular folding is stabilized by two inter-chain disulfide bridges (cysteines A7–B7 and A20–B19) and one intra-chain bridge (cysteine A6–A11). The core is sealed on one face by the B chain's C-terminal β -strand (residues B24–B28). This β -strand forms an antiparallel β -sheet at the classical dimer interface (Fig. 1*B*), a building block of zinc insulin hexamers (Fig. 1*A*) (1). Substitutions at positions B28 and B29 have enabled design of rapid-acting insulin analogs for mealtime injection or use in

pumps (15). Whereas clinical use of animal insulins gave way in the 1980s to recombinant human insulin (16), current therapeutic regimens often employ analogs with enhanced pharmacokinetic (PK) properties. Their development represented an early triumph of structure-based protein design in pharmacology (2).

Crystallographic studies of an insulin monomer bound to domain-minimized models of the insulin receptor (IR) (17, 18) demonstrated that the B chain β -strand pivots from the α -helical core to insert *in trans* between IR α -subunit domains (the N-terminal Leu-rich domain (L1) from one protomer and the C-terminal element (α CT) of the other; for review of IR domains, see Ref. 6). Such pivoting unmasks an otherwise hidden non-polar surface of the A chain (including Ile^{A2} and Val^{A3}), thereby extending the hormone's primary receptor-

binding surface (17, 18). This conformational transition rationalizes a wealth of prior biochemical data (10, 19–22), including early studies of insulin analogs modified by bifunctional reagents to cross-link the C-terminal segment of the B chain and the N terminus of the A chain (23, 24). These studies demonstrated that short cross-links markedly impair IR binding, whereas longer cross-links could, at least in part, restore activity.

Studies of SCIs began in the 1980s with the advent of microbial manufacture of insulin (16). Providing trypsin-cleavable manufacturing intermediates (mini-proinsulins), these recombinant proteins contain a peptide bond to the N terminus of the A chain (Gly^{A1}) from a residue at or near the C terminus of the B chain (Thr^{B30}) (25, 26). Constrained within a closed conformation, such SCIs (50–53 residues) exhibited favorable folding properties and hence high manufacturing yield (25, 27), but they were essentially devoid of biological activity despite native-like structures (19, 26). The T₃R^f₃ crystal structure of B29-A1 mini-proinsulin, like the “dog that did not bark in the nighttime,” was remarkable for the absence of perturbations (*central panel* in Fig. 1A) (19).

Subsequent studies of single-chain chimeric insulin-IGF proteins suggested that insertion of the 12-residue IGF-I connecting (C) domain between insulin's Thr^{B30} and Gly^{A1} could restore, at least in part, IR affinity (28). Interest in active SCIs was first stimulated by potential applications in viral gene therapy.⁸ Their biophysical properties have been investigated in relation to insulin fibrillation (29, 30), a form of physical degradation (31) that makes necessary a pharmaceutical “cold chain” of storage and distribution (32). Circumventing this complex and costly process defines a current translational goal of insulin analog design (32, 33).

Here, we describe the structure and function of an active SCI with sequence features designed to co-optimize its biophysical, biochemical, and biological properties (SCI-a in Fig. 1D and Table 1). The C domain contains 6 residues, resulting in a 57-residue protein. To enhance solubility, the protein contains acidic substitutions predicted to reduce its isoelectric point from 5.4 (wild-type (WT) insulin)⁹ to 4.9. Sites of substitution were chosen to impair binding to the mitogenic type 1 IGF receptor (IGF-1R) (34, 35) and enhance stability (29, 36). The crystal structure of SCI-a, obtained herein at 2.8 Å resolution, depicts a novel zinc-free hexamer containing native-like insulin folds with apparent disorder in the C domains. Whereas SCI-a forms classical dimers (Fig. 1B), their hexameric assembly differs from that of zinc-coordinated two-chain insulin hexamers

(Fig. 1A) (1, 37). In the accompanying article (8) we employed heteronuclear NMR methods to investigate the solution structure and dynamics of a monomeric SCI variant (SCI-b in Fig. 1D). The two SCIs differ at positions B28 and B29 in accordance with well-established structure–activity relationships underlying past design of rapid-acting clinical analogs (38–40).

This study paid particular attention to the duration of biological action as defined by the pharmacodynamic (PD) properties of the insulin analog on intravenous (i.v.) bolus injection. Because insulin clearance from the bloodstream is >20-fold more rapid than physiological attenuation of insulin signaling (41), such studies probe the duration of insulin signaling at target tissues once the IR is engaged. Previous efforts to obtain ultra-stable analogs, either by introduction of an additional disulfide bridge (33) or by design of an active SCI (29), encountered aberrant prolongation of *in vivo* glucose-lowering activity (Fig. S1). Such prolonged signaling would be unfavorable for prandial or pump-based therapy (15) and could be associated with adverse biological effects (42).

To our knowledge, SCI-a provides the first example of an ultra-stable insulin analog whose *in vivo* duration of action is similar to that of WT insulin. The analog exhibits striking resistance to thermal degradation with prolonged retention of biological activity. Moreover, its cell cycle–related transcriptional signaling resembles WT insulin and not single-chain homolog IGF-I or mitogenic insulin analogs (34). Together, these studies highlight the translational promise of ultra-stable SCIs as a platform for global distribution without the cost and complexity of a therapeutic cold chain. Such molecular technologies, motivated by the current diabetes pandemic, may be especially important in the developing world (32, 43).

Results

SCI-a and monomeric analog SCI-b incorporated multiple design elements for simultaneous optimization of biophysical and therapeutic properties. These elements were introduced based on prior studies of two-chain insulin analogs (38, 39) and a single-chain prototype (SCI-c (29)). Key sequence features and their rationale are summarized in Table 1. Whereas the C domain (EEGPRR) contains offsetting negative and positive charges, the set of substitutions in the A and B domains was expected to accentuate the proteins' net negative charge at neutral pH and thereby mitigate both native and non-native aggregation (44).

The SCIs contain exclusively WT residues at the hormone's classical (site 1) and accessory (site 2) receptor-binding surfaces (6, 18). Stabilizing acidic substitutions at residue B10 (Asp or Glu) was avoided due to their association with enhanced mitogenicity and carcinogenesis (34). Retention of His^{B10} was also intended to enable zinc-mediated hexamer assembly (1), traditionally employed to stabilize pharmaceutical formulations (45). Near the site 1 interface, His^{A8} (a feature of avian insulins (36)) has previously been shown to enhance both stability and receptor binding, presumably as a more favorable α -helical C-cap residue than Thr^{A8} (30, 36).

An overarching objective was avoidance of anomalously prolonged activity on i.v. bolus injection as observed in prior ultra-stable insulin analogs (Fig. S1). Such prolongation, first

⁷ From “The Adventure of Silver Blaze,” a Sherlock Holmes short story from The Memoirs of Sherlock Holmes by Sir Arthur Conan Doyle first published in 1892.

⁸ Clinical use of SCIs was first motivated by viral gene therapy in a study since retracted by its authors (101).

⁹ Wild-type insulin pertains to the human sequence unless otherwise stated. Insulin residues are denoted by residue type (in standard three-letter code) followed by chain and position as a superscript (e.g. Tyr^{B16} designates a tyrosine at the 16th position of the B chain). Insulin *lispro* is a clinical analog that exhibits rapid action due to interchange of residues B28 and B29. Activity of insulin formulations is standardized in International Units (IU); standard U-100 formulations contain 100 IUs per ml and correspond to a WT protein concentration of 0.6 mM. A U-1167 strength formulation would thus correspond to ~7 mM WT insulin.

Table 1

SCI sequence design features and their associated rationale

Design element	Rationale
Glu ^{C1} –Glu ^{C2}	Isoelectric shift (for enhanced solubility) and hypothesized reduction in IGF-1R cross-binding
Gly ^{C3} –Pro ^{C4}	Facilitates flexible chain reversal in direction of A1–A8 helix ^a
Arg ^{C5} –Arg ^{C6}	Mimics the charge pattern of the dibasic cleavage site at the C to A domain junction in proinsulin ^a
His ^{A8}	Stability enhancement through optimization of A1–A8 helical C-cap ^a
Glu ^{A14}	Protein stabilization through reduction in entropic penalty likely incurred by solvent exposed Tyr ^{A14} in the WT ^b
Glu ^{B29} (SCI-a)	Formation of stable dimers ^c
Asp ^{B28} –Pro ^{B29} (SCI-b)	Accelerated hexamer/dimer disassembly ^d

^a Data are as in the SCI-c prototype (29).^b A14 was hypothesized to exhibit the “reverse hydrophobic effect” (103) wherein the WT solvent-exposed and hydrophobic tyrosine side chain would destabilize the folded state. Replacement with charged (hydrophilic) and solvated Glu^{A14} would ameliorate this effect. Although a Glu^{A14}-insulin analog has been previously synthesized (59) with no significant change in hormone activity, to our knowledge, the isolated contribution of Tyr^{A14} → Glu to thermodynamic stability has not yet been determined.^c Data are as in insulin *glulisine*, a fast-acting clinical analog (40).^d Combination of the designs of fast-acting analogs insulin *aspart* (39) and insulin *lispro* (38) is shown.

reported by Vinther *et al.* (33) in studies of an insulin analog stabilized by a fourth disulfide bridge (Fig. S1, A and C), was reproducible in our hands (black triangles in Fig. S1D) and also seen in studies of a prior SCI prototype (SCI-c; blue crosses in Fig. S1D and structure in Fig. S1B). These studies employed male Lewis rats rendered diabetic by streptozotocin (30). Use of i.v. bolus injection circumvented PK issues related to the subcutaneous depot (41).

SCI-a and SCI-b each exhibited substantial activity in the i.v. rat assay (Fig. 2). Results are shown in relation to the absolute blood-glucose concentration (Fig. 2A) and relative to the starting blood-glucose level (Fig. 2B). The PD profile of SCI-a (orange circles in Fig. 2) was similar to that of insulin *lispro* (red squares), whereas SCI-b exhibited a late tail of insulin action (green triangles) similar to that of SCI-c (Fig. S1D). The native-like PD profile of SCI-a implies that its clearance and duration of cellular signaling are similar to WT insulin. Despite its native potency *in vivo*, receptor-binding studies of SCI-a *in vitro* revealed decreased affinity for the A (by 5-fold) and B (by 15-fold) isoforms of the IR with at least 10-fold reduction in affinity for the type 1 IGF receptor (IGF-1R). The respective receptor-binding affinities of SCI-b were similarly reduced (Table S2).

SCI-a retains canonical structure as a zinc-free hexamer

The crystal structure of SCI-a was determined as a zinc-free hexamer to a resolution of 2.8 Å (Fig. 3A). Diffraction statistics are given in Table 2. The asymmetric unit contained the entire hexamer, and so the structure provided six independent protomers (labeled A–F) forming three independent dimers (AB, CD, and EF). Electron density corresponding to the C domains was absent as was (to a varying extent among protomers) density corresponding to adjoining residues in the A and B domains (gray dashed lines in Fig. 3A). The most complete map pertained to protomer D (gold in Fig. 3A): weak density was observed at Pro^{B28} and Gly^{A1}, whereas intervening residues (sequence ETEEGPRR) were not visualized. The average pairwise root-mean-square deviations (r.m.s.d.) among main-chain atoms well-defined in each protomer was 0.75(±0.09) Å. Following analogous pairwise alignment and comparison of the three dimers, mean r.m.s.d. among main-chain atoms was 1.0(±0.2) Å.

Each protomer exhibited a canonical T state conformation (Fig. 3C; porcine insulin monomer 1 as reference structure). Relative to six reference T state protomers in prior crystal structures of insulin, the mean pairwise main-chain r.m.s.d. was

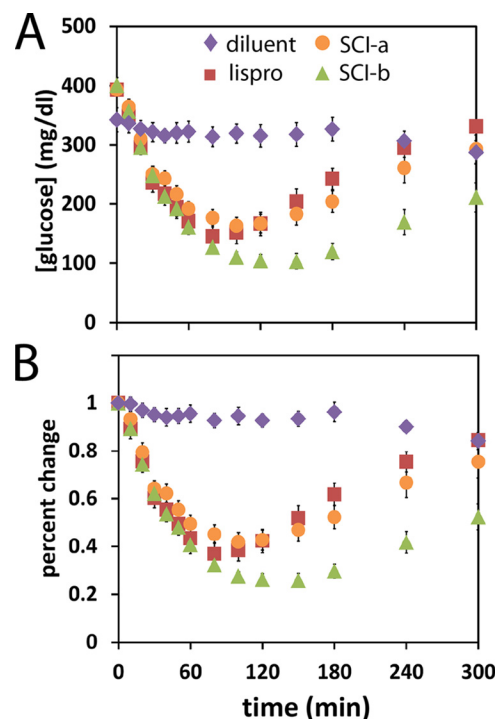


Figure 2. Rat studies of fresh insulin analog solutions. Shown are rat studies of insulin action following i.v. injection of 1.7 nmol/300-g rat of the specified SCI or control. A, blood-glucose concentration; B, percent change relative to initial blood-glucose concentration. Symbols are defined as inset in A. Samples sizes: (diluent control) $n = 12$; (insulin *lispro*) $n = 13$; (SCI-a) $n = 13$; and (SCI-b) $n = 10$.

0.71 Å (Table S14) with a range of 0.60–0.95 Å. Each dimer likewise conformed to a classical T₂ dimer (Fig. 3B). Relative to reference dimers in two prior crystal structures, the mean main-chain r.m.s.d. was 1.10 Å with range 0.96–1.33 Å (Table S1B, left). Higher r.m.s.d. were obtained on comparison of the SCI-a hexamer with classical insulin hexamers (Table S1B, right), reflecting a novel change in dimer–dimer orientation relative to zinc insulin hexamers (1). This observation, as well as the potential presence of magnesium ion coordination (Fig. 3, D and E), is further evaluated under “Discussion” (below).

The absence of C domain electron density suggests its flexibility. To probe such apparent disorder, SCI-b was prepared as a monomeric model. Whereas ¹H NMR spectra of SCI-a exhibited concentration-dependent resonance broadening (Fig. S2, A–D), the spectrum of SCI-b exhibits narrow resonances even at a concentration of 1 mM (Fig. S2E). The solution structure and dynamics of SCI-b are described in our accompanying article (8).

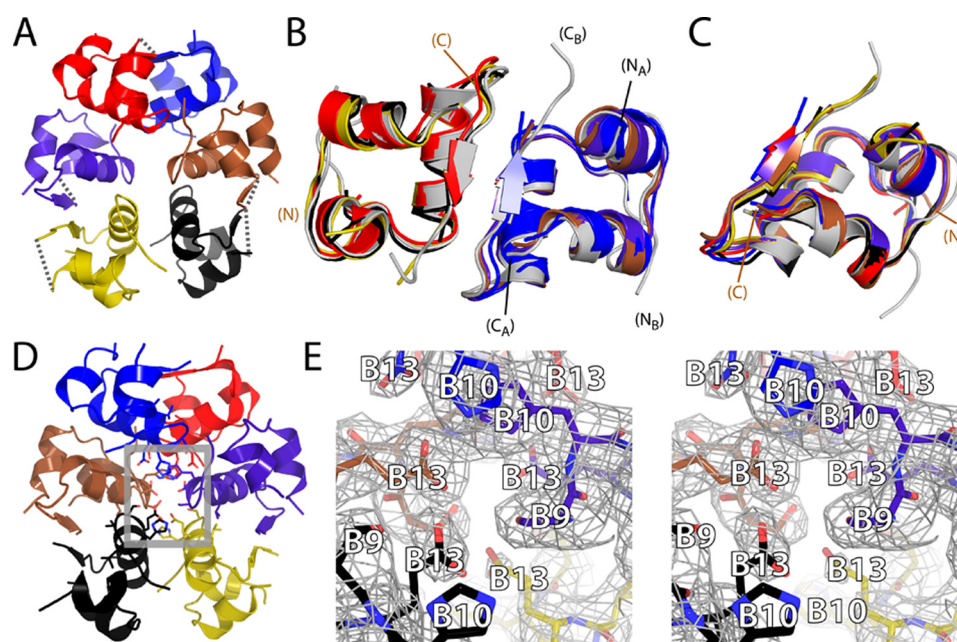


Figure 3. SCI-a crystal structure and electron density map. *A*, ribbon model of novel zinc-free hexamer. Electron densities of the B1–B4, B27–B30, and C1–C6 segments were poor in all SCI-a monomers; gray dashed lines (connecting α -carbons) in each monomer denote missing C domains in the hexamer. *B* and *C*, backbone superposition of all SCI-a dimers (color scheme as in *A*) aligned according to the main-chain atoms of residues A1–A8, A13–A19, and B9–B19 (helices) of the 4INS dimer (gray) (*B*) and all SCI-a monomers aligned according to the above main-chain atoms in 4INS monomer 1 (gray) (*C*). N_A , C_A , N_B , and C_B labels respective chain termini of the A and B chains of 4INS; the termini of one SCI-a monomer (brown) are likewise labeled by *N* and *C*. Disulfide bonds are not shown. *D*, SCI-a hexamer with residues B9, B10, and B13 within hexamer core displayed as sticks. The gray boxed region is expanded in the stereo view of the core in *E*. *E*, stick representations of residues B9–B13 and underlying electron density ($2m|F_o| - D|F_c|$; gray wire frame). Side-chain labels are provided as white text with black outlines. Atom colors: pink/red, oxygen, and blue, nitrogen; carbons are colored as in respective monomers in *D*.

Table 2
Crystallographic data collection and refinement statistics for SCI-a

Data collection	
Source	SSRL BL7-1
Wavelength (Å)	1.12709
Space group	P2 ₁
Unit cell dimensions	$a = 43.382$, $b = 85.771$, $c = 45.696$ Å
	$\beta = 110.89^\circ$
Resolution (Å)	38.22–2.80 (2.95–2.80) ^a
R_{merge} (%)	5.6 (30.9)
$I/\sigma I$	15.2 (3.6)
Completeness (%)	99.3 (96.0)
Redundancy	3.7 (3.5)
No. of reflections	7718
Refinement	
Resolution (Å)	38.22–2.80
R -factor/ R_{free} (%)	25.10/31.42
No. of protein atoms	2155
r.m.s.d. bond length (Å)	0.007
r.m.s.d. bond angle ($^\circ$)	1.098
Average B-factors (Å ²)	
Chain A	78.5
Chain B	79.5
Chain C	59.8
Chain D	69.1
Chain E	84.1
Chain F	54.4
Ramachandran plot (%)	
Most favored region	94.8
Additionally allowed regions	4.8
Generously allowed regions	0.4
Disallowed regions	0.0

^a Values in parentheses are for the highest resolution shell.

SCIs exhibited enhanced stability and resistance to physical degradation

Stability was assessed by CD-monitored guanidine denaturation. Free energies of unfolding (ΔG_U) were calculated based on two-state modeling (Table 3). SCI-a (ΔG_U 5.0($\pm$ 0.1) kcal/mol) is $\sim$ 2 kcal/mol more stable than insulin *lispro* (3.0($\pm$ 0.1)

Table 3
CD denaturation and fibrillation studies

Analog	ΔG_U^a	$m\text{-value}^b$	C_{mid}^c	Fibrillation lag time (days) ^d
	kcal/mol	kcal/(mol·M)	M	
WT insulin	3.3 $\pm$ 0.1	0.69 $\pm$ 0.01	4.8 $\pm$ 0.1	5.3 $\pm$ 0.5
<i>lispro</i>	3.0 $\pm$ 0.1	0.63 $\pm$ 0.01	4.7 $\pm$ 0.1	4.3 $\pm$ 0.2
SCI-a	5.0 $\pm$ 0.1	0.86 $\pm$ 0.01	5.8 $\pm$ 0.1	>140
SCI-b	4.7 $\pm$ 0.1	0.82 $\pm$ 0.01	5.7 $\pm$ 0.1	>110

^a Data are from two-state modeling of CD-guanidine titrations performed at 25 $^\circ$ C and pH 7.4.

^b The m -value (units of kcal/(mol·M)) is the slope of unfolding free energy ΔG_U versus molar concentration of denaturant.

^c C_{mid} is the guanidine denaturant concentration at which 50% of the protein is in the unfolded state.

^d SCI analogs at U-100 concentration were gently rocked in triplicate at 45 $^\circ$ C in modified Lilly[®] diluent containing Zn²⁺ ions, wherein Tris-HCl was substituted for sodium phosphate buffer. WT insulin and *lispro* fibrillation studies utilized gentle rocking at 45 $^\circ$ C of the pharmaceutical formulations Humulin[®] and Humalog[®], respectively; both are U-100 strength in Lilly[®] diluent. Error in associated lag times is the standard deviation of triplicate samples.

kcal/mol); under these conditions the stability of WT insulin was reported to be 3.3($\pm$ 0.1) kcal/mol, in accordance with prior studies (46). SCI-b was somewhat less stable than SCI-a ($\Delta\Delta G_U$ 0.3($\pm$ 0.2) kcal/mol) as expected based on its B28–B29 element (47). These assays predict that formulations of SCI-a and SCI-b would exhibit slower rates of chemical degradation (45).

Susceptibility to physical degradation, defined as aggregation-coupled misfolding without breakage of chemical bonds, was assessed through measurement of lag times in fibrillation assays (30, 45). The studies were performed in glass vials at a protein concentration of 0.6 mM (corresponding to WT U-100 formulations) at 45 $^\circ$ C with gentle agitation in the presence of an air–liquid interface. Such conditions mimicked insulin solu-

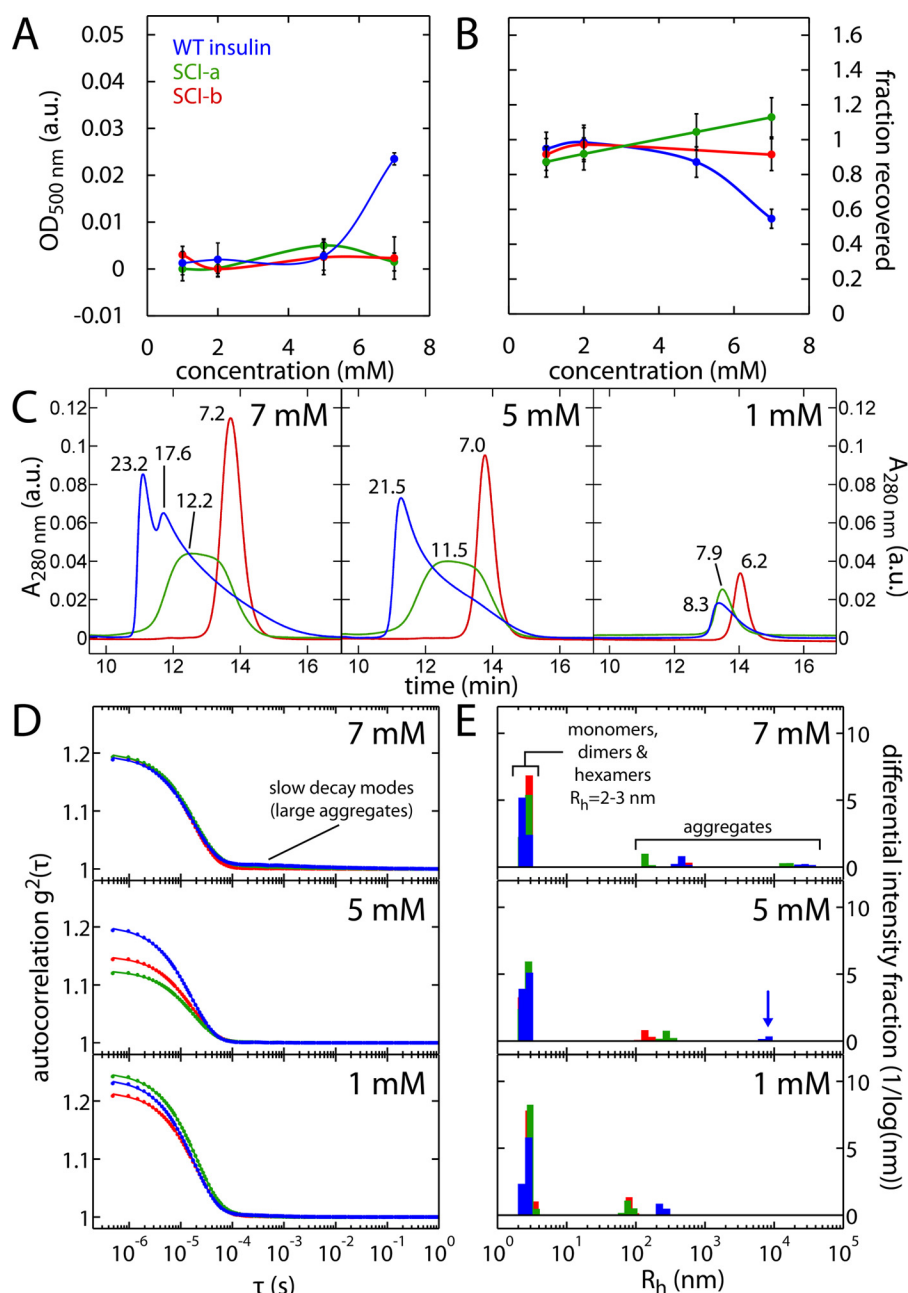


Figure 4. Studies of protein self-assembly and aggregation. A, concentration dependence of optical absorbance at 500 nm (turbidimetry (78)); insulin analogs were dissolved in Tris-HCl buffer (pH 7.4) containing zinc, phenol, and *meta*-cresol. Error bars represent the standard deviation of four measurements. B, studies of protein solubility; rp-HPLC-based estimates are presented as fraction of protein recovered after centrifugation. Error per measurement in B is fixed at 10% to reflect deviations between multiple injections of 10- μ g Humalog[®] standard (see “Experimental procedures”). C, SEC of insulin analogs at stated concentrations (7, 5, and 1 mM). Elution peaks are labeled with calculated masses (in kDa) as interpolated from a set of molecular mass standards (see under “Experimental procedures” and Fig. S5). D and E, dynamic light scattering. Field autocorrelation functions (D, solid lines are fits) and inferred size distributions (E) by the regularization method (91) for insulin analogs at stated concentrations. Blue arrow in E highlights presence of large aggregates of WT insulin at 5 mM. Color code: WT insulin, blue; SCL-a, green; and SCL-b, red.

tions transported in the developing world without benefit of a cold chain (32). Whereas WT insulin (Humulin[®]) and *lispro* (Humalog[®]) were observed to begin fibrillation within a few days, fibrillation of SCIs was not observed despite prolonged thermal stress (Table 3).

SCIs exhibited enhanced solubility with decreased aggregation

To assess solubility and aggregation, SCIs in Zn²⁺-containing solutions compatible with pharmaceutical formulation

were studied by turbidimetry (Fig. 4A) and rp-HPLC (Fig. 4B). WT insulin at pH 7.4 began to precipitate between 5 and 7 mM. Whereas at 7 mM <60% of WT insulin was HPLC-detectable after centrifugation (Fig. 4B), the SCIs at 7 mM were fully recoverable.

Protein aggregation was probed by size-exclusion chromatography (SEC; Fig. 4C). Samples were made 1–7 mM and diluted on entrance to the Zn²⁺-free mobile phase and SEC matrix. As this simulates dilution and clearance of formulation buffer upon subcutaneous injection, zinc-coordinated species

such as hexamers are not observed (46). At 5–7 mM WT insulin eluted as a heterogeneous mixture of oligomers with masses between those of the dimer (~12 kDa) and hexamer (~35 kDa); peaks were observed at 23.2 and 17.6 kDa. At 1 mM, the elution time corresponded to a mass of 8.3 kDa, providing evidence of a monomer–dimer equilibrium. Although also heterogeneous, SCI-a at 5–7 mM was less polydisperse than WT insulin; its elution time corresponding to a mass of 12.2 kDa, close to that predicted of an SCI dimer. At 1 mM, SCI-a yielded an elution profile similar to that of WT insulin. SCI-b eluted as a monomer (6.2–7.2 kDa) at all concentrations tested. For all samples, no V_0 peak corresponding to elution of aggregates larger than the column molecular weight range were observed.

We further evaluated the distribution of protein aggregates in the presence of zinc-containing formulation buffer by dynamic light scattering (DLS; Fig. 4, *D* and *E*), which is more sensitive to large aggregate formation than SEC. All analogs form hexamers in the above concentration range, as indicated by the presence of hydrodynamic radii (R_h) between 2 and 3 nm. Although it was difficult to distinguish the DLS signatures of insulin monomers and dimers (R_h ~1–2 nm) from that of hexamers (R_h = 2.8 nm) due to similar translational diffusion rates (48), at 7 mM both WT insulin and SCI-a exhibited a combined monomer–dimer–hexamer signal.

WT insulin also formed large aggregates with radii ~1000 and 10,000 nm, the signal from such higher-order aggregates was attenuated in the SCI solutions as indicated by (i) rarer bursts in count rate associated with large aggregates at each protein concentration, and (ii) comparative studies at 5 mM at which only WT insulin forms micron-sized species. Smaller aggregates (R_h ~100–150 nm) were weakly detectable in all 1 mM solutions, in part due to the disproportionate sensitivity of DLS to larger aggregates relative to smaller assemblies (48).

SCIs were refractory to thermal inactivation

To assess thermal inactivation, an SQ rat assay was employed to test activities of protein solutions following gentle agitation at 45 or 75 °C (*upper panel* in Fig. 5*A*); conditions were as described for the fibrillation lag-time assay (above). Fractional areas under the curve (AUC; *lower panel* at center in Fig. 5*A*) were extracted to estimate fractional changes (*upper right-hand panel*) and percent maintenance of activity (*lower right-hand panel*). Results demonstrated that the SCIs exhibited prolonged retention of activity under conditions wherein current clinical analogs degraded (Fig. 5, *B–E*). Thus, SCI-a and SCI-b both remain fully active when incubated at 45 °C with gentle agitation for 140 days (SCI-a) and 111 days (SCI-b), whereas essentially complete inactivation of standard analogs insulin *lispro* (after 5 days) and insulin *glargine* (after 11 days) was observed (Fig. 5, *B* and *D*). Similar trends were obtained at 75 °C (Fig. 5, *C* and *E*).

SCIs exhibited no increase in mitogenic signaling relative to WT insulin

Cellular signaling was assessed in two cell lines: rat L6 myoblasts overexpressing insulin receptor isoform A (L6-IRA (49)) and human MCF-7 breast cancer cells (50); the latter expresses

human IGF-1R and to a lesser extent IR isoforms A (IR-A) and B (IR-B) (50). Following exposure to 100 nM hormone (WT insulin, SCI, or control analogs; *top left* in Fig. 6*A*), transcriptional activation of mitogenic/proliferative pathways was assessed by real-time quantitative PCR (rt-qPCR; schematic at *bottom right* in Fig. 6*A*). In addition, ligand-dependent IR tyrosine phosphorylation and Akt serine phosphorylation were probed by Western blotting as respective probes of receptor activation and post-receptor signaling (*top right* in Fig. 6*A*; Ref. 51). A flowchart describing these assays and their rationale is provided in Fig. S3. These blots were performed to verify that analogs inducing reduced mitogenic transcriptional activation still execute a significant insulin-stimulated response from the L6-IRA and MCF-7 cells.

In the rt-qPCR assay mitogenic analogs Asp^{B10}-insulin (labeled “X10”) and IGF-I (34) effected a reduction in cyclin G2 transcription with an increase in cyclin D1 mRNA accumulation (Fig. 6, *B* and *D*). By contrast, WT insulin and *lispro* exhibited decreased cyclin D1 activation and cyclin G2 repression. The rt-qPCR signatures of SCI-a and SCI-b resembled WT insulin and *lispro* and were distinct from the rt-qPCR signatures of Asp^{B10}-insulin or IGF-I (Fig. 6, *B* and *D*). Western blotting revealed that the SCIs nonetheless stimulated IR and Akt phosphorylation to an extent similar to or greater than WT insulin or *lispro* (Fig. 6, *C* and *E*; see also Fig. S4).

Discussion

Proinsulin, the biosynthetic precursor of insulin, is a single chain with domain organization B–C–A (3). Flanked by dibasic sites, the 35-residue C domain is excised by prohormone convertases in pancreatic β -cells, yielding the mature two-chain hormone (52). Vertebrate C domains vary in length (30–35 residues) and are less well conserved than are the A and B domains (Table S3) (53). Although proinsulin binds to IR isoforms more weakly than does WT insulin (relative affinity 5%) (54), the precursor retains substantial biological activity *in vivo* (55). Its therapeutic efficacy was evaluated in the 1980s as a partial hepatoselective agonist (55). Although clinical trials were terminated due to excess cardiovascular deaths (55), these data provided proof of principle for an SCI-based regimen.

Our interests have focused on insulin analogs that are less susceptible to physical and chemical degradation than WT insulin or current clinical analogs (32). Such degradation is exacerbated above room temperature (31, 45), leading to guidelines from the United States Food and Drug Administration regarding the distribution, storage, shelf life, and disposal of pharmaceutical formulations (56). A particular problem is posed by the susceptibility of two-chain analogs (*i.e.* all current insulin products) to fibrillation (31). Because creation of an amyloidogenic nucleus (“seeds”) can occur on their transient exposure to moderately high temperatures (31) and because such seeding can accelerate fibrillation even in refrigerated solutions (57), distribution of insulin formulations presently requires a complex and costly cold chain (32, 43). Implications for global health have been deepened by an emerging pandemic of type 2 diabetes mellitus (32, 43).

In this and our accompanying article (8), we have investigated the structure and function of an ultra-stable SCI with a six-residue

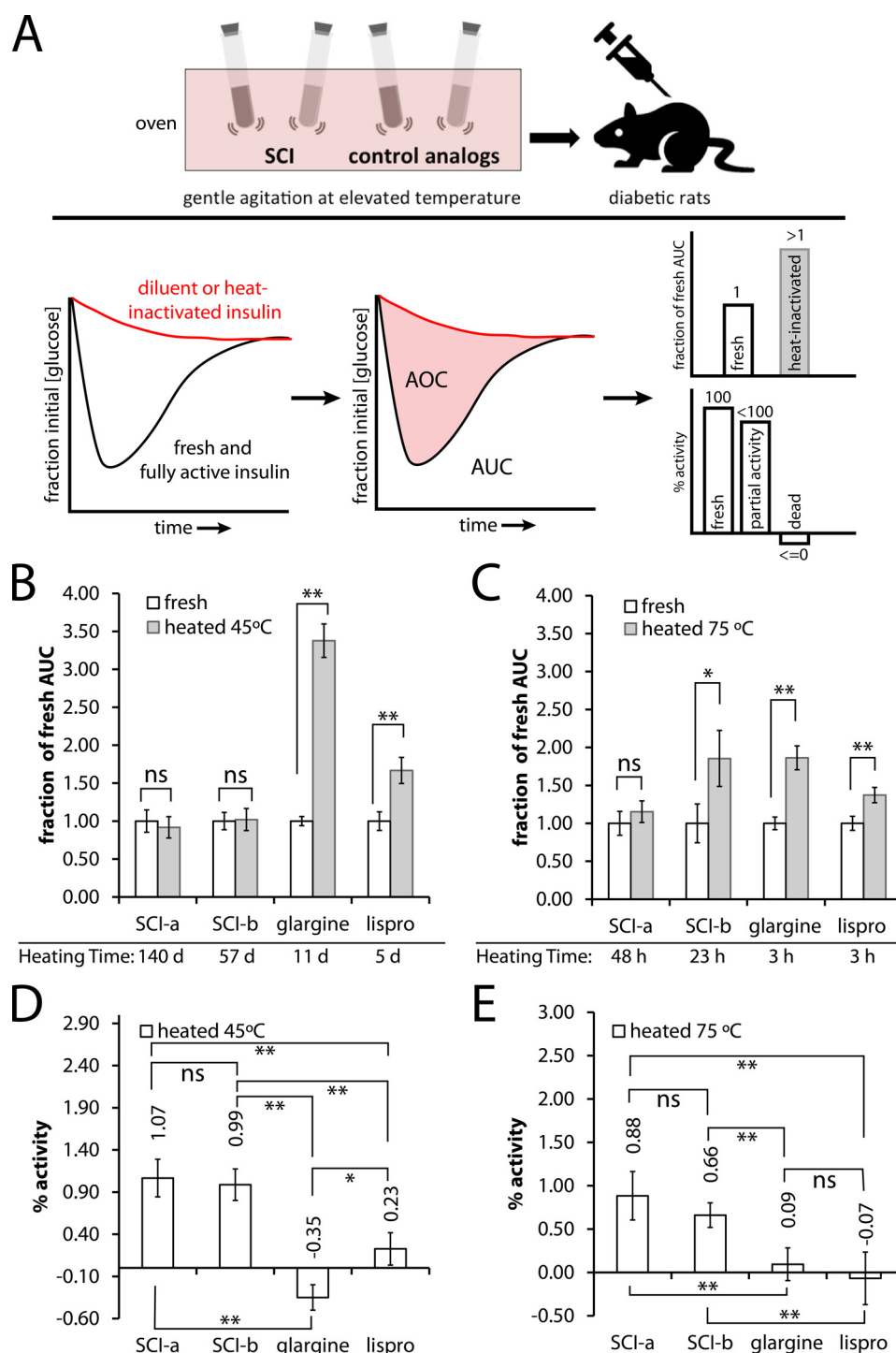


Figure 5. Rat studies of heat-stressed insulin analogs. *A*, schematic diagram of the thermal inactivation assay wherein formulations of insulin at U-100 (0.6 mM) in septum-sealed glass vials are gently rocked in an oven set to either 45 or 75 °C. At the indicated times, the vials were removed followed by dilution and SQ injection into diabetic rats to assess residual potency. *Bottom panel of A* shows the resulting glucose-response curves for a fully active (black) and completely dead analog (red), the latter of which would give an identical curve to a diluent-only control. These curves are integrated and processed to provide AUC and AOC. These metrics provide fractional AUC, a reporter of significant differences between the fresh and heated analog data, and percent activity, which illustrates degree of inactivation following heating. Fractional AUC for the stated fresh (white) or heated (gray) analogs are given for 45 °C (*B*) and 75 °C (*C*) experiments. Heated incubation times are given below the appropriate analog name. Percent (%) activities for the experiments in *B* and *C* are given in *D* and *E*. *, $p < 0.1$; **, $p < 0.05$, ns, not significant ($p > 0.1$). Sample sizes for each SQ experiment were $n = 4-5$ rats and analog doses ranged from 3.0 to 6.0 nmol/300-g rat. Doses in this range do not produce significantly different glucose-response profiles. The dose and sample size of any given analog set was the same for fresh and heated injections.

C domain. The connecting domain sequence (EEGPRR) contains offsetting negative (*bold*) and positive (*italics*) charges. The former element was introduced to disfavor binding to IGF-1R, a

receptor associated with mitogenic signaling (58), whereas the latter (mimicking the natural dibasic site) was intended to interact with Glu^{A4} (29) to stabilize the adjoining A domain

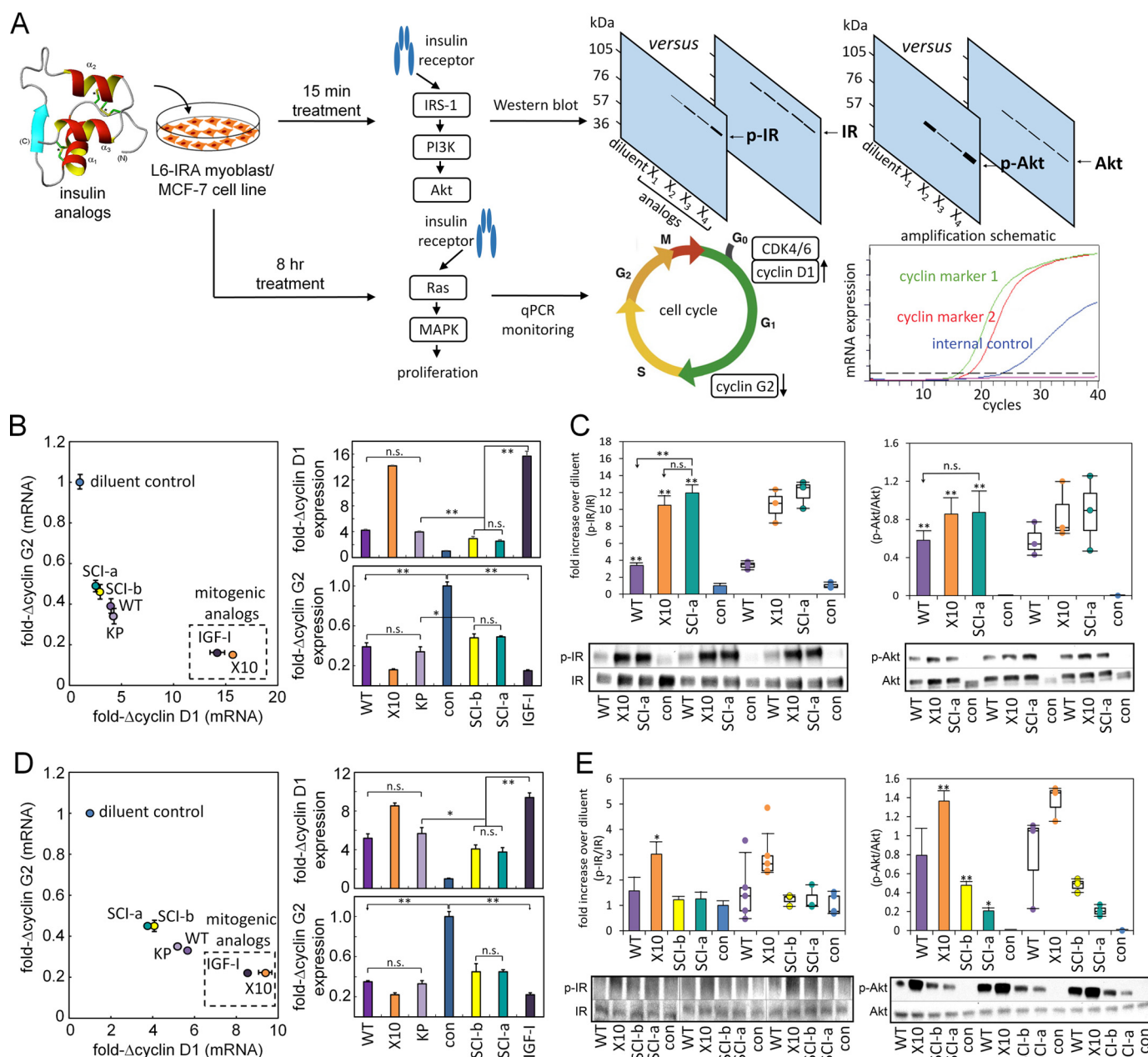


Figure 6. Cell biological assessment of insulin signaling. A, schematic outline of cell-based assays for the assessment of hormone-induced IR-A signaling and activation of mitogenic pathways. L6 rat myoblasts stably expressing human IR isoform A (IR-A) (developed by De Meyts and co-workers (49)) or MCF-7 human breast cancer cells expressing both IR isoforms (IR-A and IR-B) and high levels of IGF-1R (50) were treated with SCI analogs or control two-chain analogs. After 15 min of treatment, Tyr-phosphorylation of IR and Akt was assessed by Western blotting (51) (upper flowchart). To analyze relative mitogenic potencies, L6-IRA or MCF-7 cells treated for 8 h were collected, and the transcriptional response of cell proliferation markers cyclin D1 and cyclin G2 (cell cycle schematic adapted from Ref. 102) was assessed by rt-qPCR (schematic adapted from Bio-Rad). The increased accumulation of cyclin D1 and decreased cyclin G2 mRNA served as readouts for insulin-induced activation of cellular proliferation pathways (bottom flowchart). B, assessment of insulin-driven cyclin D1 and G2 transcription in L6-IRA cells; known mitogenic analogs (dashed box) yield increased accumulation of cyclin D1 mRNA and repression of cyclin G2. Brackets designate *p* values: *, <0.05, or **, <0.01; *ns* indicates *p* values >0.05. C, histogram representation of the average fold increase over diluent and dot-plot representation of the actual fold increase over diluent for each data point collected from L6-IRA Western blottings of p-IR/IR (left) and histogram average and actual dot-plot representations of the ratio of p-Akt to total cell Akt obtained from L6-IRA blots of p-Akt/Akt (right). D and E, studies in a human breast cancer cell line. MCF-7 cell-derived rt-qPCR (D) and phosphorylation Western blot data (E) are presented as in B and C. Histograms: *n* = 3; ± standard error. ** or * indicates *p* value < 0.01 or < 0.05. Whiskers for the dot-plot follow Tukey's box plot method: lowest data point within 1.5 Interquartile Range (IQR) of the lower quartile, and the highest data point within 1.5 IQR of the upper quartile. Abbreviations: Con, diluent control; X10, Asp^{B10}-insulin; WT insulin; KP, insulin *lispro*.

α-helix (residues A1–A8), an unstable “Achilles’ heel” in the mechanism of insulin fibrillation (30). The central Gly^{C3}–Pro^{C4} element (underlined above) was intended to promote flexible chain reversal. SCI-a also contained substitutions in the A and B domains intended to augment stability, solubility, and receptor binding in accordance with previous studies of two-

chain analogs (Table 1) (36, 59). Substitution of His^{B10} by Asp, incorporated in a previous SCI design (29) to augment stability and activity (SCI-c in Fig. 1D), was excluded due to its association with elevated mitogenicity and carcinogenesis in rats (34, 60). Because these adverse effects may, at least in part, reflect the enhanced affinity of Asp^{B10}-insulin for the IR iso-

forms and IGF-1R (relative affinities >200%) with increased residence times (34), our design criteria tolerated relative affinities <100% in cell-free assays provided native potency was maintained *in vivo*.

Previous efforts to engineer ultra-stable insulin analogs encountered a puzzling prolongation of signaling on i.v. bolus injection (Fig. S1) (33). Such extended duration of activity was observed, for example, in otherwise elegant studies of two-chain analogs stabilized by a fourth non-canonical disulfide bridge (33). This PD perturbation presumably reflected an alteration in cellular events that mediate termination of insulin signaling. Analogous perturbations have not been observed in studies of current long-acting insulin products, whose protracted action reflects conventional PK properties rather than delayed signal termination by target cells (61). Irrespective of molecular mechanism, such prolonged signaling would preclude use in insulin pumps and raise safety concerns on chronic administration (42).

Unlike the above analog (33) and our prior SCI prototype (sequence SCI-c at bottom in Fig. 1D (29, 62)), i.v. bolus injection of SCI-a in streptozotocin-induced diabetic rats was found to reproduce the PD profile similar to that of insulin *lispro* (Fig. 2). Modification of the B domain in SCI-b (intended as a monomeric NMR model; see accompanying article (8)) nonetheless conferred a delayed “tail” in blood-glucose recovery. This tail exhibited greater prominence in SCI-c (Fig. S1D). Biochemical principles relating SCI sequence to duration of action *in vivo* are not presently understood. The native-like PD properties of SCI-a motivated studies of its structure, stability, and possible mode of receptor binding as discussed in turn.

Crystal structure reveals native-like subunits in novel hexamer

The compatibility of the canonical insulin fold with direct B-A peptide bonds or with C domains of variable length reflects the native proximity of the C terminus of the B domain and N terminus of the A domain (25, 26, 63). Although Thr^{B30} is disordered in some crystal structures (37) and exhibits motional narrowing in solution structures of engineered insulin monomers (12), in a subset of crystallographic protomers a salt bridge has been observed between the C-terminal B30 carboxylate and the N-terminal amino group of Gly^{A1} (1). We thus anticipated that the crystal structure of SCI-a would depict native insulin folds with preservation of a canonical dimer interface.

Crystallographic analysis of SCI-a at moderate resolution (2.8 Å) indeed revealed an ordered and native-like α -helical subdomain (spanning residues B4–B19 and A1–A21); its dimer interface (including dimer-related antiparallel β -sheet; B24–B28 and B28'–B24') was similar to that of WT insulin (37). Unusually, the structure was obtained in the absence of zinc ions and presence of high concentrations of magnesium ions. The only existing zinc-free insulin hexamer crystallized *de novo* required Glu^{B13} → Gln substitution to alleviate electrostatic repulsion within the hexamer (64). Near-complete extraction of zinc ions from WT T₆ hexamers on soaking 2-Zn insulin crystals with EDTA yielded native-like hexamers (65). Neither of these prior structures anticipated the shift in dimer–dimer orientations observed in the present crystal structure.

SCI-a by contrast retains Glu^{B13}. Although metal-ion density in the SCI-a hexamer core (Fig. 3, D and E) was not observed due to either diffraction resolution limitations or ion static or dynamic disorder, magnesium coordination or binding is possible. Packing defects among His^{B10} and Glu^{B13} side chains were observed (top half of Fig. 3E). It is possible that protonation of the B10 imidazole rings may offset the negatively charges of Glu^{B13}; the side chains of Ser^{B9} may also participate in stabilizing hydrogen bonds (lower half of Fig. 3E). It would be of future interest to measure the pK_a values of His^{B10} and Glu^{B13} in such a metal-ion-free hexamer to allow their protonation states to be inferred. The free space surrounded by these negatively charged and polar residues could be occupied by an interacting magnesium cation, magnesium hydrate, or possibly hydronium molecules.

Evidence for disorder in the crystal state was provided by qualitative features of the electron-density maps. In this crystal form the asymmetric unit was the hexamer itself, and so three independent views of the SCI dimer were obtained, in turn providing six views of the C domain. Each dimer exhibited a canonical interface with discontinuous electron density between B27 and A1. In most monomers, density in this region was not interpretable. An exception, monomer D included some density for residues B28–B29 and C6. Although static disorder in the lattice can in principle contribute to such attenuated or discontinuous electron density, the consistency of these findings motivated design of SCI-b to enable NMR studies as described in our accompanying article (8).

The structure of the SCI-a hexamer exhibits a novel change in dimer–dimer orientations relative to canonical T₆, T₃R₃^f, and R₆ zinc-insulin hexamers (structures given in Fig. 1A) (9, 19, 37, 64). Specifically, the trimer interface (Fig. 7), which is composed of the three independent dimers (AB, CD, and EF), is unprecedented. With alignment of AB dimers (Fig. 8, A–C), the other two dimers (CD and EF) in SCI-a are rotated about 24–26° (Table 4) relative to their usual orientation in T₆ insulin. This large deviation results in high pairwise r.m.s.d. between SCI-a and classic T₆ hexamers (Table S1B, right). Alignment of EF dimers or CD dimers (Fig. 8, D and E, and Table 4) produces similar transformations for the remaining dimers, although the axis of rotation/translation shifts away from the dimer center of mass. The average rotational deviation of non-aligned dimers (Table 4) is 28(±4)°. Whereas WT T₆ hexamers are symmetric, the SCI-a hexamer is asymmetric as shown by alignment of dimer AB to CD of a replicated structure (Fig. 8F).

It is possible that the zinc-free crystal structure of SCI-a (schematically represented as ellipsoids in Fig. 9B) is of biological relevance. Proinsulin translation and oxidative folding occurs in the ER of β -cells (Fig. 9A, left). After folding and upon (i) transit through Golgi apparatus and (ii) packaging into immature secretory granules (66), the C domain is cleaved by specific prohormone convertases (52) at dibasic sites in the BC and CA junctions. The mature hormone is stored (Fig. 9A, middle) as Zn²⁺-stabilized hexamers within specialized secretory granules (67), which dissociate upon secretion into the portal circulation (Fig. 9A, right). SCI-a is the first Glu^{B13}-containing insulin to form zinc-free hexamers *de novo*. Building upon this

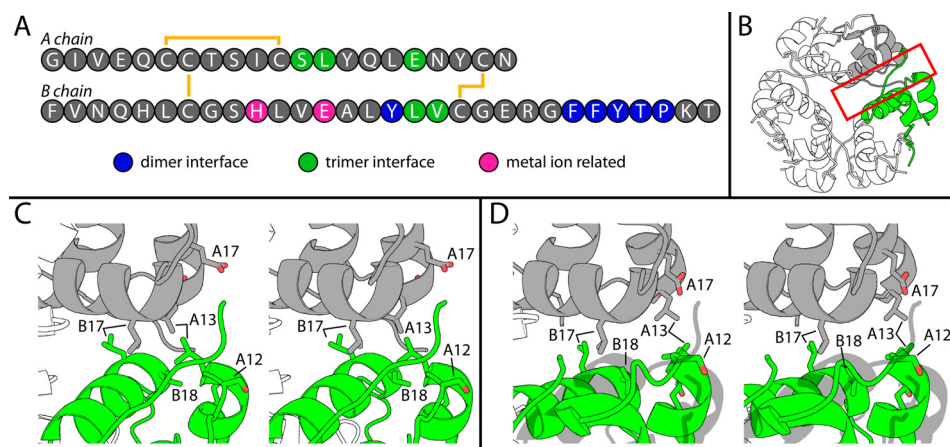


Figure 7. Self-assembly surfaces of insulin and novel SCI-a hexamer. A, color-coded sequence of WT insulin. Residues at dimer interface (blue), trimer interface (green), or metal ion binding in the hexamer (pink) are highlighted. B, classical 4INS T_6 hexamer with trimer interface of monomer B (gray) and monomer C (green) boxed in red. C, stereo view of red-boxed region in A with residues at the trimer interface (B17, B18, A12, A13, and A17) from both protomers shown as sticks. D, stereo view of SCI-a trimer interface with appropriate residues highlighted as sticks. 4INS monomer C is a gray ghost to highlight its rotation relative to its counterpart protomer C in SCI-a. For this, dimer AB of 4INS was aligned to dimer AB of SCI-a. Color code: red, oxygen; blue, nitrogen; carbons are colored as in schematic monomer representations.

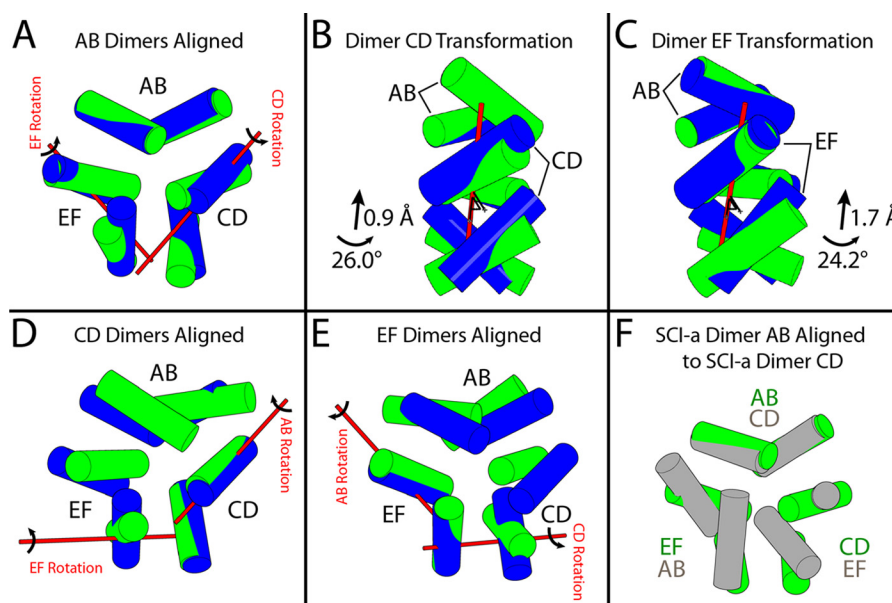


Figure 8. Structural relationship between SCI-a hexamer and classical T_6 zinc hexamer. Shown are minimized cylindrical representations showing only the B9–B19 helices of SCI-a (green) and T_6 human insulin (PDB code 1MSO (37); blue). A, alignment of dimer AB of SCI-a to dimer AB of 1MSO via the backbone of all three native helices. Red bars drawn in 3D denote the axis of rotation about which dimers CD and EF must independently be translated and rotated to align to the classic T_6 arrangement of dimers CD and EF in 1MSO. B and C, enlarged views of alignment in A with independent alignment transformations for dimers CD and EF, respectively. Alignment via main-chain atoms of the three α -helices and their dimer-related mates within dimer CD to CD (D) and dimer EF to EF (E); independent alignment vectors for remaining dimers (red bars) are illustrated in A. F, dimer CD of an SCI-a copy was aligned to dimer AB of the original (green).

observation, it is possible that proinsulin forms zinc-free hexamers in the ER and/or Golgi where zinc content is typically low ($[Zn^{2+}]_{ER} = 0.9 \mu M$ and $[Zn^{2+}]_{Golgi} = 0.6 \mu M$ in HeLa cells (68)) to enable zinc-dependent self-assembly. The novel dimer orientations (mean $\sim 28^\circ$ rotation relative to classic T_6 dimers) in the SCI-a hexamer, which is zinc-free but may contain magnesium ions that are either disordered or unseen at $2.8 \text{ \AA}$ resolution, may reflect those within the hypothetical zinc-free proinsulin hexamers in the ER and/or Golgi (Fig. 9B) that, upon entry into secretory granules via the ZnT8 transporter (67) and coordination to Zn^{2+} , acquire the dimer orientations of classic T_6 insulin (Fig. 9C).

Table 4

Orientation of SCI-a dimers relative to T_6 hexamers

Aligned dimer ^a	Transformed dimer ^b	Rotation	Translation
		$^\circ$	$\text{\AA}$
AB	CD	26.00	0.90
AB	EF	24.20	1.70
CD	AB	24.06	1.63
CD	EF	33.58	0.53
EF	AB	26.10	0.00
EF	CD	33.67	0.37
Mean ($\pm$ S.D.)		27.9 (± 4.5)	0.9 (± 0.7)

^a "Aligned dimers" in SCI-a were first aligned to the same dimer in T_6 human insulin (PDB code 1MSO) by the backbone atoms of helices A1–A8, A13–A19, and B9–B19.

^b Transformations necessary to align the "Transformed Dimer" in SCI-a to its counterpart in 1MSO were calculated in PyMOL and are presented as degrees rotation and angstrom translation (along the axis of rotation).

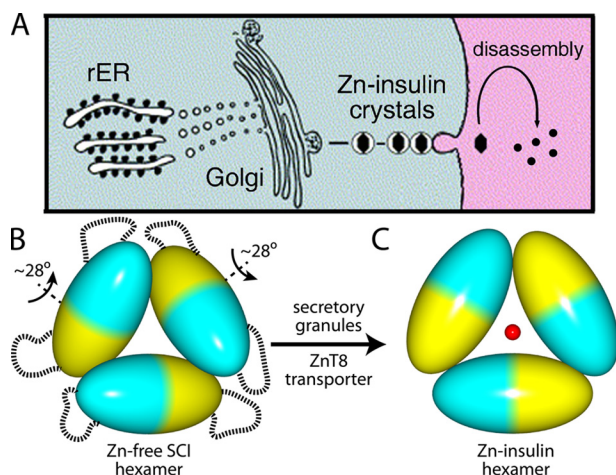


Figure 9. Insulin self-assembly in β -cell-specific biosynthetic pathway. A, proinsulin biosynthesis in the β -cells begins in the rough endoplasmic reticulum (rER) (left) followed by trafficking through the Golgi apparatus (middle) and storage into zinc-insulin granules, ultimately leading to glucose-regulated secretion with disassembly in the bloodstream (right). B, ellipsoid model of SCI-a zinc-free hexamer. Each ellipsoid represents an insulin dimer; monomers are denoted by blue or yellow. Dashed lines indicate disordered C domains on hexamer surface. Each ellipsoid is rotated an average of 28° relative to the bottom ellipsoid (see Table 5). C, corresponding model of classical T_6 zinc insulin hexamer with two axial zinc ions (red sphere; the second axial zinc ion is hidden directly behind sphere). Packaging of WT insulin hexamers into zinc-containing storage vesicles is enabled by granule-specific zinc transporter ZnT8 (67).

SCI might bind to the receptor via threading of α CT

In accordance with an extensive prior literature (19, 20, 22), recent crystallographic studies of insulin bound to a domain-minimized insulin micro-receptor (μ IR) demonstrated that the B24–B27 segment of insulin is displaced from its classical packing against the A1–A8 and B9–B19 α -helices (17, 18). Effected by changes in Ramachandran dihedral angles flanking Phe^{B24}, this “unhinging” (by $\sim 60^\circ$ relative to classical crystal structures of the free hormone dimer or hexamer (17, 18)) enables conserved non-polar surface (spanned by the side chains of Ile^{A2}, Val^{A3}, Phe^{B24}, and Phe^{B25}) to engage the receptor (17, 18). An extended B24–B27 segment lies in a groove between receptor elements L1 and α CT (17, 18). Residues B28–B30, not well conserved among vertebrate insulins and dispensable for activity (1), were presumed to be disordered in the μ IR complex. Accordingly, these three residues would amplify the orientational freedom of the C domain as a flexible tether between Thr^{B27} and Gly^{A1} at the hormone– μ IR interface.

To enable IR binding, we speculate that the B domain of SCI-a undergoes a change in conformation similar to that observed in WT insulin on μ IR binding (residues B23–B27; *black strand* in Fig. 10A). An essential feature of this co-crystal structure is the reposition of α CT (*purple ribbon* in Fig. 10A) over the surface of L1 (*powder blue*) to occupy space ordinarily taken (in insulin’s free conformation) by the C-terminal segment of the B chain (*green strand*). In the WT insulin– μ IR complex the B24–B30 segment must pivot both (a) to reposition conserved aromatic residues Phe^{B24}, Phe^{B25}, and Tyr^{B26} and (b) to expose the conserved aliphatic side chains of Ile^{A2} and Val^{A3} (data not shown). The C domain in the free hormone (*dashed line* in Fig. 10A), extended by flexible residues B28–B30, must therefore reposition to span the gap between Thr^{B27}

and Gly^{A1} at the μ IR interface (*red arrow* in Fig. 10A). Thus, we envision that in the bound state SCI-a would open a hole through which α CT would “thread.” A similar “threading model” may pertain to homologous IGF–IGF-1R complexes (69). To test the plausibility of this model, molecular dynamics simulations of an SCI bound to the μ IR complex are described in our accompanying article (8).

Enhanced stability predicts resistance to chemical and physical degradation

SCI-a exhibits enhanced thermodynamic stability relative to WT insulin ($\Delta\Delta G_U = 1.7$ kcal/mol as inferred from chemical-denaturation studies; Table 3). This estimate was based on two-state modeling (46), which may introduce baseline fitting artifacts in the presence of considerable resistance to denaturation. Nevertheless, the reliability of this method was corroborated by our companion NMR studies (8). Previous studies of the susceptibility of insulin analogs to chemical degradation suggested that such a change in ΔG_U predicts reduced rates of a variety of processes (breakage or rearrangement of disulfide bridges, scission of peptide bonds, deamidation of Asn, and formation of iso-Asp) that can impair activity and confer immunogenicity (45). Such processes are likely to be faster in the unfolded state than in the native state.

The SCIs demonstrated remarkable resistance to physical degradation as probed by fibrillation assays at elevated temperatures (Table 3) and associated rat studies of heat-stressed protein solutions (Fig. 5). Insulin fibrils (transmission electron micrograph in Fig. 10B), like pathological amyloid deposits in general (70), are thought to arise from nucleation by non-native aggregates (71). Lag times in kinetic studies of protein fibrillation reflect rare intermediate conformational fluctuations among distorted monomers, whose aggregation can provide amyloidogenic nuclei (Fig. 10C) (30). A variety of evidence suggests that splaying of the B chain C-terminal strand from the α -helical core promotes insulin fibrillation (29, 31, 71, 72). Whereas mature fibrils (*far right* in Fig. 10C) presumably represent the true thermodynamic ground state of insulin as a heteropolymer, the closed conformation of the B chain and its native self-assembly thus protect the hormone from physical degradation (*far left* in Fig. 10C).

We suggest that the foreshortened C domain in SCI-a and SCI-b provides a topological barrier to amyloid formation (29, 30). By delimiting the extent to which B30 may separate from A1, an SCI whose C domain is below a critical length imposes a partial or total block to nucleation (*blocked arrows* in Fig. 10C). This model assumes that in an insulin protofilament the A and B domains undergo a substantial $\alpha \rightarrow \beta$ transition in accordance with fiber diffraction studies (70). Because pro-insulin is susceptible to fibrillation, its long C domain (35 residues) must confer sufficient conformational flexibility to enable packing of the distorted A and B domains within the fibril’s cross- β assembly (73). It would be of future interest to determine a 3D structure of an insulin protofilament in relation to the free and μ IR-bound structures of a foreshortened SCI.

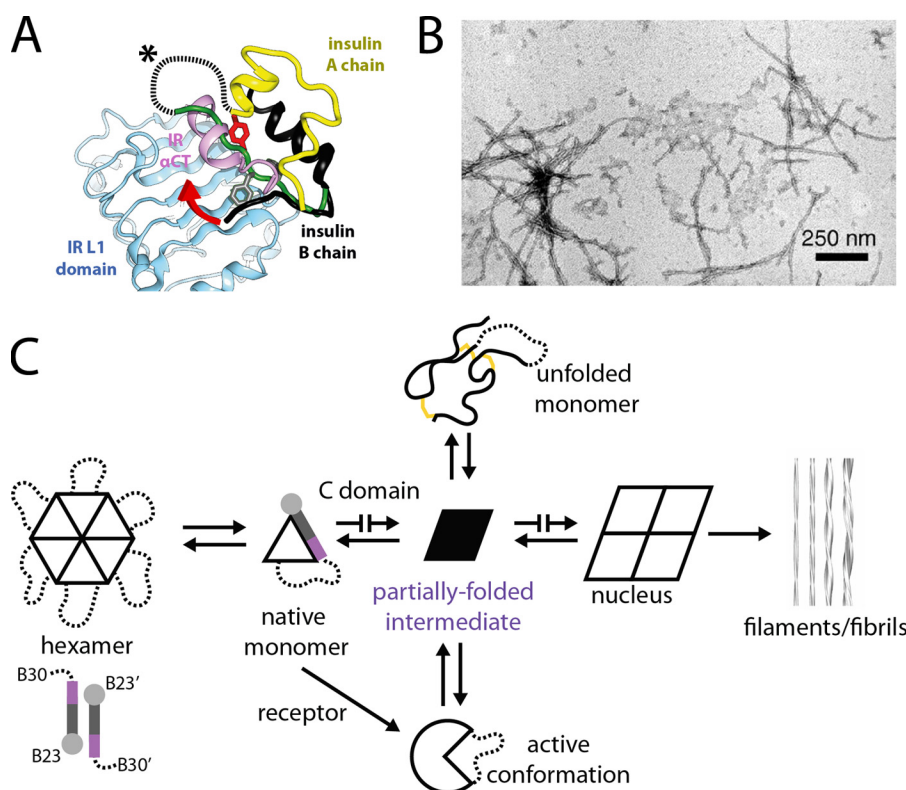


Figure 10. Open state of receptor-bound insulin and its implications for mechanism of fibrillation. *A*, crystal structure of WT insulin (A chain, yellow; B chain, black) bound to the μ IR (17). The B24–B30 segment (black; only observed up to B26) of WT insulin inserts between IR L1 (blue) and α CT (pink) domains (18). The classical (unbound) conformation of the insulin B chain is displayed in green; aromatic side chains B24–B26 in the unbound monomer are presented as sticks. Dashed line and asterisk indicate a schematic C domain in unbound SCI-a. The red embossed arrow indicates that the C domain of the bound SCI may wrap about the α CT segment of the IR in an SCI- α CT threading motif. This panel was adapted from Pandeyarajan *et al.* (90) with permission of the authors. This research was originally published in the Journal of Biological Chemistry. Pandeyarajan, V., Phillips, N. B., Rege, N. K., Lawrence, M. C., Whittaker, J., and Weiss, M. A. Contribution of Tyr^{B26} to the function and stability of insulin: structure-activity relationships at a conserved hormone-receptor interface. *J. Biol. Chem.* 2016; 291:12978–12990. © the American Society for Biochemistry and Molecular Biology. *B*, transmission electron microscopy image of mature insulin fibrils induced by heat (reproduced with permission from Ref. 72). This research was originally published in Journal of Biological Chemistry. Hua, Q. X., and Weiss, M. A. Mechanism of insulin fibrillation: the structure of insulin under amyloidogenic conditions resembles a protein-folding intermediate. *J. Biol. Chem.* 2004; 279:21449–21460. © the American Society for Biochemistry and Molecular Biology. *C*, schematic nucleation pathway of insulin fibrillation is mediated by aggregation of a distorted monomeric conformation (black trapezoid at center; adapted with permission from Yang *et al.* (30) (This research was originally published in the Journal of Biological Chemistry. Yang, Y., Petkova, A., Huang, K., Xu, B., Hua, Q. X., Ye, I. J., Chu, Y. C., Hu, S. Q., Phillips, N. B., Whittaker, J., Ismail-Beigi, F., Mackin, R. B., Katsoyannis, P. G., Tycko, R., and Weiss, M. A. An Achilles' Heel in an amyloidogenic protein and its repair: insulin dynamics, misfolding, and therapeutic design. *J. Biol. Chem.* 2010; 285:10806–10821. © the American Society for Biochemistry and Molecular Biology.); models of insulin protofilament and their higher-order assembly are shown at far right (reproduced with permission from the authors (70) (Jimenez, J. L., Nettleton, E. J., Bouchard, M., Robinson, C. V., Dobson, C. M., and Saibil, H. R. (2002) The protofilament structure of insulin amyloid fibrils. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9196–9201. Copyright (2002) National Academy of Sciences)).

SCI-a and IGFs exhibit similar structures but differ in mitogenic signaling

IGF-I and IGF-II are insulin-like peptides with ~40% sequence identity to insulin and similar 3D structures; their domain organization is similar to an SCI (74) with the addition of a C-terminal D region. Analogous to that of SCI-a in this study, crystal structures of IGF-I and IGF-II typically exhibit poorly defined density in the C region (69, 74), suggesting conformational flexibility or disorder. Furthermore, crystallization of IGF-I and IGF-II has required a binding partner, such as an IGF-binding protein, an IGF receptor fragment, anti-IGF antibody, or detergent molecule, presumably to prevent non-specific aggregation and reduce conformational flexibility of the monomer (74, 75). IGFs do not form ordered hexamers analogous to those of insulin due to Glu^{B10}, Lys^{B28}, and Pro^{B29} (residue positions 9, 27, and 28 in IGF-I). Glu^{B10} would be expected to block axial zinc coordination, whereas Lys^{B28} and Pro^{B29}

would impair dimerization as in insulin *lispro*. NMR studies of IGFs are discussed in our accompanying article (8).

The signaling activities of the SCIs (in comparison with those of WT insulin, Asp^{B10}–insulin, and IGF-I) were tested in (i) L6 rat myoblast cells with exogenous overexpression of human IR-A and (ii) MCF-7 human mammary adenocarcinoma cells (50) expressing high levels of IGF-1R and, to a lesser extent, IR-A and IR-B. The proliferative phenotype was correlated with representative gene-regulatory markers (encoding cyclin D1 and cyclin G2) as evaluated by rt-qPCR. The biological signature of cell-cycle progression, as probed by transcriptional activation (cyclin D1) or repression (cyclin G2), has previously been validated by De Meyts and co-workers (34) at the protein level; our assay monitored the analogous process at the level of mRNA accumulation. Because of its technical simplicity and reproducibility, we believe that the rt-qPCR approach enhances the utility of such assays to probe differences in mitogenic sig-

Table 5
Isoelectric points of WT insulin and SCI analogs

Analog	Isoelectric point (pI)	
	Predicted ^a	IEF ^b
WT insulin	5.4	5.8
SCI-a	4.9	5.2
SCI-b	4.8	5.1

^a Data were calculated using protein sequence and ProtParam.^b Data were as measured by IEF electrophoresis.

naling among insulin analogs (other families of growth factors). In the future, this approach may be extended from two genes to gene-regulatory networks.

SCI-a demonstrated no increased activation of mitogenic pathways relative to WT insulin and *lispro*. Notably, SCI-b showed a similarly low level of cyclin D1 accumulation (Fig. 6B) in both cell lines. Although not originally intended as a candidate therapeutic, these data suggest that SCI-b may also be safe for clinical use¹⁰ despite demonstrating delayed blood-glucose recovery (through unknown mechanisms) on i.v. injection (Fig. 2). These results correlate with (but are more informative than) *in vitro* receptor-binding affinities of SCIs for IR-A, IR-B, and IGF-1R (Table S2). This study has thus demonstrated novel ultra-stable insulin analogs that appear not to be mitogenic, raising confidence in their safety for potential human use.

Although biased agonists of G-protein-coupled receptors have been identified (76), such synthetic molecules have not yet been discovered in the context of receptor tyrosine-kinase networks, including the IR. Naturally-occurring biased agonists of IR include insulin, which favors Akt-mediated metabolic pathways, and IGF-II, which preferentially activates mitogen-activated protein kinase (MAPK)-mediated mitogenic pathways (51). An example of biased metabolic stimulation has been provided by a recent study of an insulin-mimetic peptide that favored activation of hepatic lipid metabolism in an obese rodent model of type 2 diabetes mellitus (77). As the search for metabolic-biased and/or non-mitogenic insulin therapeutics expands (2), this study has described a straightforward rt-qPCR method for screening the mitogenic signaling activities of novel analogs with minimal sample consumption. Furthermore, our results obtained from two cell lines (L6-IRA and MCF-7) suggest that the rt-qPCR method can be extended to other cellular models pertinent to insulin action, such as primary hepatocytes in culture. Future development of this technique may enable identification of analogs that preferentially stimulate Akt-mediated pathways relative to MAPK-mediated pathways.

Enhanced solubility and decreased aggregation may offer clinical advantages

The net negative charge of SCI-a at neutral pH was enhanced by substitutions Lys^{B29} → Glu and Tyr^{A14} → Glu (offset to a limited extent by His^{A8}). Its IEF-defined isoelectric point (Table 5) was reduced by ~0.6 units relative to WT insulin. This shift was associated with enhanced solubility (>7 mM by turbidimetry (78) and rp-HPLC (79)) in a buffer compatible with pharma-

ceutical formulation. Concentration-dependent precipitation of SCI-a or SCI-b was not observed, in striking contrast to WT insulin (~60% soluble at 7 mM; Fig. 4B). SEC studies suggested that, under conditions similar to those in an SQ depot (Fig. 4C), WT insulin in the concentration range of 5–7 mM forms a heterogeneous mixture of aggregates, whereas at these concentrations SCIs were predominantly dimeric (SCI-a) or monomeric (SCI-b). DLS likewise indicated that the SCIs are less prone than WT insulin to form large protein aggregates.

Aggregation and precipitation are significant general barriers to development of high-concentration solutions of therapeutic proteins. In the case of insulin there is a growing clinical need due to increasing prevalence of marked insulin resistance (defined as >200 units of insulin per day) (80). Such high insulin requirements are primarily encountered in obese patients but may also be associated with lipodystrophy and in pregnancy. Use of standard insulin formulations (0.6 mM) in such patients requires large injection volumes, which can be painful and inconsistently absorbed (80). Interest in ultra-concentrated insulin formulations has been further generated by innovative engineering efforts to miniaturize insulin pumps, including implantable devices for intraperitoneal delivery (80).

Unfortunately, aggregation of WT insulin at high concentrations markedly delays its SQ absorption (38). Thus, rather than being rapid-acting as a regular U-100 insulin formulation, Humulin® R U-500 (3 mM; Lilly) exhibits the prolonged PD properties of a twice-a-day microcrystalline insulin suspension (Neutral Protamine Hagedorn) due to delayed subcutaneous absorption (80). In small clinical trials, U-500 treatment of patients with marked insulin resistance was nonetheless associated with better glycemic control than was achieved in the same patients treated with U-100 formulations (81). Off-label use of U-500 in insulin pumps has also been described despite safety concerns regarding prolonged hypoglycemia following overdosage (80). These observations have led to the recent reformulation of insulins *lispro* and *aspart* (Asp^{B28}-insulin) at double the standard strength (U-200 or 1.2 mM (82)), near the limit of concentration as rapid-acting products.

Our results hold promise to the development of ultra-concentrated and yet rapid-acting insulin formulations. The remarkable solubility of SCI-a (≥7 mM) and its limited aggregation under these conditions in principle makes possible rapid-acting U-500 formulations and even strengths ≥U-1000. Such applications might enhance the efficacy and safety of multi-injection regimens in patients with marked insulin resistance and enable next-generation external and implanted insulin pumps (80).

Concluding remarks

The coming years will soon mark the 50th anniversary of the crystal structure of insulin (1969) and centennial of its discovery (1921). The intervening decades have been remarkable for the interplay between basic science and its translation. Pharmaceutical manufacture of recombinant human insulin, for example, has long exploited proinsulin or “mini-proinsulin” analogs as production intermediates (16, 25, 26). The latter exhibit more efficient folding and secretion in yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* than does proinsulin, enhancing

¹⁰ Our accompanying article (8) discusses the possible utility of SCI-b as a biphasic agonist with PD properties analogous to current premixed formulations.

fermentation yields (25, 27). Increased *in vitro* folding efficiency has likewise been described in a synthetic 49-residue “des-DI” polypeptide containing a peptide bond between residues B28 and A1 (63).

In this and our accompanying article (8), we have sought to extend the role of an SCI from manufacturing intermediate to therapeutic protein. This extension may provide an ultra-stable template by which to prolong shelf life and circumvent the global cold chain of insulin distribution, with particular utility in developing regions experiencing high year-round temperatures and sparse access to refrigeration (32). **Furthermore, the resistance of SCI-a to higher-order self-assembly at high protein concentrations may enable, for the first time, development of ultra-high-strength rapid-acting insulin formulations.** Such applications motivated foundational studies of structure and function with a focus on the identification of Achilles’ heels in the protein fold that, when exploited through conformational fluctuations, results in the formation of amyloid nuclei and fibrils that underlie physical degradation (30). Such weakness in native two-chain insulins is avoided with the foreshortened C domain of SCIs, which provides a topological barrier to amyloid fibril formation (29, 30), enhancing physical stability and thereby extending projected pharmaceutical shelf life.

The present findings have uncovered salient questions for further investigation. How flexible are connecting domains, and to what extent might the dynamics of the tether be coupled to conformational fluctuations in the globular portion of the structure? How do the structural and dynamic properties of an SCI enable its engagement by the insulin receptor? Finally, what controls the duration of signaling at the cellular level, and in particular why are some ultra-stable analogs associated with aberrant prolongation of signaling *in vivo*? This and our accompanying article (8) promise to provide a foundation for addressing these questions.

Experimental procedures

Construction of SCI expression vectors

A set of plasmids (pPICZ α) was constructed for expression in yeast *P. pastoris* directing secretion of SCI precursors into media (21, 83). A template plasmid for SCI precursors, designated pPICZ α TKSCI-c, was first constructed from a synthetic gene purchased from Genscript (Piscataway, NJ) encoding (from 5′ to 3′) the following: (i) an α -factor pre-pro-leader and Kex2-cleavage site (EKR) connected to (ii) spacer sequence (EEAEAEAEPK); both are N-terminal to (iii) the coding sequence of SCI-c. An XbaI site was included at the 3′-end to facilitate cloning; codon usage was optimized for expression in *P. pastoris*. The synthetic gene was cloned into the XhoI and XbaI sites (respective nucleotides 1185 and 1272) (84). Following sequence verification (Molecular Cloning Laboratories, San Francisco), this construct provided a template for synthesis (via the QuikChange site-directed mutagenesis kit from Agilent, Santa Clara, CA) of pPICZ α A plasmids encoding precursors of SCI-a, SCI-b, or variant “mini-proinsulins” (MPI); the latter encodes single-chain precursors of two-chain analogs [Asp^{B10}]insulin, 4SS-DKP, and DKP insulins (respectively designated DB10-MPI, 4SS-DKP-MPI, and DKP-MPI), where

“DKP” denotes substitutions Asp^{B10}, Lys^{B28}, and Pro^{B29}. For construction of MPI derivatives from pPICZ α TKSCI-c, a long single-stranded DNA primer (Ultamer from Integrated DNA Technologies, Coralville, IA) was amplified by PCR, and the purified product was used as a primer in the QuikChange reaction according to the manufacturer’s instructions. DKP-MPI differs from insulin *lispro* in three respects: (a) addition of His^{B10} $\rightarrow$ Asp; (b) deletion of B30; and (c) incorporation of short tripeptide sequence EWK connecting B29 to A1. 4SS-DKP-MPI has the same sequence as DKP-MPI, but with Ile^{A10} $\rightarrow$ Cys and Gln^{B4} $\rightarrow$ Cys, enabling formation of fourth disulfide linkage (cystine A10–B4 (33)). DB10-MPI possesses the WT insulin sequence except for Asp^{B10} and EWK linker. Final PCR products were amplified and isolated from *Escherichia coli* DH5 α cells as described by the vendor (EasySelect *Pichia* Expression kit; Invitrogen), followed by sequence verification (Molecular Cloning Laboratories).

Biosynthesis of SCI precursor

Biosynthesis of SCI precursors followed an established protocol (85). First, electroporation of competent *P. pastoris* strain X33 with isolated SCI-precursor pPICZ α plasmid (corresponding to either SCI-a, SCI-b, SCI-c, DKP-MPI, or 4SS-DKP-MPI) was performed with Gene Pulser II (Bio-Rad) as described in the EasySelect *Pichia* Expression kit (Invitrogen) followed by selection on sterile YPDA + Z plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 100 μ g/ml Zeocin). After 3 days of incubation at 30 °C, protein expression by transformant colonies was assessed via small-scale culture in 50-ml Falcon tubes. Colonies were inoculated into 10-ml autoclave-sterilized YPS + Z media (1% yeast extract, 2% peptone, 0.1 M sorbitol, 100 μ g/ml Zeocin) and grown for 24 h at 30 °C with stirring speed 225 rpm (C25KC Incubator/Shaker; New Brunswick Scientific, Edison, NJ). Cells were then diluted into new 10-ml volumes of YPS + Z to an $A_{600\text{ nm}}$ of 0.33 and incubated for 3 days at 30 °C with 225 rpm stirring; methanol was added to 1% after the first 24 h and to 0.5% at 48 and 72 h. After pelleting (1500 $\times g$ for 5 min), 19 μ l of each culture’s supernatant was analyzed by SDS-PAGE (NuPAGE 12% BisTris; Life Technologies, Inc.) to assess relative SCI-precursor expression levels. After verifying the presence of the SCI precursor (7601, 7587, and 7455 Da for SCI-a, SCI-b, and SCI-c precursors, respectively) in the supernatant by matrix-assisted laser-desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS; 4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA), the colony presenting the most intense SCI-precursor band (at 6–8 kDa) by SDS-PAGE was selected for post-transformational vector amplification (86). Iterative amplification, adapted for the SCI system, increased pPICZ α plasmid copy number (and thereby SCI-precursor expression (87)) by (i) growing colonies overnight in 10 ml of YPS + Z media (30 °C with 225 rpm stirring); (ii) streaking cells on YPDA selection plates containing increasing Zeocin concentration (200, 400, 1000, and finally 2000 μ g/ml); (iii) expression screening (as above) of amplified colonies; and (iv) regrowth of the highest-expressing strain followed by selection on YPDA plates containing the next Zeocin concentration step. Iterative amplifica-

tion (*i.e.* repeating steps i–iv above) was stopped once a strain resistant to 2000 $\mu\text{g/ml}$ Zeocin was obtained.

For scaled SCI-precursor production, cells were inoculated in a flask containing 100 ml of YPG + Z (1% yeast extract, 2% peptone, 2% glycerol, 100 $\mu\text{g/ml}$ Zeocin) and grown to 20 $A_{600\text{ nm}}$ at 30 °C with stirring. Cells were then added to fermentation medium (5% glycerol, 2% $(\text{NH}_4)_2\text{SO}_4$, 1.2% KH_2PO_4 , 0.47% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.036% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1% yeast extract, 1% peptone, and 0.5% fermentation *Pichia* trace metal 1 (PTM1; Bioworld, Dublin, OH)) in a fermentation vessel (BioFlo310, New Brunswick Scientific, Edison, NJ) to a final 0.25 $A_{600\text{ nm}}$. Expression of an SCI precursor was induced by adding methanol to 1%. After fermentation, supernatant containing SCI precursors was collected by centrifugation and purified (see below).

Purification of recombinant yeast product

Hydrophobic-interaction chromatography (butyl-Sepharose 4 Fast Flow resin; GE Healthcare) was used to capture the SCI precursors. Purifications by rp-HPLC were performed using 0.1% trifluoroacetic acid (TFA) in water as aqueous mobile phase (buffer A) and 0.1% TFA in acetonitrile as the organic modifier (buffer B). Preparative purifications utilized a Waters 2545 quaternary pumping system with FlexInject (Waters). The SCI precursors were isolated by preparative C4 rp-HPLC (C4 Proto 300 Å, 10- μm , 20 $\times$ 250 mm; Higgins Analytical Inc., Mountain View, CA). Eluted SCI precursors were collected and lyophilized.

Preparation of ACP-1 protease

A gene encoding *Achromobacter* protease I (ACP-1), derived from *Achromobacter lyticus* (88), was cloned into a pET15b plasmid; its sequence contained substitution $\text{Lys}^{30} \rightarrow \text{Arg}$ (to enhance stability) and a C-terminal His tag (GSGSHHHHHH; to enable affinity purification). The plasmid was transformed into competent *E. coli* cells (strain BL21(DE3) pLysS as described by the vendor; Promega, Madison, WI) with selection on lysogeny broth (LB) plates containing 100 $\mu\text{g/ml}$ ampicillin and 34 $\mu\text{g/ml}$ chloramphenicol. ACP-1-expressing cells were grown at 37 °C in LB media (with antibiotics) to 1.0 $A_{600\text{ nm}}$ followed by induction with 0.8 mM isopropyl β -D-1-thiogalactopyranoside for 20 h at 25 °C. Cells were collected by centrifugation, washed in 100 mM Tris-HCl (pH 8.0), and resuspended in buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 20% sucrose. Cells were lysed via 10 min of gentle agitation in the presence of lysozyme (0.2 mg/ml) at 25 °C followed by addition of ice-cold water to lysate for 10 min. Lysates were centrifuged at 2500 $\times g$ (Centrifuge 5810R; Eppendorf, Hauppauge, NY) for 15 min. To immobilize His-tagged ACP-1, supernatants were incubated with Ni-Sepharose Fast Flow resin (GE Healthcare) at 25 °C for 1 h, rinsed with wash buffer (50 mM sodium phosphate (pH 6.5), 300 mM NaCl, 2 mM imidazole), and resuspended in digestion buffer (25 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.1 M urea).

Enzymatic cleavage of SCI precursor

Enzymatic conversion of the SCI precursors to SCI (effected via cleavage of the EEAEAEAEK decapeptide at the C termi-

nus of lysine) was achieved by gently rocking (at 25 °C) a 2 mg/ml solution of SCI precursor in digestion buffer containing ACP-bound resin (see above). Preparative C8 rp-HPLC (C8 Kromasil 100 Å, 10- μm , 20 $\times$ 250 mm; Akzo Nobel, Amsterdam, Netherlands) isolated the target SCI. Final purification utilized a C18 column (C18 Proto 300 Å, 10- μm , 200 $\times$ 250 mm; Higgins Analytical Inc.); the mass of the eluted SCI was verified by MALDI-TOF MS (6517, 6503, and 6371 Da for SCI-a, SCI-b, and SCI-c, respectively). Purities were assessed using analytical C18 rp-HPLC (C18 Discovery BIoWide 300 Å, 5- μm , 4.6 $\times$ 250 mm; Supelco, Bellefonte, PA). Purities $\geq 98\%$ were achieved.

Enzymatic semi-synthesis of insulin analogs

Two-chain insulin analogs were prepared by trypsin-catalyzed semi-synthesis (89) using variant *des*-octapeptide(B23–B30)-insulin (DOI) fragments containing Asp^{B10} (with or without cystine A10–B4) as described (46). These fragments were generated by tryptic digestion of Asp^{B10} -MPI, DKP-MPI, or 4SS-DKP-MPI isolated from *P. pastoris* cultures followed by preparative C8 rp-HPLC purification as above. Final and in-process purities were assessed using C18 analytical rp-HPLC as above. Eluates were lyophilized. Following semi-synthetic linkage of variant DOI and octapeptide, the analogs were purified by C4 preparative rp-HPLC as above. The analog was lyophilized, and its mass was verified by MALDI-TOF MS.

Purification of control insulin analogs

WT insulin, insulin *lispro*, and insulin *glargine* were purified from respective products Humulin®, Humalog® (both from Lilly), and Lantus® (Sanofi-Aventis, Paris, France) by preparative C4 rp-HPLC as above. Eluted proteins were lyophilized and stored at –20 °C until use.

Formulation-compatible buffers

SCIs and control insulin analogs were dissolved at stated concentrations in either Lilly Diluent® (a product containing 3.8 mg/ml sodium phosphate (pH 7.4), 16 mg/ml glycerin, 1.6 mg/ml *meta*-cresol, 0.65 mg/ml phenol) or, in the case of insulin *glargine*, a solution similar to that in Lantus® as prepared in accordance with the product insert (2.7 mg/ml *meta*-cresol, 16 mg/ml glycerin, with pH adjusted to 4.0 with NaOH and HCl, but without inclusion of polysorbate 20). ZnCl_2 was then added to a ratio of 3 Zn^{2+} :1 insulin hexamer.

Isoelectric focusing

Isoelectric points (pI) of SCIs and WT insulin were measured by IEF gel electrophoresis using a 5–10- μg protein samples (79). Experimental pI values were calculated via a linear plot of migration distance *versus* pH gradient of the IEF standards (79). Estimates of pI predicted on the basis of protein sequence were generally lower than gel-derived values.

Studies of protein solubility and aggregation

Lyophilized proteins (3–5 mg) were dissolved in 1 ml of deionized water with pH adjustment to 7.4 with dilute NaOH. Protein content was measured by UV absorbance (Nanodrop

2000C spectrophotometer; Thermo Fisher Scientific, Waltham, MA). DLS samples were degassed and syringe-filtered (0.02- μm Whatman® Anotop® 10 filters; Sigma). Aliquots necessary to obtain respective 7, 5, 2, and 1 mM protein solutions in 30- μl final volumes were pipetted into separate Eppendorf tubes and lyophilized. The powder was then dissolved in “Tris diluent” (25 mM Tris-HCl (pH 7.4) containing 16 mg/ml glycerol, 1.6 mg/ml *meta*-cresol, 0.65 mg/ml phenol, and ZnCl_2 at a ratio of 3 Zn^{2+} ions per insulin hexamer; Tris-HCl was substituted for sodium phosphate (as in Lilly Diluent®; see above) to avoid interaction between phosphate anions and zinc cations to form a precipitate). For DLS samples, all buffer components (*i.e.* deionized water, 4 $\times$ Tris-HCl diluent (pH 7.4), and 10 mM ZnCl_2) were degassed via nitrogen bubbling for 15 min and then syringe-filtered as above, followed by centrifugation (5 min at 16,100 $\times g$). Gentle mixing of the filtered components with lyophilized protein was performed by repeated pipetting. Once mixed, samples were incubated at room temperature for 30 min before use.

Turbidimetry

Possible precipitation of insulin in the protein concentration range 1–7 mM in Tris Diluent (above) was assessed by 500 nm absorbance as described (78) using a Nanodrop 2000C (Thermo Fisher Scientific) with 2- μl samples per measurement.

HPLC-based assessment of solubility

Solubility was assessed by rp-HPLC as described (79). In brief, insulin analogs were made 1–7 mM in Tris Diluent (above). 2- μl aliquots were mixed with 98 μl of deionized water and analyzed by C4 analytical rp-HPLC (C4 Proto 300 Å, 5- μm , 250 $\times$ 4.6 mm; Higgins Analytical Inc.). The stock insulin solution was then centrifuged for 5 min at 16,100 $\times g$ (Centrifuge 5415D; Eppendorf) to remove any precipitates and another 2 μl was analyzed by C4 analytical rp-HPLC as above. The insulin peak in each chromatogram was integrated in the Empower HPLC software (Waters), and data were presented as the ratio of peak area of the non-centrifuged sample to that of the centrifuged sample, yielding percent recovery, which reflects the fraction of protein that is fully dissolved at stated concentrations (1–7 mM). Error presented in these measurements was obtained by replicate injection of 10 μg of Humalog® (0.6 mM insulin *lispro* at pH 7.4), which revealed ~6–10% random error between injections. Injection of known quantities of insulin *lispro* in water (quantitated by UV absorbance) provided a control for losses unrelated to solubility; percent recoveries of insulin *lispro* were typically between 85 and 90%.

Size-exclusion chromatography

Self-assembly of WT insulin and SCIs (1–7 mM) was analyzed by SEC as described previously (90). Insulins in Tris-HCl diluent (10 μl injection) at stated concentrations were analyzed by SEC (Enrich SEC-70 10 $\times$ 300-mm column; fractionation range 0.5–70 kDa; Bio-Rad) using a 1 ml/min isocratic flow of mobile phase (10 mM Tris-HCl (pH 7.4), 145 mM NaCl, and 0.01% sodium azide). Molecular weights of eluted peaks were interpolated as described (90) from a linear regression

analysis of the elution volumes, V_e , of known molecular weight standards. Briefly, thyroglobulin (669 kDa) was used to determine void volume, V_0 . The calibration curve (Fig. S5) used to determine the molecular mass of insulin samples was then obtained from a semi-log plot of the V_e/V_0 versus the known molecular mass of the following gel filtration standards (Sigma): ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa).

Dynamic light scattering

Protein-free Tris-HCl diluent or Tris-buffered insulin solution (pH 7.4) was placed in a low-volume Wyatt microcuvette (25- μl sample volume) and 90° scattering data collected for 30 min (10-s correlation averaging time) using a Wyatt miniDawn TREOs instrument equipped with a quasi-elastic light-scattering autocorrelator module with subsequent data analysis performed in ASTRA 6 (all from Wyatt Technology Corp., Santa Barbara, CA). Protein-free samples gave only shot noise, indicating that the mixing protocol does not introduce bubbles. Size distributions of insulin samples were obtained using the regularization (91) processing protocol in ASTRA 6. As more concentrated protein samples (*e.g.* WT insulin at 7 mM) had a high incidence of large aggregates resulting in spurious bursts in count rate that confound analysis, regularization analysis was applied only to regions of stable count rate; 5–10 correlation functions were averaged to obtain each distribution.

Receptor-binding assays

Affinities of insulin or insulin analogs to the A and B isoform of the insulin receptor and to the insulin-like growth factor 1 receptor (IGF-1R) were measured in a competitive-displacement assay as described (46). Data for homologous and heterologous receptor binding were analyzed as described previously (92).

Guanidine denaturation studies

Thermodynamic stabilities of all insulin peptides in KPi buffer (10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7.4) with 50 mM KCl) at 25 °C were determined by monitoring guanidine-induced unfolding at the helix-sensitive wavelength of 222 nm with a CD spectropolarimeter (Aviv Biomedical Inc., Lakewood, NJ) equipped with temperature control and an automated titration unit, as described previously (46). Using non-linear least-squares regression, plots of ellipticity versus guanidine concentration were fit to a two-state unfolding model (93) as shown in Equation 1,

$$\Theta(c) = \frac{\Theta_A + \Theta_B e^{(-\Delta G - mc)/RT}}{1 + e^{(-\Delta G - mc)/RT}} \quad (\text{Eq. 1})$$

where ΔG is the Gibbs free energy of unfolding; c is guanidine concentration; R is the ideal gas law constant; T is absolute temperature, and Θ_A and Θ_B are baseline ellipticity values representing the folded and unfolded state. Baseline ellipticities were calculated via simultaneous fitting of linear equations $\Theta_A(c) = \Theta_A + m_A c$ and $\Theta_B(c) = \Theta_B + m_B c$, as described previ-

ously (94).

Fibrillation assays

Insulin fibrillation was monitored in solutions containing 0.6 mM protein dissolved in Lilly® Diluent (pH 7.4) or, in the case of insulin *glargine*, a Lantus-like solution (see above), in each case with gentle rocking at 45 °C in the presence of an air-liquid interface. The solutions were incubated in sealed glass vials. Aliquots were taken at regular intervals and frozen to enable analysis of thioflavin T fluorescence at assay termination, which occurred on visual appearance of cloudiness (46).

NMR spectroscopy

Spectra were acquired at pH 7.4 in 10% D₂O, 90% H₂O with a Bruker AVANCE 700 MHz spectrometer equipped with a triple-resonance cryoprobe (Bruker Biospin Corp, Billerica, MA), as described (46). Data were processed using Bruker's TopSpin software.

X-ray crystallography

Lyophilized SCI-a was dissolved in 0.02 N HCl to a concentration of 10 mg/ml; crystallization trials employed sitting-drop vapor diffusion. The best diffracting crystals were obtained at 20 °C with a 1:1 volume ratio of SCI-a mixed with well solution containing 0.2 M MgCl₂, 15% w/v polyethylene glycol (PEG) 8000, and 0.1 M Tris-HCl (pH 8.5). Single crystals were harvested with a nylon cryoloop and immersed in perfluoropolyether cryoprotectant oil (Hampton Research, Aliso Viejo, CA) to remove mother liquor before submerging in liquid nitrogen for storage.

Diffraction data were collected at SSRL BL7-1 from a single cryo-cooled crystal using a Quantum 315r CCD detector (Area Detector Systems Corp., Poway, CA). Data were processed with XDS (95), and the structure was solved by molecular replacement using PHASER (Table 2) (96). A search model was constructed using a monomer from a high-resolution crystal structure of WT insulin (PDB code 1GUJ (44)) with residues renumbered 1–57 in accordance with the nomenclature of SCI-c as studied previously by NMR (PDB code 2JZQ (29)). The 57 residues thus consist of a B domain (residues 1–30), C segment (residues 31–36), and A domain (residues 38–57). Side chains of non-conserved residues in the search model were computationally truncated to Ala. Molecular replacement calculations with this monomer (residues Gln⁴–Tyr²⁶ and Gly³⁷–Asn⁵⁷) located six copies that form a hexamer in the asymmetric unit of the P₂₁ unit cell to give a calculated V_m of 2.03 Å³/Da and solvent content of 39%. After rigid body and restrained refinement in Refmac (97), side chains of non-conserved residues as well as additional residues not present in the original search model were built into electron-density maps using Coot (98). Iterative cycles of model building were alternated with refinement calculations using Phenix (Table 2) (99). Composite refined omit maps were calculated to generate density with minimal search model bias. Refinement calculations were carried out with and without non-crystallographic symmetry restraints; release of the restraints resulted in a modest decrease of the refined *R*-factor (25.1 to 24.7%) but a significant increase

in R_{Free} (31.4–33.1%). The final model was refined with non-crystallographic symmetry restraints and against $F > 0\sigma F$. In each monomer, between 7 and 9 residues in the linker (and adjoining BC and CA junctions) could not be located in density and were presumed to be flexible or disordered. The six monomers were labeled as chains A–F. Using this convention, the final model consisted of residues Gln^{A4}–Thr^{A27} and Gly^{A37}–Asn^{A57}; Gln^{B4}–Pro^{B28} and Gly^{B37}–Asn^{B57}; His^{C5}–Thr^{C27} and Gly^{C37}–Asn^{C57}; Gln^{D4}–Pro^{D28} and Arg^{D36}–Asn^{D57}; Gln^{E4}–Pro^{E28} and Arg^{E36}–Asn^{E57}; His^{F5}–Thr^{F27} and Gly^{F37}–Asn^{F57}. In total, there was a lack of continuous, interpretable density for ~20% of the expected amino acids, which were not built into the final model. These are located at the N termini (residues 1–4) and in the B and C domain segments B27–B30 and C1–C6 (together comprising residues 27–36) of each monomer and are presumed to be flexible or disordered. As these disordered residues represent a significant fraction of the total molecule that yields non-continuous electron density not accounted for in either the refinement model or the uniform density bulk solvent model, they may contribute to the slightly high R_{Free} (31.4%) which nonetheless gives a reasonable R_{Free} –*R*-factor difference of ~7%.

Biological testing in diabetic rats

The insulin analogs were dissolved at concentrations specified in the legends to Figs. S1, S2, and S5 in either Lilly® Diluent or, in the case of insulin *glargine*, a Lantus-like acidic solution. The formulations each contained ZnCl₂ at a ratio of 3 Zn²⁺:1 insulin hexamer. Insulin purity was verified by C4 analytical rp-HPLC as above. Protein-free Lilly® Diluent was used as a negative control (buffer only).

Groups of male Lewis rats (mean body mass ~300 g) rendered diabetic by streptozotocin (100) were utilized. Rats were injected either SQ or i.v. at time $t = 0$ with the specified dose of insulin (100 µl/300 g of body mass). For i.v. injection, animals were placed in an induction chamber and anesthetized using 5% isoflurane in 100% oxygen. While under anesthesia, the tail of the rat was washed with lukewarm water and further cleaned with gauze containing alcohol and/or xylene. WT insulin or insulin analogs were injected at specified doses into the lateral tail vein.

Following SQ or i.v. injection, a small drop of blood was obtained from the clipped tip of the rats' tails at $t = 0$ and then every 10 min for the 1st h, every 20 min for the 2nd h, every 30 min during the 3rd h and then once per h for the remainder of the experiment. Measurements of [glucose] were made using a clinical glucometer (EasyMax® V Glucose Meter; Oak Tree Health, Las Vegas, NV). Because of day-to-day variation (even in the same rat on different days), 4–8 rats were injected per analog formulation on each test day, and the experiments were repeated on one or more non-consecutive days to obtain a larger sample size.

Thermal inactivation assays

Heating of SCIs and control analogs was performed in an oven at either 45 or 75 °C with gentle rocking. Protein solutions were incubated in sealed glass vials at a concentration of 0.6 mM (nominal U-100 strength in the case of WT insulin,

insulin *lispro*, or insulin *glargine*) in either Lilly® Diluent or, in the case of insulin *glargine*, in an acidic solution similar to that in Lantus® (above). At stated time points in Fig. 5, samples were assessed for residual potency by diluting 10-fold (to a nominal strength of U-10; 0.06 mM) in their appropriate buffer (Lilly Diluent® (pH 7.4) or Lantus-like solution (pH 4.0)) before SQ injection at stated doses into diabetic rats (see above).

To obtain a quantitative assessment of residual insulin action, a custom MATLAB (MathWorks, Natick, MA) script was written to process rat glucose measurements in batch. AUCs were calculated for each individual data set using trapezoidal numerical integration. Two reporters of functional degradation were then determined: fractional AUC and percent activity. Fractional AUC was defined as the ratio of average AUC for a heat-stressed analog's dataset to the average AUC of the fresh (unstressed) analog. This value defined as fully active the fresh analogs, corresponding to a fractional AUC of 1.0, whereas partially or fully inactivated samples exhibited fractional AUCs >1.0. To further quantify the degree of thermal inactivation, percent activity was calculated: the average AUC of fresh or heated datasets was subtracted from the average AUC of diluent-only control injections to yield area over the curve (AOC). AOC for the heated dataset was divided by the AOC of the unheated sample, yielding a percentage. Fully active samples thus had the same AOC as the fresh sample (*i.e.* 100% active), whereas completely inactivated analogs had the same glucose-response curve as diluent-only (*i.e.* AOC = 0). Because of biological variability among rat responses, the heated insulin curves can have a lower AUC than that of the diluent-only curve, resulting in apparent negative values for percent activity; this corresponded to complete inactivation.

Signaling assays in mammalian cell culture

Signaling activities of the insulin analogs were tested in (a) L6 cells (rat myoblasts) engineered to overexpress human IR-A (49) and (b) MCF-7 human breast adenocarcinoma cells (which express IR-A, IR-B and high levels of IGF-1R (50)). L6-IRA cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and G418 antibiotic for selection. MCF-7 cells (American Type Culture Collection, Manassas, VA) were cultured in Eagle's minimum essential medium supplemented with 10% FBS, 1% penicillin/streptomycin, and sodium pyruvate (1 mM). 24-h serum-starving protocol using appropriate culture medium except FBS was applied at 70–75% cell confluence ($\sim 0.8 \times 10^6$ cells per well (L6-IRA) and 6×10^6 per 10-cm dish (MCF-7)). After serum-starving, serum-free medium containing 100 nM insulin analogs was added to each well (control wells received medium with no added insulin). Media were removed after 15 min, followed by cell lysis using RIPA buffer with protease and phosphatase inhibitors (Cell Signaling Technology, Danvers, MA). Protein concentrations in cell lysates were determined with a Pierce BCA assay kit (Thermo Fisher Scientific) for use in immunoblotting. Cells cultured for rt-qPCR assays (see below) were, after 24 h of serum starvation, treated with either a protein-free medium or medium supple-

mented with an insulin analog (to a hormone concentration of 100 nM) medium for 8 h.

Real-time qPCR transcriptional assay

Mitogenic gene transcription was measured in triplicate by rt-qPCR with samples prepared according to the One-step rt-qPCR kit (Bio-Rad).

The following sets of primers (5'–3') were used for L6-IRA cells: cyclin D1, GCCGAGTGGAACTTTTGTCTG and CGG-GAAGCGTGTACTTATCCT; cyclin G2, GCAAGAAAAGA-AGCCAAGCT and TGACCAAGAGGCAAAATAAAATCAA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GACATGCCGCCTGGAGAA and GCCCAGGATGCCCTTAGT; and TF_{II}D, CTGAGGGGGCAATGTCTAAC and GGGCAGCTAGTGAGATGAGC.

For MCF-7 cell-based assays, the following primers (5'–3') were used: cyclin D1, AATGACCCCGCACGATTTC and TCA-GGTTCAGGCCTTGAC; cyclin G2, ATCGTTTCAAGGC-GCACAG and CAACCCCTCAGGTATCG; GAPDH, AG-CCGAGCCACATCGCT and TGGCAACAATATCCACTT-TACCAGAGT; and TF_{II}D, GCACAGGAGCCAAGAGTGAA and TCACAGCTCCCCACCATGTT.

Insulin-stimulated protein phosphorylation

Samples contained 2–5 µg (for L6-IRA) or 25 µg (for MCF-7) of protein from cell lysate in each loading well (as quantitated by BCA assay). Cell lysates were dissolved in Laemmli buffer (Bio-Rad) with 10% β-mercaptoethanol, heated at 100 °C for 5 min (for all Akt/p-Akt blotting), 50 °C for 10 min (for L6-IRA IR/p-IR), or 100 °C for 8 min (for MCF-7 IR/p-IR) and then centrifuged at $9300 \times g$ for 1 min (Biofuge A; Baxter Scientific, Soddy Daisy, TN). Samples were loaded into 10% Mini-PROTEAN TGX gels (Bio-Rad). Proteins were transferred to 0.2-µm PVDF membrane and then blocked in 5% bovine serum albumin (or 10% milk for p-Akt data) for 1 h. All antibodies were purchased from Cell Signaling Technology unless otherwise stated. Membranes were incubated overnight at 4 °C with insulin receptor β (4B8) rabbit monoclonal antibody (mAb) or an equal mixture of phospho-insulin receptor β (Tyr^{1150/1151}) rabbit mAb; phospho-insulin receptor (Tyr¹¹⁵⁸) polyclonal antibody (Thermo Fisher Scientific); phospho-insulin receptor (Tyr¹³³⁴) polyclonal antibody (Thermo Fisher Scientific); phospho-insulin receptor β (Tyr¹³⁴⁵) rabbit mAb; and anti-insulin receptor (phospho-Tyr⁹⁷²) antibody (Abcam, Cambridge, UK). Dilutions for these antibodies were 1:5000 in 5% bovine serum albumin. Antibodies for Akt blotting were p-Akt1/2/3 antibody (Ser⁴⁷³) (1:1000) and Akt1/2/3 antibody (H-136) (1:2000). For MCF-7 samples, the following anti-Akt antibody dilutions in 10% milk were used: p-Akt1/2/3 antibody (Ser⁴⁷³) (1:1000) and Akt1/2/3 antibody (H-136) (1:2000). After the primary antibody incubation, membranes were washed with Tris-buffered saline with 0.1% Tween 20 and incubated in goat anti-rabbit HRP-conjugated secondary antibody diluted 1:10,000 in 5% bovine serum albumin (or 10% milk for p-Akt data) for 1–2 h at room temperature. All membranes were washed three times with 1× Tris-buffered saline, 0.1% Tween 20 and incubated in

Luminata Crescendo Western HRP Substrate (Merck Millipore, Billerica, MA) for 45 s and developed.

Author contributions—Plasmids were designed and constructed by J. W. and M. P. Biochemical and biophysical assays were performed by M. D. G. and N. B. P. Rat studies were performed by K. C., M. P., N. R., M. S., and N. B. P. under the guidance of F. I.-B. with data analysis by M. D. G., K. C., and F. I.-B. Crystallization trials and structure determination were performed by K. A., Y. P., and V. C. Y.; analysis of the crystal structure was undertaken by M. D. G., V. C. Y., and M. A. W. with advice from M. C. L. NMR studies were performed by N. P. W., Y. Y., and M. A. W. Receptor-binding studies were performed by J. W. and N. R. Cell biological studies were performed by K. C. and Y.-S. C. The overall program of research was directed by M. A. W.

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