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Substitution of an Internal Disulfide Bridge with a Diselenide Enhances both Foldability and Stability of Human Insulin

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Abstract

Insulin analogues, mainstays in the modern treatment of diabetes mellitus, exemplify the utility of protein engineering in molecular pharmacology. Whereas chemical syntheses of the individual A and B chains were accomplished in the early 1960s, their combination to form native insulin remains inefficient because of competing disulfide pairing and aggregation. To overcome these limitations, we envisioned an alternative approach: pairwise substitution of cysteine residues with selenocysteine (Sec, U). To this end, Cys^{A6} and Cys^{A11} (which form the internal intrachain A6-A11 disulfide bridge) were each replaced with Sec. The A chain-[C6U, C11U] variant was prepared by solid-phase peptide synthesis; while sulfitolysis of biosynthetic human insulin provided wild-type B chain-di-S-sulfonate. The presence of selenium atoms at these sites markedly enhanced the rate and fidelity of chain combination, thus solving a long-standing challenge in chemical insulin synthesis. The affinity of the Se-insulin analogue for the lectin-purified insulin receptor was indistinguishable from that of WT-insulin. Remarkably, the thermodynamic stability of the analogue at 25 °C, as inferred from guanidine denaturation studies, was augmented ($G_u \approx 0.8 \text{ kcal mol}^{-1}$). In accordance with such enhanced stability, reductive unfolding of the Se-insulin analogue and resistance to enzymatic cleavage by Glu-C protease occurred four times more slowly than that of WT-insulin. 2D-NMR and X-ray crystallographic studies demonstrated a native-like three-dimensional structure in which the diselenide bridge was accommodated in the hydrophobic core without steric clash.

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Conflict of interest

A provisional patent application covering this work has recently been filed. M.A.W. has equity in Thermalin Diabetes, LLC (Cleveland, OH) where he serves as Chief Scientific Officer; he has also been a consultant to Merck Research Laboratories and DEKA Research & Development Corp.

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Keywords

chemical protein synthesis; insulin; oxidative protein folding; protein engineering; protein modifications; selenocysteine

Introduction

Diabetes mellitus (DM) is one of the most prevalent diseases in the world, and it is approaching pandemic status in both the developed and developing worlds. The discovery of insulin in 1921 revolutionized the treatment of DM.^[1] Since the advent of solid-phase peptide synthesis (SPPS),^[2,3] development of new and more efficient methods for its chemical synthesis has become a major focus in peptide chemistry.

The mature form of insulin contains two chains: an A chain (21 residues) and a B chain (30 residues) (Figure 1 a).^[4] The protein is stabilized by three disulfide bridges, two between chains (A7-B7 and A20-B19), and one within the A chain (A6-A11). Although the chemical synthesis of the individual chains was reported more than 50 years ago,^[5] their combination remains inefficient. Yields of insulin are thus low (typically < 20% in relation to the starting B-chain concentration) because of the formation of non-native disulfide isomers, covalent polymers, noncovalent aggregates and fibrils.^[5–6] In recent decades, insulin analogues have been sought with enhanced chain-combination efficiency^[7] or stability;^[8] many of these excellent approaches are based on orthogonal Cys protection and regioselective disulfide formation^[9] or the use of tethers as “proinsulin” mimics. For example Wade, Hossain and co-workers prepared a human insulin analogue in which the intrachain A6-A11 disulfide bond was replaced with cystathionine to obtain enhanced thermal stability.^[8] Kent and co-workers engineered a labile ester bond between the side chains of Glu^{A4} and Thr^{B30} in the reduced and unfolded chains. This tether, connecting side chains that are nearby in the three-dimensional (3D) structure of insulin, creates a “mini-proinsulin” with enhanced folding efficiency.^[10] DiMarchi and colleagues used an internal dike-topiperazine tether to achieve similar efficiencies.^[11] “DesDi,” a synthetic Lys^{B28}-modified 49-residue single-chain insulin, inspired by proinsulin but lacking residues B29 and B30, was found to fold with higher efficiency than longer single-chain precursor polypeptides.^[12] Wade, Hossain and co-workers adopted a similar approach for a one-pot cleavable bis-linker tether for the synthesis of proteins based on heterodimeric peptides.^[13]

The present study describes an alternative approach to prepare an active insulin analogue based only on the favorable chemical properties of selenocysteine (Sec, U), the 21st-encoded amino acid.^[14] Sec, a near isostere of Cys, has become a useful tool in peptide chemistry, both for chemical ligation approaches and for replacing disulfide bonds with diselenide or selenylsulfides.^[15] The selenol group of Sec has a lower pK_a (near 5.2)^[16] and a lower reduction potential ($E_0 = -386$ mV)^[17] than does the thiol group of Cys. Pairwise Cys-to-Sec substitutions have been found in several cases to enhance the oxidative folding of proteins^[15a–d,h] as well as the efficient use of Se-containing reagents.^[18] Furthermore, because diselenide bonds are more stable than disulfides (relative to their respective reduced forms), substituting Sec residues in strategic positions can improve folding efficiency even

via non-native pairing followed by protein structure-driven rearrangement.^[15d,f] This counterintuitive outcome was demonstrated in the case of the bovine pancreatic trypsin inhibitor (BPTI), a classical model of protein folding: pairwise replacement of Cys5 and Cys14 with Sec yielded a non-native diselenide bond that was associated with enhanced folding efficiency because trapped intermediates that otherwise confound the folding of WT-BPTI were sidestepped.^[15d,e]

Unlike previous studies of pairwise Cys-to-Sec substitution in proteins, this Report focuses on a folding reaction that is coupled to the specific pairing of the two-polypeptide chains. A novel analogue of human insulin is described in which the Cys residues at positions 6 and 11 in the A chain were replaced with Sec; cysteines were retained at positions A7 and A20. The resulting A chain[C6U, C11U] analogue was then combined with the wild-type (WT) B chain, obtained by sulfitolysis of human insulin; the (B7, B19) di-S-sulfonate B-chain derivative is refractory to intramolecular disulfide pairing and resists aggregation.^[6,20]

Segmental folding of the N-terminal α -helix of the A chain, although critical to the overall structure and stability of insulin, is not required for chain combination.^[21] Nonetheless, previous folding studies of porcine proinsulin (porcine and human insulin differs only at position B30) suggested that “locking in” the A6-A11 crosslink may facilitate correct formation of the remaining two disulfide bonds. Such a conformational constraint may “steer” the folding pathway to the upper route (Figure 1 b), which would thereby avoid formation of kinetic traps and off-pathway disulfide isomers.^[19,22] These speculations suggest that replacing the internal A6-A11 disulfide bridge with a diselenide could: 1) orient and maintain a favorable conformation of the A chain, in turn facilitating interchain disulfide bonding and 2) enhance thiol/selenol-disulfide/diselenide exchange reactions, hence improving the rate and yield of insulin production (Figure 1 c). Evidence supporting this rationale was recently published.^[23] We therefore envisaged that preferred preformation of the A6-A11 bond via Se-Se pairing would 1) accelerate on-pathway mechanism and 2) disfavor off-pathway traps (Figure 1 b). We report herein that the Se-insulin[C6U^A, C11U^A] was obtained in high yield relative to control reactions in which human insulin was prepared by recombination of sulfitolized A and B chains. Remarkably, with this strategy, the Se-insulin-[C6U^A, C11U^A] analogue was obtained in a high yield *without intermediate steps*, and was purified in a single step.

The analogue exhibited an affinity for the lectin-purified insulin receptor (IR) that was indistinguishable from that of WT-insulin, whereas its stability was augmented. To our knowledge, this is the first time that a modification of the core of insulin has been observed to enhance its stability.

While our work was in progress,^[24] Arai et al. exploited Sec to prepare an analogue of bovine insulin^[25] with a diselenide bridge on the surface of insulin.^[26] Containing substitutions [C7U^A, C7U^B], the insulin analogue was obtained with a yield of up to 27%.^[25] Bioactivity similar to that of WT bovine insulin was demonstrated in a cell-based assay but only at high hormone concentration (1 μ M), beyond the physiological range (0.1–10 nM); the relative affinity of the analogue for the insulin receptor was not reported. The interchain diselenide bonds likewise improved the efficiency of chain combination and

increased resistance to degradation by insulin-degrading enzyme (IDE) (relative to bovine insulin).^[25] We note in passing that bovine insulin is remarkable for its lower stability and higher propensity to form fibrils (relative to human or porcine insulins).^[27,28]

The recent study of [C7U^A, C7U^B]-bovine insulin and the present study of [C6U^A, C11U^A] human insulin are complementary. Although these studies differ in detail, each opens new directions for future investigations. From the perspective of synthetic simplicity, we note that the present use of an intrachain disulfide bond (A6-A11) requires one modified troublesome Sec-containing chain, which is a striking synthetic economy.

Results and Discussion

The synthesis of the A chain[C6U, C11U] employed stepwise Fmoc-SPPS, wherein Sec residues were inserted manually^[29] (Figure S1), and A chain[C6U, C11U] was isolated in 8% yield, similar to that achieved in the synthesis of A chain[C7U] by Iwaoka and co-workers.^[25]

Although the 30-residue B chain can readily be obtained by standard stepwise Fmoc-SPPS, for convenience we decided to obtain it by oxidative sulfitolysis of commercially available human insulin. Therefore, the WT human B chain was isolated from human insulin following its oxidative sulfitolysis using equal concentrations of sodium sulfite and sodium tetrathionate (see the Experimental Section). The reaction was monitored by HPLC and ESI-MS, which indicated completion after 4 h (Figure S2).

We first compared the solubility of the A chain[C6U, C11U] analogue with that of the WT A chain. Given that both human A and B chains are prone to aggregation,^[6b] it is preferable to store them as S-sulfonate derivatives. Unexpectedly, however, we found that the unmodified A chain[C6U, C11U] was as soluble as the sulfitolized form of the A chain (with four sulfonate groups) (Figure S3). This finding suggests that preformation of fully oxidized A chain[C6U, C11U] weakens interactions between chains that would otherwise lead to aggregation and fibrillation.

We next evaluated the combination of the B chain-S-sulfonate with the A chain[C6U, C11U] in relation to chain combination of WT-insulin.^[21] The latter employed equal concentrations of the S-sulfonated A and B chains (1.2 mM each), dissolved in 0.1 M glycine at pH 10.6 in the presence of DTT, which was added at a stoichiometric concentration relative to the sulfonate groups present in the two chains (i.e., 7.2 mM).^[21] The reactions were each carried out at 4°C with exposure to air to permit oxidation.^[21] Aliquots, quenched with 0.1 % TFA in water, were taken at successive time points. Under these conditions, chain combination of WT-insulin was observed after 48 h at low yield (ca. 39% by HPLC integration; 1–2% isolated yield; Figure 2a).

For Se-insulin[C6U^A, C11U^A] preparation, a similar reaction was carried out using equal concentrations (1.2 mM) of the B-chain-S-sulfonate and A chain[C6U, C11U] *not* sulfitolized but oxidized (as two selenylsulfides or a disulfide and diselenide, see Figure S1 in the SI); DTT was added at stoichiometric equivalents to the concentration of the sulfonate groups (2.4 mM in this case). Strikingly, the combination product was observed after only 2

h, and the reaction was completed after ca. 8h (Figure 2 b), at which point the Se-insulin[C6U^A, C11U^A] was the predominant product (ca. 83% by HPLC). The Se-insulin analogue was isolated in 31% yield, and characterized by HPLC, ESI-MS and HR-MS analyses.

The observed mass corresponded to the expected mass with three crosslinks (two disulfides and one diselenide bonds, see below). The yield of human Se-insulin[C6U^A, C11U^A] was similar to that reported by Iwaoka and co-workers in the synthesis of bovine Se-insulin[C7U^A, C7U^B], except that our reaction used 1.2 mM concentrations of the two chains versus 184 μ M concentration in the synthesis of bovine Se-insulin-[C7U^A, C7U^B].^[25]

After initial characterization (Figure S4 and S5), far-ultraviolet CD spectroscopy was employed to compare the overall structures of the human Se-insulin[C6U^A, C11U^A] analogue and the WT-human insulin. Their spectra are essentially identical (Figure 3a), suggesting only minimal differences in secondary structure. CD was also used to monitor guanidine denaturation (at helix-sensitive wavelength 222 nm) (Figure 3 b). Application of a two-state thermodynamic model implied that the Se-insulin[C6U^A, C11U^A] was 0.8($\pm$ 0.2) kcalmol⁻¹ more stable than WT human insulin (G_u ; Table 1).

In accordance with the CD studies, the overall ¹H NMR spectrum of Se-insulin[C6U^A, C11U^A] was similar to that of WT-insulin. 2D ¹H-NMR COSY spectra^[30] of WT human insulin and Se-insulin[C6U^A, C11U^A] were obtained at 500 MHz under identical conditions (Figure S6; see Experimental Section);^[31] cross-peak assignments were based on previous studies.^[32] Despite an overall concordance of chemical shifts, differences were prominent at positions A6 and A11, the sites of Se modification. Also altered are CysB7 (presumably affected by the adjacent A6-A11 diselenide bond) and the side-chain resonances of Leu^{B11} (which is within ca. 3.5 Å of the diselenide bond). Chemical-shift differences were also observed at Val^{A3} and Ser^{A9}, proximate to A6 in the helical structure. These and other changes in main-chain ¹H-N chemical shift (Table S1 in the Supporting Information) are illustrated in relation to the canonical structure of human insulin (PDB: 3w7y, Figure S6).

The detailed 3D structure of Se-insulin[C6U^A, C11U^A] was determined by X-ray crystallography. The Se-insulin analogue was crystallized by the hanging-drop vapor-diffusion methodology. One such crystal (ca. 0.3×0.3×0.2 mm, *PI* space group) was selected for structure determination, yielding a well-resolved X-ray diffraction to a resolution of 1.82 Å (PDB code 6H3M, Table S2). The structure was determined by the single anomalous dispersion (SAD) method, using the anomalous signal obtained from the Se atoms in the introduced Sec residues. Structure determination revealed eight independent Se-insulin monomers in the crystallographic asymmetric unit, organized in an oval-shaped octameric assembly (Figure 4a). No zinc ions were detected in the structure, despite their inclusion in the crystallization buffer. To our knowledge, this is a novel crystal form for an insulin analogue.

Clear and unequivocal electron density was obtained for the diselenide bridges (Figure 4c) of all eight protomers, with an average Se—Se length of 2.4($\pm$ 0.1) Å. The octameric assembly obtained in the crystal structure contains four monomers forming two Se-insulin

dimers at the periphery, and four independent monomers at the center (Figure S7). The conformations of the outer Se-insulin dimers differ when compared to the conformations of the four inner monomers, adopting different conformational states that are notated here as State-1 and State-2, respectively. These states differ primarily in the positions of the C-termini of their B chain, where in State-1 the terminal residues starting from Gly^{B23} extend linearly to form a β -strand (as in the WT-insulin dimers), while in State-2 these residues bend outward (Figure S7). Despite these differences, both states resemble generally the WT-structure, specifically the T state insulin rather than the R state (Figure S8).^[33,34] The two independent T₂ Se-insulin dimers in the present crystal form closely resemble WT zinc-free T₂ insulin dimers (e.g., PDB entry 1DPH)^[35] and the component TT' dimer within the classical 2-Zn insulin hexamer (PDB entry 4INS).^[36] These similarities demonstrate that the diselenide bridge at A6-A11 does not prevent formation of a native B-chain dimer interface, including its prominent B24-B28 antiparallel β -sheet.

To test whether the minor changes in structure indicated by both the NMR and X-ray diffraction studies might affect biological activity of Se-insulin[C6U^A, C11U^A], we characterized the affinity of our analogue for the isolated insulin receptor (IR).^[37] The binding assay employed detergent-solubilized and lectin-purified IR (isoform B) as immobilized in a plate assay.^[38] A control was provided with a semisynthetic version of insulin, Orn^{B29} insulin,^[39] which is known to have the same activity as WT-insulin. The results indicated that the two hormones had essentially identical affinity for IR, with $K_d = 0.08(\pm 0.02)$ nM (Figure 5).

Two kinetic studies of the Se-insulin analogue were undertaken as surrogate probes of pharmaceutical degradation: respective time courses of chemical change due to 1) cleavage of either peptide bonds by a protease or 2) disulfide bonds by a thiol reagent. We describe these assays in turn.

1) Susceptibility to proteolysis:

The stability of insulin analogues in the presence of a typical protease, endoproteinase Glu-C (V8 protease), was determined by following their overall rate of cleavage. Equal concentrations of the Se-insulin[C6U^A, C11U^A] and WT-insulin were treated separately with V8 protease at 37°C in an ammonium buffer (see the Experimental Section).^[40] At successive time points, small aliquots were placed on ice and quenched with 0.1 % TFA in water, and the digestion process was analyzed by HPLC. The results indicate that the protease did not disrupt either the disulfide or diselenide bonds. As expected (Figure S9), four fragments were obtained for both Se-insulin[C6U^A, C11U^A] and WT-insulin. These were characterized by ESI-MS (Figure S10 and S11), confirming that the structures of both hormones are similar, with canonical pairing.^[11a] The degradation rate of Se-insulin was approximately four times slower than that of WT-insulin ($t_{1/2} = 92 \pm 3$ min for Se-insulin vs. 25 ± 2 min for the WT-insulin Figure 6a).^[41]

2) Susceptibility to reduction:

It has been shown that replacing a disulfide with a diselenide bridge increases protein stability under reductive unfolding conditions.^[15a] To examine such an effect in the present

case, equal concentrations of WT-insulin and Se-insulin[C6U^A, C11U^A] were dissolved separately in buffer (pH 7.4) containing 300 μ M reduced glutathione (GSH) at 37°C (see the Experimental Section),^[42] and the degree of reduction of insulin was analyzed by HPLC and ESI-MS. The results indicate that the Se-insulin[C6U^A, C11U^A] analogue was fourfold more stable than WT-insulin under the same conditions ($t_{1/2} = 40 \pm 3$ h for Se-insulin vs. 11 ± 1 h for the WT-insulin, Figure S6b), highlighting the enhanced stability of the diselenide bond relative to the corresponding disulfide bridge.

The present study has presented a novel strategy for the rapid and efficient preparation of an active Se-insulin analogue with enhanced stability. We anticipate that this approach will have broad utility for the preparation of other nonstandard insulin analogues that are not readily obtained by biosynthetic protocols. Based on the observed efficient chain combination for Se-insulin in comparison to WT-insulin (Figure 2 b vs. 2a), it is plausible to assume that the use of an internal diselenide bridge can circumvent off-pathway reactions that confound classical insulin chain combinations. On a mechanistic level, our results support the hypothesis that the intrachain diselenide bond forms first and then favors a productive orientation of the two chains in an encounter complex, thereby facilitating the formation of the two subsequent disulfides bonds. This reaction scheme suggests a predominance of the upper route in the folding mechanism of Se-insulin (Figure 1 b).

The rationale underlying the present approach was based on fundamental properties of Se. Given that the atomic radius of Se is larger than that of S, however, it was difficult to predict from first principles whether an internal diselenide bridge would stabilize or destabilize the 3D structure of the analogue, once its native conformation was achieved. Furthermore, because the A1-A8 α -helix plays a critical role in receptor binding, its potential structural perturbation by the modified A6-A11 bridge could, in principle, have impaired activity. Such possibilities were systematically addressed in the course of our studies. Crystallographic, NMR and CD studies demonstrated that the diselenide bridge is readily accommodated within the hydrophobic core of insulin without transmitted structural perturbations. Such accommodation presumably reflects subtle conformational adjustments in neighboring side chains, which, in turn, are enabled by the flexibility of the native insulin structure. Some gaps between side chains are ubiquitous in the cores of globular proteins, and these presumably facilitate local conformational adjustments. We suggest that the larger diselenide bridge at A6-A11 enhances the overall core packing that leads to the observed increase in protein stability (ΔG_u ca. 0.8 kcal/mole). The position and orientation of the A1-A8 α -helix remains compatible with the mode of binding of the hormone to the insulin receptor.^[43]

Analogous principles were uncovered in a recent study of an insulin analogue in which Tyr^{B26} (which packs within a crevice near the side chains of Val^{B12}, Ile^{A2}, and Val^{A3}) was substituted with 3-iodo-Tyr. The large iodine atom was shown to fill a cryptic packing defect near cystine A6-A11 in the core.^[44] This modification enhances the biophysical properties of the modified insulin while preserving its biological activity; indeed, receptor binding was enhanced, presumably by formation of a halogen bond to the hormone-receptor interface as the modified ring pivots from the core to engage the receptor. In the present case, the diselenide bridge underlies the receptor-binding surface of insulin and so would be unlikely to provide novel contacts.

Conclusions

The present study has demonstrated that human Se-insulin was stabilized when the intrachain disulfide bridge (A6-A11) was replaced with a diselenide bond. This synthetic scheme is simpler than that described by Arai et al.^[25] because only one selenopeptide chain is required (the shorter A chain). Relative to classical insulin chain combination, the intrachain diselenide bond markedly enhanced the rate of the combination between the A and B chains, giving the desired product in higher yield and after only 30 minutes. The Se-insulin analogue retained native-like structure and activity, with enhanced thermodynamic stability and resistance to both reductive and enzymatic digestion.

Our results showcase the utility of diselenide bonds as favorable substitutes for disulfide bonds in therapeutic proteins, which enhances folding and stability of the modified proteins, while preserving their general structure and biological activity. Together, our data provide a new approach for the rapid and efficient preparation of insulin analogues, especially synthetic insulins with unnatural entities. Such an approach now opens promising horizons for the development of new insulin-related drugs for the treatment of DM, which will have improved biophysical properties and an enhanced shelf life. This approach is likely to be generalized further to include other disulfide-rich proteins.

Experimental Section

Fmoc-SPPS synthesis of chain A[C6U, C11U] analogue (21 AA)

The amino acid sequence: G-I-V-E-Q-U-C-T-S-I-U-S-L-Y-Q-L-E-N-Y-C-N: Chain A[C6U, C11U] analogue was prepared with an automatic peptide synthesizer (CS136XT, CS Bio Inc. CA) on a 0.25 mmol scale using Fmoc-Asn(Trt)-TentaGel[®]R PHB resin (loading of 0.17 mmol g⁻¹). Fmoc-amino acids (2 mmol in 5 mL DMF) activated with HCTU (2 mmol in 5 mL DMF) and DIEA (4 mmol in 5 mL DMF) for 5 min and allowed to couple for 25 min, with constant shaking. Fmoc-deprotection was carried out with 20% piperidine in DMF (2×5 min). Fmoc-Sec(Mob)-OH was manually coupled for 2 h by using the DIC/OxymaPure activation method (2.9 equiv/3.0 equiv, respectively).

The peptide-resin was washed with DMF, DCM and dried under vacuum. The dried peptide-resin was cleaved in the presence of 2 equiv of DTNP,^[56] using a TFA/water/thioanisole/triisopropylsilane/ethanedithiol (92.5:1.5:1.5:1.5:1.5) cocktail for 4 h. The cleavage mixture was filtered and TFA was evaporated with N₂ bubbling to minimum volume, to which an eightfold volume of cold ether was added dropwise. The precipitated crude peptide was centrifuged (5000 rpm, 10 min), ether was removed, and the crude peptide was dissolved in ACN/water (1:1) containing 0.1% TFA and was further diluted to ca. 25% ACN with water and lyophilized, giving 208 mg of crude material.

The crude peptide was dissolved in 25% ACN in water containing 0.1% TFA and purified by multiple injections of 100 mg each on prep RP-HPLC (XSelect C18 column, 5 µm, 30× 250 mm) using a gradient of 25–50% B over 50 min, to give 49 mg of chain A[C6U, C11U] analogue (8% yield, based on resin loading), which was characterized by UPLC using

ACQUITY UPLC XSelect C18 column (3.5 μm , 130 $\text{\AA}$, 4.6 $\times$ 150 mm), and a gradient of 5% B over 2 min then 5–70% B over 20 min and ESI-MS (Figure S1).

Isolation of sulfitolized A and B chains by oxidative sulfitolysis of recombinant human insulin

Human insulin was reduced and A and B chains were sulfitolized using 0.2 M Na_2SO_3 and 0.2 M $\text{Na}_2\text{S}_4\text{O}_6$ in 20 mM Tris-HCl, 1 mM EDTA, 8 M urea buffer.^[45] Protein concentration was 10–15 mg mL^{-1} and the pH was adjusted to 6–7. The reaction was monitored by analytical HPLC (XSelect C18 column, 3.5 μm , 130 $\text{\AA}$, 4.6 $\times$ 150 mm), and ESI-MS and was completed in 4 h. Semipreparative RP-HPLC XBridge Prep C8 column (5 μm , 10 $\times$ 150 mm) was used to purify the products. Chain A, with four sulfonate groups (with a coeluting peak for three sulfonate groups), and chain B, with two sulfonate groups, were obtained, one group linked to each cysteine residue (Figure S2).

Preparation of WT-insulin by recombination of sulfitolized A and B chains

A solution of sulfitolized chain A (final concentration in the recombination mixture was 1.2 mM) was dissolved in glycine buffer (0.1 M, pH 10.6) and then mixed with a solution of sulfitolized chain B (final concentration 1.2 mM) in the same buffer.^[21] DTT initiated the reaction and was added in quantity stoichiometric to the concentration of S-sulfonate groups (7.2 mM). The reaction was carried out at 4°C with open sample reaction to permit air oxidation. The progress of the reaction was followed by taking small aliquots (10 μL) of the reaction solution and quenched by addition of water containing 0.1 % TFA (15 mL) and analyzed by analytical HPLC (XSelect C4 column, 3.5 μm , 130 $\text{\AA}$, 4.6 $\times$ 150 mm). Samples (20 μL) were injected onto the HPLC C4 analytical column using a gradient of 5% eluent B in eluent A for 1 min then 5–70% B over 21 min. HPLC analysis using absorbance at 220 nm was used to detect the folded species. After 48 h, the WT-insulin was isolated in low yield (1–2% based on the amounts of sulfitolized chains used).

Combination of chain A[C6U, C11U] and sulfitolyzed chain B

A solution of chain A[C6U, C11U] analogue (3.5 mg, final concentration in the combination experiment was 1.2 mM) was dissolved in a glycine buffer (0.1 M, pH 10.6), then mixed with a solution of sulfitolized chain B (4.85 mg, final concentration in the combination experiment was 1.2 mM) in the same buffer.^[21] DTT initiated the reaction and was added in quantity stoichiometric to the concentration of S-sulfonate groups (2.4 mM in this case). The reaction was carried out at 4°C with open sample reaction to permit air oxidation. The progress of the reaction was followed by taking small aliquots (10 μL) of the reaction solution and quenched by addition of water containing 0.1 % TFA (15 μL) and analyzed by injecting 20 μL of the sample onto an analytical HPLC (XSelect C4 column, 3.5 μm , 130 $\text{\AA}$, 4.6 $\times$ 150 mm) using a gradient of 5% eluent B in eluent A for 1 min then 5–70% eluent B over 21 min, and detection at 220 nm. At the end of the recombination reaction, the Se-insulin[C6U^A, C11U^A] was isolated with a XBridge BEH300 analytical C4 column (3.5 μm , 130 $\text{\AA}$, 4.6 $\times$ 150 mm) and lyophilized to yield 31 % (2.6 mg) based on the amounts of the chains used (Figure 2).

Digestion of human insulin and Se-insulin[C6U^A, C11U^A] by Glu-V8 protease

Stock solutions of human insulin and Se-insulin[C6U^A, C11U^A] (1.0 mg mL⁻¹) were prepared in 0.1 M NH₄HCO₃ buffer and the protease was dissolved in water to obtain a concentration of 1.0 mg mL⁻¹.^[40] The reaction was initiated by adding 5 μ L of the protease solution to a volume of 250 μ L of insulin solution. The reaction was incubated at 37 °C. To monitor the degradation progress, 30 μ L of the reaction aliquots were taken and quenched with 20 μ L 0.1 % TFA in water and were placed in ice to stop the reaction. The aliquots were analyzed by analytical HPLC XSelect C18 column (3.5 μ m, 130 Å, 4.6× 150 mm) and eluted using a gradient of 10% B in eluent A for 2 min then 10–60% B over 20 min.

Reductive unfolding of human insulin and Se-insulin[C6U^A, C11U^A] by reduced glutathione (GSH)

Stock solutions of human insulin and Se-insulin[C6U^A, C11U^A] (1 mg mL⁻¹) were prepared in phosphate buffer (20 mM, pH 7.4, 0.1 M NaCl),^[42] and a stock solution of reduced glutathione (1.62 mM) was prepared in the same buffer. The reaction was initiated by adding 92.6 μ L of the GSH in 125 μ L of protein solution; buffer was added to a volume of 500 μ L. The concentration of insulin was 0.25 mg mL⁻¹ and 300 μ M for GSH. Denaturation was carried out at 37°C. To monitor the reductive unfolding progress, 60 μ L of the reaction aliquots were taken and quenched with 5 μ L 0.1 % TFA in water. The aliquots were analyzed by analytical HPLC XSelect C4 column (3.5 μ m, 130 Å, 4.6× 150 mm) and eluted using a gradient of 5% B in eluent A for 2 min then 5–70% B over 20 min.

2D-NMR studies of WT-insulin and Se-insulin[C6UA, C11UA]

Samples of WT-insulin and Se-insulin[C6UA, C11UA] were prepared under identical conditions.^[31] Proteins were dissolved in 286 μ L TDW filtered and pH was corrected to ca. 3.6 using solutions of NaOH 0.1 M and HCl 0.15 M. Acetonitrile-*d*₃ (ACN-*d*₃) was added to get a final ratio 65%:35%, TDW/ACN-*d*₃. The final concentration of the Se-insulin[C6U^A, C11 U^A] was 0.93 mM and that of the WT-insulin 0.72 mM. NMR experiments were performed with a Bruker AVII 500 MHz spectrometer operating at a proton frequency of 500.13 MHz, using a 5-mm selective probe equipped with a self-shielded *xyz*-gradient coil at 27.2 °C. The transmitter frequency was set on the water signal and calibrated at 4.811 ppm. Correlation spectroscopy (COSY)^[30] experiments were acquired using gradients for water saturation under identical conditions. Spectra were processed and analyzed with TopSpin (Bruker Analytische Messtechnik GmbH) and SPARKY3 software (Figure S6).^[46]

X-ray crystallography

Lyophilized Se-insulin was dissolved in 20 mM HCl to obtain a ca. 8 mg mL⁻¹ protein solution, which was used subsequently for crystallization experiments. Crystals of Se-insulin were obtained through the hanging-drop vapor diffusion method, appearing after ca. 3 weeks in drops containing 2 μ L protein and 1 μ L reservoir, equilibrated over a 500 μ L reservoir solution containing 1.5 M NaCl, 35 mM Na citrate, 0.5 mM Zn acetate, and 0.3 M Tris pH 7.5. The crystals were then soaked for ca. 10 s in a cryogenic solution containing 20% glycerol and 80% reservoir solution prior to their flash-freezing in liquid nitrogen for X-ray diffraction data collection. X-ray diffraction data were collected at the Se peak (λ = 0.976 Å,

$T = 100$ K) with a Pilatus 6M detector in the ID29 beamline of the ESRF synchrotron (Grenoble, France). The raw diffraction images were processed, integrated, and scaled with the XDS software,^[47] indicating a complete data set to 1.82 Å resolution belonging to the P1 space group (Table S2). These data allowed the structure determination of Se-insulin by the single anomalous dispersion (SAD) methodology, using the anomalous data that was obtained from the two Sec residues incorporated into the protein. The AutoSol software^[48] identified 20 Se atoms with a figure of merit of 0.379, producing phases of sufficient quality to automatically build 213 residues in the asymmetric unit, corresponding to respective R_{work} and R_{free} values of 36.00% and 37.84%, respectively. This initial structure was later improved by iterative cycles of manual building with the program Coot,^[49] and concomitant refinement with the program phenix.refine.^[50] The final structure was shown to contain only 16 Se atoms, corresponding to eight independent Se-insulin monomers in the asymmetric unit and 402 modeled protein residues, producing final R_{work} and R_{free} values of 18.96% and 22.92%, respectively (Figure S11). Geometric validation of the structure was conducted with Procheck^[51] and Molprobit,^[52] confirming a model of good structural and stereochemistry parameters, as demonstrated by root mean square deviation (RMSD) values of 0.013 Å and 1.491° for the bond lengths and bond angles, respectively. The final Se-insulin structure was deposited in the RCSB protein data bank^[53] under accession code 6H3M. Further details regarding data collection, data processing, and structure determination and refinement of the Se-insulin structure can be found in Table S2.

In vitro receptor-binding assays

Analogue affinities for detergent-solubilized IR-B holoreceptor^[54] were measured in a competitive-displacement assay.^[38] Successive dilutions of analogues were incubated overnight with WGA-SPA beads (PerkinElmer Life Sciences®), receptor, and radiolabeled tracer before counting.^[44] To obtain dissociation constants, competitive binding data were analyzed by nonlinear regression by the method of Wang.^[55]

Supplementary Material

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Figure 1.

Structure of WT human insulin, the proposed disulfide folding pathway, and synthetic approach of Se-insulin. a) 3D structure of human insulin, composed mainly of α -helical structures, and its three disulfide bonds A6-A11, A7-B7 and A20-B19 indicated as sticks (PDB: 3w7y). b) The proposed folding pathway of proinsulin as suggested by Feng and co-workers.^[19] The intrachain A6-A11 disulfide is replaced with a diselenide (shown in red) in our Se-insulin analogue, which would suggest that the upper path is more predominant and also avoids kinetic traps and off-pathway intermediates formation. c) Our synthetic approach

for Se-insulin[C6U^A, C11U^A] based on a simple chain combination of A chain[C6U, C11U], prepared by SPPS, and sulfitolized B chain, prepared by sulfitolysis of WT-insulin.

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Figure 2.

Combination assays for the preparation of WT-insulin and Se-insulin[C6U^A, C11U^A]. The combination reactions were performed in 0.1M glycine buffer (pH ca. 10.6) at 4°C, in the presence of DTT at stoichiometric equivalents to the concentration of sulfonate groups, and were followed by analytical HPLC. a) Recombination of WT-insulin preparation, [A chain(SSO₃²⁻)₄]=[B chain(SSO₃²⁻)₂]=1.2 mM, and [DTT]=7.2 mM. Combination product was observed after 48 h. b) Combination for Se-insulin[C6U^A, C11U^A] preparation, A chain[C6U, C11U]=[B chain(SSO₃²⁻)₂]=1.2 mM, and [DTT]=2.4 mM; c) Kinetic traces of the combination reactions for human insulin (WT-Ins, ■) and Se-insulin[C6U^A, C11U^A] (Se-Ins, ●).

Figure 3.

Structural characterization and thermodynamic stability of insulin analogues. A) CD spectra of Se-insulin[C6U^A, C11U^A] (Se-Ins, red) and human insulin (WT-Ins, blue). B) Guanidine denaturation assays of insulin analogues monitored by ellipticity at 222 nm; color code as in Panel A. The resulting stability values are given in Table 1.

Figure 4.

The X-ray crystal structure of Se-insulin[C6U^A, C11U^A] (PDB code 6H3M). a) The octameric assembly of Se-insulin obtained in the asymmetric unit of the crystal (each protomer is shown in a different color). b) A representative structure of a Se-insulin monomer, displaying the diselenide bridge formed between Sec6 and Sec11 of chain A (Se atoms shown in gold). c) The electron-density map corresponding to the region surrounding the A6-A11 diselenide bridge, demonstrating a very good fit of the model of this segment to the corresponding experimental density.

Figure 5. Receptor binding assay for insulin receptor (IR)^[37] for the Se-insulin analogue (■) compared to a semisynthetic version of WT-insulin (Orn^{B29} insulin, ●). The two proteins present a similar binding affinity to IR with $K_d = 0.08 \pm 0.02$ nM.

Figure 6.

Stability comparison between WT-insulin and Se-insulin[C6U^A, C11U^A]. a) Digestion of WT-insulin and Se-insulin[C6U^A, C11U^A] by V8 protease. The seleno-analogue is roughly four times more stable than the WT-insulin, with $t_{1/2} = 92 \pm 3$ min vs. 25 ± 2 min, respectively. b) Reductive unfolding of WT-insulin and Se-insulin[C6U^A, C11U^A] by glutathione. Se-insulin-[C6U^A, C11U^A] was more resistant to reductive conditions with $t_{1/2} = 40 \pm 3$ h vs. 11 ± 1 h for the WT-insulin. Standard deviation was within $\pm 5\%$. The data points

were fit to a first-order decay function using KaleidaGraph to estimate the rate constants, which were used to calculate $t_{1/2}$.

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Table 1.

Stabilities of insulin analogues.

Analogue	$G_u^{[a]}$ [kcal mol ⁻¹]	$C_{mid}^{[a]}$ [M]	$m^{[b]}$ [kcal mol ⁻¹ M ⁻¹]	Digestion $t_{1/2}^{[c]}$ [min]	Red. unfold $t_{1/2}^{[c]}$ [h]
WT-Ins.	3.6±0.1	4.9±0.1	0.74±0.01	25±2	11±1
Se-Ins.	4.4±0.1	5.7±0.1	0.77±0.02	92±3	40±3

^[a]Parameters were inferred from CD-detected guanidine denaturation data (Figure 3b) by application of a two-state model; uncertainties represent fitting errors for a given data set.

^[b]The m -value (slope $(G)/(M)$) correlates with extent of hydrophobic surfaces exposed on denaturation.

^[c]The $t_{1/2}$ for V8 protease digestion (Figure 6a) and reductive unfolding (Figure 6b) of WT-insulin and Se-insulin[C6U^A, C11U^A] were calculated based on a first-order decay function (for details see the Experimental Section).