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Biomimetic Synthesis of Lispro Insulin via a Chemically Synthesized "Mini-Proinsulin" Prepared by Oxime-Forming Ligation

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Abstract: Here we report a proof-of-principle study demonstrating the efficient folding, with concomitant formation of the correct disulfides, of an isolated polypeptide insulin precursor of defined covalent structure. We used oxime-forming chemical ligation to introduce a temporary "chemical tether" to link the N-terminal residue of the insulin A chain to the C-terminal residue of the insulin B chain; the tether enabled us to fold/form disulfides with high efficiency. Enzymatic removal of the temporary chemical tether gave mature, fully active insulin. This chemical tethering principle could form the basis of a practical, high yield total synthesis of insulin and analogues.

Introduction

An efficient chemical synthesis of human insulin would enable researchers to incorporate a wide variety of non-natural structures into the molecule and would thus have advantages for the full exploration of the medicinal chemistry of this important therapeutic molecule.[1] However, although the total chemical synthesis of insulin has been reported by several groups,[1−5] synthesis is still difficult in practical terms both for use in an industrial process and for research applications. This severely limits the application of synthetic chemistry to insulin. The principal difficulty originates in the two-chain nature of the insulin molecule. In mature insulin, the A and B chains are linked by interchain disulfide bonds; in addition, the A chain has an intramolecular disulfide. Folding of equimolar amounts of insulin A and B chains ("A and B chain recombination") results in a complex mixture of oxidation products containing only a low yield of the correctly fold protein molecule.[6] In nature, insulin is biosynthesized by ribosomal translation as a single long polypeptide chain, termed proinsulin, in which the B-chain is connected via a C-peptide sequence to the N-terminal residue of the A chain.[7] The 86 amino acid residue proinsulin polypeptide chain folds efficiently, and subsequent enzymatic removal of the C-peptide gives mature insulin.[8] The proinsulin-based approach has been successfully adopted in the recombinant production of insulin, and is now the standard method for the commercial production of insulin.[9,10]

A practical total chemical synthesis of insulin must address the folding of the synthetic A and B chains to give correctly folded protein in high yield. Based on the work of Katsoyannis, the individual chains are prepared as the S-sulfonates, to improve solubility and ease of handling.[3] Using isolated insulin A and B chains, it has been found that optimal folding/disulfide formation requires an ∼3-fold stoichiometric excess of A-chain over the B-chain, and gives only a ∼25% folding yield based on the limiting amount of B chain.[6] An alternative methodology with chemically directed formation of each of the three disulfide bonds has been reported.[11,12] These approaches involve rather complex protecting group schemes and are not truly practical for routine analogue production.[1] Recently, the Novo Nordisk group reported the folding of a desB30 "mini-proinsulin" molecule prepared by stepwise solid phase peptide synthesis.[13] By altering the hydrophilicity of the short connecting peptide, estimated folding yields as high as 20−25% were obtained, although it was not possible to isolate the pure reduced form of the mini-proinsulin polypeptide. However, this mini-proinsulin approach has not been applied to total chemical synthesis of mature human insulin, probably because of the need for a laborious synthesis of a long (∼60 amino acid residue) polypeptide including the connecting peptide.

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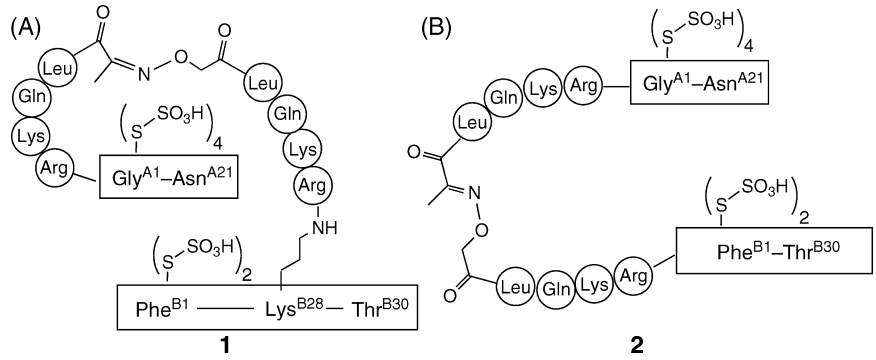

Figure 1. Designed "mini-proinsulin" precursors **1** (A) and **2** (B). The tetrapeptide -LeuGlnLysArg- corresponds to the C-terminal sequence of proinsulin's C-peptide.

We envisioned a total chemical synthesis of insulin based on the principles of the natural biosynthetic process, in which intramolecular folding/disulfide formation occurs in the natural single chain precursor proinsulin molecule.[7] One way of surmounting the challenge of making the necessary longer polypeptide would be to make use of modern chemical ligation methods. We have previously developed a modular total chemical synthesis of the proinsulin-like molecule IGF-1 (insulin-like growth factor-1) by modern methods.[14] In principle, it would be possible to use total chemical synthesis to make a similar mini-proinsulin polypeptide construct. But, there is no absolute requirement for a linear polypeptide chain precursor molecule containing only peptide bonds: Wollmer and Brandenburg et al.,[15] Obermeier and Geiger,[16] and Busse and Carpenter[17] independently reported efficient folding of intermediates in which the insulin A and B chains were covalently linked by artificial suberoyl,[15] sulfonylbis(ethoxycarbonyl),[16] or carboxyl(bismethionine)[17] chemical tethers between the epsilon amino group of Lys^{B29} and the N-terminal residue of the A chain.

The goal of the work reported here was to carry out a proof-of-principle study for the efficient folding of a chemically tethered precursor polypeptide of human insulin, in purified form and of defined covalent structure. We introduced a temporary chemical tether to covalently link the A chain to the B chain; the tether enabled us to fold/form disulfides with high efficiency. Modern chemical ligation methods were used to generate several possible forms of the tethered polypeptide chains, and the efficiency of folding/disulfide formation was explored. An oxime-linked mini-proinsulin polypeptide containing the insulin A and B chains was found to give high efficiency folding with concomitant formation of the correct disulfides. Subsequently, we showed that mature insulin with full biochemical activity was obtained by enzymatic removal of the temporary chemical tether.

Results and Discussion

Design of Chemically Tethered "Mini-Proinsulin" Precursors. We designed several covalently linked {A+B chain} intermediates, and explored the efficiency of folding/disulfide formation for each of them. Based on the work of Wollmer and Brandenburg et al.,[15] Obermeier and Geiger,[16] and Busse and Carpenter,[17] a mini-proinsulin precursor **1** (Figure 1A) was designed in which the side chain of Lys^{B28} was linked to the N-terminal residue of the A chain by means of short peptide sequences and an oxime bond. Construct **1** was prepared by oxime-forming ligation (Figure S1, for synthesis see Supporting Information). Under standard redox conditions, the oxime-linked polypeptide **1** folded and formed disulfides in relatively good yield (Figure 2A), which agrees with the reported high-yield refolding of N$^{\alpha A1}$,N$^{\varepsilon B29}$-suberoylinsulin,[15] N$^{\alpha A1}$,N$^{\varepsilon B29}$-2,2′-sulfonylbis(ethoxycarbonyl)insulin[16] and N$^{\alpha A1}$,N$^{\varepsilon B29}$-carbonylbis-(methionyl)insulin.[17] When folded **1** was treated with trypsin (after solid phase extraction and lyophilization), the Arg residue at the ε-amino group of Lys^{B28} was retained (Figure S2,

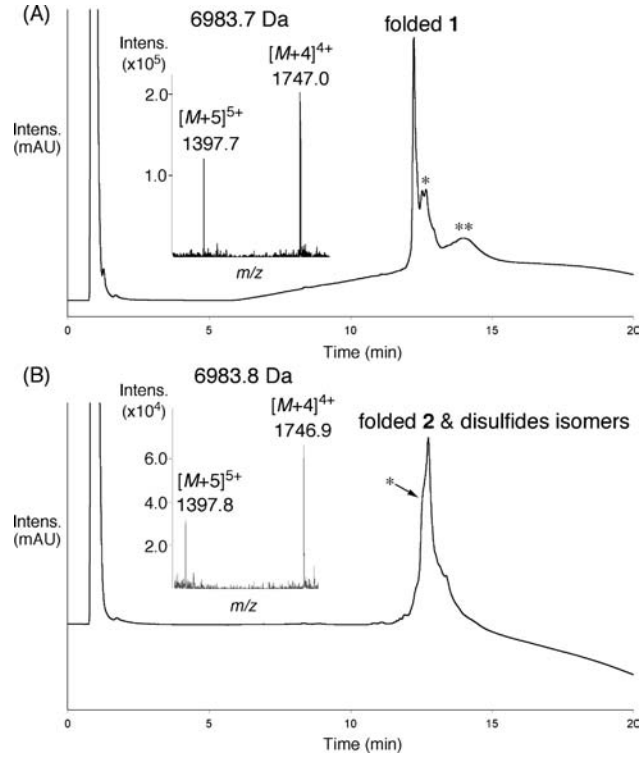

Figure 2. Folding of mini-proinsulin precursors **1** (A) and **2** (B). Conditions were: polypeptide **1** or **2**, 0.2 mg mL^{-1}; Tris: 20 mM; Cys: 8 mM; cystine: 1 mM; GnHCl 1 M; pH = 8.0. *Cys adduct, **nonpeptidic compound. (Inset) Online ESMS spectra taken at the top of the main peak in each chromatogram. (A) **1**, T = 16 h; (B) **2**, T = 3 h. The chromatographic separations were performed using a linear gradient (5−65%) of buffer B in buffer A over 15 min (buffer A = 0.1% TFA in water; buffer B = 0.08% TFA in acetonitrile) (UV profiles at 214 nm).

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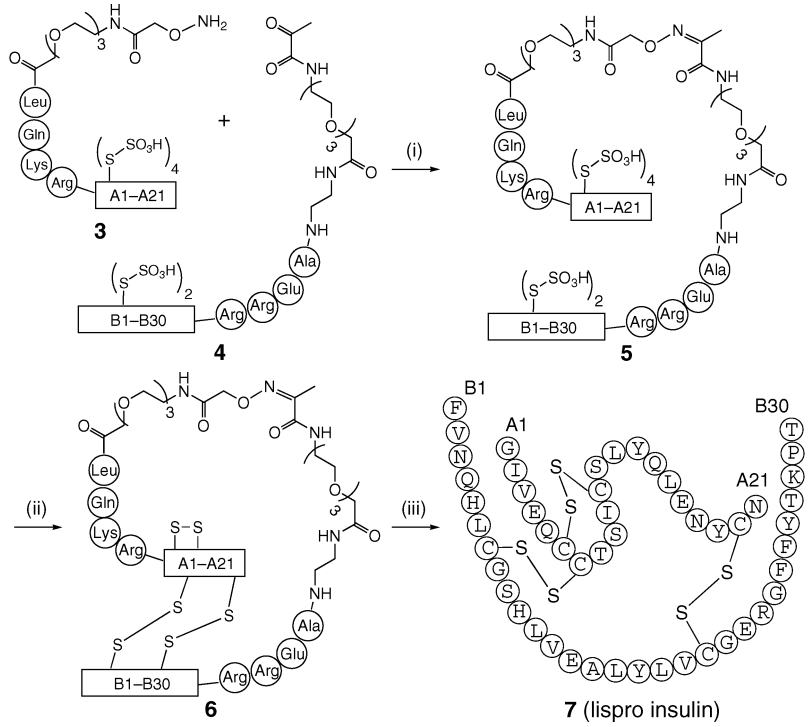

Figure 3. Strategy for the synthesis of lispro insulin, using an optimized chemical tether in the mini-proinsulin precursor **5**: (i) oxime-forming ligation; (ii) folding/disulfide formation; (iii) enzymatic cleavage. Lispro insulin is a fast-acting analogue of native human insulin in which the Pro^{B28}−Lys^{B29} sequence is inverted to Lys^{B28}−Pro^{B29}.[19]

Supporting Information), indicating that as expected the amide bond between Arg and Nε-Lys^{B28} was not cleaved by the trypsin and thus **1** did not lead to native insulin.

The Lilly group reported that "inverted" human proinsulin where the A-chain was connected to the N-terminal residue of the B-chain by a connecting peptide derived from proinsulin folded readily with correct disulfide pairings.[18] We were interested in the folding behavior of a conceptually related proinsulin analogue in which the N-terminal residues of both A-and B-chains are connected by oxime-linked peptide sequences (Figure 1B). Such a mini-proinsulin analogue **2** would be impossible to make by recombinant means due to the change of direction of the polypeptide chain, which gives a construct with two carboxy terminal residues and no N-terminus. Our synthetic target was based on "lispro insulin", a fast-acting analogue of native human insulin in which the ProB28-LysB29 sequence is inverted to LysB28-ProB29.[19] The total chemical synthesis of mini-proinsulin precursor **2** by oxime-forming ligation (Figure S3, for synthesis see Supporting Information) enabled us to perform a folding study of this unique insulin analogue. Interestingly, under standard folding conditions compound **2** gave a complex mixture dominated by broadened peaks apparently containing several disulfide isomers (Figure 2B). Online ESMS analysis taken at the top of the peak indicated an observed mass of 6983.8 Da, consistent with the formation of three disulfide bonds. While the elution time of the newly formed peak overlapped with that of starting **2**, online ESMS indicated that **2** was quantitatively consumed. The broad nature of the peak could result from formation of disulfides isomers, although LCMS analysis indicated that Cys-adducts byproducts were also present. Treatment of the folded mixture with trypsin (after solid phase extraction and lyophilization) gave at least three peaks with masses identical to lispro insulin and separated A- and B-chain derivatives (Figure S4, Supporting Information), also indicating that the folding/disulfide formation from **2** involved several disulfide isomers. The poor folding of construct **2** is consistent with the work of Wollmer and Brandenburg et al., which implied unfavorable folding of NA1,NB1-suberoylinsulin due to distortion of the molecule.[15]

Finally, based on these initial observations, we designed a mini-proinsulin precursor **5** in which the A- and B-chains are connected via a chemical tether -ArgArgGluAla-$NHCH_2CH_2$-NH−CO(ethyleneglycol)$_3$-$NHCOC(CH_3)$═$NOCH_2CO$-NH(ethyleneglycol)$_3$CO-LeuGlnLysArg- (Figure 3). Ethylenediamine and the commercially available NH_2(ethyleneglycol)$_3$COOH were used as a soluble spacer to introduce the moieties necessary for oxime-forming ligation. The tetrapeptides -ArgArgGluAla- and -LeuGlnLysArg- correspond to the N- and C-terminal sequence of proinsulin's C-peptide, respectively. In our synthetic scheme (Figure 3), **5** was formed from aminooxyacetic acid (AOA)-functionalized A-chain **3** and pyruvic acid-functionalized B-chain **4**.

Synthesis of Chemical "Mini-Proinsulin" Precursor 5. The A-chain derivative **3** was constructed using *in situ* neutralization Boc chemistry stepwise SPPS,[20] coupling of Boc-AOA by DIC (*N*,*N*′-diisopropylcarbodiimide)-NHS (*N*-hydroxysuccinimide), then HF cleavage followed by sulfonation[3] of four cysteine residues at A6, A7, A11, and A20 (Scheme S1, Supporting

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Information). In the synthesis of pyruvic acid-B-chain **4**, the protected peptide chain (Boc-Phe^{B1}-Thr(*t*Bu)^{B30}-Arg(Pbf)Arg-(Pbf)Glu(*t*Bu)Ala) was constructed using stepwise Fmoc SPPS on one nitrogen of the ethylenediamine linker of NovaTag resin (Scheme S2, Supporting Information).[21] Removal of the 4-methoxytrityl (Mmt) protecting group of the other nitrogen of the ethylenediamine linker enabled subsequent pegylation at the newly revealed amine. Then, the pyruvic acid was coupled by DIC-NHS after the removal of the Fmoc group. Desired peptide **4** was obtained by final cleavage with a TFA-scavenger cocktail, followed by sulfonation of the side chain thiols of Cys^{B7} and Cys^{B19}.

The oxime-forming ligation[22−24] of **3** (1.2 mM) with **4** (1.0 mM) in acetonitrile-H_2O (7:3) containing 0.1% TFA was approximately 70% complete at t = 30 min (Figure 4A). Purification by HPLC provided pure full-length mini-proinsulin precursor **5**, as the hexa *S*-sulfonate (Figure 4B). Note that the S-sulfonate bonds were unstable under the MS conditions used, thus giving a range of partially desulfonated species for each charge state, as shown in Figure 4B.

Folding/Disulfide Formation of Chemical "Mini-Proinsulin". Subsequent folding/disulfide formation of **5** was performed under the following conditions, previously identified as optimal for the folding of IGF-1:[14] 0.2 mg mL^{-1} **5**, 20 mM Tris, 8 mM Cys, 1 mM cystine, 1 M GnHCl, pH = 8.0. Folding, as monitored by LC, was rapid and was complete within four hours (Figure 4C). Following oxidation, the product **6** had an observed mass of 7390 ± 1 Da (calculated mass: 7391.2 Da, average isotopes), consistent with the formation of three disulfide bonds. The chemical mini-proinsulin precursor **5** folded with an HPLC yield of 60 ± 2%. Our folding study used pure polypeptide **5**, isolation of which was made possible by the use of chemical ligation technologies, and gave a folding yield 2−3 times greater than the Novo Nordisk group's constructs,[13] and thus gives more precise insights into the folding of insulin. This yield of 60% should be compared with the yield of optimized insulin A plus B chain recombination of 25% (based on limiting B chain, from 3 mol A chain + 1 mol B chain).[6] This result indicates that the "chemical tether" made the polypeptide of mini-proinsulin precursor as favorable for folding/disulfide formation as the C-peptide (35 amino acids) in proinsulin: the folding yield was similar to that of His_8-Arg-proinsulin under optimized folding conditions (60−70% yield).[25]

Enzymatic Cleavage Reaction of Chemical "Mini-Proinsulin". To show that the folded/oxidized mini-proinsulin **6** had the correct disulfides of native lispro insulin, the oxidized mini-proinsulin was enzymatically cleaved. After solid-phase extraction of the folding mixture and lyophilization, **6** was redissolved in 1.5 M Urea, and to the solution trypsin and carboxypeptidase B (1/60 of **6** each, *w*/*w*) were added (pH = 8.1). The enzymatic reaction, as monitored by LC, was complete in two hours, and lispro insulin **7** and the linker peptide were obtained as the only significant polypeptide products (Figure 5A). After purification, lispro insulin **7** was obtained in 15% yield from compound **5** (Figure 5B). The synthetic protein had the expected mass and

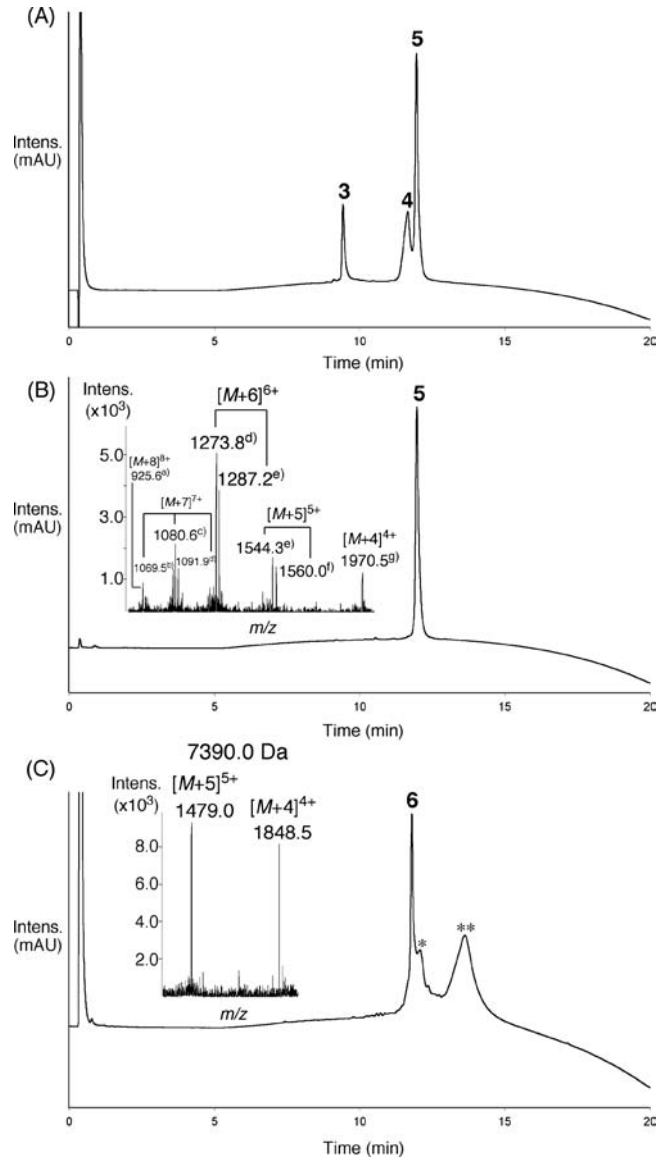

Figure 4. Synthetic data for the preparation of the oxime-linked mini-proinsulin **6**. (A) Oxime-forming ligation of **3** (1.2 mM) with **4** (1.0 mM) in acetonitrile-H_2O (7:3) + 0.1% TFA. At t = 30 min, solvents were evaporated on a speedvac, and the products were redissolved in 0.2 M Na_2HPO_4/6 M GnHCl (pH 4.6) and an aliquot analyzed by HPLC. (B) Purified **5** (Inset) Online ESMS spectra taken at the top of the main peak. The product was detected as -(S-SO_3H)$_n$ (a) n = 0, (b) n = 1, (c) n = 2, (d) n = 3, (e) n = 4, (f) n = 5, (g) = 6. (C) Folding of **5** to give the oxime-linked mini-proinsulin **6**; folding conditions were polypeptide **5**: 0.2 mg mL^{-1}, Tris: 20 mM, Cys: 8 mM, cystine: 1 mM, GnHCl: 1 M, pH = 8.0, *Cys adduct, **nonpeptidic compound, T = 4 h. Chromatographic separations were performed as described in Figure 2 legend.

was also characterized by measurement of the relative binding affinity to the insulin receptor (Figure 5C). Within experimental uncertainy, the activity of synthetic lispro insulin obtained from folded mini-proinsulin **6** was the same as that of an authentic sample of lispro insulin obtained from Eli Lilly and Co.

Conclusions

In this article, we have demonstrated a new principle for the chemical synthesis of insulin via the convergent synthesis of chemical mini-proinsulin molecules. We designed and made three mini-proinsulins using oxime-forming ligation in the chemical tether regions. Mini-proinsulin precursor **1**, the design

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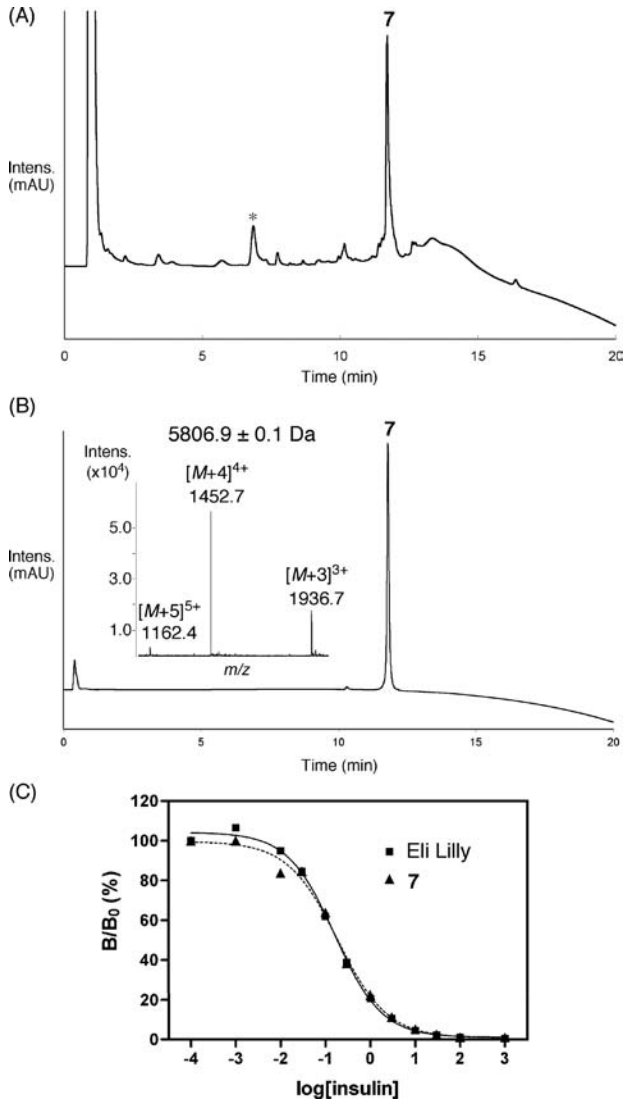

Figure 5. Enzymatic cleavage of mini-proinsulin (**6**) to give mature lispro insulin (**7**). (A) At $T = 22$ h (similar data were obtained at $T = 2$ h). Reaction conditions were trypsin: 1/60 of **6** (*w*/*w*), carboxypeptidase B: 1/60 of **6** (*w*/*w*), Urea: 1.5 M, pH = 8.1. *derived from tether fragment. Chromatographic separations were performed as described in Figure 2 legend. (B) Purified synthetic lispro insulin **7**. (Inset) Online ESMS spectra taken at the top of the main peak in each chromatogram. (C) Binding affinities of synthetic lispro insulin **7** and authentic lispro insulin (purchased from Eli Lilly and Co) to the insulin receptor.

of which was inspired by the works of Wollmer and Brandenburg et al.,[15] Obermeier and Geiger,[16] and Busse and Carpenter,[17] folded cleanly and in relatively good yield, but enzymatic cleavage necessarily gave an insuln with a residual Arg on the side chain of Lys^{B28}. Mini-proinsulin precursor **2**, in which the chemical tether joined the N-terminal residues of the A-and B-chains, did not fold properly. Finally, we designed and made mini-proinsulin precursor **5** in which the oxime chemical tether had the same connectivity as natural proinsulin. This molecule folded with great efficiency to give a single major product **6** with three disulfides. Enzymatic cleavage of this construct **6** gave native lispro insulin with full biochemical activity. This chemical mini-proinsulin approach combines the advantage of synthesis of relatively short peptides with the folding advantages of a single chain proinsulin-type molecule.

How can we improve the design of the mini-proinsulin used to promote more efficient insulin A and B chain recombination? The construct used here involves a relatively long/complicated chemical tether (i.e., eight amino acids, two polyethylene glycol moieties and an oxime bond) which makes the synthesis laborious and inefficient. This strategy is still far from practical for the efficient generation of insulin analogues or for use on an industrial scale. A much simpler type of chemical tether is needed. Because it is necessary to remove the chemical tether in a separate step to give mature insulin, simple chemical cleavage would be preferable to use of an enzyme cocktail. Therefore, we are currently developing a simpler and more practical synthesis of human insulin based on the proof-of-principle study reported here. When fully developed, this chemical tethering principle could form the basis of a practical, high yield total synthesis of insulin analogues.

Experimental Section

AOA-(ethyleneglycol)$_3$-LQKR-(Gly^{A1}-Cys(-SSO$_3$H)^{A6,A7,A11,A20}-Asn^{A21}) (3). Boc-LQKR-(Gly^{A1}-Asn^{A21}) was prepared manually by "*in situ* neutralization" Boc chemistry stepwise solid phase peptide synthesis[20] on -OCH$_2$−Pam-resins (at a 0.1 mmol scale). Side-chain protection for amino acids was as follows: Arg(Tos), Asn(Xan), Asp(OcHex), Cys(4-CH$_3$Bzl), Glu(OcHex), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(Br-Z). After the removal of the Boc group from the N-terminal residue by treatment with neat TFA, the resulting LQKR-(Gly^{A1}-Asn^{A21})-resin was reacted with Boc-11-amino-3,6,9-trioxaundecanoic acid·DCHA (0.35 mmol) in DMF in the presence of HBTU (0.32 mmol) and DIEA (0.91 mmol) for 30 min (preactivation: 30 s). After the removal of the Boc group followed by neutralization with 10% DIEA in DMF for 2 min, 1.0 mmol of (Boc-aminooxy)acetic acid was added to the resin after activation (5 min) with 1.0 mmol DIC and 1.0 mmol NHS in DMF, and coupled for 45 min. The Boc group was then removed by treatment with neat TFA for 2 min. The peptide was deprotected and cleaved from the resin support by treatment with anhydrous HF containing *p*-cresol (90:10, v/v) for 1 h at 0 °C. After evaporation of the HF under reduced pressure, crude peptide products were precipitated and triturated with chilled diethyl ether, and the peptide products were dissolved in 50% aqueous acetonitrile containing 0.1% TFA. A portion (66 mg) of the lyophilized powder was dissolved in 6 M GnHCl/0.1 M Tris (pH 8.8, 6.6 mL) in the presence of Na_2SO_3 (1.6 mmol) and $Na_2S_4O_6 \cdot 2H_2O$ (0.31 mmol), and stirred for 1 h. The crude products were purified by preparative HPLC, immediately frozen at −78 °C, and lyophilized. The disulfide bond of -S-SO$_3$H was partly labile during ESMS analysis and the product was detected as -(S-SO$_3$H)$_n$; masses: obsd, 3170.4 Da ($n = 0$), 3250.2 Da ($n = 1$), 3330.0 Da ($n = 2$), 3411.0 Da ($n = 3$), 3491.2 Da ($n = 4$); calcd (average isotopes), 3171.5 Da ($n = 0$), 3251.6 Da ($n = 1$), 3331.7 Da ($n = 2$), 3411.8 Da ($n = 3$), 3491.9 Da ($n = 4$).

(Phe^{B1}-Cys(-SSO$_3$H)^{B7,B19}-Thr^{B30})-Arg-Arg-Glu-Ala-NHCH$_2$CH$_2$NH-(ethyleneglycol)$_3$-COCOCH$_3$ (4). The peptide was prepared manually by Fmoc chemistry stepwise solid phase peptide synthesis on NovaTag resin (at a 0.4 mmol scale). Side-chain protection for amino acids was as follows: Arg(Pbf), Asn(Trt), Cys(Trt), Gln(Trt), Glu(*t*Bu), His(Trt), Lys(Boc), Ser(*t*Bu), Thr(*t*-Bu), Tyr(*t*Bu). After the removal of Fmoc group of NovaTag resin by treatment with 20% piperidine in DMF (20 min), Fmoc-Ala-OH (4.1 mmol) was coupled in the presence of HATU (3.7 mmol) and DIEA (11 mmol) in DMF for 40 min (preactivation: 30 s). The peptide chain was assembled by the sequential coupling of each activated N^{α}-Fmoc-amino acid (4.1 mmol) in DMF in the presence of HBTU (3.7 mmol) and DIEA (11 mmol) with a reaction time of 20 min. N^{α}-Fmoc deprotection was carried out by treatment with 20% piperidine (5 min, 2x). After the Boc-Phe^{B1} was introduced as an N-terminal residue, the linker Mmt group was removed by treatment with 1 M HOBt in TFE/CH$_2$Cl$_2$ (1:1) for 1 h. Subsequently, Fmoc-11-amino-3,6,9-trioxaundecanoic acid (2.8

mmol) was coupled in DMF with HBTU (2.5 mmol) and DIEA (3.3 mmol) for 2 h. Then, after the Fmoc group was removed by 20% piperidine, pyruvic acid (2.0 mmol) was coupled in DCM with DIC (2.0 mmol) and NHS (2.0 mmol) for 1 h (preactivation: 5 min). After the peptide-resin was washed with methanol and dried *in vacuo*, the peptide was cleaved from the resin and simultaneously deprotected by treatment with TFA in the presence of thioanisole, 1,2-ethanedithiol and distilled water (87.5:5:5:2.5) for 2.5 h at room temperature, concentrated *in vacuo*, and precipitated with chilled diethyl ether followed by centrifugation. The product was dissolved with 5% aqueous acetonitrile containing 0.1% TFA and lyophilized. A portion (500 mg) of the lyophilized powder was dissolved in 6 M GnHCl/0.1 M Tris (pH 8.8, 50 mL) in the presence of Na_2SO_3 (11.9 mmol) and $Na_2S_4O_6 \cdot 2H_2O$ (2.2 mmol) and stirred overnight. The crude products were purified by preparative HPLC, immediately frozen at −78 °C, and lyophilized. LCMS gave: mass obsd, 4403.8 Da; calcd, 4403.7 Da.

Synthesis of "Mini-Proinsulin" Precursor (5) by Oxime-Forming Ligation of 3 and 4. Oxime-forming ligation was performed in acetonitrile-H_2O (7:3) containing 0.1% TFA at a concentration of 1.2 mM **3** (30 mg, 8.5 μmol) and 1.0 mM **4** (32 mg, 7.2 μmol). After 30 min reaction time, solvents were evaporated on a speedvac, and the products were dissolved in 0.2 M Na_2HPO_4/6 M GnHCl (pH 4.6). HPLC purification afforded the linear polypeptide-$(SSO_3H)_6$ (**5**). Yield: 12.5 mg, 1.5 μmol, 21%; The disulfide bond of -S-SO_3H was partly broken during ESMS analysis and the product was detected as -(S-$SO_3H)_n$; masses obsd, 7396.8 Da (n = 0), 7479.5 Da (n = 1), 7557.2 Da (n = 2), 7636.5 Da (n = 3), 7716.8 Da (n = 4), 7795.0 Da (n = 5), 7878.0 Da (n = 6); calcd, 7397.6 Da (n = 0), 7477.6 Da (n = 1), 7557.6 Da (n = 2), 7637.6 Da (n = 3), 7717.6 Da (n = 4), 7797.6 Da (n = 5), 7877.6 Da (n = 6).

Folding/Disulfide Formation of 5. The purified polypeptide chain-$(SSO_3H)_6$ **5** (10.8 mg, 1.3 μmol) was folded in 1.0 M GnHCl, 20 mM Tris, 8 mM cysteine, 1 mM cystine·HCl, pH 8.0, at a concentration of <0.2 mg mL^{-1} with exclusion of air. During the folding reaction, no stirring was performed. After 4 h, HPLC analysis showed the folding was complete, and the folding buffer was diluted with 10% aqueous acetonitrile containing 0.1% TFA. After solid-phase extraction and lyophilization, the crude resulting peptide (~6.0 mg) was used for the next reaction without further purification. **6** (LCMS gave: mass obsd, 7390.0 Da; calcd, 7391.2 Da).

Enzymatic Cleavage of 6. The folded crude product (~3.0 mg) was dissolved in 1.5 M Urea (13 mL). To this solution, trypsin (1/60 of **6**, *w*/*w*) and carboxypeptidase B (1/60 of **6**, *w*/*w*) were added and the pH was adjusted to 8.1. After 2 h, HPLC analysis showed the reaction was complete, and the mixture was acidified by addition of 0.1% aq TFA, and purified by preparative HPLC to afford pure lispro insulin (**7**). Yield: 0.59 mg, 0.10 μmol, 15% (from **5**). LCMS gave: mass obsd, 5806.9 ± 0.5 Da; calcd, 5807.5 Da.

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Supporting Information Available: General procedures and materials, synthesis of **1** and **2**, procedure of receptor-binding assay, Scheme S1 and S2, and Figure S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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