Supplemental Material

for

Design of an Active Ultra-Stable Single-Chain Insulin Analog.
SYNTHESIS, STRUCTURE, AND THERAPEUTIC IMPLICATIONS

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Purpose of Supplement

This supplement describes our synthetic methods in detail and contains five additional figures and six archival tables. In Figure S1 are shown 1D ^{1}H-NMR spectra of SCI-57, 2CA, and the well-characterized engineered insulin monomer DKP-insulin. Figure S2 contains representative 2D-NMR spectra of SCI-57 and 2CA under two conditions (pD 7.6 at 32 ^{o}C and pD 7.0 at 25 ^{o}C). In Figure S3 are shown initial TOCSY spectra of SCI-57 and 2CA, obtained just after dissolving the proteins in 80% D_2O and 20% deuterioacetic acid; the two spectra exhibit similar patterns of protected amide resonances. Summaries of sequential assignment for SCI-57 and 2CA are provided in Figures S4 and S5. The six supplemental tables provide a summary of selected NMR NOEs (Tables S1 and S2), resonance assignments and chemical shifts (Tables S3 and S4), differences in chemical shifts between SCI-57 and 2CA (Table S5), and restraint information and statistical parameters related to the DG/RMD structure of SCI-57.

Experimental Procedures

Materials. Human insulin was provided by Novo-Nordisk (Copenhagen, DK). 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from AnaSpec, Inc. (San Jose, CA) and Novabiochem (Darmstadt, Germany); 1,2-diisopropycarbodiimide and N-hydroxybenzotriazole (recrystallized from 95% ethanol) were obtained from Sigma-Aldrich (St. Louis, MO). Chromatography resins were pre-swollen microgranular carboxymethylcellulose (CM-cellulose; CM52), diethylaminoethyl-cellulose (DE53; both from Whatman, Clifton, NJ) and Cellex E (Ecteola cellulose; Sigma-Aldrich, St. Louis, MO). Columns for reverse-phase (rp) high-performance liquid chromatography (HPLC) were semi-preparative C4 and C8 (dimensions 2.2 x 25 cm; Vydac, Inc., Hesperia, CA) and analytical C18 (dimensions 0.5 x 25 cm; Vydac). Solvents were HPLC grade.

Synthesis of Insulin Chains. To obtain the two-chain control analog 2CA, its component A- and B-chains (21 and 30 residues, respectively) were individually prepared by the solid-phase method using an automated peptide synthesizer (Omega model 396, Advanced Chem Tech, Louisville, KY) using Fmoc chemistry with standard side-chain protecting groups. Syntheses were performed by coupling amino-acid esters of 1-hydroxybenzotriazole (HOBt) using 1,3 diisopropylcarbodiimide (DIPC) as coupling agent. A three- or six-fold excess of N^{α}-Fmoc amino-acid esters of HOBt in N,N-dimethylformamide (DMF) was employed at each step with a 1:1 ratio of amino acid to DIPC. Deprotection of the Fmoc group was effected by 25% piperidine in DMF twice (5 min and 25 min). After completion of the syntheses, the peptides were cleaved from the solid support and de-protected using a modified reagent K® cocktail consisting of 88% trifluroacetic acid (TFA), 3% thioanisole, 5% ethanedithiole, 2% water and 2% phenol. The cleavage cocktail (4 ml) was added to the dried peptide-resins in a 15-ml glass vial blanketed with nitrogen; cleavage was carried out for 2.5 hrs with gentle magnetic stirring. Following cleavage, the mixture was filtered on a Quick-Snap column (IsoLabs Inc., Akron, OH). The filtrate was collected in 20-ml ice-cold butane ether. The peptides were allowed to precipitate for an hour at -20 ^{o}C, centrifuged, and washed twice with ice-cold methyl-*tert*-butyl ether. The precipitate was dissolved in 25% acetonitrile and lyophilized. The crude peptides were analyzed by analytical rp-HPLC (Beckman, System Gold HPLC) and mass spectrometry (MS) as determined by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF; Applied Biosystem model 4800; Foster City, CA). Sulfitolysis of the crude peptides was in each case accomplished by dissolving the material (typically 100-300 mg) in 25 ml of a buffer consisting of 0.1 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris·HCl; pH 8.8), 8 M guanidine hydrochloride to which was added 500 mg of sodium tetrathionate dihydrate ($Na_2O_6S_4$) and 750 mg of anhydrous sodium sulfite (Na_2SO_3). The solution was allowed to react for 3.5 hr at room temperature, dialyzed against distilled deionized water (ddH_2O) for 24 hours, and lyophilized.

His^{A8}-A-chain Analog. Fmoc-Asn(Trt) Wang resin; (Novabiochem, Gibbstown, NJ) was used as solid support. After deblocking and sulfitolysis, the S-sulfonated chain was purified by chromatography on a Cellex E column (1.5 x 47 cm) equilibrated with 0.1 M Tris·HCl (pH 7.0). After application of the crude A-chain and an initial wash with 100 mL of the above buffer, elution was carried out with a linear NaCl gradient (from 0-1M) using 0.1

M Tris-HCl (250 mL) and 1 M NaCl in the above buffer (250 mL). The eluted sulfonated A-chain was dialyzed against ddH_2O and lyophilized. 61 mg of the A-chain analog were obtained from 143 mg of the crude S-sulfonated material.

[Asp^{B10}, Asp^{B28}, Pro^{B29}]-B-chain Analog. Fmoc-L-Thr (tBu-protected) Wang resin (AnaSpec Inc., San Jose, CA) was used as solid support. After deblocking and sulfitolysis, the crude S-sulfonated B-chain analog was purified by chromatography on a cellulose DE52 column (1.5 cm x 47 cm) equilibrated with 0.1 M Tris·HCl (pH 7.5). Elution was carried out with a linear NaCl gradient formed by adding to the above buffer (250 ml) 0.8 M NaCl in the same buffer (250 ml). The effluent corresponding to the major peak was collected, dialyzed against ddH_2O (four changes over 24 hrs) and lyophilized. 117 mg of the B-chain analog were obtained from 124 mg of the crude S-sulfonated material.

Synthesis of [His^{A8}, Asp^{B10}, Asp^{B28}, Pro^{B29}]-Insulin. To obtain 2CA, chain combination (1) was effected by interaction of the S-sulfonated derivative of the A-chain analog (30 mg) and B-chain analog (15 mg) in 0.1 M glycine buffer (pH 10.6, 10 ml) in the presence of dithiothreitol (5.2 mg) as described (2,3). CM-52 cellulose chromatography of each combination mixture enabled partial isolation of the hydrochloride form of the protein contaminated by free B-chain (combined weight 4.3 mg). Final purification was accomplished by C18 rp-HPLC, yielding 2.8 mg of the insulin analog. Re-chromatography by analytical rp-HPLC gave a single peak; its predicted molecular mass (5808.5 Da) was verified (5807.6 Da) by MALDI-TOF MS. The overall yield of 2CA was similar to that obtained in control syntheses of wild-type human insulin.

Synthesis of single-chain insulin analog. SCI-57 was synthesized by native chemical ligation (4) of three peptides: *segment I* (B1-B6; polypeptide residues 1-6), *segment II* (B7-A6; polypeptide residues 7-42), and *segment III* (A7-A21; polypeptide residues 43-57). Segments III and II were first ligated; their product was then ligated to segment I to yield the reduced and unfolded 57-residue polypeptide. This synthetic scheme, exploiting cysteines at positions B7 and A7 as ligation sites, thus employs peptides of nominal lengths 6, 36, and 15 residues exclusive of C-terminal thioester extensions (segments I and II; see below). Assembly of peptide segments I and II employed t-Boc chemistry and an *in situ* neutralization protocol as described (5); peptide III was assembled by Fmoc chemistry on polyethylene glycol-polystylene graft polymer (PEG-PS). Each segment was synthesized by a manual solid-phase protocol as described in turn below. Analyses of purity by rp-HPLC (C4 or C18, 2.1 x 50 mm, Microsorb, packed in-house) from Varian, (Palo Alto, CA) and subsequent verification of peptide masses were performed using an Agilent 1100 liquid chromatography/mass spectrometry (LC/MS) system. Preparative HPLC was performed using C4 or C8 columns (1.0 x 25 cm or 2.2 x 25 cm) from Vydac Inc. (Hesperia, CA).

Synthesis of Segment I. Segment I was synthesized as a thioester containing a C-terminal 3-mercaptopropionyl-Leu (βMp-Leu) extension. The synthesis was started from *t*-Boc-Leu-phenylacetamido-methyl (PAM) resin (0.66 g; 0.5 mmol; Applied Biosystems; Foster City, CA), and the peptide chain was extended stepwise to the N-terminal residue. The Boc group was treated with neat TFA 3 times (each for 1 min), washed twice with DMF, and condensed with S-trityl-βMp (0.75 mmol) dissolved in 0.5M 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU/DMF; 0.675 mmol) and diisopropylethylamine (DIEA; 1.5 mmol). After 20 min with occasional mixing, the solvent was drained, and the resin was washed once with DMF. To remove the S-trityl group, a mixture of TFA/water/triisopropyl silane (95:2.5:2.5 v/v/v) was applied 3 times (each for 1 min) followed by 2 washes with DMF. The next Boc-amino acid (2 mmol), activated with 0.5 M-HBTU (1.9 mmol) and DIEA (2.6 mmol; prepared 1 min before addition), was then added. After 30 min, another portion of DIEA (2.6 mmol) was added and the mixture was left for another 30 min. Removal of the Boc group by neat TFA and subsequent coupling reactions were repeated until the last residue, but the coupling reaction time was reduced to 10 min. The N-terminal Boc group was removed, and the peptidyl resin (dried from dichloromethane; DCM) was treated with anhydrous hydrofluoric acid (HF), with 10% *p*-cresole (v/v) added as scavenger, at 0 ^{o}C for 60 min. HF was evaporated in vacuo, and the residue was washed 4 times with diethyl ether. The peptide was extracted with a mixture of acetonitrile/water/TFA (50:50:0.1 v/v/v) and freeze-dried (wt 465 mg). A portion of crude peptide (150 mg) was purified by preparative C8 rp-HPLC in a solvent system of

water with 0.1% TFA (solvent A) and acetonitrile with 0.08% TFA (solvent B). A linear elution gradient (10-35%, solvent B) was applied over 50 min at the flow rate of 10 ml/min with optical detection of peptide bonds at 215 nm. Purified segment I thus obtained was 73 mg (over-all yield, 47%). MS gave a single compound with molecular mass 958.0 Da (calculated 958.2 Da).

Synthesis of Segment II. Boc-Gly-OCH_2-Pam resin (0.64g; 0.5 mmol) was used for the synthesis of segment II, a βMp-thioester containing C-terminal extension Arg-Arg-Gly. This charged moiety enhanced the solubility of the segment prior to its ligation. The N-terminal amino acid of segment II (Cys^{B7}) was protected as 1,3-thiazolidine-4-carboxylic acid (thioproline; Thz) as described (6,7). The peptide chain was elongated as above to the N-terminal Thz and cleaved by anhydrous HF (crude weight 544 mg). A portion (136 mg) was purified by preparative rp-HPLC using a C4 column in a solvent system of A/B (see above) from 20-35% solvent B in 45 min at the flow rate of 10 ml/min. The yield of purified peptide was 29.7 mg (16.4% relative to the starting Gly-OCH_2-Pam resin). MS gave an observed molecular mass (4335.9 Da) similar to that predicted (4335.96 Da).

Synthesis of Segment III. Fmoc-Asn(trityl)-PEG-PS resin (0.5 mmol, 2.94 g; PerSeptive Biosystems, Framingham, MA), solvated in DMF, was treated with 20% piperidine in DMF for 20 min. After 3 DMF washings, the next Fmoc-amino acid (2 mmol) was activated with 0.5 M HBTU/DMF (3.8 ml), and DIEA (0.35 ml; 2 mmol) was added. The mixture was allowed to react for 20 min. An aliquot was tested for negative ninhydrin reaction prior to the subsequent step. After 3 washes with DMF, the same procedure was repeated at each cycle. The completed peptide on resin was dried from DCM after removal of the final Fmoc group (resin weight 2.12 g). A portion (1.09 g) was stirred with a cleavage mixture of 95% TFA, 2.5% water, and 2.5% ethanedithiol (v/v/v) (8 ml) at 0 ^oC for 5 min and then at room temp for 2 hr. The reaction mixture was filtered into diethyl ether (140 ml); the peptide precipitate was separated by centrifugation and washed twice more with diethyl ether. After drying in vacuo, 190 mg of crude segment III was obtained. This material was purified by preparative rp-HPLC using a C4 column at the flow rate of 10 ml/min; the linear gradient used was 10-25% (solvent B) over 45 min. Segment III thus obtained was 70 mg (overall yield 31.3%). MS demonstrated that the observed molecular mass (1789.3 Da) was in accord the predicted molecular mass (1790.02 Da).

Synthesis of SCI (1-57) by Native Chemical Ligation. Segment II, as a C-terminal-extended thioester ((Thz^7-42)-βMp-RRG; 18.5 mg (4 μmoles)), segment III (an unmodified peptide, 7.7 mg (4 μmol)), and tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl; 12 mg (0.04 mmole)) were dissolved in 1.8 ml of a ligation buffer consisting of 0.1 M sodium phosphate (pH 6.9) and 6 M guanidine·HCl. After dissolution, the pH of the solution was re-adjusted to 7.0 with 2 M sodium hydroxide (NaOH), and an aliquot of ligation buffer was added to a final volume of 2.0 ml. Argon was flushed through the solution for 1 min, and thiophenol (10 μl, 0.5% v/v) was added. The solution was tightly capped, stirred vigorously until the mixture became cloudy, placed on a shaker, and gently mixed at room temperature. After 8 hrs, additional aliquots of segment II (2 mg) and thiophenol (5 μl) were added, and the pH of the mixture was re-adjusted to 7.0. After 12 hrs, CH_3ONH_2·HCl (49 mg) was added to a final concentration of 0.2 M (6,7) and the solution was kept for 9 hrs. The reaction mixture was transferred to a dialysis tube (Spectra/Por membrane with molecular-weight cut-off 1,000 Da; Fisher Scientific, Pittsburg, PA) and dialyzed against water (1 L x 5) for 1 day at room temperature. The dialyzed mixture containing precipitates was lyophilized. The dried powder was dissolved in the same ligation buffer (2 ml) and mixed with thioester peptide segment I ((1-6)-βMp-Leu; 4.2 mg (4.3 μmol)) and TCEP·HCl (17.2 mg); the pH was re-adjusted to 6.9; argon was flushed, and thiophenol (15 μl) was added (total volume, 3ml). After 20 hr, additional segment I (3.2 mg) was added; the pH was re-adjusted to 6.9, and additional thiophenol (5 μl) was added. The reaction was continued for 8 more hrs, yielding a clear solution.

Redox-Coupled Protein Folding. To the above solution was added an oxidation-reduction-coupled (redox) buffer (0.4 ml) consisting of 100 mM reduced glutathione (GSH) and 10 mM oxidized glutathione (GSSG); the pH was adjusted to 8.6 with 2 N NaOH. This solution was immediately diluted with ddH_2O to 40 ml. Soon after, precipitates appeared, which after 16 hr were centrifuged and separated from the supernatant (*folding mixture 1*).

The precipitate was dissolved in the same ligation buffer containing redox mixture (4 ml); the pH was adjusted to 8.6, and ddH_2O was added to a final volume of 40 ml. Precipitates again appeared, and the mixture was centrifuged to separate the supernatant (*folding mixture 2*). Precipitates were once more dissolved, and the above procedure repeated to generate *folding mixture 3*. Each folding mixture was gently stirred for 1-2 days at room temperature. The extent of folding was periodically monitored by LC/MS. When almost no further folding was observed, the mixture was acidified to pH 2 with 10% TFA, and acetonitrile was added to a final concentration of 10% (v/v). The solutions were subjected to rp-HPLC using a C4 column (1 x 25cm); a gradient elution was applied from 15-35% (solvent B) over 40 min at the flow rate of 4 ml/min. Pure fractions corresponding to SCI-57 were pooled and lyophilized. In total, 6.6 mg was obtained, representing an overall yield of 24.4 % for the 2-step ligation procedure, subsequent folding, and purification. MS of the final product gave an observed molecular mass (6370.8 Da) in accord with the predicted molecular mass (6371.2 Da)

Supplemental Abbreviations

βMp, 3-mercaptopropionyl; Boc, butoxycarbonyl; ddH_2O, distilled de-ionized water; Da, Daltons; DCM, dichloromethane; DIPC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; GSH, reduced glutathione; GSSG, oxidized glutathione; HBTU, 1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; kDa, kilo-dalton of mass; LC/MS, liquid chromatography coupled MS; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight; MS, mass spectrometry; PAM, phenylacetamido-methyl resin; PEG-PS, polyethylene glycol-polystylene graft polymer; pI, isoelectric point; rp-HPLC, reverse-phase HPLC; TCEP·HCl, tris-(2-carboxyethyl)-phosphine hydrochloride; TFA, trifluroacetic acid; Thz, thiazolidine-4-carboxylic acid (thioproline); and Wang resin, *p*-benzyloxybenzyl alcohol resin.

Supplemental References

1. Chance, R. E., Hoffman, J. A., Kroeff, E. P., Johnson, M. G., Schirmer, W. E., and Bormer, W. W. (1981) The production of human insulin using recombinant DNA technology and a new chain recombination procedure. In: Rich, D. H., and Gross, E. (eds). *Peptides: Synthesis, Structure and Function; Proceedings of the Seventh American Peptide Symposium*, Pierce Chemical Co., Rockford, IL
2. Hu, S. Q., Burke, G. T., Schwartz, G. P., Ferderigos, N., Ross, J. B., and Katsoyannis, P. G. (1993) *Biochemistry* **32**, 2631-2635
3. Hua, Q. X., Hu, S. Q., Frank, B. H., Jia, W., Chu, Y. C., Wang, S. H., Burke, G. T., Katsoyannis, P. G., and Weiss, M. A. (1996) *J. Mol. Biol.* **264**, 390-403
4. Dawson, P. E., and Kent, S. B. H. (2000) *Annu. Rev. Biochem.* **69**, 923-960
5. Schnolzer, M., Alewood, P., Jones, A., Alewood, D., and Kent, S. B. (1992) *Int. J. Pept. Protein Res.* **40**, 180-193
6. Villain, J., Vizzavona, K., and Rose, K. (2001) *Chem. Biol.* **8**, 673-679
7. Bang, D., and Kent, S. B. (2004) *Angew. Chem. Int. Ed. Engl.* **43**, 2534-2538

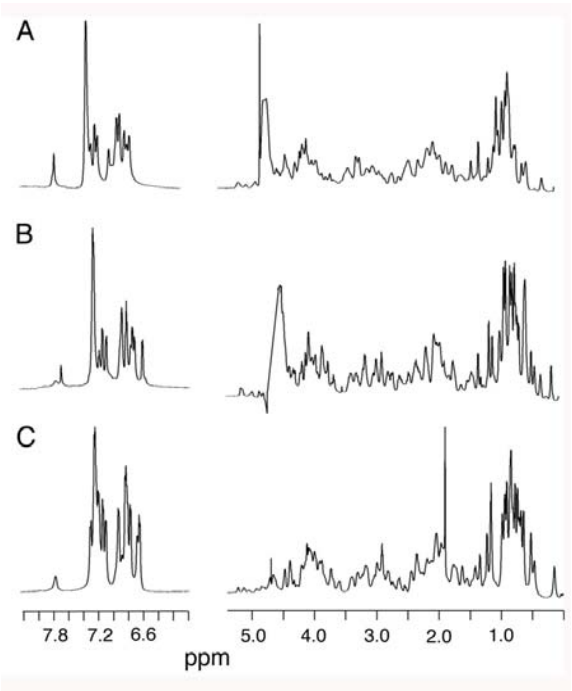

Figure S1. **Comparison of NMR spectra.** 1D ^{1}H-NMR spectra of SCI-57 (*A*), its two-chain analog 2CA (*B*) and standard engineered monomer DKP-insulin (*C*). Single-chain analog retains favorable line widths and chemical-shift dispersion. Spectra were obtained at 700 MHz in D_2O at pD 7.6 (direct meter reading) and 32 °C.

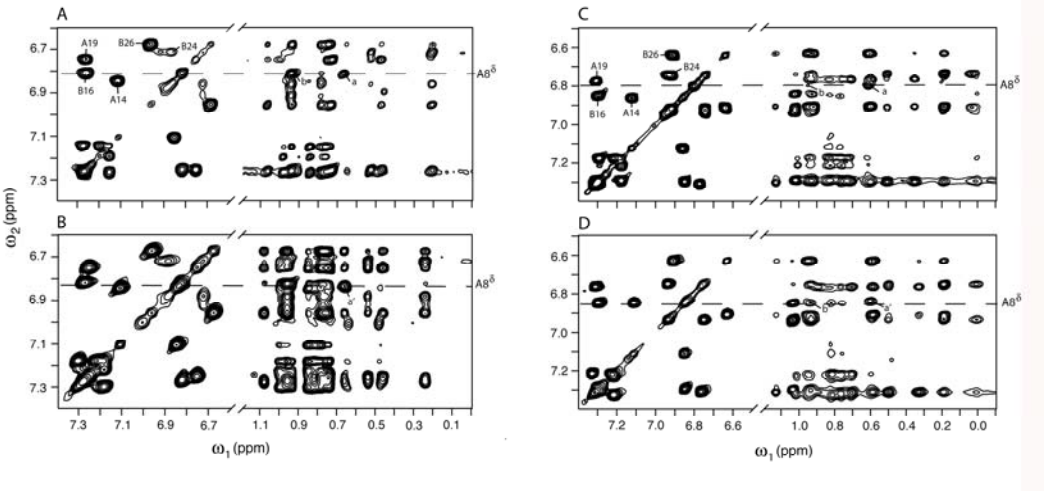

Figure S2. **2D-NMR studies of SCI-57 and 2CA in D_2O under two conditions.** (*A* and *B*) NMR studies of SCI-57. (*C* and *D*) NMR studies of 2CA. In each panel (boxed) the TOCSY spectrum of the aromatic region is shown at left, and the NOESY spectrum of aromatic-aliphatic contacts is shown at right; TOCSY and NOESY mixing times were 55 and 200 ms, respectively. Spectra in panels *A* and *C* were acquired at pD 7.6 (direct meter reading) and 32 °C whereas spectra in panels *B* and *D* were acquired at pD 7.0 and 25 °C. Selected assignments are as labeled. Corresponding long-range NOEs from the Hδ of His^{A8} to A3 γ1,2-CH_3 are labeled a and b (panels *A* and *C*) or a' and b' (panels *B* and *D*).

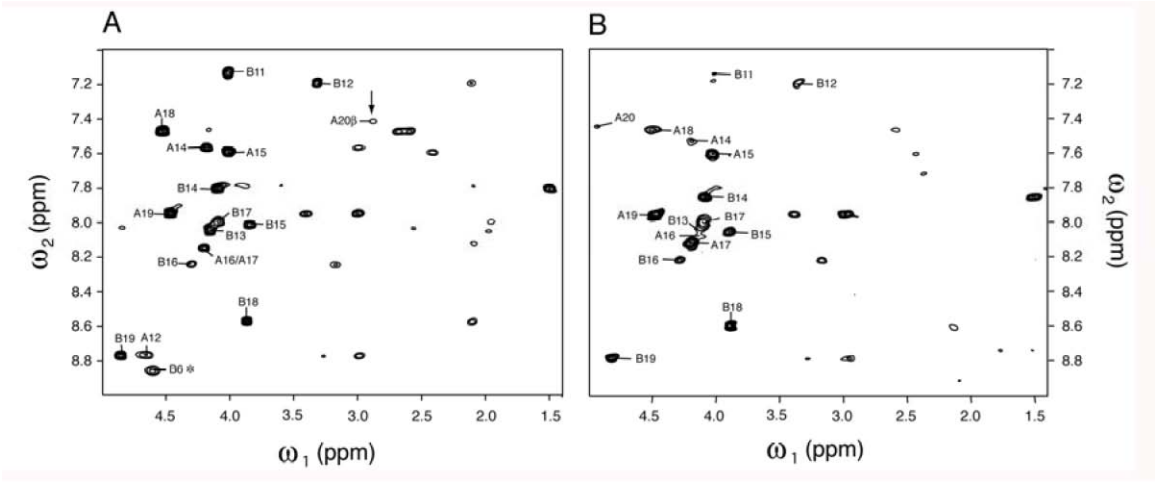

Figure S3. **SCI-57 and 2CA exhibit corresponding sites of amide proton protection in D_2O.** TOCSY spectra of SCI-57 (*A*) and its two-chain analog 2CA (*B*) obtained within two hours after sample dissolved in 20% (v/v) deuterioacetic acid at 25 °C. The sample was first dissolved in H_2O and then lyophilized as powder. 1D spectra, obtained within five minutes of initial idssolution in the deuterated solvent, provide evidence for additional sites of subglobal protection, including in each case Cys^{A11} and Leu^{B6} (sites of inter-chain or inter-domain hydrogen bonds in crystallographic T-state protomers, data not shown).

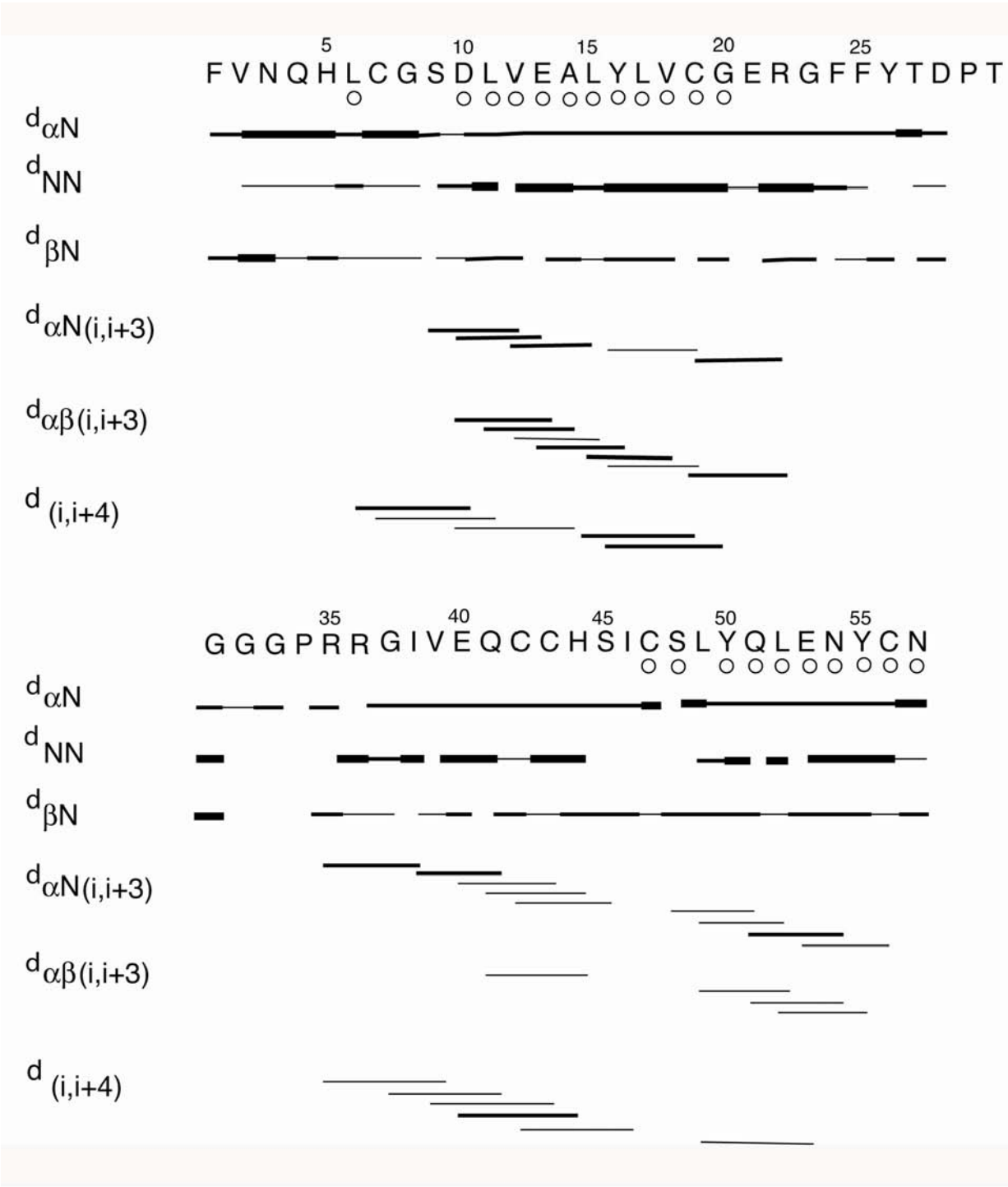

Figure S4. **Summary of sequential assignment in Wuthrich format for SCI-57.** NOE intensities are represented in schematic fashion by line thickness. Open circle indicate site of slowly exchanging amide resonances in D_2O. The residue numbering is 1 - 30 for B-domain (residues B1-B30 in insulin), 31 - 36 for C-domain (C1-C6), and 37 - 57 for A-domain (A1-A21).

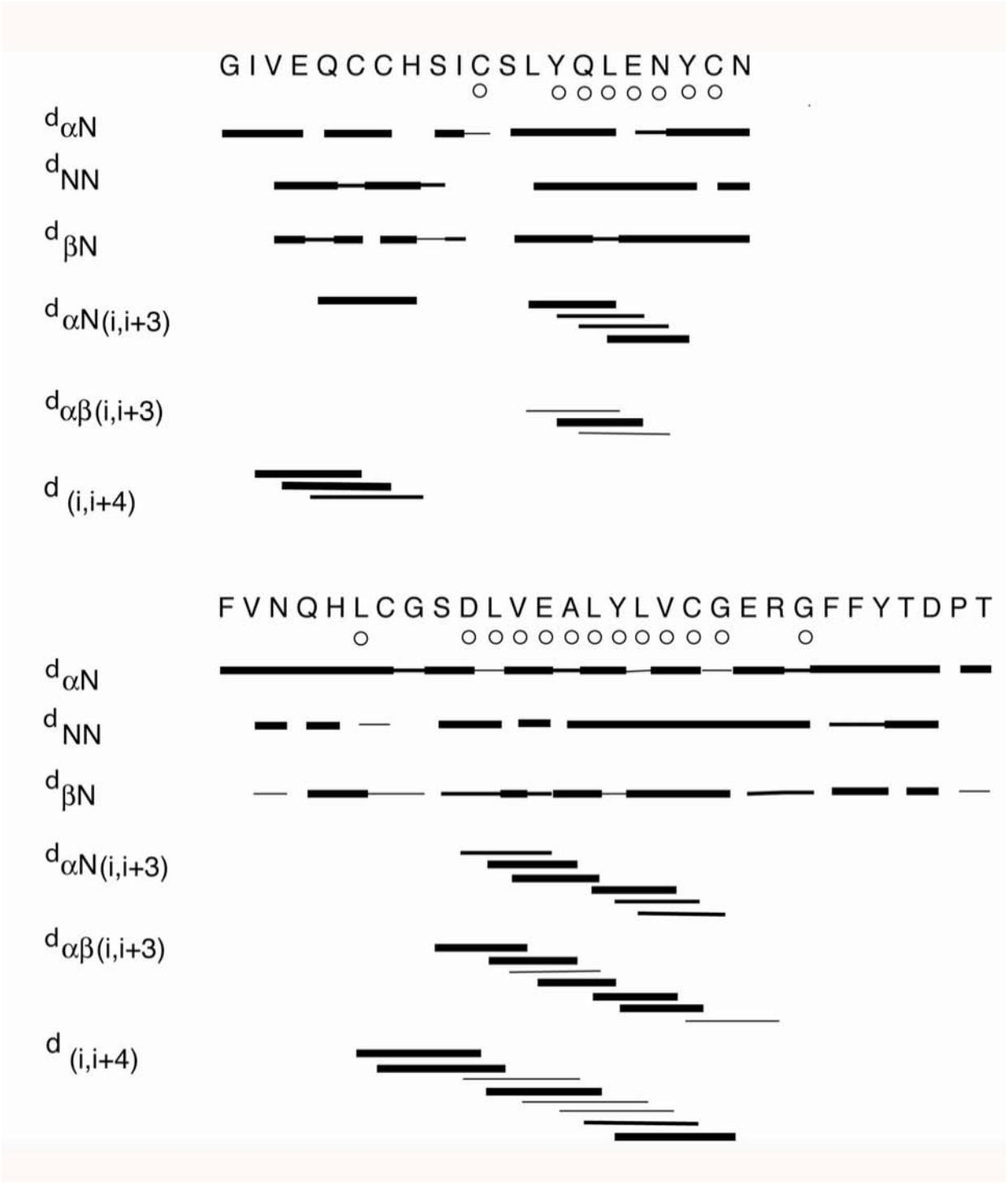

Figure S5. **Summary of sequential assignment in Wuthrich format for two-chain analog 2CA.** NOE intensities and protected amide protons are represented as in Figure S3.

Table S1. His^{A8}-related NOEs in SCI-57 and 2CA.

A8 proton	SCI-57	2CA
A8 H_N	A4 H_α	A4 H_α
	A5 H_α	A5 H_α
A8 H_α	A4 H_α	
		B5 H_α
A8 H_β	A4 H_α	A4 H_α
	A4 H_γ	
	A5 H_α, H_β	
A8 H_δ	A3 H_γ	A3 H_γ
	A4 H_α, H_β	A4 H_α
	A5 H_α, H_β	
A8 H_ε		A3 H_γ
		A4 H_β, H_γ

Table S2. NOE comparison between SCI-57 and 2CA

A, Additional NOEs in SCI-57

helix-related NOEs	long-range NOEs	inter-chain NOEs
A1 H_N - A4 H_β	A6 H_N - A10 $H_{\gamma'}$	A6 H_α - B5 H_β
A1 H_N - A5 H_β	A9 H_N - A6 H_α	A7 H_α - B5 H_β
A5 H_N - A2 H_α	A11 H_β - A5 H_β	A11 H_N - B5 H_α
A7 H_N - A4 H_α	B6 H_α - B14 H_β	A21 H_N - B19 H_α
A15 H_N - A12 H_α	B23 H_N - B19 H_β	A21 H_N - B19 H_β
A15 H_N - A12 H_β	B4 H_ε - A13 $H^{\cdot}_{1,2}$	
A19 HE - A15 H_β	B8 H_N - A7 H_β	
A20 H_N - A17 H_α	B14 H_α - A13 $H^{\cdot}_{1,2}$	
B11 H_γ - B15 $H^{\cdot}_{1,2}$	B18 H_γ - A16 $H^{\cdot}_{1,2}$	
B11 $H^{\cdot}_{1,2}$ - B15 $H^{\cdot}_{1,2}$	B19 H_α - A17 H_α	
B12 H_N - B9 H_α	B25 H_N - A19 H_β	
B14 H_N - B10 H_α		
B15 $H^{\cdot}$ - B11 $H^{\cdot}_{1,2}$		
B17 H_N - B14 H_β		

B. Additional NOEs in 2CA

helix-related NOE	long-range NOE	inter-chain NOE
none	A3 $H_{\gamma 2}$ - A19 H_ε	A21 H_δ - B23 H_α

Table S3-A. Chemical shifts of ^{1}H-NMR resonances of SCI-57 (pH 7.4 and 25 ^{o}C)

Residue	NH	$C_{\alpha}H$	$C_{\beta}H$	others
B1 Phe		4.00	3.03, 2.96	$C_{2,6}H$ 7.18, $C_{3,5}H$ 7.31
B2 Val	8.18	4.00	1.92	$C\gamma H_3$ 0.79,0.79
B3 Asn	8.62	4.63	2.82, 2.73	
B4 Gln	8.37	4.51	2.09,1.93	$C\gamma H_2$ 2.20, 2.15
B5 His	8.45	4.31	3.44, 3.04	C2H , C4H 6.89
B6 Leu	9.08	4.60	1.75, 0.86	CγH 1.66,$C\delta H_3$ 0.74, 0.71
B7 Cys	8.80	4.97	3.20, 2.96	
B8 Gly		3.98, 3.86		
B9 Ser	9.16	3.99	4.02, 3.90	
B10 Asp	7.92	4.38	2.99, 2.63	
B11 Leu	6.88	3.89	1.83, 1.13	CγH 1.22, $C\delta H_3$ 0.75, 0.65
B12 Val	6.96	3.12	2.03	$C\gamma H_3$ 0.94, 0.78
B13 Glu	7.98	4.04	2.13, 2.06	$C\gamma H_2$ 2.43, 2.30
B14 Ala	7.54	4.02	$C\beta H_3$ 1.34	
B15 Leu	7.94	3.68	1.00, 0.07	CγH 1.26, $C\delta H_3$ 0.52, 0.21
B16 Tyr	8.10	4.33	3.14, 3.14	$C_{2,6}H$ 7.27, $C_{3,5}H$ 6.82
B17 Leu	7.64	4.04	1.98, 1.37	CγH 1.75, $C\delta H_3$ 0.95,0.95
B18 Val	8.44	3.73	1.94	$C\gamma H_3$ 0.98, 0.84
B19 Cys	8.80	4.80	3.26, 2.90	
B20 Gly	7.70	3.98, 3.85		
B21 Glu	9.16	4.16	2.18, 2.04	$C\gamma H_2$ 2.34
B22 Arg	8.13	4.16	2.16, 2.04	$C\gamma H_2$ 1.85, $C\delta H_2$ 3.38, 3.32
B23 Gly	7.31	4.12, 3.83		
B24 Phe	7.63	5.09	3.32, 2.87	$C_{2,6}H$ 6.72, $C_{3,5}H$ 6.88, C_4H 6.95
B25 Phe	8.50	4.84	3.18	$C_{2,6}H$ 7.23, $C_{3,5}H$ 7.31
B26 Tyr		4.60	2.94, 2.94	$C_{2,6}H$ 6.96, $C_{3,5}H$ 6.68
B27 Thr	8.19	4.33	4.04	$C\gamma H_3$ 1.06
B28 Asp	8.50	4.75	2.76, 2.61	
B29 Pro		4.42	2.25, 1.97	$C\gamma H_2$ 1.97, $C\delta H_2$ 3.83
B30 Thr	8.33	4.32	4.29	CγH3 1.22
31 Gly				
32 Gly				
33 Gly	8.26	4.10		
34 Pro		4.29	2.10, 1.96	$C\gamma H_2$ 1.96, $C\delta H_2$ 3.60
35 Arg	8.37	4.28	1.90, 1.82	$C\gamma H_2$ 1.66, 1.62, $C\delta H_2$ 3.13, 3.07
36 Arg		4.22	1.88, 1.68	$C\gamma H_2$ 1.68, $C\delta H_2$ 3.19
A1 Gly				
A2 Ile		3.82	0.96	$C\gamma H_2$ 0.54, $C\gamma' H_3$ 0.74, $C\delta H_3$ 0.46
A3 Val	7.98	3.50	1.94	$C\gamma H_3$ 0.94, 0.66
A4 Glu		4.03	2.13, 2.13	$C\gamma H_2$ 2.49, 2.39
A5 Gln		3.93	2.11	$C\gamma H_2$ 2.32
A6 Cys	8.20	5.10	3.38, 2.81	
A7 Cys	8.21	4.88	3.78, 3.16	
A8 His	8.09	4.56	3.34, 3.29	C2H 6.82, C4H
A9 Ser	7.32	4.69	3.96, 3.83	
A10 Ile	7.80	4.27	1.52	$C\gamma H_2$ 1.01, 0.22, $C\gamma' H_3$ 0.62, $C\delta H_3$ 0.46
A11 Cys	9.98	5.18	3.22	
A12 Ser	8.59	4.62	4.20, 4.02	
A13 Ieu	8.75	3.90	1.44, 1.44	CγH 1.52, $C\delta H_3$ 0.84,0.77
A14 Tyr	7.67	4.31	3.02, 2.98	$C_{2,6}H$ 7.11, $C_{3,5}H$ 6.87
A15 Gln	7.49	3.94	2.21, 2.03	$C\gamma H_2$ 2.40, 2.34, $N\varepsilon H_2$ 7.57,7.05
A16 Leu	7.91	4.12	1.98, 1.40	CγH 1.74, $C\delta H_3$ 0.74, 0.74
A17 Glu	8.12	4.16	2.05, 1.98	$C\gamma H_2$ 2.36, 2.19
A18 Asn	7.31	4.46	2.61, 2.52	$N\delta H_2$ 7.21, 6.52
A19 Tyr	7.96	4.36	3.32, 2.89	$C_{2,6}H$ 7.26, $C_{3,5}H$ 6.75
A20 Cys	7.37	5.06	3.31, 2.84	
A21 Asn	8.12	4.48	2.80, 2.67	NδH2 7.48, 6.42

[a]Chemical-shifts relative to 5,5-dimethylsilapentanesulfonate (0 ppm).

Table S3-B. Chemical shifts of ^{1}H-NMR resonances of SCI-57 (20% deuterioacetic acid and 25 ^{o}C)

Residue	NH	$C_\alpha H$	$C_\beta H$	others
B1 Phe		4.31	3.20, 3.20	$C_{2,6}H$ 7.25, $C_{3,5}H$ 7.38
B2 Val	8.22	4.14	1.94	$C\gamma H_3$ 0.88, 0.88
B3 Asn	8.52	4.73	2.76, 2.70	$N\delta H_2$ 7.59, 6.97
B4 Gln	8.43	4.48	2.11, 1.92	$C\gamma H_2$ 2.26, 2.20, $N\varepsilon H_2$ 7.35, 6.85
B5 His	8.61	4.58	3.57, 3.27	C2H 8.50, C4H 7.40
B6 Leu	8.82	4.58	1.72, 0.98	$C\gamma H$ 1.60, $C\delta H_3$ 0.90, 0.77
B7 Cys	8.37	4.92	3.22, 2.98	
B8 Gly	9.08	4.02, 3.89		
B9 Ser	9.05	4.17	3.97, 3.97	
B10 Asp	8.24	4.52	3.22, 2.86	
B11 Leu	7.12	4.00	1.93, 1.22	$C\gamma H$ 1.34, $C\delta H_3$ 0.84, 0.75
B12 Val	7.17	3.30	2.10	$C\gamma H_3$ 0.95, 0.95
B13 Glu	8.03	4.15	2.20, 2.11	$C\gamma H_2$ 2.55
B14 Ala	7.80	4.08	$C\beta H_3$ 1.48	
B15 Leu	8.02	3.83	1.29, 0.55	$C\gamma H$ 1.45, $C\delta H_3$ 0.65, 0.42
B16 Tyr	8.24	4.28	3.16, 3.16	$C_{2,6}H$ 7.16, $C_{3,5}H$ 6.80
B17 Leu	7.98	4.08	1.97, 1.66	$C\gamma H$ 1.90, $C\delta H_3$ 0.95, 0.95
B18 Val	8.55	3.86	2.09	$C\gamma H_3$ 1.04, 0.88
B19 Cys	8.76	4.84	3.26, 2.97	
B20 Gly	7.77	4.01, 3.89		
B21 Glu	8.70	4.19	2.22, 2.12	$C\gamma H_2$ 2.56
B22 Arg	8.06	4.22	2.02, 1.83	$C\gamma H_2$ 1.80, $C\delta H_2$ 3.32, $N\varepsilon H_2$ 7.14
B23 Gly	7.52	4.07, 3.86		
B24 Phe	7.72	4.94	3.22, 2.97	$C_{2,6}H$ 6.90, $C_{3,5}H$ 7.04, C_4H 7.06
B25 Phe	8.41	4.71	3.14, 3.08	$C_{2,6}H$ 7.23, $C_{3,5}H$ 7.28
B26 Tyr	8.03	4.63	2.95, 2.95	$C_{2,6}H$ 7.07, $C_{3,5}H$ 6.78
B27 Thr	7.74	4.37	4.16	$C\gamma H_3$ 1.14
B28 Asp	8.31	4.92	2.96, 2.76	
B29 Pro		4.47	2.27, 2.06	$C\gamma H_2$ 2.02, $C\delta H_2$ 3.84
B30 Thr	7.92	4.40	4.37	CγH3 1.22
31 Gly	8.14	4.06, 4.01		
32 Gly	8.22	4.03, 4.03		
33 Gly	8.25	4.21, 4.08		
34 Pro		4.47	2.26, 2.00	$C\gamma H_2$ 1.92, $C\delta H_2$ 3.69, 3.63
35 Arg	8.34	4.31	1.94, 1.84	$C\gamma H_2$ 1.72, 1.64, $C\delta H_2$ 3.19, $N\varepsilon H_2$ 7.21
36 Arg	8.19	4.32	1.92, 1.85	$C\gamma H_2$ 1.70, 1.68, $C\delta H_2$ 3.22, $N\varepsilon H_2$ 7.24
A1 Gly	8.46	4.09, 4.09		
A2 Ile	7.83	3.87	1.17	$C\gamma H_2$ 1.38, 0.82, $C\gamma' H_3$ 0.76, $C\delta H_3$ 0.62
A3 Val	7.80	3.59	2.05	$C\gamma H_3$ 0.96, 0.86
A4 Glu	8.18	4.08	2.14, 2.14	$C\gamma H_2$ 2.53, 2.53
A5 Gln	8.28	4.09	2.16, 2.12	$C\gamma H_2$ 2.49, 2.44, $N\varepsilon H_2$ 7.53,6.94
A6 Cys	8.37	4.94	3.37, 2.91	
A7 Cys	8.00	4.82	3.73, 3.14	
A8 His	8.13	4.58	3.55, 3.36	C2H 8.70, C4H 7.30
A9 Ser	7.49	4.78	4.14, 3.94	
A10 Ile	7.91	4.47	1.63	$C\gamma H_2$ 1.20, $C\gamma' H_3$ 0.69, $C\delta H_3$ 0.57
A11 Cys	9.69		3.16	
A12 Ser	8.77	4.64	4.34, 4.03	
A13 Ieu	8.68	3.89	1.40, 1.40	$C\gamma H$ 1.46, $C\delta H_3$ 0.85, 0.79
A14 Tyr	7.57	4.18	3.00, 2.93	$C_{2,6}H$ 7.09, $C_{3,5}H$ 6.86
A15 Gln	7.59	4.00	2.05	$C\gamma H_2$ 2.47, 2.40, $N\varepsilon H_2$ 7.55, 7.00
A16 Leu	8.05	4.14	1.96, 1.52	$C\gamma H$ 1.77, $C\delta H_3$ 0.88, 0.83
A17 Glu	8.15	4.18	2.14, 2.05	$C\gamma H_2$ 2.59, 2.39
A18 Asn	7.46	4.51	2.66, 2.60	$N\delta H_2$ 7.26, 6.53
A19 Tyr	7.95	4.44	3.38, 2.99	$C_{2,6}H$ 7.31, $C_{3,5}H$ 6.80
A20 Cys	7.42	4.97	3.28, 2.87	
A21 Asn	8.27	4.72	2.88, 2.73	$N\delta H_2$ 7.52, 6.57

[a]Chemical-shifts relative to 5,5-dimethylsilapentanesulfonate (0 ppm).

Table S4-A. Chemical shifts of ^{1}H-NMR resonances of 2CA (pH 7.4 and 25^{o}C)

Residue	NH	$C_\alpha H$	$C_\beta H$	others
A1 Gly				
A2 Ile		3.69	0.90	$C\gamma H_2$ 0.70, 0.59, $C\gamma' H_3$ 0.50, $C\delta H_3$ 0.34
A3 Val	8.34	3.40	1.82	$C\gamma H_3$ 0.90, 0.60
A4 Glu	8.46	3.85	2.16, 2.16	$C\gamma H_2$ 2.58, 2.29
A5 Gln	8.04	4.03	2.07	$C\gamma H_2$ 2.53, 2.42
A6 Cys	8.30	5.01	3.21, 2.79	
A7 Cys	8.17	4.86	3.81, 3.21	
A8 His	8.10	4.52	3.48, 3.31	C2H 6.83, C4H 7.84
A9 Ser	7.16	4.71	4.05, 3.87	
A10 Ile	7.76	4.38	1.50	$C\gamma H_2$ 1.01, 0.65, $C\gamma' H_3$ 0.48, $C\delta H_3$ 0.28
A11 Cys	9.92			
A12 Ser		4.61	4.17, 3.99	
A13 leu	8.73	3.88	1.44, 1.44	$C\gamma H$ 1.51, $C\delta H_3$ 0.81,0.76
A14 Tyr	7.64	4.32	3.02, 2.98	$C_{2,6}H$ 7.11, $C_{3,5}H$ 6.85
A15 Gln	7.47	3.94	2.21, 2.02	$C\gamma H_2$ 2.39, 2.34, $N\varepsilon H_2$ 7.57,7.07
A16 Leu	7.87	4.11	1.94, 1.44	$C\gamma H$ 1.72, $C\delta H_3$ 0.77, 0.71
A17 Glu	8.01	4.19	2.04, 1.98	$C\gamma H_2$ 2.36, 2.21
A18 Asn	7.30	4.44	2.60, 2.49	$N\delta H_2$ 7.13, 6.54
A19 Tyr	7.96	4.35	3.41, 2.81	$C_{2,6}H$ 7.31, $C_{3,5}H$ 6.76
A20 Cys	7.32	5.13	3.31, 2.81	
A21 Asn	8.13	4.48	2.76, 2.64	$N\delta H_2$ 7.44,6.26
B1 Phe		4.11	3.12, 3.12	$C_{2,6}H$ 7.22, $C_{3,5}H$ 7.33
B2 Val		4.01	1.91	$C\gamma H_3$ 0.83,0.83
B3 Asn	8.57	4.59	2.81, 2.72	
B4 Gln		4.49	1.94,1.94	$C\gamma H_2$ 2.16, 2.16
B5 His	8.44	4.29	3.46, 3.06	C2H , C4H 6.95
B6 Leu	9.12	4.57	1.74, 0.81	$C\gamma H$ 1.64,$C\delta H_3$ 0.72, 0.68
B7 Cys	8.72	5.00	3.20, 2.92	
B8 Gly	9.55	3.98, 3.86		
B9 Ser	9.18	4.18	3.99, 3.99	
B10 Asp	7.90	4.38	3.03, 2.63	
B11 Leu	6.88	3.87	1.83, 1.05	$C\gamma H$ 1.12, $C\delta H_3$ 0.60, 0.60
B12 Val	7.03	3.18	2.07	$C\gamma H_3$ 1.02,0.92
B13 Glu	7.97	4.09	2.08, 2.08	$C\gamma H_2$ 2.47, 2.31
B14 Ala	7.55	4.08	$C\beta H_3$ 1.36	
B15 Leu	7.95	3.66	0.91, 0.02	$C\gamma H$ 1.19, $C\delta H_3$ 0.50, 0.19
B16 Tyr	8.20	4.40	3.17, 3.17	$C_{2,6}H$ 7.30, $C_{3,5}H$ 6.85
B17 Leu	7.68	4.08	1.91, 1.78	$C\gamma H$ 1.78, $C\delta H_3$ 0.94,0.94
B18 Val	8.43	3.77	1.97	$C\gamma H_3$ 1.00, 0.86
B19 Cys	8.88	4.88	3.29, 2.92	
B20 Gly	7.75	3.98, 3.85		
B21 Glu	9.18	4.18	2.20, 2.05	$C\gamma H_2$ 2.37
B22 Arg	8.13	4.12	2.16, 2.08	$C\gamma H_2$ 1.89, $C\delta H_2$ 3.40, 3.32
B23 Gly	7.31	4.10, 3.80		
B24 Phe	7.52	5.14	3.38, 2.91	$C_{2,6}H$ 6.75, $C_{3,5}H$ 6.92, C_4H 6.94
B25 Phe	8.69	4.87	3.21, 3.18	$C_{2,6}H$ 7.28, $C_{3,5}H$ 7.32
B26 Tyr	8.18	4.61	2.99, 2.89	$C_{2,6}H$ 6.92, $C_{3,5}H$ 6.63
B27 Thr	7.56	4.29	4.08	$C\gamma H_3$ 1.13
B28 Asp	8.21	4.84	2.74, 2.47	
B29 Pro		4.50	2.20, 2.04	$C\gamma H_2$ 1.94, $C\delta H_2$ 3.79
B30 Thr	7.90	4.22	4.09	$C\gamma H3$ 1.18

[a]Chemical-shifts relative to 5,5-dimethylsilapentanesulfonate (0 ppm).

Table S4-B. Chemical shifts of $_1$H-NMR resonances of 2CA (20% deuterioacetic acid and 25^{o}C)

Residue	NH	CαH	C_βH	others
A1 Gly				
A2 Ile	8.57	3.88	1.13	CγH$_2$ 0.94, 0.81, Cγ'H$_3$ 0.74, CδH$_3$ 0.60
A3 Val	8.14	3.58	1.95	CγH$_3$ 0.95, 0.83
A4 Glu	8.22	4.04	2.16, 2.16	CγH$_2$ 2.56, 2.46
A5 Gln	8.26	4.08	2.12, 2.12	CγH$_2$ 2.52, 2.44, NεH$_2$
A6 Cys	8.42	4.96	3.34, 2.87	
A7 Cys	8.00	4.85	3.78, 3.18	
A8 His	8.19	4.58	3.58, 3.40	C2H 8.73, C6H 7.31
A9 Ser	7.44	4.78	4.14, 3.96	
A10 Ile	7.88	4.42	1.61	CγH$_2$ 1.18, Cγ'H$_3$ 0.70, CδH$_3$ 0.58
A11 Cys	9.74			
A12 Ser	8.77	4.64	4.34, 4.03	
A13 leu	8.67	3.89	1.38, 1.38	CγH 1.45, CδH$_3$ 0.83,0.77
A14 Tyr	7.54	4.19	3.00, 2.92	C$_{2,6}$H 7.08, C$_{3,5}$H 6.85
A15 Gln	7.60	4.02	2.05	CγH$_2$ 2.49, 2.41, NεH$_2$ 7.54,6.98
A16 Leu	8.10	4.17	1.94, 1.62	CγH 1.76, CδH$_3$ 0.83, 0.81
A17 Glu	8.13	4.21	2.13, 2.06	CγH$_2$ 2.59, 2.38
A18 Asn	7.45	4.50	2.62, 2.56	NδH$_2$ 7.20, 6.56
A19 Tyr	7.93	4.46	3.38, 2.98	C$_{2,6}$H 7.34, C$_{3,5}$H 6.81
A20 Cys	7.44	4.92	3.27, 2.85	
A21 Asn	8.31	4.75	2.89, 2.77	NδH$_2$ 7.53,6.66
B1 Phe		4.30	3.15, 3.15	C$_{2,6}$H 7.24, C$_{3,5}$H 7.37
B2 Val	8.23	4.15	1.94	CγH$_3$ 0.88,0.88
B3 Asn	8.52	4.74	2.76, 2.76	NδH$_2$ 7.60,6.96
B4 Gln	8.45	4.49	2.10,1.92	CγH$_2$ 2.27, 2.20, NεH$_2$ 7.36,6.86
B5 His	8.63	4.54	3.56, 3.28	C2H 8.62, C6H 7.41
B6 Leu	8.85	4.59	1.74, 0.95	CγH 1.63,CδH$_3$ 0.91,0.79
B7 Cys	8.37	4.93	3.21, 2.96	
B8 Gly	9.13	4.03, 3.89		
B9 Ser	9.05	4.18	4.02, 4.02	
B10 Asp	8.22	4.53	3.22, 2.86	
B11 Leu	7.12	4.01	1.91, 1.22	CγH 1.37, CδH$_3$ 0.82, 0.77
B12 Val	7.19	3.35	2.11	CγH$_3$ 0.96,0.96
B13 Glu	8.02	4.15	2.20, 2.11	CγH$_2$ 2.55
B14 Ala	7.83	4.09	CβH$_3$ 1.49	
B15 Leu	8.04	3.88	1.34, 0.70	CγH 1.50, CδH$_3$ 0.70, 0.52
B16 Tyr	8.20	4.28	3.17, 3.17	C$_{2,6}$H 7.16, C$_{3,5}$H 6.80
B17 Leu	7.98	4.09	1.94, 1.66	CγH 1.87, CδH$_3$ 0.95,0.95
B18 Val	8.58	3.88	2.12	CγH$_3$ 1.04, 0.90
B19 Cys	8.78	4.82	3.28, 2.96	
B20 Gly	7.78	3.96, 3.96		
B21 Glu	8.50	4.24	2.21, 2.11	CγH$_2$ 2.53
B22 Arg	8.03	4.24	1.97, 1.82	CγH$_2$ 1.76, CδH$_2$ 3.28, NεH$_2$ 7.15
B23 Gly	7.69	4.04, 3.86		
B24 Phe	7.74	4.85	3.18, 2.94	C$_{2,6}$H 6.95, C$_{3,5}$H 7.09, C$_4$H
B25 Phe	8.34	4.70	3.13, 3.04	C$_{2,6}$H 7.21, C$_{3,5}$H 7.28
B26 Tyr	8.01	4.64	2.94, 2.94	C$_{2,6}$H 7.04, C$_{3,5}$H 6.78
B27 Thr	7.79	4.33	4.12	CγH$_3$ 1.14
B28 Asp	8.31	4.90	2.96, 2.75	
B29 Pro		4.51	2.26, 2.03	CγH$_2$ 2.03, CδH$_2$ 3.81
B30 Thr	7.97	4.49	4.40	CγH3 1.22

[a]Chemical-shifts relative to 5,5-dimethylsilapentanesulfonate (0 ppm).

Table S5-A. Chemical-Shift Differences between SCI-57 and 2CA at pH 7.4 and 25 °C[a]

residue	NH	$C_\alpha H$	$C_\beta H$	others
B1 Phe		-0.11		
B8 Gly				
B9 Ser		-0.19		
B11 Leu				C_γ H 0.10, CδH_3 0.15
B12 Val				$C_\gamma H_3$,-0.14
B16 Tyr	-0.10			
B20 Gly	-0.12			
B24 Phe	0.11			
B25 Phe	-0.19			
B27 Thr	0.63			
B28 Asp	0.29		0.14	
B30 Thr	0.43	0.10	0.20	
A2 Ile		0.13		$C_\gamma H_2$ -0.16
A3 Val	-0.36	0.10	0.12	
A4 Glu				$C_\gamma H_2$, 0.10
A5 Gln	-0.10			$C_\gamma H_2$, -0.10
A6 Cys	-0.10		0.17	
A9 Ser	0.16			
A10 Ile		-0.11		Cγ'H_3 0.14, CδH_3 0.18
A17 Glu	0.11			

Table S5-B. Chemical-Shift Differences between SCI-57 and 2CA (20% deuterioacetic acid and 25°C)

residue	NH	$C_\alpha H$	$C_b H$	others
B5 His		0.10		C_2H -0.12
B15 Leu				$C_\delta H3$ -0.10
B21 Glu	0.20			
B23 Gly	-0.17			
A2 Ile	-0.74			$C_\gamma H_2$ 0.44
A3 Val	-0.34		0.10	

[a]Chemical Shifts relative to 5,5-dimethylsilapentanesulfonate (0 ppm). Only $\Delta\delta > 0.1$ppm listed.

Table S6. DG/RMD Statistical parameters for SCI-57 Ensemble (± 2σ)

NOE restraints:		Average restraint violations	
total	783	NOE-violation	0.038 Å
sequential	287	Dihedral angle-violation	1.72°
medium	167		
long-range	202		
Intra-residue	127		
Dihedral angle restraints:	47	Deviations from idea covalent geometry	
φ-angles	34	bond length	0.004 Å
χ_1-angles	13	bond angle	0.69 °
H-bonds:	22		
Main chain (RMSD[1])		Empirical energy function[3] (kcal/mol)	
α-helices[2]	0.23	NOE restrain energy	59.18 ± 9.6
A-domain	0.33	van der Waals	29.64 ± 2.77
B-domain	0.20	improper dihedral angles	16.76 ± 1.76
Side chain (RMSD[1])		constrained dihedral angles	8.59 ± 2.07
α-helices[2]	0.56	covalent bond lengths	15.09 ± 1.96
A-domain	0.79	bond angles	81.81 ± 6.24
B-domain	0.50	total	241.10 ± 21.61

(1) RMSD were calculated according to C_α atoms of residues A1-19 and B5-25.
(2) Helical segments employed in RMSD calculation span residues A2-7, A13-19 and B9-19.
(3) NOE and dihedral force constants were 40 kcal A^{-2} and 40 kcal $radian^{-2}$, respectively (1 kcal = 4.18 kJ)