Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Comparative Properties of Insulin-like Growth Factor 1 (IGF-1) and [Gly7D-Ala]IGF-1 Prepared by Total Chemical Synthesis

Youhei Sohma, Brad L. Pentelute, Jonathan Whittaker, Qin-xin Hua, Linda J. Whittaker, Michael A. Weiss, and Stephen B. H. Kent*

General procedures and Materials Boc-amino acids was obtained from Peptide Institute, Inc. (Osaka, Japan). S-Trityl-*β*-mercaptopropionic acid and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Peptides International, Inc. (Louisville, KY). *N,N*-Diisopropylethylamine (DIEA) was obtained from Applied Biosystems (Foster City, CA). *N,N*-Dimethylformamide (DMF), dichloromethane (DCM), and acetonitrile were purchased from Fisher (Chicago, IL). *p*-Cresol was purchased from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) was from Halocarbon (New Jersey). All molecular biological procedures including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation and DNA sequencing were performed by standard methods.[1] All oligonucleotides were purchased from Integrated DNA Technologies. Restriction and modifying enzymes were from New England Biolabs. Recombinant human insulin and HPLC-purified monoiodinated ^{125}I-[Tyr 31] IGF-1 were from NovoNordisk A/S.[2] HPLC-purified monoiodinated ^{125}I-[Tyr A14] insulin was from Amersham. Recombinant human IGF-1 was from GroPep. Protease inhibitors were from Roche Molecular Biochemicals. PEAK Rapid cells were purchased from Edge Biosystems. Medium and serum for tissue culture were from Cellgro. The mammalian expression vector pcDNA3.Zeo+ was from InVitrogen. cDNAs for the insulin and IGF-1 receptors were as previously described.[3,4] Both were modified for subcloning into the epitope tag expression vectors by introduction of a Bam HI site encoding an in-frame C-terminal Gly-Ser linker at their 3' ends just prior to the stop codon by site-directed mutagenesis.[5] Monoclonal anti-FLAG IgG was purchased from Sigma-Aldrich.

Peptide Segment Synthesis (peptide-$^{\alpha}$carboxylate or peptide-$^{\alpha}$thioester) Peptides were prepared manually by "in situ neutralization" Boc chemistry stepwise solid phase peptide synthesis,[6] on -OCH_2-Pam-resins (free $^{\alpha}$carboxyl peptides) or on $HSCH_2CH_2CO$-Ala-OCH_2-Pam-resin[7] or $HSCH_2CH_2CO$-$(Arg)_4$-Ala-OCH_2-Pam-resin ($^{\alpha}$thioester peptides).[8] Side-chain protection for amino acids was as follows: Arg(Tos), Asn(Xan), Asp(OcHex), Cys(4-CH3Bzl), Glu(OcHex), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(Br-Z). After completion of the chain assembly, peptides were 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 30–50% aqueous acetonitrile containing 0.1% TFA. Measured masses were; IGF-1[1-17]$^{\alpha}$thioester-$(Arg)_4$-Ala-OH (obsd, 2545.2 ± 0.3 Da; calcd (average isotopes), 2545.9 Da), [Gly7D-Ala]IGF-1[1-17]$^{\alpha}$thioester-$(Arg)_4$-Ala-OH (obsd, 2559.0 ± 0.3 Da; calcd, 2559.9 Da), IGF-1[Thz18-47]$^{\alpha}$thioester (obsd, 3439.6 ± 0.4 Da; calcd, 3440.7 Da), IGF-1[Cys48-70] (obsd, 2658.8 ± 0.3 Da; calcd, 2659.1 Da).

Analytical HPLC. Peptide compositions were evaluated by analytical reverse phase LC-MS using a gradient of 0.08% TFA in acetonitrile versus 0.1% TFA in water. For all the work reported in this paper, analytical HPLC was carried out as follows: Vydac C4 2.1 X 150 mm column using a linear gradient of 5-65% buffer B over 15 min at 40 °C with a flow rate of 0.5 mL/min (buffer A= 0.1% TFA in H_2O; buffer B = 0.08% TFA in acetonitrile). The eluent was monitored at 214 nm, with on-line electrospray mass spectrometry (MS) using an Agilent 1100 LC-ion trap.

Preparative HPLC: Peptides were purified on C8 silica with columns of dimension 10 X 250 mm. The silica used was TP Vydac. Crude peptides were loaded onto the prep column and eluted at a flow rate of 10 mL per minute with a shallow gradient (e.g. 5%B-55%B over 100 minutes) of increasing concentrations of solvent B (0.08% TFA in acetonitrile) in solvent A (0.1% TFA in water). Fractions containing the pure target peptide were identified by analytical LC and/or MALDI MS, and were combined and lyophilized. A full description of preparative HPLC methods, similar to those used in this work, can be found in Reference 9.

Synthesis of IGF-1[18-70] by Native Chemical Ligation of IGF-1[Thz18-47]$^{\alpha}$thioester and IGF-1[Cys48-70]: Native chemical ligation reaction was performed in aqueous solution containing 6 M GnHCl, 200 mM phosphate, 10 mM (4-carboxymethyl)thiophenol (MPAA), 20 mM tris-(2-carboxyethyl)phosphine (TCEP), pH 6.7, at concentration of 2 mM for each peptide (IGF-1[Thz18-47]$^{\alpha}$thioester: 60.3 mg, 17.5 μmol; IGF-1[Cys48-70]: 46.5 mg, 17.5 μmol).[10] The ligation reaction was completed after 14 h, then conversion of Thz- to Cys-peptide was performed by adding 0.2 M methoxylamine·HCl and the pH of the reaction was adjusted to 4 (reaction time = 3 h). After solid-phase extraction and lyophilization, the crude resulting peptide (105.4 mg) was used for the next ligation without further purification. IGF-1[18-70] (LCMS gave: obsd, 5910.5 ± 0.7 Da; calcd, 5910.7 Da).

Synthesis of linear polypeptide IGF-1[1-70] by Native Chemical Ligation of IGF-1[1-17]$^{\alpha}$thioester-$(Arg)_4$-Ala-OH and IGF-1[Cys18-70]: Native chemical ligation reaction was performed in aqueous solution containing 6 M GnHCl, 200 mM phosphate, 200 mM MPAA, 20 mM TCEP, pH 6.9, at concentration of 2 mM for each peptide (IGF-1[1-17]$^{\alpha}$thioester-$(Arg)_4$-

Ala-OH: 22.7 mg, 8.9 μmol, IGF-1[Cys18-70]: 52.8 mg, 8.9 μmol) (reaction time = 18 h). HPLC purification of the ligation solution afforded the linear polypeptide IGF-1$(SH)_6$. Yield: 22.3 mg, 2.9 μmol, 32% (LCMS gave: obsd, 7655.2 ± 0.4 Da; calcd, 7654.6 Da).

[Gly7D-Ala]IGF-1[1-70] was obtained in a similar manner using [Gly7D-Ala]IGF-1[1-17]$^{\alpha}$thioester-$(Arg)_4$-Ala-OH (17.3 mg, 6.7 μmol). Yield: 18.6 mg, 2.4 μmol, 35%, (LCMS gave: obsd, 7666.4 ± 0.8 Da; calcd, 7668.7 Da)

Folding: The purified polypeptide chain IGF-1$(SH)_6$ (22.3 mg, 2.9 μmol) was folded in 0.5 M GnHCl, 20 mM Tris, 8 mM cysteine, 1 mM cystine·HCl, pH 7.8, at a concentration of ~0.5 mg/mL with exclusion of air. During the folding reaction, no stirring was performed. After 1 h, HPLC showed the folding was complete, and the folding buffer was acidified with 0.1% aq. TFA (this caused the pH of the solution to drop to ≈2) and purified by preparative HPLC to afford pure IGF-1 and IGF-swap. Yield: IGF-1 (4.8 mg, 0.62 μmol, 21%); IGF-swap (1.8 mg, 0.23 μmol, 8.1%). The folded products were characterized by LC-MS; IGF-1 (obsd, 7648.6 ± 0.6 Da; calcd, 7648.6 Da), IGF-swap (obsd, 7647.7 ± 0.2 Da; calcd, 7648.6 Da). [Gly7D-Ala]IGF-1 was also obtained in a similar manner by folding 16.0 mg (2.0 μmol) of [Gly7D-Ala]IGF-1$(SH)_6$. [Gly7D-Ala]IGF-1 (3.0 mg, 0.39 μmol, 18%, LCMS gave: obsd, 7661.8 ± 0.3 Da; calcd, 7662.7 Da); [Gly7D-Ala]IGF-swap (3.0 mg, 0.39 μmol, 18%, LCMS gave: obsd, 7662.4 ± 0.6 Da; calcd, 7662.7 Da).

Construction of Receptor Expression Plasmids To produce plasmids for the expression of receptor with a C-terminal epitope tag, in-frame coding sequences for a triple repeat of FLAG (DYKDDDDK), were introduced between the BamHI and XbaI sites of the pcDNA3.Zeo+ polylinker by cassette mutagenesis. In-frame BamHI restriction sites were introduced at the 3' end of the coding regions of the receptor cDNAS, just upstream from the stop codons. These were then subcloned into the Hind III and Bam HI sites of the FLAG epitope tag modified expression plasmid.

Expression of Receptor cDNAs DNA for transfection was prepared as previously described by others.[5] The receptor cDNAs were expressed transiently in PEAK rapid cells using Polyethyleneimine.[11] Cells were harvested by lysis in 0.15M NaCl 0.1M Tris pH 8 containing 1% (v/v) Triton X-100 and protease inhibitor cocktail three days post-transfection. Lysates were stored at -80^{0}C until assay.

Guanidine.HCl Denaturation by Circular Dichroism. CD spectra were acquired at 4 ^{o}C with an Aviv spectropolarimeter (Aviv Biomedical, Inc., Lakewood, NJ) and normalized by mean residue ellipticity. Estimates of secondary structure were obtained by deconvolution. Denaturation data for wild-type IGF-I, wild-type IGF-swap, and D-Ala7-IGF-I were fitted by non-linear least squares to a two-state model. In brief, CD data $\theta(x)$, where x indicates the concentration of denaturant, were fitted by a nonlinear least-squares program according to

$$\theta(x) = \frac{\theta_A + \theta_B e^{(-\Delta G^o_{H_2O} - mx)/RT}}{1 + e^{-(\Delta G^o_{H_2O} - mx)/RT}}$$

where x is the concentration of guanidine hydrochloride and where θ_A and θ_B are baseline values in the native and unfolded states. These baselines were approximated by pre- and post-transition lines $\theta_A(x) = \theta_A^{H_2O} + m_A x$ and $\theta_B(x) = \theta_B^{H_2O} + m_B x$. Fitting CD data and baselines simultaneously circumvents artifacts associated with linear plots of ΔG as a function of denaturant according to $\Delta G^{\circ}(x) = \Delta G^{\circ}_{H_2O} + m^o x$. Fitting of this standard two-state model to the denaturation transition of D-Ala7-IGF-swap yields anomalously high estimates of DG_u due to its rightward shift and markedly increased steepness near the midpoint of the transition. For this reason only a conservative lower bound is provided for the gain in stability.

Receptor Binding Assays Insulin and IGF-1 competitive receptor binding assays were performed by a modification of the microtiter plate antibody capture assay that Whittaker and colleagues have described previously.[12] Microtiter strip plates (Nunc Maxisorb) were incubated overnight at 4 $^{\circ}$C with anti-FLAG IgG (100 ml/well of a 40 mg/ml solution in PBS). Washing, blocking, receptor binding and competitive binding assays with labeled and unlabeled peptides were performed as described. Binding data were analyzed by a 2 site sequential model with homologous or heterologous labeled and unlabeled ligands to obtain dissociation constants.[13,14]

References

1. J. Sambrook, D. W. Russell, (2001) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. L. Schaffer, U. D. Larsen, S. Linde, K. R. Hejnaes, L. Skriver, *Biochim. Biophys. Acta* **1993**,*1203*, 205-209.
3. D. C. Mynarcik, P. F. Williams, L. Schaffer, G. Q. Yu, J. Whittaker, *J. Biol. Chem.* **1997**, *272*, 18650-18655.
4. J. Whittaker, A. K. Okamoto, R. Thys, G. I. Bell, D. F. Steiner, C. A. Hofmann, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 5237-5241.
5. R. D. Kirsch, E. Joly, E. *Nucleic Acids Res.* **1998**, *26*, 1848-1850.
6. M. Schnolzer, P. Alewood, A. Jones, D. Alewood, S. B. Kent, *Int. J. Peptide Protein Res*. **1992**, *40*, 180-193.
7. T. M. Hackeng, J. H. Griffin, P. E. Dawson, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10068-10073.
8. E. C. B. Johnson, S. B. H. Kent, *Tetrahedron Lett.* **2007**, *48*, 1795-1799.
9. Synthesis, purification and characterization of biologically active peptides. Clark-Lewis I., Kent S.B.H., in Receptor Biochemistry and Methodology Vol. 14 (J.C. Venter, L.C. Harrison, series eds.) - The use of HPLC in Protein Purification and Characterization, A.R. Kerlavage, vol. ed., Alan R. Liss, Inc., New York, 1989, pp 43-79.
10. E. C. B. Johnson, S. B. H. Kent, *J. Am. Chem. Soc.* **2006**, *128*, 6640-6646.
11. P. L. Pham, A. Kamen, Y. Durocher, *Mol.Biotechnol.* **2006**, *34*, 225-237.
12. J. Whittaker, A. V. Groth, D. C. Mynarcik, L. Pluzek, V. L. Gadsboll, L. J. Whittaker, *J. Biol. Chem*. **2001**, *276*, 43980-43986.
13. P. De Meyts, *Diabetologia* **1994**, *37*, S135-S148.
14. Z. X. Wang, *FEBS Lett.* **1995**, *360*, 111-114.

Figure S1. Amino acid sequence of IGF-1. The dotted lines show the disulfide bond connectivity of folded IGF-1.

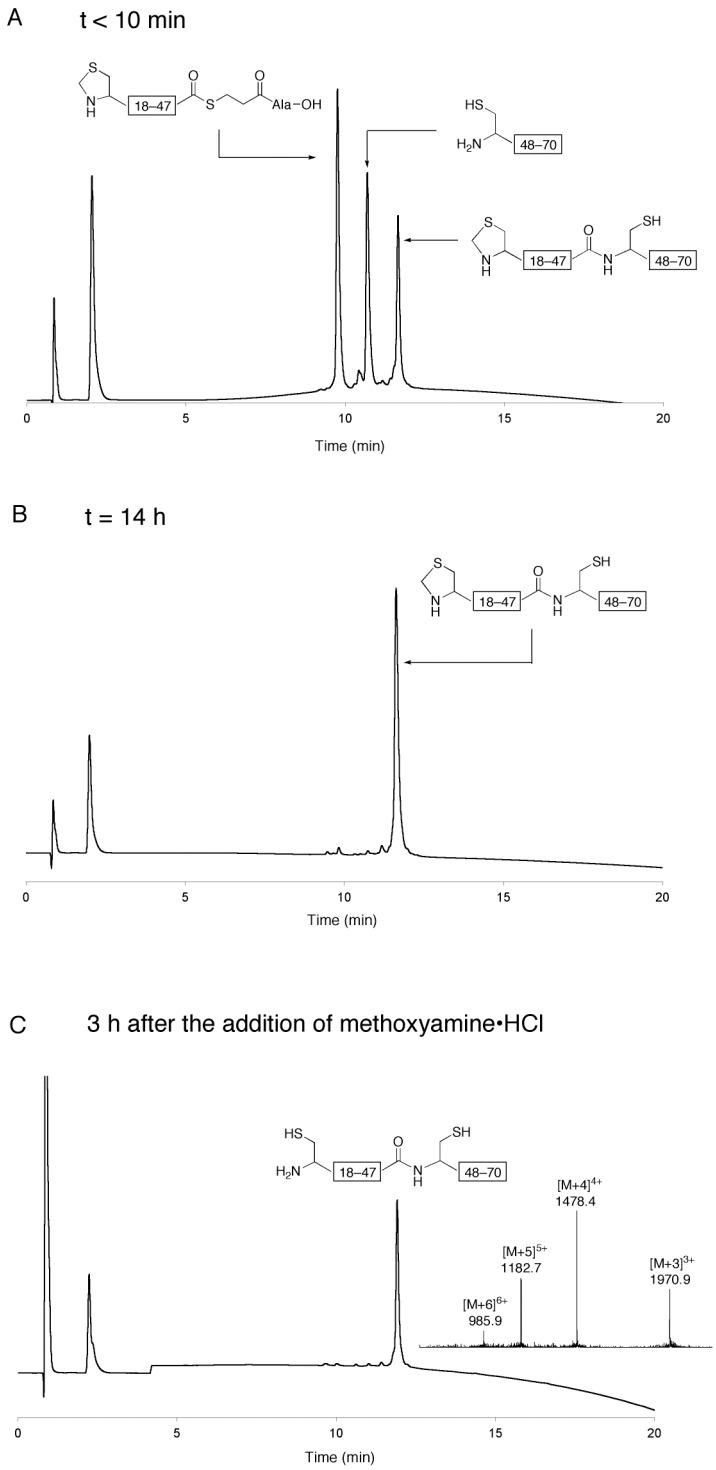

Figure S2. Ligation reaction of IGF-1(Thz^{18}-47)-$^{\alpha}$thioester with IGF-1(Cys^{48}-70): (A) At t < 10 min, (B) At t = 14 h; Conversion of Thz- to Cys-peptide: (C) At t = 3 h after the addition of methoxylamine·HCl to convert Thz- to Cys-peptide, showing total crude products. (Inset) On-line ESMS spectra of the principal peak. Reactions were monitored by analytical HPLC (UV profiles at 214 nm are shown). 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). Peaks were identified by on-line ESMS.

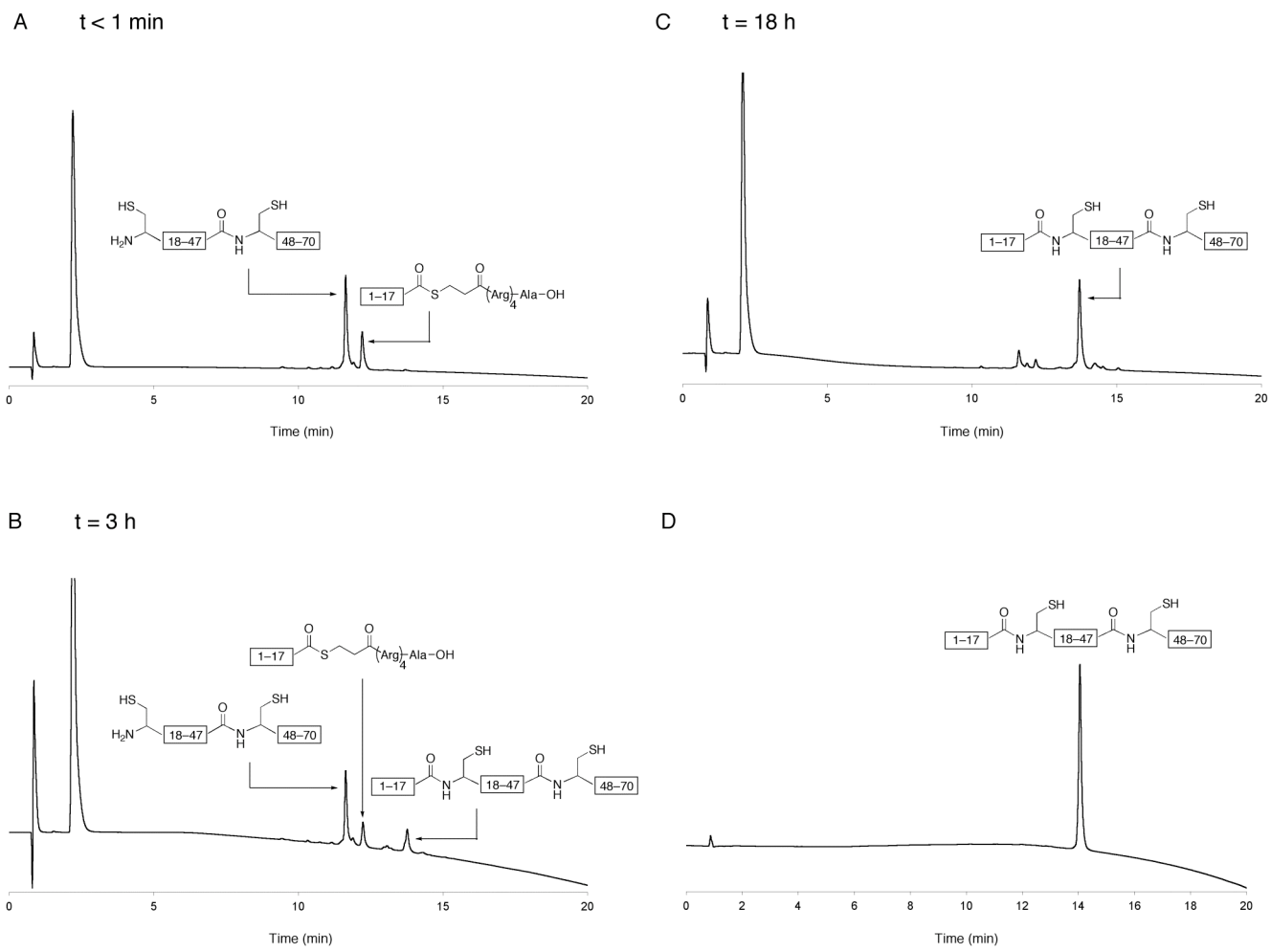

Figure S3. Ligation reaction of IGF-1[1-17]-$^{\alpha}$thioester-$(Arg)_4$-Ala-OH with IGF-1[Cys^{18}-70] was monitored by analytical HPLC (UV profiles at 214 nm are shown). (A) At $t < 1$ min, (B) At $t = 3$ h, (C) At $t = 18$ h, (D) purified IGF-1 polypeptide (observed mass: 7655.2 ± 0.4 Da; calculated mass (average isotopes): 7654.6 Da). 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). Peaks were identified by on-line ESMS.

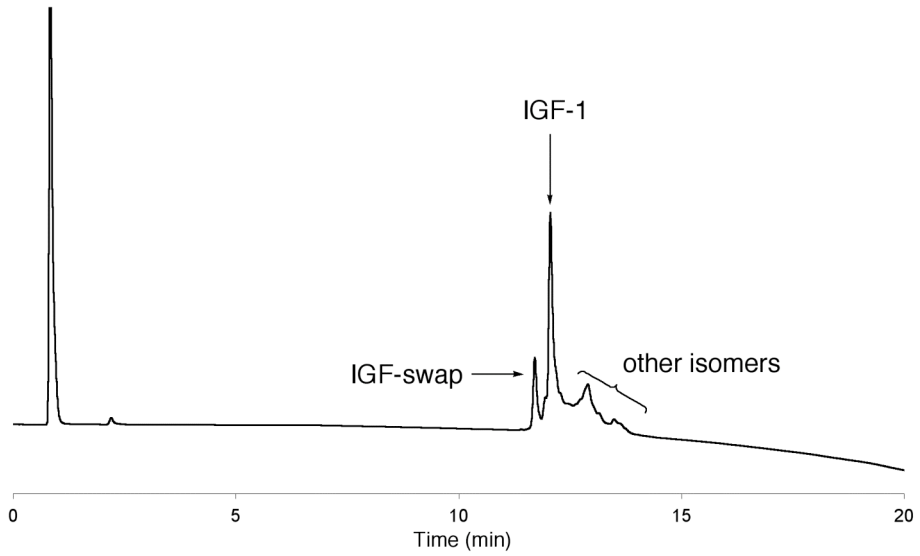

Figure S4. Folding was monitored by LC after 1 h (UV profiles at 214 nm are shown). Similar data were obtained after 6 h. Folding conditions were IGF-1$(SH)_6$: ~0.5 mg mL^{-1}, Tris: 20 mM, Cysteine: 8 mM, Cystine·HCl: 1 mM, GnHCl: 0.5 M, pH = 7.7. The chromatographic separations were performed using 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). Peaks were identified by on-line ESMS.

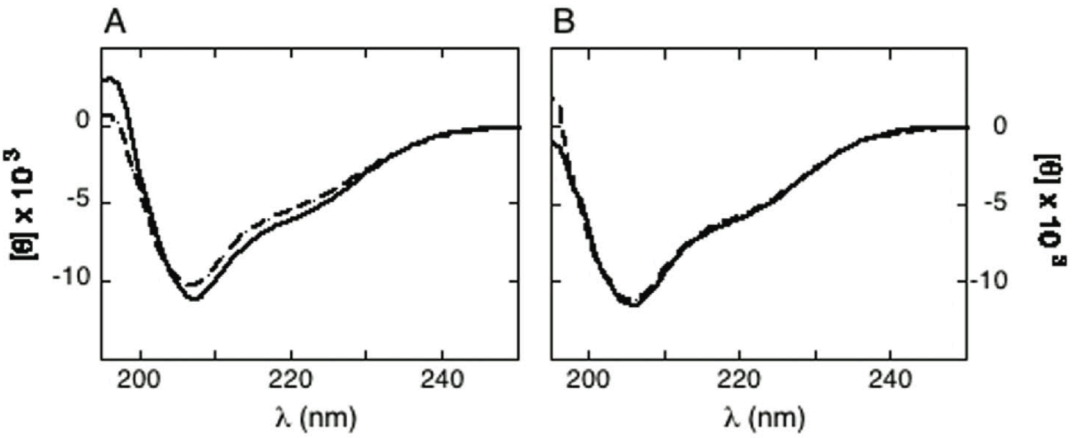

Figure S5. CD spectra of synthetic proteins: (A) native IGF-1 (dash line) and [Gly7D-Ala]IGF-1 (solid line); (B) IGF-swap (dash line) and [Gly7D-Ala]IGF-swap (solid line). Spectra were obtained by use of an Aviv spectropolarimeter, and normalized by mean residue ellipticity. Samples with protein concentration of ~25 μM were dissolved in PBS buffer at pH 7.4 and 25 ˚C.

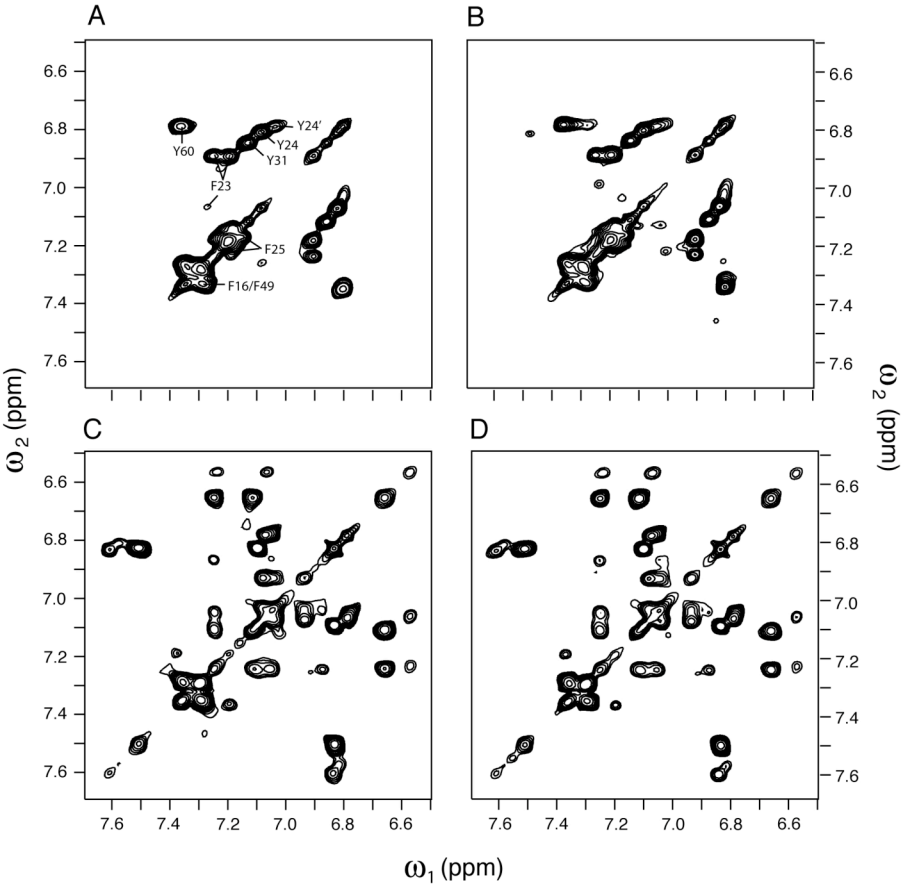

Figure S6. 2D TOCSY ^{1}H NMR spectra of the aromatic regions of IGF-1 and IGF-'swap'.
(A) recombinant IGF-1; (B) synthetic IGF-1;
(C) recombinant IGF-swap; (D) synthetic IGF-swap.

IGF-1 spectra were acquired in 10%perdeuterated acetic acid / D_2O pH3.0 at 40C; IGF-swap spectra were obtained in PBS D_2O buffer at 25C.

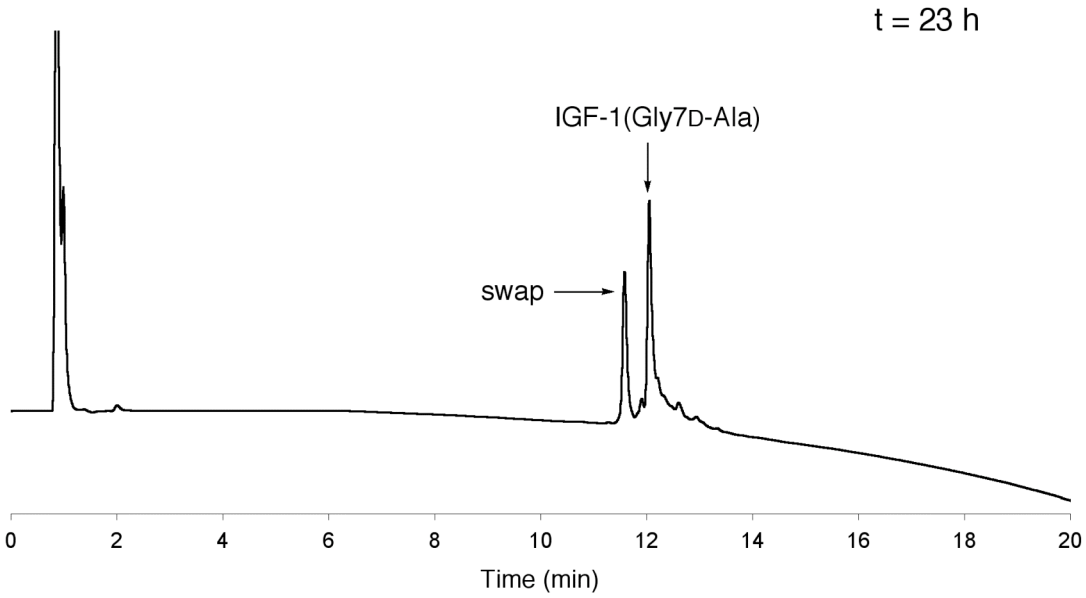

Figure S7. Crude products obtained from folding [Gly7D-Ala]IGF-1$(SH)_6$: ~0.4 mg mL^{-1}, Tris: 20 mM, Cysteine: 8 mM, Cystine·HCl: 1 mM, GnHCl: 0.5 M, pH = 7.8. Folding was monitored by LC (UV profiles at 214 nm are shown). The chromatographic separations were performed using a linear gradient (5-65%) of buffer B in buffer A over 15 min (buffer A = 0.1% TFA in water; buffer B = 0.08% TFA in acetonitrile). Peaks were identified by on-line ESMS.

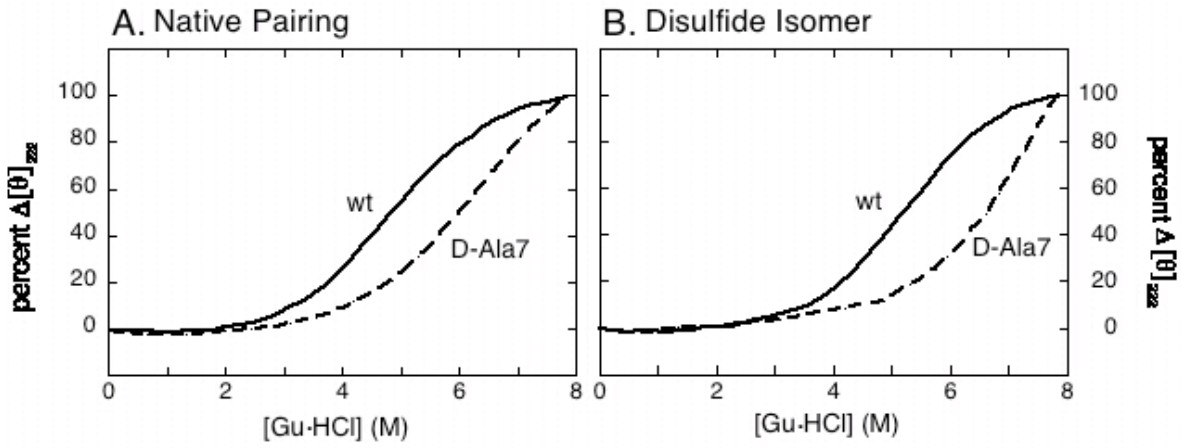

Figure S8. Guanidine.HCl denaturation curves for the four synthetic proteins. Panel A: IGF-1 ('wt') and [Gly7D-Ala]IGF-1 ('D-Ala7'); Panel B: IGF-swap ('wt') and [Gly7D-Ala]IGF-swap ('D-Ala7').