Supporting information

for

An insight into structural and biological relevance of the T/R transition of the B-chain N-terminus in human insulin

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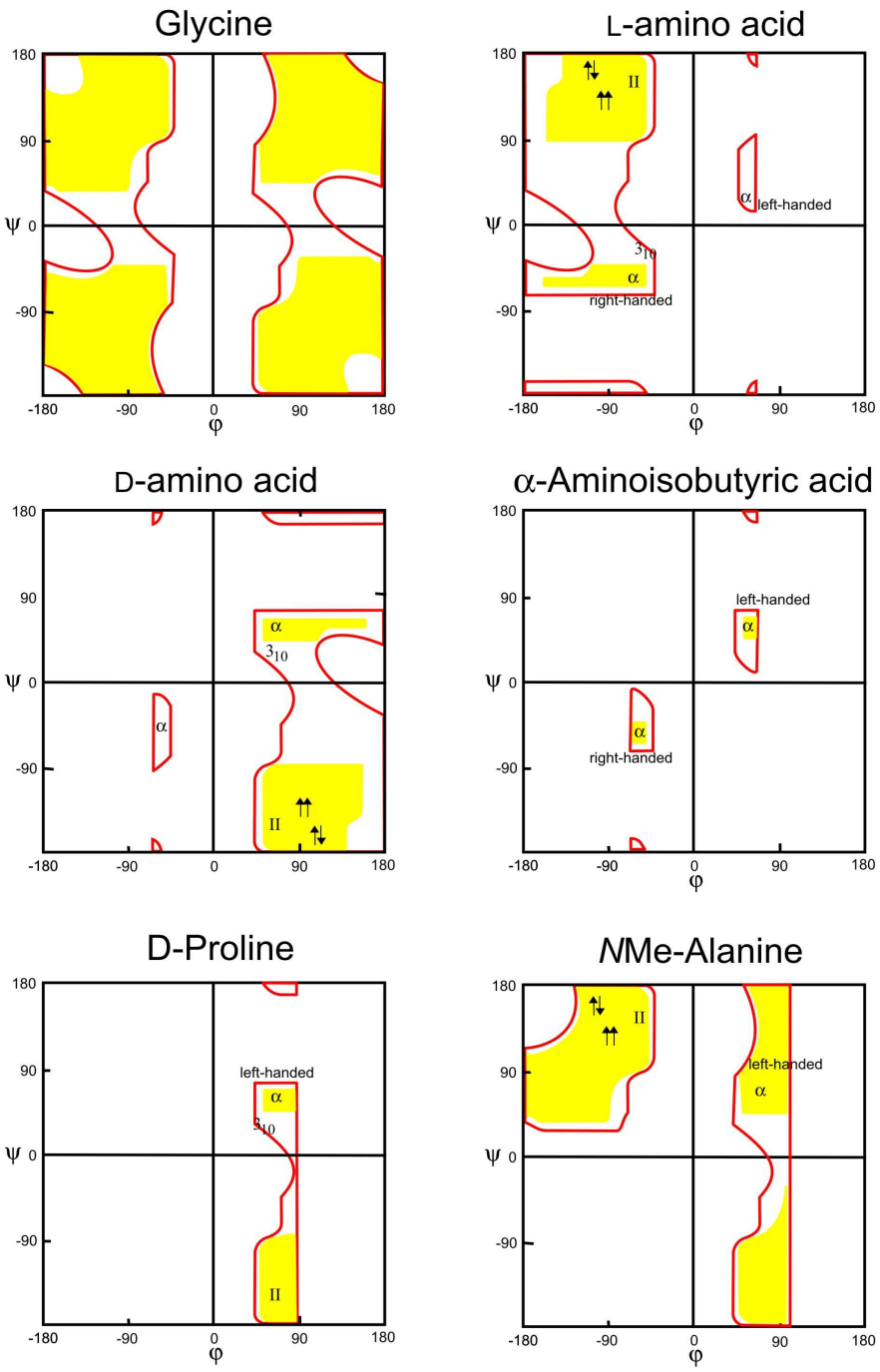

Figure S1. Schematic representations of Ramachandran plots showing approximate values of allowed dihedral angles for selected amino acids. The plots were created based on different scientific reports (*1-3*). Abbreviations: α - α-helix, 3_{10} - 3_{10} helix, II - type II β-turn, ↑↓ - anti-parallel, and ↑↑- parallel β-sheet.

Table S1. Data collection and refinement statistics

	[*D*-ProB8]-insulin	[*N*MeAlaB8]-insulin[I]	[*N*MeAlaB8]-insulin[II]
PDB Code	4CXL	4CXN	4CY7
Data collection			
Beamline/Detector	DLS*, I04, Pilatus 2M	DLS*, I02 Pilatus 6M	DLS*, I04 Pilatus 2M
Wavelength (Å)	0.9200	0.9795	0.9200
Space group	$I2_13$	$I2_13$	$P2_12_12_1$
Cell dimensions			
a, *b*, *c* (Å)	78.66 78.66 78.66	79.17 79.17 79.17	44.30 46.19 51.76
α, β, γ (°)	90.0 90.0 90.0	90.0 90.0 90.0	90.0 90.0 90.0
Resolution (Å)	39.33 - 1.50 (1.54-1.50)	56.0 – 1.70 (1.74-1.70)	32.0 – 1.40 (1.44-1.40)
R_{sym}	0.052 (0.656)	0.041 (0.769)	0.040 (0.636)
$\langle I / \sigma(I) \rangle$	31.8 (5.0)	36.1 (4.4)	22.3 (2.7)
Completeness (%)	100.0 (100.0)	100.0 (100.0)	98.9 (98.5)
Redundancy	20.0 (20.0)	19.4 (20.2)	6.5 (6.6)
Wilson B ($Å^2$)	29.7	30.5	14.2
Refinement			
Resolution (Å)	39.33 - 1.50	56.0 – 1.70	32.0 – 1.40
No. reflections	12441	8872	20063
R_{work} / R_{free}	0.167/0.197	0.173/0.209	0.179/0.199
No. atoms	480	445	976
Protein	427	406	816
Ligand/ion	1	-	9
Water	57	39	151
B-factors			
Protein	25.7	34.2	18.3
Ligand/ion	78.6	-	45.4
Water	44.1	46.4	29.2
R.m.s. deviations			
Bond lengths (Å)	0.033	0.030	0.027
Bond angles (°)	2.784	2.175	2.537
Ramachandran (%)			
Preferred/Allowed	97.5/2.5	95.4/4.6	93.2/6.8
Crystallization			
insulin: 10 mg/mL in 0.025M HCl, hanging drop method, 1:1 or 1:2 protein: well drop ratio, (1-2 µL drops) no cryoprotection: direct flash-cooling in liquid N_2	40% v/v MPD, 0.2 M NaCitrate, 0.1M Tris/HCl pH 8	40% v/v MPD, 0.2 M NaCitrate, 0.1M Tris/HCl pH 8	0.0375 M Na_2SO_4 pH 4.0

*DLS – Diamond Light Source, Didcot, UK
**Values in parentheses are for highest-resolution shell

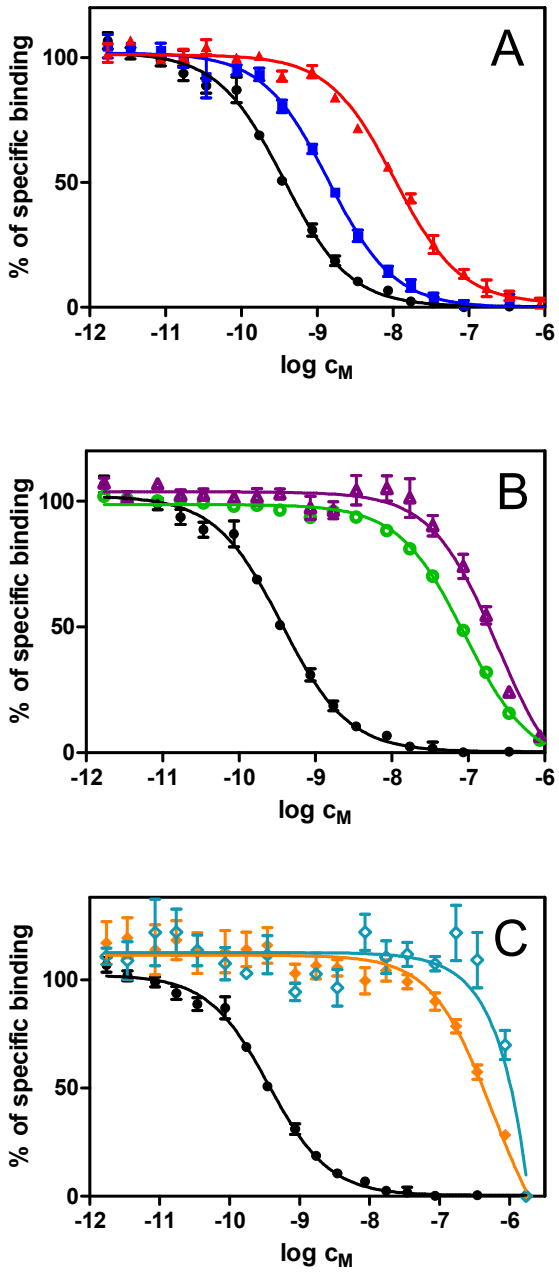

Figure S2. Inhibition of binding of human [^{125}I]-insulin to IR-A in IM-9 cells by human insulin and insulin analogues. (A) ● – human insulin, ■ – [AibB3]-insulin, ▲ – [AibB5]-insulin; (B) ● – human insulin, ○ – [AibB8]-insulin, Δ – [AibB8,LysB28,ProB29]-insulin; (C) ● – human insulin, ♦– [D-Pro]-insulin, ◊ - [*N*MeAlaB8]-insulin. c_M is a molar concentration in mol.l^{-1}.

Determination of receptor binding affinity for the isoform B of human IR (IR-B)

Receptor binding affinity was determined according to Frasca et al (*4*) using mouse embryonic fibroblasts derived from IGF-1R knock-out mice (*5*) and transfected with the human IR-B isoform. The cells were a kind gift of Prof. Antonino Belfiore (Catanzaro, Italy). The cells were grown at 37°C in a humid atmosphere (5% CO_2) in 87.6% DMEM containing glucose (4.5 $g.l^{-1}$), 10% fetal bovine serum, L-glutamine (2 $mmol.l^{-1}$), penicillin (100 $U.ml^{-1}$) streptomycin (100 $\mu g.ml^{-1}$) and puromycin (3 $\mu g.ml^{-1}$).

For the assay, the cells (about 38,000 per well) were washed twice with the binding buffer (100 $mmol.l^{-1}$ HEPES pH 7.6, 100 $mmol.l^{-1}$ NaCl, 5 $mmol.l^{-1}$ KCl, 1.3 $mmol.l^{-1}$ $MgSO_4$, 1 $mmol.l^{-1}$ EDTA, 10 $mmol.l^{-1}$ glucose, 15 $mmol.l^{-1}$ sodium acetate and 1% bovine serum albumine). The cells were incubated and stirred with increasing concentrations of insulin/analogue and human [^{125}I]monoiodotyrosyl-A14-insulin (PerkinElmer Life Science, 2200 $Ci.mmol^{-1}$, 43,000 cpm, 0.043 nM) for 16 h at 5° C in the binding buffer (total volume 250 µl). After incubation, the cells were washed twice with the cold binding buffer and solubilized with 0.1 $mol.l^{-1}$ NaOH. The solutions of solubilized cells were counted for cell-associated radioactivity. Each point was determined in duplicates. Binding data were analyzed by GraphPad Prism 5 using a non-linear regression and one-site fitting program, which takes the potential ligand depletion into account. The dissociation constant of human ^{125}I-insulin was set up to 0.3 nM.

Determination of receptor binding affinity for human IGF-1 receptor (IGF-1R)

Receptor binding affinity for IGF-1R was determined by the same methodology as for receptor binding affinity for IR-B according to Frasca et al (*4*) but using mouse embryonic fibroblasts derived from IGF-1R knock-out mice (*5*) and transfected with the human IGF-1R. The cells were a kind gift of Prof. Antonino Belfiore (Catanzaro, Italy).

The cells were grown to about 21,000 per well. As a radiotracer human [^{125}I]-IGF-1 was used (PerkinElmer Life Science, 2497 $Ci.mmol^{-1}$, 44,000 cpm, 0.039 nM). The dissociation constant of human ^{125}I-IGF-1 was set up to 0.2 nM. The concentration of human IGF-1 (Tercica) was determined using an extinction coefficient (ε) 4560 $M^{-1}.cm^{-1}$at 280 nm.

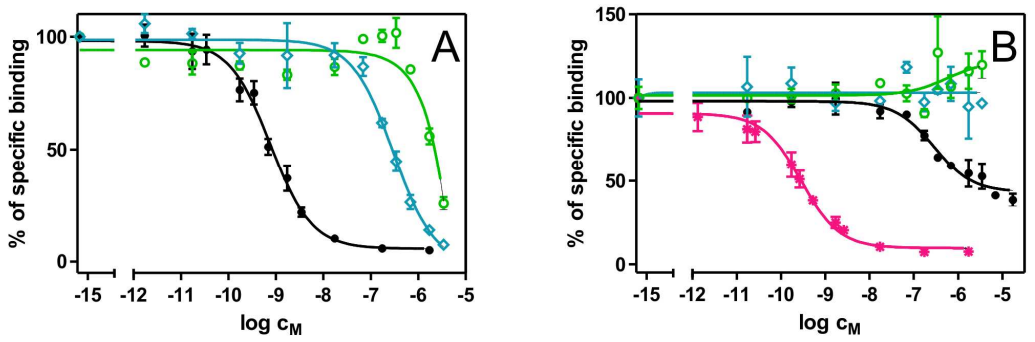

Figure S3. (A) Inhibition of binding of human [^{125}I]-insulin to IR-B in mouse embryonic fibroblasts by human insulin and insulin analogues; ● – human insulin, ○ – [AibB8]-insulin and ◊ – [*N*MeAlaB8]-insulin. (B) Inhibition of binding of human [^{125}I]-IGF-1 to IGF-1R in mouse embryonic fibroblasts by human IGF-1 and insulin analogues; * – human IGF-1, ● – human insulin, ○ – [AibB8]-insulin and ◊ - [*N*MeAlaB8]-insulin. c_M is a molar concentration in mol.l^{-1}.

Table S2. Values of K_d and relative binding affinities of human insulin, human IGF-1 and insulin analogues to the isoform B (IR-B) of human IR or to human IGF-1R in membranes of mouse embryonic fibroblasts.

	IR-B		IGF-1R	
Analogue	K_d ± S.D.[a] [nM] (n)	Potency[b] [%]	K_d ± S.D.[a] [nM] (n)	Potency[b] [%]
Human insulin	0.670 ± 0.167	100 ± 25	292 ± 54	0.08 ± 0.01
Human IGF-1	n.d.[c]	n.d.[c]	0.240 ± 0.104	100 ± 43
[AibB8]-insulin	248 ± 59 (3)	0.27	>2000 (2)	< 0.01
[*N*MeAlaB8]-insulin	>1500 (3)	< 0.05	>2000 (2)	< 0.01

[a]Each value represents the mean ± S.D. of multiple determinations (n).

[b]Relative receptor binding affinity (potency) is defined as (K_d of human insulin or IGF-1/K_d of analogue) × 100.

[c]n.d., not determined.

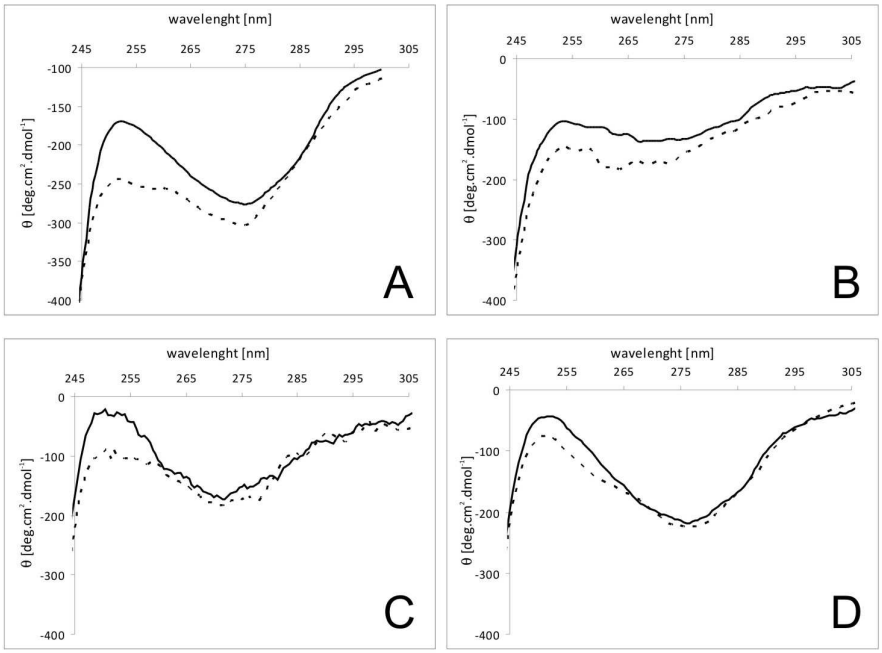

Figure S4. Near-UV CD spectra of insulin analogues in the absence (solid line) and the presence (dashed line) of phenol; (A) human insulin, (B) [AibB8]-insulin, (C) [D-ProB8]-insulin, (D) [*N*MeAlaB8]-insulin.

Table S3. Secondary structures content (in %) in human insulin or insulin analogues with or without phenol calculated from CD spectra (200-260 nm) using CD Spectra Deconvolution software version 2.11.developed in 2001 by Dr. Gerald Böhm, from Institut für Biotechnologie Martin-Luther-Universität Halle-Wittenberg in Germany.

	Insulin (%)	**Insulin + phenol (%)**
Helix	22.2	30.5
Antiparallel	18.7	14.0
Parallel	5.9	6.0
Beta-Turn	16.7	14.9
Random Coil	32.0	29.5
Total Sum	95.4	95.0

	[AibB8]-insulin (%)	**[AibB8]-insulin + phenol (%)**
Helix	26.9	30.2
Antiparallel	11.0	8.9
Parallel	10.4	10.3
Beta-Turn	17.5	17.5
Random Coil	36.2	33.3
Total Sum	102.0	100.2

	[AibB3]-insulin (%)	**[AibB3]-insulin + phenol (%)**
Helix	26.5	27.0
Antiparallel	10.1	10.0
Parallel	9.5	9.3
Beta-Turn	17.4	17.4
Random Coil	36.0	35.2
Total Sum	99.5	98.9

	[DProB8]-insulin (%)	**[DProB8]-insulin + phenol (%)**
Helix	22.2	27.1
Antiparallel	18.9	15.9
Parallel	5.9	6.0
Beta-Turn	16.9	15.8
Random Coil	32.1	30.7
Total Sum	96.1	95.5

	[*N*MeAlaB8]-insulin (%)	**[*N*MeAlaB8]-insulin + phenol (%)**
Helix	26.0	26.2
Antiparallel	16.4	16.2
Parallel	6.0	5.9
Beta-Turn	16.1	16.1
Random Coil	31.0	31.0
Total Sum	95.5	95.4

Table S4. NMR constraints and structural statistics for [AibB8,LysB28,ProB29]-insulin

Non-redundant distance constrains	
Total number of NOE constraints	488
Short-range NOEs (*i, i*+1)	329
Medium-range NOEs (*i,i>1 i*≤4)	80
Long-range NOEs (*i, i* ≥5)	79
Distance constraints violations	
In six or more structures > 0.2 Å	0
In six or more structures > 0.3 Å	0
r.m.s. (Å)	0.003 ± 0.002
Ramachandran plot	
Residues within the most favored region	84.2%
Residues within the additionally allowed region	12.5%
Residues within generously allowed region	2.6%
Residues within the disallowed region	0.7%
Cing ROG scores	
ROG (%)	16/16/69
r.m.s.d. to the mean structure (residues chain A_{1-21} and chain B_{1-23})	
Backbone heavy atom (Å)	1.49 ± 0.34
All heavy atom (Å)	2.21 ± 0.15

Table S5. Relative affinities of some insulin analogues with modification at the N-terminus of the B chain reported in literature.

analogue	relative affinity (%)	reference
insulin	100	
[AlaB1]-insulin	79	(*6*)
[AlaB2]-insulin	110	(*6*)
[AlaB3]-insulin	134	(*6*)
[ProB3]-insulin	54	(*7*)
[SerB3]-insulin	97	(*7*)
[AlaB4]-insulin	54	(*6*)
[Cys(B4-A10),desB30]-insulin	132	(*8*)
[AlaB5]-insulin	31	(*9*)
[ArgB5]-insulin	40	(*10*)
[ThrB5]-insulin	20-24	(*7, 11*)
[AsnB5]-insulin	46	(*12*)
[AspB5]-insulin	0.4	(*12*)
[AlaB5]DKP-insulin[1]	129	(*13*)
[AlaB6]-insulin	1.4	(*6, 14*)
[GlyB6]-insulin	0.052	(*14*)
[MetB6]-insulin	15	(*14*)
[ValB6]-insulin	3.3	(*14*)
[PheB6]-insulin	10	(*14*)
[AlaB8]-insulin	1.0 - 3.0	(*6, 15, 16*)
[D-AlaB8]-insulin	0.11	(*15, 16*)
[AlaB8]DKP-insulin[1]	3.2 - 3.6	(*15, 16*)
[D-AlaB8]DKP-insulin[1]	0.17	(*15, 16*)
[SerB8]DKP-insulin[1]	90	(*15, 17*)
[D-SerB8]DKP-insulin[1]	1.1	(*15, 17*)
[D-ArgB8]DKP-insulin[1]	0.05	(*15*)
[SerB8,desB30]-insulin	23	(*18*)
[ThrB8,desB30]-insulin	< 0.2	(*18*)
[LeuB8,desB30]-insulin	< 0.2	(*18*)
[D-LysB8]-insulin	< 0.5	(*19*)
[D-TrpB8]-insulin	< 0.6	(*19*)
[GluB9]-insulin	21	(*20*)
[AlaB9]-insulin	80	(*6*)

[1]DKP-insulin, monomeric insulin analogue containing three substitutions in the B-chain (AspB10, LysB28, ProB29). Relative affinity of DKP-insulin is 160% (*15*)

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