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NMR STRUCTURE NOTE

Recombinant $A22^{G}$–$B31^{R}$-human insulin. A22 addition introduces conformational mobility in B chain C-terminus

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Biological context

Wilde type insulin consists of two chains, having 21 and 30 aminoacids in chain A and B, respectively, held together by two disulfide bridges, namely $A7^{C}$–$B7^{C}$ and $A20^{C}$–$B19^{C}$. There is also intra strand bridge in chain A; $A6^{C}$–$A11^{C}$. Insulin and its various derivatives are used in large amounts in the treatment of diabetes mellitus and are often manufactured on a large industrial scale. The modifications are aimed at altering the kinetics of drug release, often by favouring monomer over dimer (Hoeg-Jensen et al. 2005), the aim being to find a preparation that would maintain a constant level of glucose for an extended period of time. Commercial preparations can be long-acting [basal insulins such as glargine: $A21^{N} \rightarrow G$, $B31^{R}$, $B32^{R}$ (Younis et al. 2002) or detemir: $B29^{K^{\epsilon}} \rightarrow$ C14 aliphatic acid, des-$B30^{T}$ (Kurtzhals 2004)] or fast-acting [aspart: $B28^{P} \rightarrow D$ (Mudaliar et al. 1999), lyspro: $B28^{P} \rightarrow K$, $B29^{K} \rightarrow P$ (Howey et al. 1994; Ciszak et al. 1995) or glulysine: $B3^{N} \rightarrow K$, $B29^{K} \rightarrow E$ (Ciszak et al. 1995; Owens 2007)]. The problem of controlling aggregation in solution is related to the general problem of amyloid formation (Dobson and Karplus 1999; Hua and Weiss 2004; Mauro et al. 2007).

Insulin pharmaceuticals are mainly based on insulins engineered in the B chain. The influence of the A chain on insulin aggregation properties has received less attention. Here we characterize a novel insulin with an additional amino acid at the C terminus of the A chain, $A22^{G}$, which interacts with the β-turn environment of the B chain resulting in high flexibility of the B chain C-terminus. There is also an additional arginine at the C terminus of the B chain. This protein, $A22^{G}$–$B31^{R}$-human insulin (insulin GR), is a new analogue of human insulin with a stable profile of action (Borowicz et al. 2009). We have shown that these modifications result in a shift of isoelectric point to pH 6.2–6.4 and thus a decreased solubility at physiological pH. This probably causes precipitation of a microdeposit at the injection site and then its gradual, slow release to blood, as a result of which a therapeutic level is maintained for a longer time. It is also important that insulin GR starts its action almost immediately. This means that the compound exhibits a combination of rapid onset as well as a prolonged time of action (Borowicz et al. 2009). This observation is very worthy mentioning in a context of our motivation to undertaking structural modification studied in this work. We were concerned to preserve the effect of 'long-acting depot' (KR) and simultaneous introducing another structural modification, which may modify different biological property.

In this account we present the NMR-derived structure of recombinant $A22^{G}$–$B31^{R}$-human insulin (insulin GR) (PDB ID—**2lgb**) in H_2O/CD_3CN, 65/35 vol%, pH 2.4 (see ^{1}H NMR in Fig. 1S) and compare it with the available X-ray structure of human insulin (Smith et al. 2003) (PDB ID—**1mso**), NMR solution structures of a human

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insulin in H_2O/CD_3CN, 65/35 vol%, (Bocian et al. 2008b) (PDB ID—**2jvl**) and modified insulin $B31^K$–$B32^R$ (PDB ID—**2rn5**) (Bocian et al. 2008a). Earlier (Bocian et al. 2008d) we have also compared the wilde type insulin structure in H_2O/CD_3CN, 65/35 vol%, solvent with the tertiary structure of wild type insulin established in 20% acetic acid. (Hua et al. 1995) (PDB ID—**1 hiu**). We have shown that human insulin exists in H_2O/CD_3CN, 65/35 vol% solution (Cringus et al. 2004) (see to Supplementary Information for more discussion on a solvent composition) as a monomer (Bocian et al. 2008b) with a tertiary structure similar to but not identical with the one established in 20% acetic acid (Hua et al. 1995). Detailed analysis using PFGSE NMR, dilution experiments [see Fig. 2S, the linear decrease in Di value with concentration is accounted for by friction due to crowding of the monomers (Price et al. 1999)] and CSI [see Figs 3S and 4S, note the lack of the β-sheet motif in C-terminus of B chain which is characteristic for a dimer interface domain (B24–B28)] proves the existence of monomer in the concentration range 0.1–3 mM also for the recombinant $A22^G$–$B31^R$-human insulin in water/acetonitrile solution at pH 2.4.

Here we present a novel intramolecular phenomenon in insulins, namely $A22^G$ addition, which leads to substantial change of intramolecular conformation in the C-terminus of chain B.

Methods and results

Synthesis

Insulin GR has been manufactured in a fermentation process using an *Escherichia coli* strain with a DNA fragment encoding the insulin GR precursor. In the fermentation conditions the GR analogue was produced in intracellular inclusion bodies in LB water medium. After the end of fermentation the broth was concentrated and subsequently treated with lysozyme, and the bacterial cells were broken open. The raw inclusion bodies were purified, finally obtaining an inclusion body homogenate, which was then subjected to renaturation and trypsin digestion in order to cleave the leader peptide out and to cleave the insulin chains. The solution after digestion was purified using low pressure liquid chromatography on DEAE Sepharose FF gel, and was subsequently diafiltered and concentrated by a second low pressure liquid chromatography step on Q Sepharose FF gel. The main fraction was further purified by high pressure liquid chromatography. The fraction containing the desired protein was concentrated to 30–40 mg/ml using dialysis and purified insulin GR was separated by crystallization. The product structure has been confirmed by mass spectroscopy, peptide mapping, sequencing and amino acid composition.

NMR experiments

All NMR spectra were recorded in H_2O/CD_3CN (65/35 vol%) or D_2O/CD_3CN (65/35 vol%) on a Varian INOVA 500 MHz using a Nalorac probe, at 25.3°C. TSP was used as internal standard for chemical shift calibration.

NOESY spectra (Jeener et al. 1979) were recorded as a DPFGSE_NOESY experiment with water suppression by gradient echo and ZQ artifact suppression during mixing, using the States-TPPI method (Bodenhausen et al. 1984; States et al. 1982), mixing time 200 ms.

TOCSY spectra (Braunschweiler and Ernst 1983; Griesinger et al. 1988) were acquired as WGTOCSY with a selective H_2O one-lobe sinc pulse with flipback; the mixing times for TOCSY spectra were 80 ms with a DIPSI-2 spin-lock field of 8 kHz.

HSQCAD spectra (Summers et al. 1986) were acquired as the echo-antiecho phase sensitive $^1H/^{13}C$-HSQC (heteronuclear single quantum coherence, adiabatic version) with a relaxation delay of 1.2 s and $^1J(C,H) = 135$ Hz.

HSQCAD-TOXY (Varian software implemented package) spectra were acquired as the echo-antiecho phase sensitive ^1H–^{13}C HSQCAD-TOXY (heteronuclear single quantum coherence, adiabatic version with DIPSI-2 spin lock), with a relaxation delay of 1.2 s and ^1J (C, H) = 135 Hz. The mixing time was 80 ms with spin-lock field of 8 kHz.

Calculation procedures

The 1368 NOE cross-peaks, 42 coupling constants ^3J (H^α, NH) and 275 ^1H NMR assigned chemical shifts were used in the automatic NOESY assignment procedure '*noeassign*' of CYANA (Güntert et al. 1997). The 100 structures with lowest CYANA target function values were used for further MD refinement in AMBER 9 (Case et al. 2006). The Generalized Born solvent model (Onufriev et al. 2000; Xia et al. 2002) was used in the refinement protocol using molecular dynamics simulated annealing (for more details see Supplementary Information).

Table 1 shows the structural statistics of recombinant $A22^G$–$B31^R$-human insulin in comparison to human insulin standard (HIS) and recombinant $B31^K$–$B32^R$-human insulin (insulin KR) engineered only in chain B.

Discussion and conclusions

Figure 1 shows a stereo view of an ensemble of the 20 lowest energy structures of recombinant $A22^G$–$B31^R$-human insulin. There are two noticeable differences in the two domains of the structure as compared to human insulin (see Fig. 2 for comparison). First, conformational

Table 1 Structural statistics of the recombinant $A22^{G}$–$B31^{R}$-human insulin (insulin GR) for 100 structures, PDB—**2lgb**. Comparison with human insulin standard HIS, PDB—**2jv1** and $B31^{K}$–$B32^{R}$-human insulin, PDB—**2rn5**

Experimental NOEs[d]	AMBER_GB[a] $A22^{G}$–$B31^{R}$-human insulin	AMBER_GB[b] $B31^{K}$–$B32^{R}$-human insulin 1251 HIS 1451 $B31^{K}$–$B32^{R}$ 1368 $A22^{G}$–$B31^{R}$	AMBER_GB[c] HIS-human insulin standard
Experimental restraints[e]			
Total inter-proton	578	815	680
Intra-residue	210	195	177
Sequential	207	289	251
Medium range	81	188	142
Long-range ($\|j-i\| > 5$)	73	143	110
Disulfide restraints	9	9	9
Chirality restraints	124	53	51
Trans-(ω) restraints	51	52	50
Torsion restraints (ψ and φ)	247	255	72(φ)
Number of distance restraint violations in calculated structures per model[f]			
Total	23.6 (0.060 ± 0.036)	18.3 (0.053 ± 0.021)	34.4 (0.057 ± 0.028)
Intra-residue	4.74 (0.072 ± 0.056)	3.8 (0.070 ± 0.032)	4.7 (0.069 ± 0.037)
Sequential	5.99 (0.069 ± 0.046)	5.3 (0.046(±0.019)	19.2 (0.057 ± 0.029)
Medium range	6.03 (0.051 ± 0.012)	5.2 (0.050 ± 0.012)	7.2 (0.047 ± 0.011)
Long range	6.87 (0.053 ± 0.009)	4.1 (0.049 ± 0.012)	3.2 (0.061 ± 0.025)
RMSD from mean structure[g]			
All atoms in ensemble	4.23(1.28)	2.501 (1.501)	1.828 (1.265)
Backbone heavy atoms	3.36 (0.52)	1.465 (0.726)	0.991 (0.482)
Long range restraints			0.8883
RMSD from X-Ray structure of HIS[g,h]			
All atoms in ensemble	(2.30)	(2.338)	2.526 (1.814)
Backbone heavy atoms	(0.96)	(0.956)	1.313 (0.703)
Long range restraints			1.3718
Ramachandran statistics (% residues included in)			
Most favored regions	90.6	89.8	92.4
Additionally allowed regions	7.2	8.2	6.9
Generously allowed regions	1.1	1.2	0.2
Disallowed regions	1.1	0.8	0.5

[a] $A22^{G}$–$B31^{R}$-human insulin in solvent. [b] $B31^{K}$–$B32^{R}$-human insulin in solvent, structure deposited in PDB—**2rn5**. [c] HIS in solvent, structure deposited in PDB—**2jv1**, BRMB accession no. **15464**. [d] The numbers of manually assigned NOE cross peaks. Only unambiguous cross peaks were considered as input for insulin KR and HIS. From the 1368 total experimental NOEs, only1201 cross peaks were automatically assigned for insulin GR. [e] Converted by CYANA into distance restraints. [f] The 578 constraints, 100 models, in $A22^{G}$–$B31^{R}$-human insulin, 815 constraints in $B31^{K}$–$B32^{R}$-human insulin, 100 models, and 680 constraints in 200 models considered in HIS. Values in parentheses show average violation, in Å. [g] 20, 50 and 50 low energy structures are compared for insulin GR, insulin KR, and HIS, respectively. Values in parentheses refer to statistics with excluded terminal residues in both chains, i.e.; residues 2–20 and 3–29 in chain A and B, respectively for HIS and insulin KR, but 3–20 and 5–20 residues in insulin GR. [h] PDB—**1mso** (X-Ray); **2rn5** (KR); **2jv1** (HIS)

heterogeneity in the C-terminus of chain B is clearly visible. This is likely to influence dimer formation due to disorder in the dimer interface domain (B24–B28). In physiological conditions, in water, this may result in weaker monomer–monomer association and faster kinetics of the dissociation of higher aggregates (tetramers, hexamers) as compared to native insulin (Bocian et al. 2008c). Secondly, in insulin GR the second α-helix in the A chain terminates at A20 rather than at A19 as in native insulin (see CSI graphs, Figure 3S and Fig. 4S, and the graph of secondary structure motifs, Fig. 6S in Supplementary Information). The extended helix at A chain C-terminus introduces a rigidity which in turn results in detachment of the B chain C-terminus from the hydrophobic core of the B

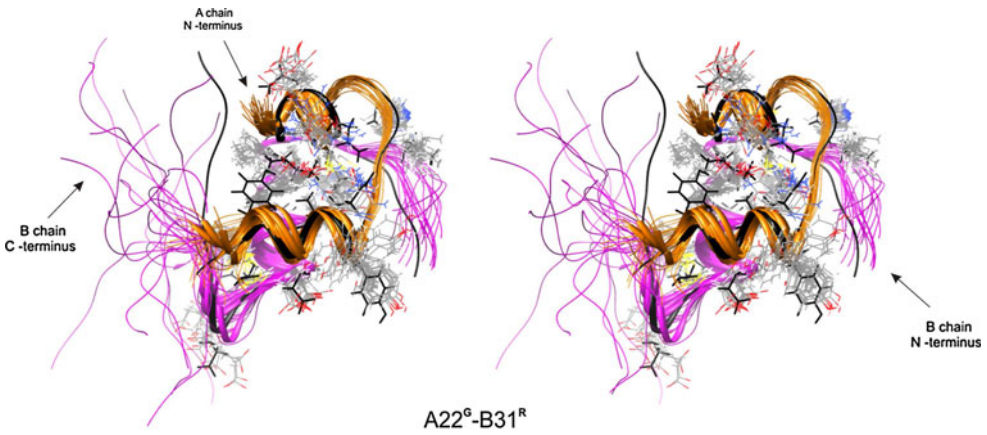

Fig. 1 Stereo view (*parallel view*) of an ensemble of 20 structures of recombinant $A22^{G}$–$B31^{R}$ human insulin analogue. The A chain is shown in *gold* and B chain in *magenta*. The *black string* represents the backbone of human insulin monomer from the X-ray structure of a hexamer (PDB ID—**1mso**)

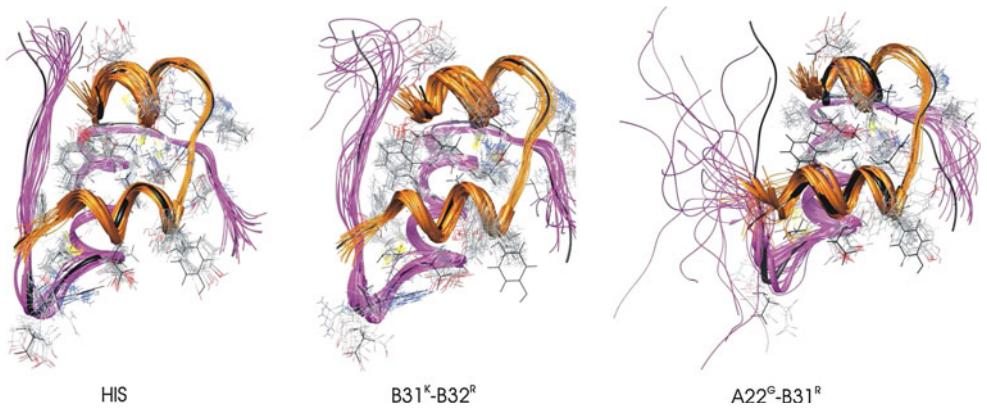

Fig. 2 Comparison of an ensemble of 20 structures of insulin GR with the human insulin standard HIS, and insulin KR in H_2O/CD_3CN, 65/35 vol%, pH 2.4. The A chain is shown in *gold* and B chain in *magenta*. The *black string* represents the backbone conformation of the X-ray structure of human insulin monomer as seen in the hexamer (PDB ID—**1mso**)

chain α-helix. The result is a weakening of the hydrophobic attractive interactions between the domains and increased mobility in the B chain C-terminus. Also the β-turn motif is affected by this modification which can be seen in Fig. 2 by comparison to HIS or the $B31^{K}$–$B32^{R}$-human insulin analogue.

Basing on performed biological tests (activation of insulin receptor's β-subunit autophosphorylation, IRS proteins phosphorylation, activations of cascades including protein kinase B PKB/Akt, mitogen activated protein kinases, MAPK, and glycogen synthase kinase, GSK-3) it can be assumed that cellular function of insulin GR is the same as for WT human insulin.

We note that $31B^{K}$–$32B^{R}$-human insulin, which lacks the $A22^{G}$ mutation in the A chain, has a B chain C-terminus essentially identical to native human insulin (Bocian et al. 2008a). Our results therefore imply that the alteration to the mobility of the B chain C terminus is due to the $A22^{G}$ mutation.

A comparison of the three ensembles in Table 1 (RMS from mean structure) supports the conclusion that all three structures are solved with the same precision if B chain C-termini were excluded from comparison. A comparison of distribution of NOE restraints in the three insulins (see Fig. 5S-1) also suggests a uniform distribution of medium and long range NOEs along B chain in insulins HIS and KR whereas they are less abundant in insulin GR in C-terminus of B chain. This strengthens our confidence in the genuine high mobility of the B chain C-terminus in insulin GR rather than a lack of restraints. An additional support to this provides the analysis of RCI (Random Coil Increment) graph in Fig. 3. This type of data analysis is recently proposed as a means of studying the protein segment flexibility in cases where relaxation parameters are not available because of lack of ^{15}N enriched molecule, as in a present case. (Berjanskii and Wishart 2008) This analysis relies essentially on the tenet that ensemble of CSI (Chemical Shift Index) (Wishart et al. 1995) values reflects the dynamic state of a motif. Displaced in Fig. 3 analysis was based on CSI values of $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $N^{1}H$ nuclei using algorithm given in web server at http://wishart.biology.ualberta.ca/rci. (Berjanskii and Wishart 2008) using parameters of Schwarzinger. (Schwarzinger et al. 2000)

It is seen in Fig. 3 that monomer embedded in hexamer has low value of RCI parameter along the whole B chain reflecting rigidity and lack of dynamics in hexamer studied in solution. The same rigidity is observed in a motif B7–B19 (α-helix) of human insulin (Bocian et al. 2008b),

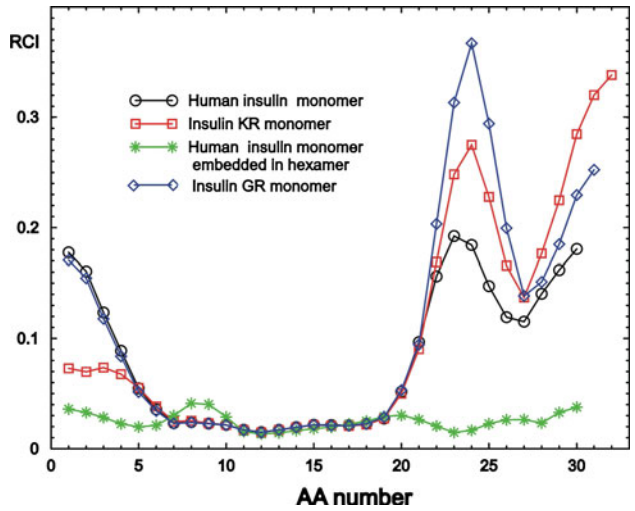

Fig. 3 RCI plot of chain B motifs reflecting comparison of dynamics in monomer of insulins studied in H_2O/CD_3CN (72/27 vol%, pH 2.5) versus human insulin monomer embedded in hexamer studied in water at pH 8.0. (Chang et al. 1997)

insulin KR (Bocian et al. 2008a) and insulin GR (present work) in a monomer of each studied in H_2O/CD_3CN (73/27 vol%, pH 2.5). The most significant changes in motif dynamics are observed at $B24^F$, i. e. an anchor of B-chain terminus to B chain helix. (Smith et al. 2003; Hua et al. 2009) This is a site where additional $A22^G$ aminoacid interferes with the B-chain. It is apparent from Fig. 3 that the dynamics at this site is much higher in insulin GR, than in human insulin and insulin KR, which are lacking mutation of $A22^G$. It is also seen in Fig. 3 that the C terminus of chain B has, in all insulins, another anchor at $B27^T$ which is due to hydrogen bonding between NH's of $A2^I$ and $A3^V$ and OH and C=O of $B27^T$.

In summary, we present here the tertiary structure of $A22^G$–$B31^R$ modified insulin which has been characterized by ^1H and ^{13}C NMR (Table 2S) at natural isotopic abundance using NOESY, TOCSY, $^1H/^{13}C$-HSQCAD, and $^1H/^{13}C$-GHSQC-TOCSY spectra. Modified insulin forms aggregates in water over a range of μM to mM concentration and therefore structural studies can not be conducted in water. Like human insulin, the modified insulin is in the same aggregation state, i.e. monomer, in the range of concentrations 0.1–3 mM in water/acetonitrile (65/35 vol%) solution at pH 2.4. We present here a novel phenomenon in insulins, namely, the $A22^G$ addition which introduces conformational mobility in the B chain C-terminus. Further studies on recombinant A22 human insulins are in progress with a goal to characterize this phenomenon further.

A Table of ^1H, ^{13}C chemical shift assignments has been deposited in the BioMagResBank (http://www.brmb.wisc.edu) database under accession number 17803. Structure deposited in PDB under accession no. **2lgb**, RCSB ID code rcsb102357.

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