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X-ray investigation of gene-engineered human insulin crystallized from a solution containing polysialic acid

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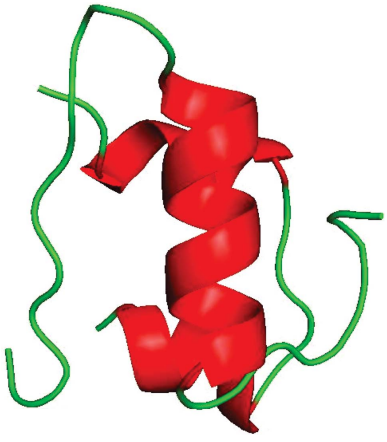
Attempts to crystallize the noncovalent complex of recombinant human insulin with polysialic acid were carried out under normal and microgravity conditions. Both crystal types belonged to the same space group, $I2_13$, with unit-cell parameters $a = b = c = 77.365$ Å, $\alpha = \beta = \gamma = 90.00^\circ$. The reported space group and unit-cell parameters are almost identical to those of cubic insulin reported in the PDB. The results of X-ray studies confirmed that the crystals obtained were cubic insulin crystals and that they contained no polysialic acid or its fragments. Electron-density maps were calculated using X-ray diffraction sets from earth-grown and microgravity-grown crystals and the three-dimensional structure of the insulin molecule was determined and refined. The conformation and secondary-structural elements of the insulin molecule in different crystal forms were compared.

1. Introduction

Insulin is a polypeptide hormone that modulates glucose levels in the blood and is a medical compound that is widely prescribed for the treatment of insulin-dependent diabetes. Prolonging the therapeutic effect of insulin is an essential task. The use of slowly dissociating complexes of insulin with polymeric compounds (*e.g.* polyethylene glycol) has been suggested to enhance the stability and prolong the therapeutic effect of the recombinant protein (Veronese & Pasut, 2005). Unlike polyethylene glycol, polysialic acid is not accumulated in the organism and is instead recognized by sialidases and degraded to monomeric residues. Bezuglov *et al.* (2009) have synthesized noncovalent complexes of insulin and polysialic acid (PSA) and have characterized them using atomic force microscopy. In aqueous solution and at a complex concentration of 0.1 g ml^{-1}, about 60% of the PSA molecules carry a single insulin molecule. Furthermore, the insulin molecules are irregularly spread along the PSA chain but bind predominantly to one of the PSA termini. Hypoglycaemic testing has demonstrated a prolonged action of the complex in animals. After a single insulin injection glucose levels remain at a low level for up to 3 h, while insulin–PSA injection regulates the glucose concentration for longer than 6 h. The prolonged pharmacological effect of insulin complexed with PSA makes it a potential therapeutic agent and therefore necessitates structural studies. In the present work, we used the noncovalent complex of insulin with PSA for crystallization but obtained pure insulin crystals. The crystals obtained were analyzed using X-rays and the conformations of the insulin molecule in different crystal forms were compared.

2. Materials and methods

Recombinant human insulin was manufactured on the Institute of Bioorganic Chemistry experimental production line. Polysialic acid 23 kDa was obtained from FDS Pharma (Great Britain). The insulin–PSA complex was prepared as described in Bezuglov *et al.* (2009). The content and molar ratio of protein and polysialic acid were determined by the bicinechoninic acid (Stoscheck, 1990) and resorcin (Svennerholm, 1963) methods, respectively.



2.1. Crystallization of the insulin–PSA complex

Crystallization screening of the insulin–PSA complex was performed by solvent vapour diffusion in a hanging drop using the Hampton Research Index HR2-144 reagent kit. Crystals grew in samples containing PEG 3350 in 0.1 *M* Tris pH 8.5, 0.2 *M* NaCl (precipitant 1) and PEG 3350 in 0.2 *M* trisodium citrate dehydrate (precipitant 2).

Earth-grown crystals were obtained by hanging-drop vapour diffusion from an aqueous solution of the insulin–PSA complex (10–20 mg ml^{-1}) using precipitants 1 and 2 with a PEG 3350 concentration of 10–15%(*w*/*v*). Crystals reached a maximum size of 0.2 mm.

The microgravity experiment was performed in the Modul′-1 protein-crystallization apparatus. Modul′-1 (Fig. 1) was designed for free-diffusion crystallization experiments in microgravity (Smirnova *et al.*, 2009). The apparatus consists of two coaxial cylinders that contain eight capillary channels of 140 µl volume each that are used as crystallization cells. The outlets of the channel cells are hermetically closed by screw-on stoppers. Each cell may be divided into two equal parts by rotation of one of the cylinders around its axis. To charge the apparatus, the cells are divided and subsequently loaded by unlocking the outer openings. One half of the cell is filled with protein solution and the other half with precipitant solution. The charged apparatus is then placed into the container with the rotating lid. Turning the lid enables the separation or connection of both halves of the cells inside. The container with the Modul′-1 apparatus is packed into a transportation vessel for delivery to the International Space Station. When microgravity is reached, the two halves of the cells are connected as described and the protein and precipitant solutions start to diffuse. The counter-diffusion of the protein and precipitant solutions results in nucleation and crystal growth.

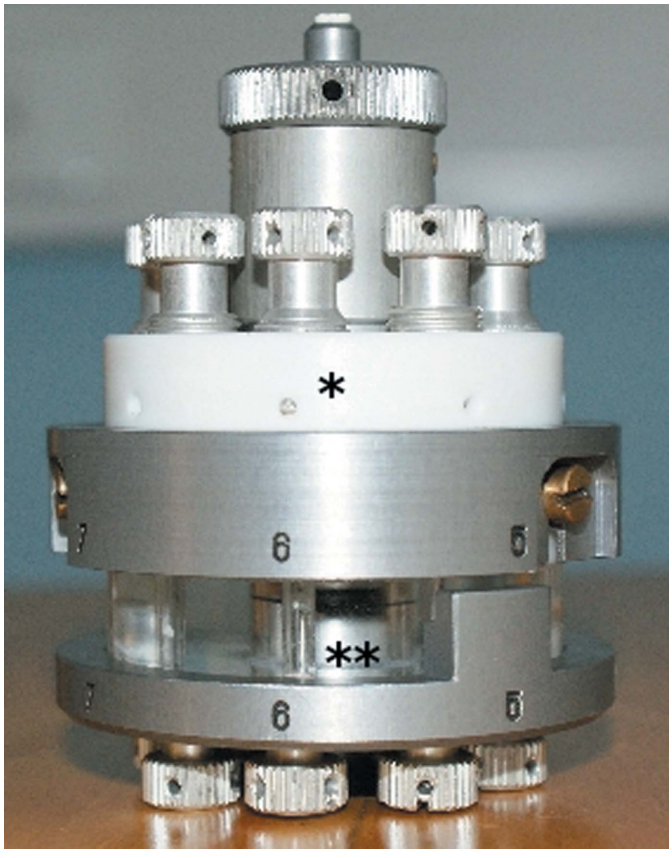


Figure 1
The Modul′-1 apparatus for protein crystallization. Glass and Teflon cylinders are indicated by single and double asterisks, respectively. Cells are numbered.

Table 1
Characteristics of the X-ray diffraction data sets collected from the space-grown (PDB code 3i3z) and Earth-grown (PDB code 3i40) insulin crystals.

PDB code	3i3z	3i40
Space group	$I2_13$	$I2_13$
Unit-cell parameters (Å, °)	$a = b = c = 77.365$, $\alpha = \beta = \gamma = 90.00$	$a = b = c = 77.220$, $\alpha = \beta = \gamma = 90.00$
Molecular weight (kDa)	5800	5800
No. of molecules per asymmetric unit	1	1
Wavelength (Å)	1.54	1.54
Resolution (Å)	54.718–1.550	54.636–1.840
No. of measured reflections	21709	13808
Crystal-to-detector distance (mm)	180	80
Oscillation range (°)	0.3	0.5
Rotation range (°)	90	90
No. of independent reflections	11366	6703
Redundancy	1.91	2.06
Completeness of data (%)	96.78	97.92
Mosaicity (°)	0.133	0.175
$R_{\rm merge}$ (%)	13.2	8.4

During the International Space Station experiment (flight ISS-17, 3 September–27 October 2008), eight cells of the Modul′-1 apparatus were filled with insulin–PSA solution (10 mg ml^{-1}) and precipitants 1 (four cells) and 2 (four cells) containing 20–25% PEG 3350. Simultaneously, a control experiment was set up under the same conditions at normal gravity. After completion of the flight and delivery of the apparatus to the Earth both the Earth and space apparatuses were opened and the contents of all cells were transferred to conservation solution.

Crystals were found in all cells of the space apparatus. The largest crystals (>0.4 mm) were obtained using precipitant 1 with 25% PEG 3350 and precipitant 2 with 20% PEG 3350 (Fig. 2). The control Earth experiment in the Modul′-1 produced only small (∼0.1 mm) crystals within amorphous precipitate.

2.2. Data collection and processing

X-ray diffraction data sets were collected from the Earth-grown and space-grown crystals. An X-ray data set was collected from the space-grown crystal to 1.55 Å resolution on EMBL beamline X11 at the DESY synchrotron (Hamburg, Germany) at room temperature. An X-ray diffraction set was collected from the Earth-grown crystal to 1.84 Å resolution using a Bruker AXT X-ray analysis system composed of a Microstar X-ray source and a Platinum 135 CCD detector at the Institute of Protein Research, Puschino, Russia.

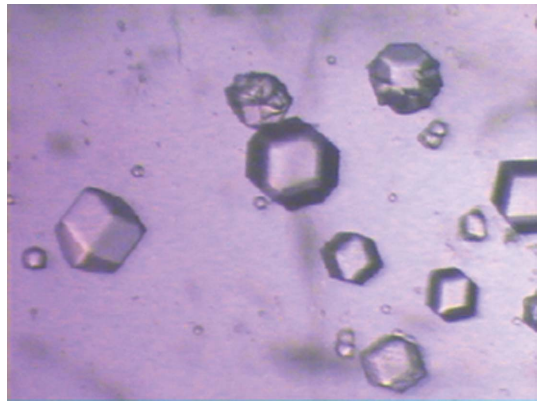


Figure 2
Insulin crystals grown in microgravity in the Modul′-1 crystallization apparatus (precipitant 2).

The X-ray diffraction data from the Earth-grown crystal were measured by the rotation method at a crystal-to-detector distance of 180 mm with an oscillation angle of 0.3° and a total rotation of 90°. The space-grown crystal data were collected at a crystal-to-detector distance of 80 mm with an oscillation angle of 0.5° and a total rotation of 90°. The experimental X-ray intensity data sets were processed using the *DENZO* and *SCALEPACK* program packages (Otwinoski & Minor, 1997) for the space-grown crystal and the *PROTEUM* (http://www.bruker-axs.com/x8_proteum.html) program package for the Earth-grown crystal. X-ray diffraction data-collection statistics are presented in Table 1.

2.3. Structure solution and refinement

The unit-cell parameters of the crystals obtained match the unit-cell parameters of the known cubic insulin crystal. The structures of the crystals obtained were solved at 1.6 Å (space-grown crystal) and 1.85 Å (Earth-grown crystal) resolution by the molecular-replacement method. The known structure of cubic insulin at 1.9 Å resolution (PDB code 2g4m; Mueller-Dieckmann *et al.*, 2007) was used as the starting model. The calculations were carried out using the *Phaser* program (McCoy *et al.*, 2005). The best solutions had R factors of 0.264 and 0.271 for the structures at 1.6 and 1.85 Å resolution, respectively. There is one insulin monomer per asymmetric unit. The polypeptide chains of the insulin monomer in the PDB file are designated *A* and *B*.

Structure refinement was carried out using the *PHENIX* (Adams *et al.*, 2002) and *REFMAC* (Murshudov *et al.*, 1997) program packages. Manual rebuilding was performed with the *Coot* interactive graphics program (Emsley & Cowtan, 2004) based on electron-density maps calculated with $(2|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ coefficients. Water molecules were also identified from electron-density maps using the *Coot* program (Emsley & Cowtan, 2004). The quality of the refined parameters was assigned using the *PROCHECK* (Laskowski *et al.*, 1993) and *WHAT_CHECK* (Hooft *et al.*, 1996) programs. The structures were deposited in the Protein Data Bank (PDB codes 3i3z and 3i40). The structure-refinement statistics based on the two diffraction data sets for insulin are given in Table 2.

Table 2
Refinement statistics for the insulin structures based on X-ray diffraction data sets collected from the space-grown (PDB code 3i3z) and Earth-grown (PDB code 3i40) insulin crystals.

PDB code	3i3z	3i40
Resolution (Å)	1.60	1.85
No. of reflections in refinement	10233	6640
No. of reflections in the test set (5%)	498	316
Completeness of data (%)	99	99
No. of refined residues in the protein molecule	51	51
Matthews coefficient (Å^3 Da^{-1})	2.32	2.32
Solvent volume (%)	47	47
No. of refined non-H atoms in the protein molecule	411	411
No. of refined water molecules	57	36
R_{cryst} (%)	0.1737	0.2003
R_{free} (%)	0.1953	0.2276
R.m.s.d. bond lengths (Å)	0.006	0.005
R.m.s.d. angles (°)	0.911	0.760
Average temperature factors (Å^2)		
Main chain	22.631	26.381
Side chain	25.712	33.599
Protein-bound water	43.435	42.314
Ramachandran statistics (%)		
Most favoured regions (%)	90.7	90.7
Additionally allowed regions (%)	9.3	9.3
Generously allowed regions (%)	0	0
Disallowed regions (%)	0	0

3. Results and discussion

As shown by Bezuglov *et al.* (2009), formation of a complex with polysialic acid prolongs the therapeutic effect of insulin. It was suggested that complex formation between insulin and PSA proceeds *via* noncovalent (probably electrostatic and hydrogen-bonding) interactions. The resulting complex is water-soluble and suitable for crystallization trials.

Aqueous solutions of a noncovalent PSA–insulin complex were used to grow crystals by free-interface diffusion in the Modul′-1 apparatus on Earth and in microgravity. Larger crystals (above 0.4 mm in size) were obtained in the microgravity environment. Crystals grown in the Modul′-1 apparatus on Earth were not suitable for structural investigation because of their small size. Crystals at normal gravity suitable for X-ray study were obtained by the hanging-drop vapour-diffusion method. The unit-cell parameters of both crystals coincided with the parameters for crystals of cubic

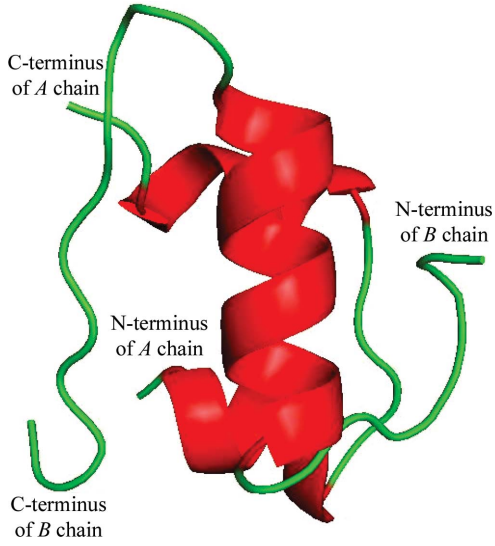


Figure 3
General view of the insulin monomer (the asymmetric unit of the cubic crystal; $a = b = c = 77.375$ Å, $\alpha = \beta = \gamma = 90°$).

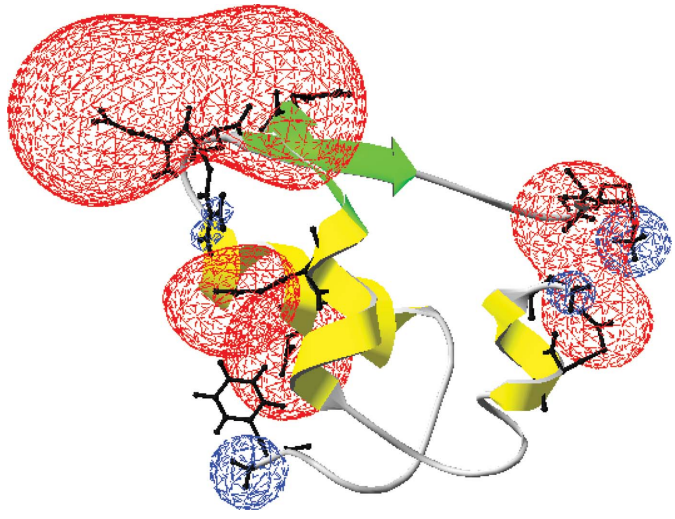


Figure 4
Distribution of electrostatic potential on the surface of the insulin molecule in the cubic crystal packing. Negatively charged sites are shown in red and positively charged sites are shown in blue.

insulin (PDB code 2g4m). This suggests that the crystals grown from the insulin–PSA complex are free insulin crystals. Insulin is known to crystallize in a cubic or (in the presence of zinc ions) rhombohedral space group with a monomer or a dimer in the asymmetric unit, respectively (Bin & Caspar, 1998; Smith *et al.*, 1996). The crystals studied in this study belonged to the cubic syngony and contained insulin monomers in the asymmetric unit (Fig. 3). Polysialic acid or its fragments were not localized in the electron-density maps.

Insulin molecules consist of two polypeptide chains (*A* and *B*) that are covalently bound *via* two disulfide bridges (*A*7–*B*7 and *A*20–*B*19), and contain 21 and 30 amino-acid residues, respectively. Fig. 4 shows the distribution of charge along the insulin-molecule surface; several positively and negatively charged sites are observed. The positively charged sites include residues Arg22*B*, Lys29*B* and the N-terminal amino groups of Gly1*A* and Phe1*B*. A small number of positively charged residues that are accessible to PSA on the insulin-molecule surface may promote the instability of the insulin–PSA complex. We have compared Earth-grown and space-grown insulin models using the *LSQKAB* program (Kabsch, 1976) from the *CCP*4 software package (Collaborative Computational Project, Number 4, 1994). The root-mean-square deviation (r.m.s.d.) between main-chain atoms of the two structures is 0.093 Å, showing the identity of the course of the main polypeptide chain. The r.m.s.d. between the side-chain atoms is 0.259 Å. Dual positions of residues Val12*B* and Arg22*B* in the space-grown crystal structure and Tyr14*B* in the Earth-grown crystal were detected. For some residues located on the surface of the molecule (Phe1*B*, Val12*B*, Glu21*B*, Arg22*B* and Lys29*B*), the deviation between their positions in the two structures exceeds 0.5 Å. The higher resolution, lower *R*-factor values and better electron-density maps indicate the better quality of the crystals grown under microgravity conditions.

A comparison of the determined structures with 22 existing cubic insulin structures from the PDB showed that they all are very similar: the average r.m.s.d. does not exceed 0.2 Å.

Comparison of the secondary-structure elements of insulin in cubic and rhombohedral crystals has revealed that the conformations of the insulin monomer in cubic and in rhombohedral packing show little difference (Figs. 5 and 6). The r.m.s.d. between backbone atoms is 0.294 Å and the r.m.s.d. between side-chain atoms is 0.564 Å.

A major conformational difference is observed in the regions that form the contact area (interface) between monomers of each dimer in the hexameric insulin form. The structure of the N-terminal part of the *B* chain of the cubic insulin monomer closely follows that of the T monomer in T_3R_3 rhombohedral Zn-insulin (Smirnova *et al.*, 2009). Little difference is observed in the positions of the backbone and side chains of Phe1*B* and Val2*B*. This can be explained by the interaction of the Val2*B* NH group in rhombohedral crystals with the main-chain O atom of Leu17*D* of the neighbouring dimer (with a corresponding distance of 2.6 Å), while in cubic crystals the N-terminal part of the *B* chain is free. Also, residues 24–26 of each *B* chain in the hexameric Zn-insulin form a β-strand that is not observed in the cubic crystals. Unlike the cubic insulin, residues *B*24–*B*26 in the rhombohedral Zn-insulin hexamer are involved in the formation of a two-stranded antiparallel β-sheet that represents the monomer–monomer interface of the dimer and stabilizes the dimeric structure (Fig. 5). In the presence of Zn ions, which coordinate His*B*10 of three dimers, a more stable hexameric Zn-insulin is formed by rotation of the dimer around the threefold-symmetry axis. In the C-terminal part of the *B* chains, the position of Lys29*B* is different in the cubic and rhombohedral forms (Fig. 6).

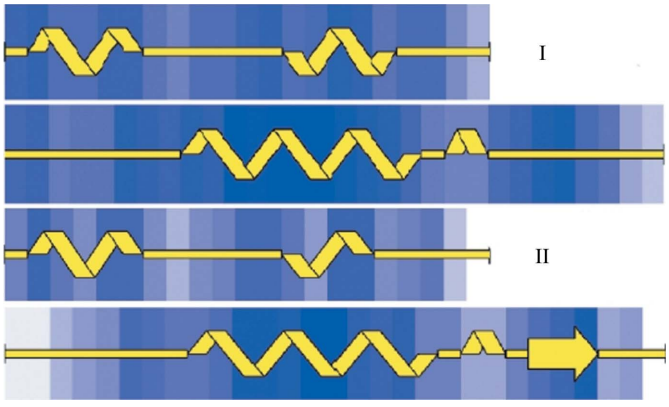


Figure 5
Comparison of secondary-structure elements of the insulin monomer in two different crystal forms: I, cubic crystals; II, rhombohedral crystals.

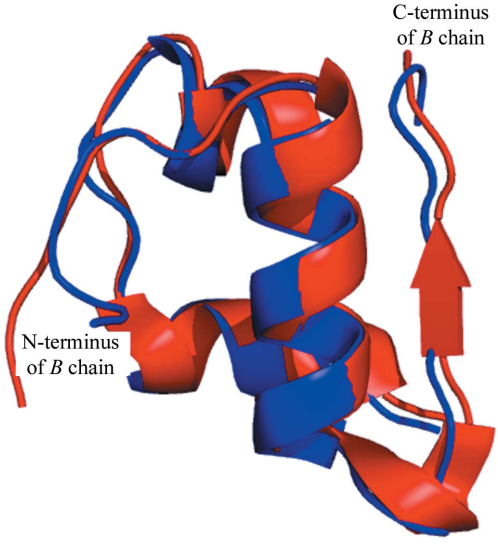


Figure 6
Comparison of insulin-monomer conformations in cubic (blue) and rhombohedral (red) crystals.

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