CROATICA CHEMICA ACTA
CCACAA, ISSN-0011-1643, ISSN-1334-417X

Croat. Chem. Acta **82** (2) (2009) 433–438
CCA-3332

Original Scientific Paper

Structure of the T_6 Human Nickel Insulin Derivative at 1.35 Å Resolution*

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RECEIVED NOVEMBER 3, 2008; REVISED FEBRUARY 13, 2009; ACCEPTED FEBRUARY 16, 2009

Abstract. This paper presents results of the structural investigation on the human insulin hexamer derivative stabilised by nickel coordination. Single crystals of the Ni-insulin derivative were prepared by the hanging drop vapour diffusion crystallisation method using metal-free insulin and nickel(II) acetate tetrahydrate. The low-temperature crystal structure was determined by the single crystal X-ray crystallographic method with data extending to 1.35 Å spacing. The investigated insulin derivative exhibits the T_6 form of insulin and crystallizes in the trigonal system in space group *R*3, with the unit cell parameters $a = b =$ 81.41 Å and $c = 33.75$ Å. There are two nickel atoms per insulin hexamer and both are octahedrally coordinated by three $N^{\varepsilon 2}$ atoms of three symmetry-related HisB10/HisD10 and three oxygen atoms of three symmetry-related water molecules.

Keywords: insulin derivative, nickel, X-ray structure

INTRODUCTION

Insulin is a hormone synthesized in humans and other mammals within the β-cells of the islets of Langerhans in the pancreas. It regulates carbohydrate metabolism and is also involved in the metabolism of fat and proteins. Insulin is used to control the level of blood sugar in patients with Type 1 diabetes mellitus and occasionally in some patients with Type 2 diabetes mellitus.

The molecule is structured of two polypeptide chains, chain A consisting of 21 and chain B of 30 amino acids. Chain A and chain B are linked by two disulphide bridges, while the third disulphide bridge links residues A6 and A11 by an intra-molecular bond. In the presence of zinc ions and some other divalent cations, insulin forms hexamers. Although insulin is accumulated in the pancreas as a Zn^{2+} containing hexamer, it is active as a monomer that does not contain zinc.[1]

The native insulin monomer forms multimers at the concentrations necessary for crystal growth; thus the crystal structure analysis of the active native monomeric molecule is impossible. In the absence of metal ions native insulin crystallizes as a dimer. There are three forms of insulin hexamers named T_6, $T_3R_3^f$ and R_6. These notations refer to the folding of the N-terminal part of the B chains in insulin hexamers.[2] All of these forms of insulin are used in therapeutic preparations for the control of diabetes.

The first crystal structure of insulin was described by Adams *et al.*[3] in 1969 and two years later in more details by Blundell *et al.*[4] This form of insulin was originally called 2Zn insulin since two zinc ions are bound by the hexamer. In 2Zn insulin both of the zinc ions are octahedrally coordinated by three HisB10 ligands and three water molecules. Later, when the T/R conformational notation was introduced the 2Zn form was called T_6 form. The T_6 insulin form was also investigated in details at a resolution of 1.0 Å.[5] The second form of insulin hexamer was originally called 4Zn insulin and according to the T/R conformational notation that form is T_3R_3.[6] In that form, the N-termini of three out of the six B chains show a conformational change from extended strand to an α-helix. This form of insulin crystallizes in the presence of high chloride or thiocyanate concentration. It was found that in the T_3R_3 type of hexamer there can be a different number of Zn ions per hexamer with different coordination.[7–9]

* Dedicated to Professor Emeritus Drago Grdenić, Fellow of the Croatian Academy of Sciences and Arts, on the occasion of his 90th birthday.

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The third insulin hexamer form is the R_6 form, which crystallizes in the presence of phenol and phenolic derivatives.[10] In the R_6 form both zinc ions lie on the molecule's 3-fold axis and are tetrahedrally coordinated.

It has been known for many years that different metal ions can substitute zinc ions in the 2Zn hexamer, however only three such complexes have been structurally characterized. Two are heavy metal derivatives Cd and Pb,[11] while the third is a Co-derivative, which was published recently.[12]

Nickel plays numerous biological roles in microorganisms and plants. Several nickel-containing enzymes are known: ureases, NiFe-hydrogenases, Ni-tetrapyrrole coenzyme, superoxide dismutase, glyoxolases, aci-reductone dioxygenase, acetyl-CaA decarbonylase/synthase.[13] Also, Ni-specific sensing and transport systems have been characterized in numerous microorganisms.[14]

Nickel is not an essential element in higher organisms. In humans it even presents a health hazard. Contacts with Ni-compounds can cause a variety of adverse effects on human health: Ni allergy in the form of contact dermatitis, lung fibrosis, cardiovascular and kidney diseases, and cancer of respiratory tract.

In the present study the Zn^{2+} ions in insulin were substituted with Ni^{2+}. We report here the crystal structure of the human insulin derivative with nickel in the hexamer T_6 form. The investigated structure provides some details on the metal binding and its coordination in the insulin hexamer, and on the conformations of the insulin molecules.

EXPERIMENTAL

Crystalization

Crystals of the human Ni-insulin derivative were grown by the hanging drop vapour diffusion method originally established for insulin by Cutfield[15] and improved by Xiao.[16] Biosynthetic human Zn-free insulin was supplied by Lilly Research Laboratories. Buffers, salts and other reagents were purchased and used without further purification. Optimum crystallization conditions are as follows: the protein solution consisted of 7.5 mg mL^{-1} of Zn-free insulin in 0.02 mol L^{-1} HCl, while the reservoir solution was at pH = 6.4, containing 1 mmol L^{-1} sodium citrate, volume fraction of aceton φ = 10 %, 15 mmol L^{-1} nickel(II) acetate tetrahydrate and redistilled water. Each drop consisted of 1μL of protein solution and 1μL of reservoir solution. It took about 7 days at 291 K for crystals to grow to a suitable size.

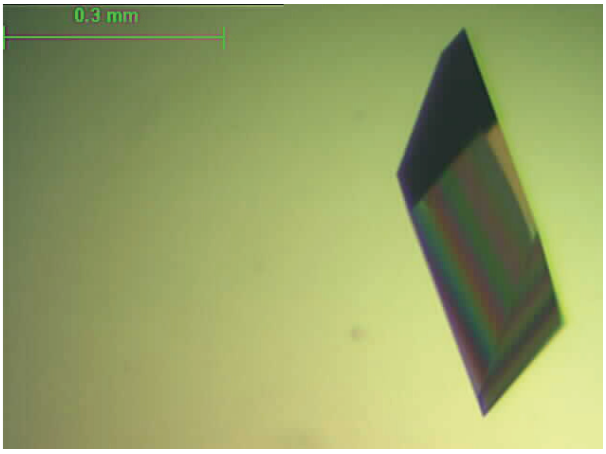


Figure 1. Single crystal of T_6 human nickel insulin derivative.

Diffraction Data Collection

Intensity data for the human nickel insulin derivative were collected on a single crystal of dimensions 0.2 × 0.2 × 0.4 mm^3 (Figure 1). The single crystal was cryoprotected by immersing it in the solution consisting of volume fraction φ = 70 % reservoir solution and φ = 30 % ethylene-glycol. The diffraction data were collected at the ELETTRA Synchrotron Light Laboratory, beamline XRD-1 (wavelength, λ = 1.00 Å), equipped with a CCD detector from MarResearch and a Oxford Cryosystems cryocooler. The 1.35 Å data set was collected by means of a combination of high- and low-resolution sweeps, with oscillation angles of 1.0°. The data were processed with MOSFLM[17] and then scaled and merged

Table 1. Data measurement statistics for T_6 nickel human insulin derivative

Space group	*R*3
Unit cell parameters	
$a = b$ / Å	81.41
c / Å	33.75
$\alpha = \beta$ / °	90
γ / °	120
Temperature / K	100
$\langle B_{iso} \rangle$ / Å^2	15.2
No. of frames	240
Resolution / Å	16.41–1.35
Total data	29352
Unique data	18240
No. of free reflections	935
Completeness / %	99.7
$\langle \sigma(F) \rangle \langle F \rangle$	0.03

by use of the CCP4 suite. Data statistics are given in Table 1.

Structure Determination and Refinement

The protein data bank (PDB) entry 1mso[5] from which all water molecules, zinc ions and alternate side chains were omitted, was taken as a starting model for structure refinement. Initially only 4 Å resolution data were included in the rigid body refinement and *R*-factors at that point were $R = 0.316$ and $R_{free} = 0.342$. Refinement was carried out using maximum-likelihood minimization implemented in REFMAC[18] with 5 % of the total data being excluded for the purpose of R_{free} refinement. The structure was then refined at a higher resolution and throughout the refinement $2F_o - F_c$ and $F_o - F_c$ maps were calculated and examined by the COOT program.[19] Water molecules were added by the ARP/wARP program.[20] The final model included 146 water molecules. The refinement was carried out using anisotropic displacement parameters of the metal ions (two nickel and two sodium ions) and amino acid atoms. Water molecules were refined isotropically, except for those bound to Ni. Low electron density was observed for the terminal atoms of the side chain of PheD1 so it was given an occupancy of 0.5. Some side chains were found to be disordered, and alternate conformations were added for IleA2, SerA9, GlnB4, ValB12, LeuB17, CysC6, CysC11, SerD9 and LysD29. Refinement converged at residual of $R = 0.126$ and $R_{free} = 0.170$. The model has a good stereochemistry, the r.m.s. deviations for bond lengths and bond angles are 0.028 Å and 2.150°, respectively. The geometry of the model was monitored with PROCHECK.[21] In the Ramachandran plot 93.0 % of the residues are in the most favoured region, 5.8 % in additionally allowed regions, and 1.2 % on the border of additionally allowed and generously allowed regions (SerA9). The refinement statistics are given in Table 2.

Table 2. Data refinement statistics for T_6 nickel human insulin derivative

Resolution range / Å	16.93–1.35
No. of reflections	18240
R	0.126
R_{free}	0.170
Cross-validated σ_A estimated error / Å	0.127
R.m.s. deviations from ideal	
Bond lengths / Å	0.028
Bond angles / °	2.150
Dihedral angles / °	19.52
Isotropic thermal model restraints / Å^2	
Main-chain bonds	2.78
Main-chain angles	3.90
Side-chain bonds	5.04
Side-chain angles	6.72

RESULTS AND DISCUSSION

Coordination of Nickel Atoms

It was already shown that the axial zinc binding cavities in the insulin hexamer can accommodate different metal ions which prefer imidazole and water molecule ligands.[2,3,5,7–9,11,12]

In the human nickel-insulin derivative the insulin molecules are organised in a hexamer very similary to that in 2Zn insulin (Figure 2). Both nickel atoms are octahedrally coordinated by three $N^{\varepsilon 2}$ atoms from three symmetry-related His imidazole ligands (HisB10/HisD10) and three oxygen atoms of three symmetry-related water molecules. The Ni-N bond lengths are both 2.06 Å, and Ni-O are 2.15 and 2.18 Å. Bond lengths in the 2-Ni insulin are slightly shorter than in the Zn-insulin derivative (Zn-N 2.09 and 2.10 Å; Zn-O 2.20 and 2.23 Å). The two nickel atoms lie 16.58 Å apart on the three-fold crystallographic axis. The separation of the two metal ions is slightly greater than in the T_6 human Zn-insulin[5] and in 2-Co porcine insulin[12] (both 16.42 Å), which also possess octahedrally coordinated metal ions. There was no evidence of metal binding to

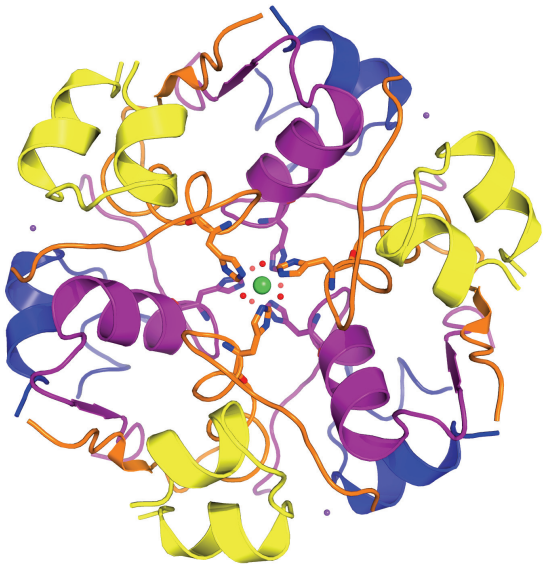
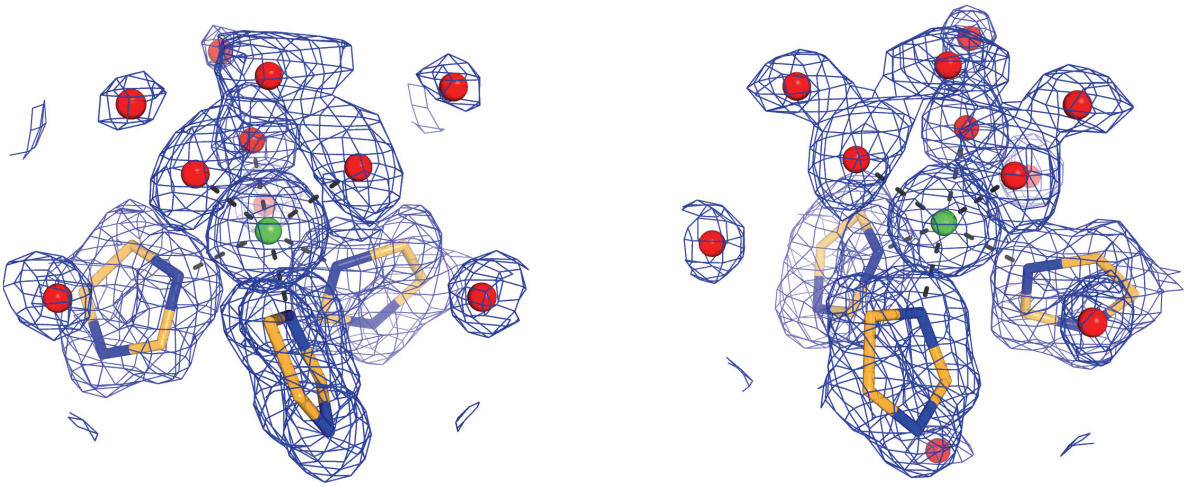


Figure 2. T_6 human nickel insulin derivative viewed down the crystallographic three-fold axis. A chains are colored yellow, B chains orange, C chains blue and D chains purple. Ni ions (green), Na ions (pink) and water molecules (red) bound to nickel are presented as spheres. HisB10 and HisD10 are presented as sticks.



a) b)

Figure 3. Coordination around nickel atoms showing the electron density: a) Ni (green) coordinated by HisB10 and water molecule O1 (red); b) Ni (green) coordinated by HisD10 and water molecule O2 (red). The contacts to ligands are shown by grey dashed lines. The $2F_o - F_c$ maps are contoured at 1.4σ. The second sphere of water molecules that form hydrogen bonds to the ones bound to nickel is also presented.

the GluB13 or GluA17, reported in the structure of the insulin derivative with cadmium and lead.[11] PyMOL[22]drawings of the nickel coordination sites with included electron density ($2F_o - F_c$) are illustrated in Figures 3a and 3b. The second sphere of water molecules that form hydrogen bonds to the ones bound to nickel was also modeled. One water molecule lies on the three-fold axis and its hydrogen atoms are therefore disordered, while the other is farther from the three-fold axis and forms hydrogen bonds to the water lying on the axis and those coordinating the nickel. The pattern is similar around both metal atoms. In contrast, in the Zn-insulin structure there is a difference in the second sphere of water molecules above the two coordination sites. In one there are three water molecules residing above the three zinc-coordinating waters, while in the other there is a continuous electron density above the zinc ion and its coordinating waters. This density was modelled as disordered water molecules. There is an assumption that even a disordered citrate anion might occupy that site.

An analysis of small molecule crystal structures containing octahedrally coordinated nickel ion with three N-donor ligands and three water molecules was carried out by searching the Cambridge Structural DataBase[23] (CSDB). 21 such structures were found with the mean Ni-N and Ni-O bond lengths of 2.080 Å, and 2.077 Å, respectively. The Protein DataBank[24] search using the Metalloprotein Database and Browser[25] (MDB) gave 52 structures (resolution ≤ 2.7 Å, $R \leq 0.22$) with octahedrally coordinated nickel of which 29 had histidine as a ligand, and 31 had water bound to the Ni ion. The coordination sites in the insulin hexamers are therefore well suited for nickel ions.

For comparison it is interesting to analyze the structure of an endopeptidase, astacin from crayfish, where zinc was also replaced by nickel in order to probe the role of metal for catalysis and structure. The catalytic zinc is coordinated by five ligands: three histidines, a tyrosine and a water molecule. Interestingly upon replacement with nickel the coordination changes to a deformed octahedral since one more water molecules enters the coordination sphere. The distances in this endopeptidase structure are similar as in the Ni-insulin, Ni-N(His) 2.07, 2.09 and 2.18 Å, Ni-O(H_2O) 2.22, 2.30 Å. Loss of activity was found in the substituted enzyme.[26]

In insulin the role of the metal ion is only structural. Since the octahedral coordination is common for both Zn and Ni the structural changes upon replacement of Zn with Ni are only minor.

Dimer and Hexamer Conformation

In the crystal structure of human Ni-insulin derivative there are three T_6 insulin hexamers in the unit cell, and asymmetric unit consists of one T_2 dimer. Insulin molecules (monomers) form a hydrogen bonded anti-parallel β-sheet in the dimer with hydrogen bond contacts between insulin momomers at: PheB24···TyrD26 and TyrB26···PheD24. In the Ni-insulin derivative the dimer is asymmetrical because the B25Phe side chain turns away from the two-fold axis and contacts D25Phe across the two-fold axis, as in the 2Zn insulin. The C^{α} root mean square difference between the T_2 dimer of human Ni-insulin derivative and T_2 human Zn-insulin[5]

Table 3. Root mean square differences (in Å) between the Zn-insulin, Co-insulin and Ni-insulin models; Residues D1-D3 are excluded

	Co-insulin		Ni-insulin	
Residue range	All atoms	Main chain	All atoms	Main chain
Insulin dimer	0.81	0.39	0.97	0.17
A chain	0.68	0.18	0.85	0.23
B chain	0.92	0.31	1.02	0.14
C chain	0.67	0.33	0.61	0.16
D chain	0.80	0.45	0.60	0.10

calculated using the CCP4 program LSQKAB[27] was 0.17 Å (Table 3), indicating that the zinc and nickel insulin structures are similar within limits of experimental error. The analogous differences between the Co-insulin and Zn-insulin structures is 0.39 indicating slightly greater difference than for the nickel derivative. In the Co-derivative the greatest main chain difference was found in the D chain whereas in the Ni-derivative it is for the A chain. The side chains which show significant differences in conformation in the nickel and zinc structures include GlnB4, PheB25, ThrB27 and LysD29. This is in agreement with the greatest root mean square difference of all atoms in the B chain of the Ni-derivative.

An interesting difference is the disordered disulphide bond between CysC6 and CysC11 which was not found in the Zn-derivative.

The hexamer three identical insulin dimers (related by the three-fold axis) assemble together around the 3-fold axis is stabilized by the metal coordination, the burial of non-polar residues along the N-terminus and hydrogen bonds between PheB1 and GluC17, GlnB4 and LeuD17, GluB21 and AsnD3.

CONCLUSION

The described structure shows that nickel ion can replace the zinc ion in the T_6 form of human Zn-insulin. The two structures were found to be highly similar with differences only in a few side chains. Coordination of the metal ion is octahedral in both structures (three His and three water molecules). Slightly shorter Ni-N and Ni-O bond lenghts were found than the analogous ones in the Zn derivative. A difference between the Ni- and Zn-insulin structures regarding the second sphere of water molecules above the metal coordination site was also found.

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SAŽETAK

Struktura T_6 niklovog derivata ljudskog inzulina pri rezoluciji od 1,35 Å

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U ovom su radu prikazani rezultati strukturnog istraživanja niklovog derivata ljudskog inzulina. Monokristali Ni-derivata inzulina priređeni su metodom difuzije para u visećoj kapi korištenjem inzulina koji nije sadržavao metalne ione i niklovog(II) acetat tetrahidrata. Kristalna struktura navedenog derivata inzulina riješena je metodom difrakcije rentgenskog zračenja na monokristalnom uzorku do rezolucije od 1,35 Å. Navedeni inzulinski derivat pripada T_6 formi heksamera inzulina i kristalizira u trigonskom sustavu u prostornoj grupi *R*3 s parametrima jedinične ćelije $a = b = 81{,}41$ Å i $c = 33{,}75$ Å. Svaki heksamer inzulina sadrži po dva atoma nikla, oba oktaedarski koordinirana s tri atoma $N^{\varepsilon 2}$ iz triju simetrijski ovisnih HisB10/ HisD10 i tri kisikova atoma iz triju simetrijski ovisnih molekula vode.