Orthorhombic crystal structure of an insulin mutant B9Asp at 0.20 nm resolution*

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Abstract Human B9(Ser→Asp) insulin, a fast-absorption insulin analogue, was obtained by site-directed mutagenesis at B9 position. The orthorhombic crystal structure of B9Asp insulin was analyzed by crystallography at 0.20 nm resolution. Although no significant change of its overall conformation was observed, the local conformation flanking B9 site differed greatly from native T6 human insulin. The substitution of serine at B9 position by aspartic acid resulted in obvious alteration of local hydrophobic and hydrophilic interactions. As a result, the insulin dimer became unstable and the capability of the hexamer formation was diminished extensively. All these properties contribute to the fast-absorption of B9Asp. In addition, the open state of N-terminus of B-chain, which differs from T- or R- state, might suggest a new conformational state in the monomer or dimer insulin.

Keywords: crystal structure, insulin mutant, fast-absorption, B9Asp analogue.

Insulin is one of the most important protein hormones that regulates the cellular uptake of glucose and affects the normal cell growth and proliferation *in vivo*. It is observed that insulin can exist in various aggregate state in response to different environmental condition. In mammals, insulin is synthesized in the cells of pancreas and stored as zinc-containing hexameric aggregates. However, this hormone is a monomer as it circulates in the blood stream and interacts with its receptor. Therefore, a complex dissociation process is needed for insulin from inactive hexamer to active monomer prior to binding to its receptor[1]. Binding and activity assay of several insulin analogues suggest that this dissociation process should be the rate-limiting step during the insulin's absorption and activation.

As the strongest subunit interaction takes place between dimerising monomers, the association of the molecules can be most effectively counteracted by preventing of dimer formation. The residues involved in the monomer-monomer interaction in the dimer consist of residues B8, B9, B12, B13, B16 and B23-B28[2,3]. The B9(Ser→Asp) human insulin reported in this paper, with the effect of fast-absorption, is a potential one in therapeutic use of these analogues. It retains 79% biological activity of native insulin in mouse blood glucose assays and can be absorbed 3-fold faster than native one after subcutaneous injection *in vivo*[4].

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1 Materials and methods

Human insulin mutant B9(Ser→Asp) (abbreviated to B9D HI) was obtained by site-directed mutagenesis in NOVO Research Institute in Denmark[4]. The crystals were grown by batch method at 14℃. The preliminary crystallographic analysis performed by a Mar Research Image Plate System revealed that they belonged to space group $P2_12_12_1$, with cell parameters of $a = 4.522$ nm, $b = 4.681$ nm, $c = 5.164$ nm, $\alpha = \beta = \gamma = 90°$.

The data were collected by a SIEMENS X-200B area detector at room temperature, using graphite-monochromated Cu Kα radiation generated by a Rigaku RU-300 rotating anode. The data were processed with XENGEN software[5] with a resolution cutoff of 0.20 nm. There were overall 24 744 reflections observed, which were merged into 10384 unique reflections. The whole data were processed as a result of R_{merge} 5.72%, with 97.39% completeness.

The native 2Zn insulin[6], with removal of flexible B1Phe-B4Gln of B-chain, was used as the structure model to calculate the initial phase. The rotation and translation functions of the molecules in the unit cell were determined by the molecule replacement technique, with AMoRe program from CCP4[1]. The X-PLOR program was used to refine the structure. The 2Fo-Fc map was calculated and examined in order to adjust some of unfavorable residues. The final R value and free-R value are 16.75% and 20.74%, respectively (table 1).

Table 1 Result of the structure refinement

Resolution limits	1.0—0.20 nm, 2.0 σ cutoff
Final R-factor	0.1675
free-R	0.2074
No. of reflections used	6927
No. of non-hydrogen atoms	978
No. of protein atoms(non-hydrogen)	814
No. of water molecules	164
RMS deviation from standard geometry	
Bond lengths/nm	0.000 9
Bond angles/(°)	1.371
Dihedral angles/(°)	25.34
Improper angles/(°)	0.700
Average B factor/nm^2	0.253 8
Protein	0.225 8
Water	0.392 7

2 Results

2.1 Quality of the model

The PROCHECK program was used to analysis the dihedral angles of main-chain of the model. Totally, 95.3% residues were located in the most favored region and 4.7% residues were located in

1) Computer programs for protein crystallography, Jorge Navaza, 1994

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the additional allowed region. The position of atoms fitted the 2Fo-Fc electronic density map very well at B9 site (figure 1).

Fig. 1. The 2Fo-Fc electron density map of B9Asp, contoured at 1.0 σ.

2.2 Specific packing in B9D HI crystal

Compared to the 2Zn insulin, the B9D HI crystal is constructed by dimer unit, which is not further assembled into hexamer by symmetric or asymmetric 3-fold axis. Two molecules in the dimer (designated as molecule 1 and molecule 2 respectively) are located in crystal lattice one by one. As a result, every molecule 1 contacts three molecules 2.

Of three monomer-monomer contact interfaces, except for dimerised monomer-monomer interface, the other two are different from the interface of 2Zn insulin crystals. One interface, involving the residues A10-A17, B2-B4, B6, B10, B14 and B17-B18 (marked L in fig. 2), approximately corresponds to the dimer-dimer interface in 2Zn insulin hexamer. The third interface consisting of residues A1, A4-A5, A15, A18-A21, B25 and B29-B30, is more hydrophilic and changeable (marked M in figure 2).

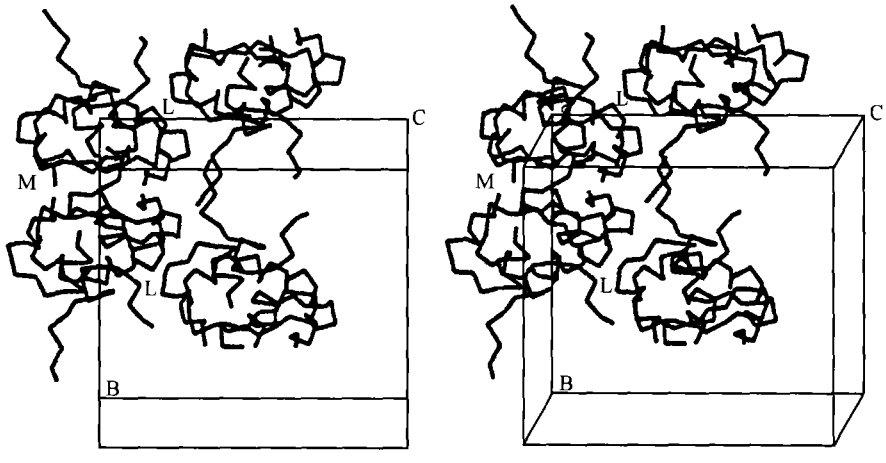

Fig. 2. Crystal packing of B9D HI.

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2.3 Structure features of B9D HI dimer

Compared to the 2Zn insulin dimer, there are some significant changes in the conformation of the B9D HI dimer.

(i) One significant rearrangement of the B9D HI is located on N-terminus of B-chain. The open state of B-chain N-terminus, which is different from the T- , R^f or R- state[7,8] described previously, drives the strand B1-B4 to extend towards the third dimer across the vicinity of the first and the second dimers, resulting in the relocation of B1 Phe side-chain in the space that is surrounded by the side-chain of A17 Glu and B22 Arg (see fig. 3). This open state of B-chain N-terminus was previously observed in the crystals of despenta-insulin (DPI)[9] and deshepta-insulin (DHPI)[10] and their relationship with insulin's assembly and potency have not been interpreted.

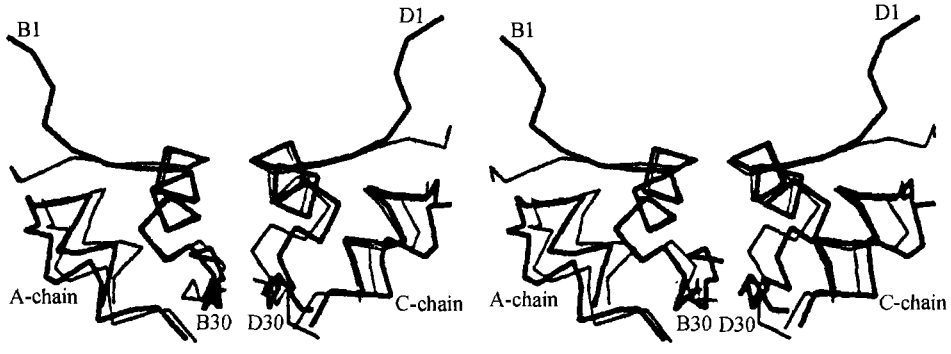

Fig. 3. The Cα superposition of the dimer in B9D HI (thick line) and 2Zn-insulin (thin line).

(ii) Molecule 1 and molecule 2 are more symmetric than that in 2Zn insulin. Both α-helix of A1-A5 in B9D HI dimer are similar to the corresponding conformation of molecule 1 in 2Zn insulin. There are some significant rearrangements on the dimerised monomer-monomer interface. One difference comes from the side-chain of B25 Phe. In comparison with the 2Zn insulin hexamer, the conformation of side-chain of B25 Phe of molecule 1 differs from that of molecule 2. In the molecule 1, of which the B25 side-chain extends inside the molecule and an H-bond formed between B25 Phe NH and A19 Tyr O, the side-chain of B25 Phe of molecule 2 extends across the dimer interface making a local asymmetry[11]. In the case of B9D HI, in contrast, the side-chains of B25 Phe of both molecule 1 and molecule 2 are nearly symmetric, which orientate to the C-terminus of B-chain. The close symmetry of two molecules is also indicated by some H-bond alterations. The H-bond formed by B25 NH and Y19 O in 2Zn insulin molecule 1 is disrupted by a distance change from 0.316 nm to 0.393 nm, resulting in a local more symmetric conformation.

2.4 Conformational change at B9 and B13 sites

At B9 and B13 positions, compared with the corresponding sites of the 2Zn native insulin, the local conformational displacement was observed. In B9D HI dimer, both the spatial and electrostatic interactions are altered due to the substitution of aspartate for serine at B9 position. The longer side-chain and negative charge possessed by aspartate lead to four acidic carboxylic groups, two of which are contributed by B13 Glu, coming together in a space of about 0.16 nm^3. At neutral pH, the elec-

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trostatic repulsion caused by four carboxylic groups on the monomer-monomer interface of the dimer results in a considerable local conformational change. The side-chain of D13 Glu turns back to the molecular body, which is H-bonded by B10 His ND1. In 2Zn insulin, there exists an H-bond formed by atom OE2 of B13 and the corresponding atom of D13 in the other monomer (the distance is 0.259 nm), which links two monomers together. In the case of B9D HI, because of charge repulsion, the side-chain of D13 Glu shifts away from the monomer-monomer interface, protruding into the solvent to reduce the effect of charge repulsion. Correspondingly, the H-bond between two atoms OE2 of glutamate is disrupted, making a new distance of 0.599 nm (fig. 4). In contrast to the dimer of 2Zn insulin, the dimer of B9D HI is more unstable, especially under neutral or basic condition.

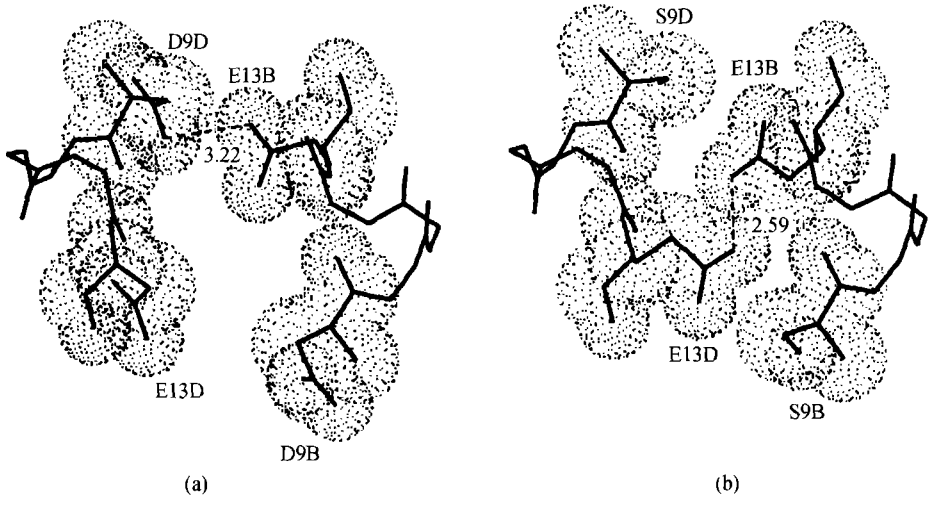

Fig. 4. Monomer-monomer interface in the dimer of B9D HI (a) and 2Zn insulin (b).

3 Discussion

The mutation from neutral residue to acidic residue on the monomer-monomer interface will result in the fast absorption due to rapid dissociation. But according to the structure of B9D HI, it can be concluded that the fast absorption of this insulin analogue can only be achieved under the neutral or basic condition. In the acidic solution, the carboxylic group will be protonated and lose the charge repulsion. As a result, the stable dimer can be assembled into crystal. In addition, the rhombohedral crystal can grow up in the presence of zinc ions and phenol at pH 8.5. It suggests that the monomer of B9D HI should only exist in the neutral or basic solution which lacks zinc ions and phenolic derivatives.

Our experiments have proved that the hexamer can be assembled in the presence of zinc ions and phenol at basic pH, with the concentration close to pharmaceutical preparation (~ 0.6 mmol/L). Naturally, according to the analysis made above, the hexamer seems to be loose and unstable. In spite of various attempts, the collection of diffraction data of the hexamer crystal failed owing to the fragility and weak diffraction ability of this crystal. However, the observation of the formation of B9D HI hexamer is inspiring for the potential pharmaceutical application of B9D HI. It is well known that

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the monomer and dimer are unstable in therapeutical preparation, while hexamer can yield both suitable chemical and physical stability. Therefore, the capability of aggregation of B9D HI to hexamer in the presence of zinc ions and phenolic derivatives contained in the therapeutical preparation is essential for the application of B9D HI in clinic. After a subcutaneous injection, the unbound phenolic preservatives are rapidly absorbed into the surrounding tissue, shifting the phenol-binding equilibrium to the direction of phenol derivatives unbinding. The loss of phenol, in accordance with the structural change at B-chain N-terminus, makes the unstable hexamer dissociate into the more unstable dimer, resulting in the yield of monomer within a short time. Hence, the fast action of the preparation is achieved.

In insulin hexamer, the strand B1-B8 can conform T- or R- or R^f- state, which correspond to the extended strand or α-helix or a frayed α-helix of local conformation. Nevertheless, the B1-B8 is rearranged in B9D HI. Unlike the T- or R- state, it extends out from rest of the molecule and takes up a conformation nearly perpendicular to the A8-A12, the middle region of A-chain. This new conformation of N-terminus of B-chain, distinguished from T- or R- state reported before, is named O-state (open state). A series of insulin analogues, in spite of different mutation types, have been crystallized and analyzed in our laboratory. These crystals, which are all grown up at acidic pH with various precipitants, belong to the same orthorhombic space group with an open state of B1-B8. Therefore, it is proposed that the acidic pH plays the key role in obtaining these series of crystals. The O-state conformation is also observed in the crystals of despenta-insulin (DPI) and deshepta-insulin (DHPI). Therefore, the O-state is supposed to be a new conformation of N-terminus of B-chain in the monomer or dimer of insulin.

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