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An abnormal pK_a value of internal histidine of the insulin molecule revealed by neutron crystallographic analysis

Takuya Ishikawa [a], Toshiyuki Chatake [a], Yukio Morimoto [a], Mitsuru Maeda [b], Kazuo Kurihara [b], Ichiro Tanaka [c], Nobuo Niimura [d,*]

[a] Kyoto University Research Reactor, Asashironishi 2, Kumatori, Osaka 590-0494, Japan
[b] Neutron Structural Biology, Japan Atomic Energy Agency, Shirakata-shirane, 2-4, Tokai, Ibaraki 319-1195, Japan
[c] Faculty of Engineering, Ibaraki University, Naka-Narusawa, 4-12-1, Hitachi, Ibaraki 316-8511, Japan
[d] Frontier Research Center for Applied Atomic Sciences, Ibaraki University, Naka-Narusawa, 4-12-1, Hitachi, Ibaraki 316-8511, Japan

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ABSTRACT

Insulin is stored in pancreatic β-cell as hexameric form with Zn^{2+} ions, while the hormonally active form is monomer. The hexamer requires the coordination of Zn^{2+} ions to the His*B*10. In order to reveal the mechanism of the hexamerization of insulin, we investigated the Zn^{2+} free insulin at pD6.6 and pD9 by neutron crystallographic analyses. His*B*10 is doubly protonated not only at pD6.6 but also at pD9, indicating an abnormal pK_a of this histidine. It is suggested that His*B*10 acts on a strong cation capture and contributes to the high stability of the hexameric form in pancreas.

Insulin is an animal hormone for glucose metabolism, composed of two chains, A (21 residues) and B (30 residues), linked by two disulphide bridges. In a solution of metal-ion-free, insulin exists as a mixture of monomer, dimer, tetramer, hexamer and higher aggregates depending on its concentration [1]. The hexamer is the stored form in pancreatic β-cell while the hormonally active form is the monomer. Zn^{2+} ion promotes the hexamerization of insulin in pancreas, and the other divalent cations such as Ni^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+} also have the same effect to the hexamerization in solution [2].

In the presence of Zn^{2+} ion, porcine insulin was crystallized as the hexamer in a rhombohedral crystal [3], while the dimeric form could be obtained without Zn^{2+} ions [4]. The hexamer, arranged as three dimers, has two Zn^{2+} ions. Both of the Zn^{2+} ions are located at the center of the threefold symmetry related dimers, and coordinate to three His*B*10, respectively. His*B*10 has been considered to be an important residue to bind metal**s** for the hexamerization [5]. Human insulin mutant, in which this residue was substituted for Asp, lacked the ability to form hexamer themselves [6] and increased hormonally activity as the monomer [7]. In general, histidine has an imidazole side chain that has a relatively neutral pK_a. However, the pK_a value of amino acid side chain involved in protein molecule sometimes shifts according to the electrostatic environment. Unfortunately, since it is very hard to determine the hydrogen atoms in protein molecules using X-rays alone, a discussion of protonation can only be speculated so far. In contrast, it is well known that neutron analysis provides an experimental method of directly locating hydrogen atoms [8–12]. In the present study, we investigated the dimeric porcine insulin by neutron crystallographic analysis at pD6.6 [13] and pD9, respectively. Our result clearly showed that His*B*10 kept the doubly protonated state even in the alkaline condition, indicating that His*B*10 would be a strong binding site for cation.

Materials and methods

Crystals of porcine insulin were grown in D_2O solutions in order to avoid high backgrounds due to the incoherent neutron scattering from H atoms. Insulin was dissolved in D_2O with 0.5 M NH_3 0.2 ml and after added 0.8 ml D_2O. Total concentration of protein and NH_3 were 20 mg/ml and 0.1 M, respectively. The protein solution was filtered through 0.22 μm Millipore-filter. A crystal was obtained by dialysis method using a 1000 μl micro-dialysis chamber with dialysis membrane attached to the bottom. Insulin solution (500 μl) was put into a chamber. The outer solution were placed in a vessel containing 2.5 ml dialysis solution containing 0.2 M Na_2HPO_4 and 0.01 M Na_3EDTA at pD9 and placed in an incubator at 298 K [14]. Three week**s** after, a large single crystal grew to the size of 4.0 × 4.0 × 1.3 mm^3.

* Corresponding author.
E-mail address: niimura@mx.ibaraki.ac.jp (N. Niimura).

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Table 1
Statistics of data collection and refinement at pD9

Data collection	
Space group	$I2_13$
Cell dimensions (Å)	$a = b = c = 79.04$
Beam port/radiation	1G, JRR-3
Wave length (Å)	2.9
Diffractometer	BIX-3
Temperature (K)	293
Exposure time (min/frame)	55
Total frames	311
Maximum resolution (Å)	2.3
Observed reflections (80–2.7 Å)	19,312
Unique reflections (80–2.7 Å)	3,747
Redundancy	5.2 (3.9)
Completeness (%)	98.9 (98.1)
R_{merge} (%)	14.5 (29.0)
Structural refinement	
Resolution (Å)	20–2.5
R_{work} (%)	22.2
R_{free} (%)	26.0
Number of D atoms	75
Number of water molecules	34
PDB ID	2ZPP

Values in parentheses are for the outer shell (2.38–2.30 Å).

The neutron diffraction experiment was carried out at room temperature with monochromatic neutron beam ($\lambda = 2.9$ Å) using the BIX-3 diffractometer at port 1G-B in the reactor hall, JRR-3, at Japan Atomic Energy Agency in Japan [15]. The data collection protocol employed the step-scan method ($\Delta\omega = 0.3°$) with 55 min exposure per frame. The crystal was rotated through 93.3° and a total of 311 frames were collected. The HKL software package, DENZO and SCALEPACK [16] were used for data processing and scaling. The observed data were collected 2.3 Å resolution range. A total of 3747 independent reflections were obtained with an overall R_{merge} of 14.5% from 19,312 observed reflections. The completeness of the data set was 98.9% in the 80–2.3 Å resolution range and 98.1% for the outermost resolution shell (2.38–2.30 Å). Structure refinement was carried out using the program of CNS [17] and Xtalview [18], which were modified for neutron analysis. The X-ray structure of porcine insulin at pH 9.0 (PDB ID:1B2G) was used for initial model [19]. The resolution for structure refinement and map calculation were 2.5 and 2.3 Å, respectively. Deuterium atoms bonded to nitrogen and oxygen atoms were not included in the initial model. They were identified by using $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ Fourier maps. Water molecules (D_2O) were estimated by higher peaks than 2.5σ level in $|F_o|-|F_c|$ Fourier map. In this analysis the final R-factor and R_{free} were 22.2% and 26.0%, respectively. The statistics of data collection and refinement were summarized in Table 1.

Results and discussions

In the present crystal, two insulin moieties are related by a two-fold crystallographic symmetry, therefore one insulin molecule exists in an asymmetric unit [4]. Crystal structures of pD6.6 and pD9 were found to be almost same. The overall root-mean-square-differences of non-hydrogen atoms and all atoms between pD6.6 [13] and pD9 are 0.67 and 1.04 Å^2, respectively. The protonation states of the amino acid residues (ex. carboxyl and amino groups etc.) are same as expected except disordered residues and histidine side chains.

Porcine insulin has two histidines (His*B*5 and His*B*10) in the B chain. As for His*B*5, both of $N\pi$ (N^{δ}) and $N\tau$ (N^{ε}) atom of imidazole ring are protonated at pD6.6 and only the N_τ atom was protonated at pD9 (Fig. 1). This fact indicates that the pK_a value of HisB5 has the range between pD6.6 and pD9. That is plausible because of the pK_a value (=6.5) of a simple histidine moiety. However, His*B*10 was confirmed to be double protonation of both N_π and $N\tau$ atoms at both pDs. Fig. 2(a) shows that His*B*10 is doubly protonated and positively charged even at alkaline pH. There are two possible reasons of this double protonation state; a hydrogen bonding network and an electrostatic potential. In His*B*10, two D-atoms of the imidazole ring are anchored with water molecules and amino acids around there by hydrogen bonds. At both pD6.6 and pD9, the N_τ-D group was hydrogen bonded to a carbonyl group of main chain of Tyr*A*14 (dD-O were 2.3 and 2.1 Å for pD9 and pD6.6, respectively.), while the N_π-D group interacted with a carbonyl group of His*B*5 mediated by a water molecule. Moreover, His*B*10 is located on the surface of insulin and near a negative electrostatic potential. Glu*B*13 was about 7 Å away from His*B*10 and the carboxyl group of this residue was deprotonated, indicating negatively charged states of this functional group. In order to investigate electrostatic effect for His*B*10, molecular surfaces and electrostatic grid potentials were calculated by GRASP2 [20]. His*B*10 was not on a negative potential surface however, the negative potential derived from Glu*B*13 was in the vicinity of the imidazole ring of His*B*10. On the other hand, there was no negative potential near His*B*5, which has a normal pK_a value. In the previous X-ray study, it was reported

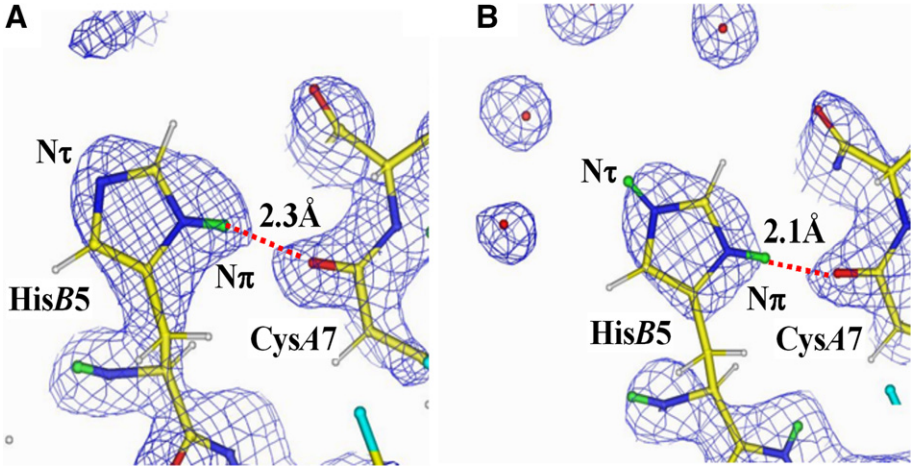

Fig. 1. $2|F_o|-|F_c|$ neutron density maps (contoured at 1.5σ) around His*B*5 at pD9 (a) and pD6.6 (b).

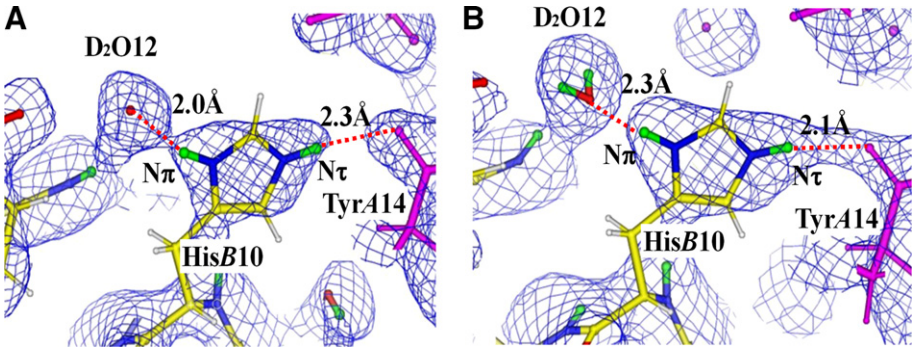

Fig. 2. $2|F_o|-|F_c|$ neutron density maps around His*B*10 at pD9 (a) and pD6.6 (b). The double protonation states are confirmed at N_τ and N_π atoms at both pDs.

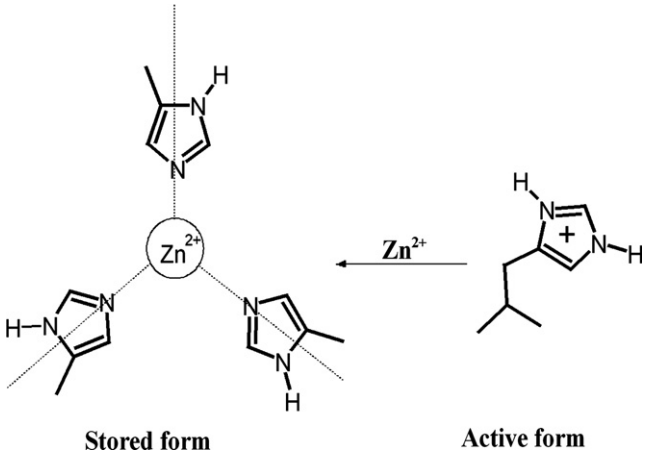

Fig. 3. Structural transition of His*B*10 between Zn^{2+} included insulin (stored form) and divalent cation free insulin (dimeric form).

that GluB13 affected a structural change of insulin by pH shift, supporting our hypothesis in the present neutron study [20]. These hydrogen bonds and electrostatic potentials seem to stabilize the double protonation state of His*B*10.

The abnormal pK_a value of HisB10 indicates the high affinity for positive ions. This property would be effective to the polymerization of insulin depending on various cations. Our hypothesis of the mechanism of the structural transition of insulin was summarized in Fig. 3. In the presence of Zn^{2+} ions, symmetry—related three N_τ atoms of His*B*10 are coordinated to one Zn^{2+} ion (Fig. 3) [3]. On the other hand, the present neutron analysis revealed the N_τ atom of His*B*10 in dimeric insulin was covalently bonded to a hydrogen atom (Fig. 3). In other words, His*B*10 captured D^+ atom instead of the divalent metal cation in the absence of Zn^{2+} ion. This structure would be the active form of insulin at biological pH. This mechanism would be achieved by the cation capturing of HisB10, therefore, HisB10 is essential for polymerization of insulin in organisms.

Conclusion

In the present neutron diffraction study of insulin, we found the abnormal pK_a value of His*B*10. The imidazole ring of His*B*10 was doubly protonated at both of pD6.6 and pD9. His*B*10 is the binding site for Zn^{2+} ion in the hexameric form, which is stored in pancreas, and it has been thought that His*B*10 was essential for the hexamerization promoted by the divalent metal cations. The abnormally high pK_a of histidine indicates that the His*B*10 molecules and its surroundings have very high affinity with cations and His*B*10 can bond to the various kinds of cation depending on the environment. It can be concluded that His*B*10 acts on a strong cation capture and contributes to the trigger of polymerization of insulin.

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