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Crystal structure of destripeptide (B28–B30) insulin: implications for insulin dissociation[1]

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Abstract

Destripeptide (B28–B30) insulin (DTRI) is an insulin analogue that has much weaker association ability than native insulin but keeps most of its biological activity. It can be crystallized from a solution containing zinc ions at near-neutral pH. Its crystal structure has been determined by molecular replacement and refined at 1.9 Å resolution. DTRI in the crystal exists as a loose hexamer compared with 2Zn insulin. The hexamer only contains one zinc ion that coordinates to the B10 His residues of three monomers. Although residues B28–B30 are located in the monomer–monomer interface within a dimer, the removal of them can simultaneously weaken both the interactions between monomers within the dimer and the interactions between dimers. Because the B-chain C-terminus of insulin is very flexible, we take the DTRI hexamer as a transition state in the native insulin dissociation process and suggest a possible dissociation process of the insulin hexamer based on the DTRI structure.

Keywords: Insulin; Insulin analogue; Association; Crystal structure

1. Introduction

Recent research on the association behavior and the corresponding structural basis of insulin has sought to design various modified insulins to treat diabetes [1–8]. Insulin that is synthesized and stored as hexamers performs its physiological activity as monomers. Insulin is secreted into the blood within a few minutes after a normal person eats. However, for a diabetic patient, injected insulin will take 30 min or more to reach the maximum concentration in the blood [2], which is less than ideal. Although the process by which subcutaneously injected insulin enters the bloodstream from the injection site is very complex, it can be simply stated that the rate of this process is correlated to the rate of the insulin hexamer dissociation into monomers [9]. Therefore, various mutants have been designed to accelerate the insulin dissociation in order to provide rapid-acting insulin.

Recognizing that the interactions between dimerizing monomers are the main cause of insulin association, Brange et al. [1] designed a series of insulin mutants which destroy these interactions by charge repulsion. Their results showed that the absorption of some of these mutants was several times faster than native insulin, and their biological activities were largely preserved. B-chain C-terminal residues, especially B28 Pro, are crucial to insulin assembly.

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[1] Coordinates have been deposited in the Protein Data Bank (ID code 1HTV).

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Whether B28 Pro was replaced by polar amino acids Asp and Lys or by neutral Ala, the association ability of the mutants all decreased [3]. Brems et al. [3] also investigated the association behavior of a series of B-chain C-terminus truncated insulin analogues by equilibrium ultracentrifugation. They found that the removal of one or two amino acid residues from the B-chain C-terminus had little effect on aggregation, but the removal of B28 Pro which resulted in destripeptide (B28–B30) insulin (DTRI) caused a sudden decrease of association ability. Its association ability was almost the same as despentapeptide (B26–B30) insulin which could be crystallized as a monomer [10]. DTRI still retains a fairly high biological activity [11]. Further understanding of the cause of the decreased association ability of DTRI should be of great interest.

The crystal structures of several C-terminal B-chain residue mutated insulins have been determined, including $Lys^{B28}Pro^{B29}$ insulin [12] and B28 Pro→Asp insulin [13]. The results showed that the hydrophobic interactions between B28 Pro and the other molecule were the main force stabilizing the dimer. The two mutants were crystallized in the presence of phenolic derivatives. It is well known that phenolic derivatives can increase the stability of hexamers [14–16] as they introduce extra interactions in the hexamer. Here we report the crystal structure of DTRI which was crystallized in the absence of phenolic derivatives.

2. Materials and methods

2.1. Crystallization

The preparation and crystallization of recombinant DTRI was as described earlier [11,17]. The crystals were grown by the hanging drop vapor diffusion method. The protein solution contained 8 mg/ml DTRI dissolved in 0.02 M HCl. The reservoir solution contained 0.1 M trisodium citrate, 15% (v/v) dimethylformamide, and 0.13% (w/v) zinc acetate, adjusted to pH 6.8 by 1 M HCl. Each hanging drop contained 3 μl of the protein solution and 3 μl of the reservoir solution and was equilibrated against 1 ml reservoir solution. It took about 2 weeks for crystals to grow to the final size.

2.2. Data collection and data processing

DTRI X-ray diffraction data were collected from three single crystals at room temperature using a MAR Research image-plate system with radiation ($\lambda = 1.5418$ Å) generated by a Rigaku rotating-anode generator. The resolution reached 1.9 Å. The data set was processed and scaled using DENZO and SCALEPACK [18]. Data statistics are shown in Table 1.

2.3. Structure determination and refinement

The DTRI crystal structure was determined by molecular replacement using the AMoRe package [19]. At first the 1.2 Å 2Zn insulin hexamer structure [20] was directly used as the search model after the removal of B-chain C-terminal residues B28–B30. The highest peak gave a correlation coefficient (C_c) value of 35.6% and an R-factor of 50.0% using data of resolution 12–4 Å and an integration radius of 22

Table 1
Data processing and refinement statistics

Space group	$P2_12_12_1$
Cell dimensions (Å)	$a = 49.81$
	$b = 51.55$
	$c = 100.6$
Resolution limits (Å)	20–1.9
No. of observations	481 068
No. of unique reflections	20 516
R_{merge} (on I)[a,b]	0.055 (0.189)
Completeness (%)[a]	97.5 (91.3)
No. of the reflections used for the refinement ($I > 2\sigma$)	17 539
R/R_{free}	0.196/0.240
No. of protein atoms	2292
No. of Zn^{2+} ions	1
No. of water molecules	171
Rmsd bond lengths (Å)	0.006
Rmsd bond angles (°)	1.2
Average B-factor (Å^2):	
Main chain	23.9
Side chain	28.4
Zinc ion	18.1
Water molecules	42.3
Ramachandran plot: No. of residues in:	
Most-favored region (%)[c]	92.3
Additional allowed region (%)[c]	7.7

[a]Statistics for highest resolution shell (1.97–1.90 Å) are given in parentheses.
[b]$R_{merge} = \Sigma_h\Sigma_i |I(h)_i - \langle I(h)\rangle| / \Sigma_h\Sigma_i I(h)_i$.
[c]Evaluated by PROCHECK [25].

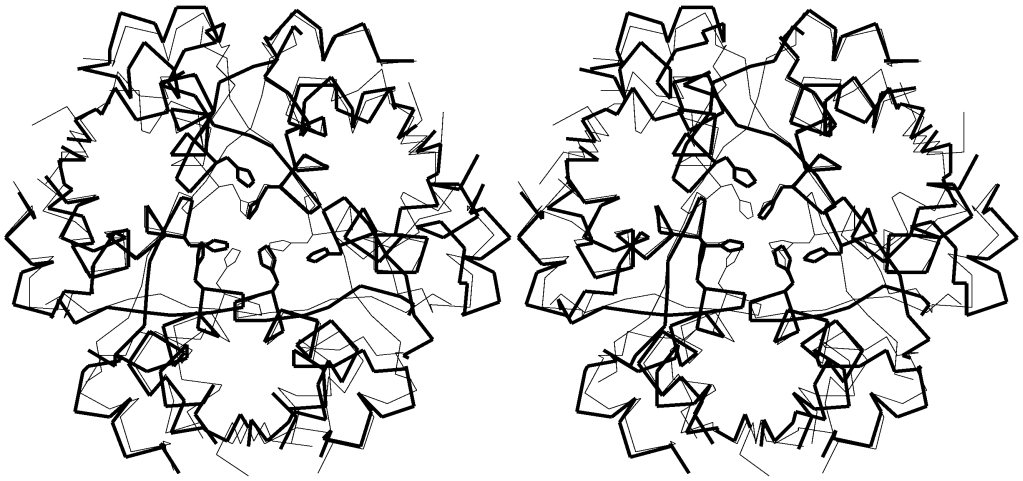

Fig. 1. Stereoview of a superposition of the DTRI hexamer (heavy lines) and the 2Zn insulin hexamer (light lines). Cα positions are shown together with B10 histidyl side chains. The DTRI hexamer is looser than the 2Zn insulin hexamer. All figures are drawn with TURBO-FRODO [22].

Å. The high *R*-factor indicated the solution might not be reliable. So we used a trimer consisting of three molecules I from the 2Zn insulin hexamer as the first search model. The highest peak gave a C_c value of 24.6% and an *R*-factor of 51.4%. Then a monomer was used as the second search model. The orientation and position of the fourth, fifth and sixth molecules were obtained using the *n*-body translation function of AMoRe. From these six molecules a new hexamer was rebuilt as a search model. With the same resolution (12–4 Å) and integration radius (22 Å), the highest peak gave a C_c value of 49.6% and an *R*-factor of 44.7%. The results indicated the correctness of this solution and the rebuilt hexamer. It is obvious that DTRI molecules do not associate as 2Zn insulin in crystals.

X-PLOR [21] was used for structural refinement and TURBO-FRODO [22] and O [23] for model rebuilding. During the refinement, a randomly selected 10% of the data was used to calculate R_{free} [24] and was excluded from the refinement. No non-crystallographic symmetry restraints or constraints were used. R/R_{free} dropped from 0.486/0.514 to 0.458/0.472 after the rigid-body refinement in the resolution range 15–2.5 Å. The model was rebuilt according to the $(2F_o-F_c)$ and (F_o-F_c) electron density map. Some residues with poor density were replaced by Ala. Several cycles of positional refinement reduced *R*/

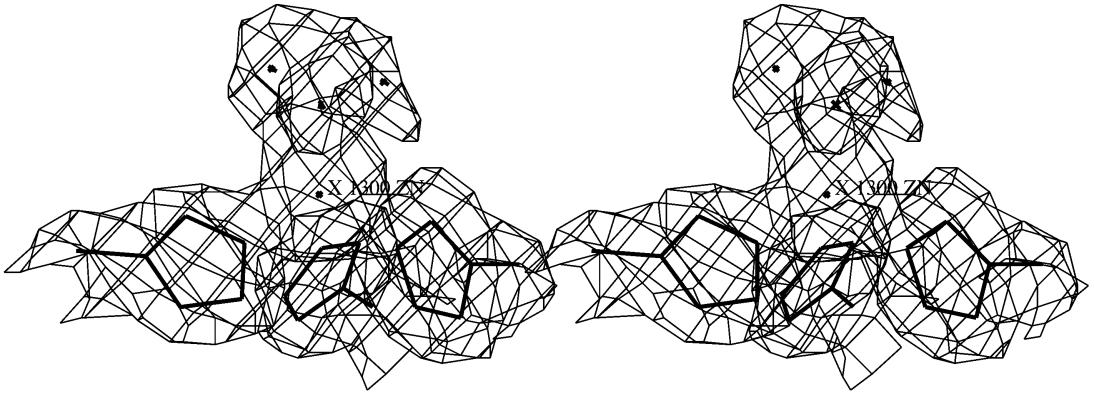

Fig. 2. Coordination of the only zinc ion in DTRI hexamer. The final 1.9 Å electron density map $(2F_o-F_c)$ is superimposed on the structure and contoured at 1σ.

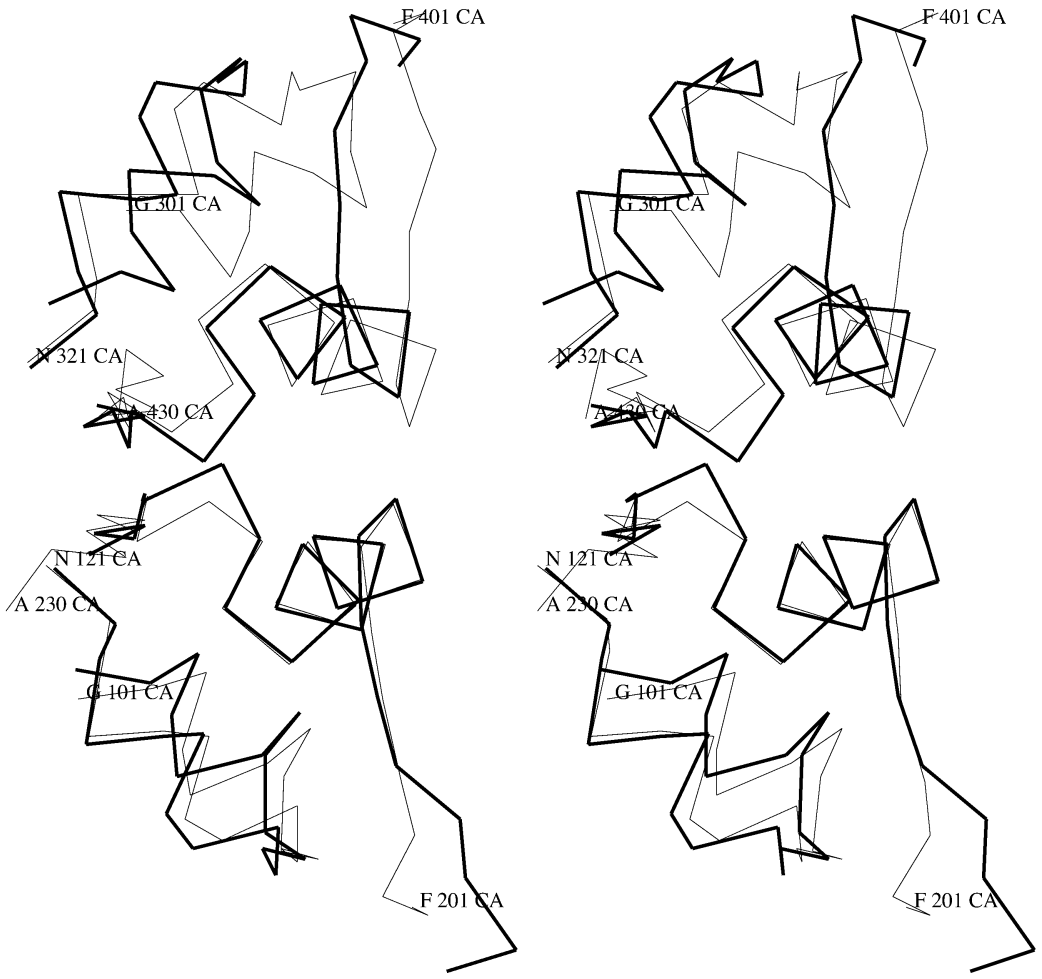

Fig. 3. Stereoview of a superposition of the DTRI dimer (heavy lines) and the 2Zn insulin dimer (light lines). Only Cα positions are shown.

R_{free} to 0.340/0.398. The replaced residues were rebuilt according to the $(2F_o-F_c)$ and (F_o-F_c) map calculated with 10–1.9 Å data. After several cycles of positional refinement, R/R_{free} dropped to 0.295/0.353. Individual *B*-factor refinement yielded an *R*-factor of 0.262 and an R_{free} of 0.328. Finally, one zinc ion and 171 water molecules were added and R/R_{free} decreased to 0.196/0.240. The refinement details were shown in Table 1.

3. Results and discussion

3.1. DTRI structure

DTRI exists as hexamers in crystals, but its association behavior is different from previously observed 2Zn or 4Zn insulin hexamers. In the DTRI hexamer, three molecules (molecule I as in 2Zn insulin) which coordinate to the only zinc ion roughly keep their positions as in 2Zn insulin. The other three molecules (molecule II as in 2Zn insulin) rotate 10.1° away from the pseudo-three-fold axis and lose the possibility of coordinating to a second zinc ion. Thus a 'distorted' hexamer forms. One end of the central cylinder channel consisting of three dimers as in 2Zn insulin opens to look like a bugle (Fig. 1). The three dimers in the DTRI hexamer still obey pseudo-three-fold symmetry, but not strictly.

In previously obtained crystal structures of zinc containing insulin or its analogues, a hexamer contained two or more zinc ions. However, only one zinc

ion was found in the orthorhombic DTRI hexamer. This is the first time a single zinc ion hexamer has been observed in zinc containing crystal structures of insulin and its analogues. In the DTRI hexamer, the zinc ion is coordinated to three nitrogens of B10 His residues of three DTRI molecules and three water molecules (Fig. 2). B10 His residues of the other three molecules are distant from each other and cannot coordinate to a zinc ion because of the 'distortion' of the hexamer. Furthermore, the coordination of the only zinc ion is also 'distorted' and not as normal as those on the crystallographic axis in 2Zn insulin.

The DTRI dimer in orthorhombic form is very different from the 2Zn insulin dimer consisting of molecule I and molecule II. Based on the superposition of the backbone of the α-helix B9–B19 of DTRI molecule I and the corresponding backbone of 2Zn insulin molecule I (Fig. 3), the orientations of the two molecules II differ by 10.1°. This suggests that the DTRI dimer is also 'distorted'. In this form of the DTRI dimer, the B-chain α-helices (B9–B19) of the two molecules move slightly away from each other and the conformation of the B20–B23 β-turn changes so that it comes closer to the C-terminal B-chain of the other molecule.

3.2. DTRI association behavior

DTRI was crystallized in a near-neutral solution containing zinc. It is very similar to the crystallization conditions of 2Zn insulin. However, their association behaviors are very different. In the 2Zn insulin hexamer, each molecule I and a molecule II form a dimer. Three molecules I and three molecules II coordinate to two zinc ions. This is a stable association form of the hexamer. But in the DTRI hexamer, only three molecules (molecules I) coordinate to one zinc ion. The other three molecules (molecules II) cannot coordinate to a second zinc ion and are reluctantly kept in the hexamer only through the interactions with molecules I. This loose hexamer form is obviously not as stable as the 2Zn insulin hexamer.

The buried solvent-accessible surface during the formation of the hexamer from monomers can be divided into two kinds of surfaces: the surface between the two monomers within a dimer and the surface between dimers. The areas of these two kinds of surfaces in the DTRI hexamer are much less than those in the 2Zn insulin hexamer (Table 2). In the DTRI dimers, the loss of B28–B30 and the 'distortion' of the dimers causes the number of van der Waals interactions (<4 Å) between two monomers to decrease from 81 in the 2Zn insulin dimer to an average of 59 in the DTRI dimers. The number is reduced by 27% which leads to the stability decrease of the DTRI dimers.

Previous research has shown that B28 Pro plays a critical role in dimer formation and stabilization [12]. However, DTRI without B28 Pro can remain as a 'distorted' dimer which has not dissociated, because the B20–B23 β-turn moved closer to the C-terminal B-chain of the other monomer. Moreover, in all three dimers, new hydrogen bonds are formed between the side chain of B21 Glu of one monomer and that of B26 Tyr of the other monomer (Fig. 4). To some extent these compensate for the loss of association ability with the removal of B28–B30. This reflects the complexity of the association behavior of insulin.

Although residues B28–B30 are located in the monomer–monomer interface in a dimer, the removal of them can weaken the dimer–dimer interactions in the DTRI hexamer. The effect is embodied in the decrease of the buried solvent-accessible surface, especially the hydrophobic area, between dimers (Table 2). In addition, the hydrogen bond between the OH of A14 Tyr of one dimer and that of the A14 Tyr of another dimer in 2Zn insulin hexamer does not exist

Table 2
Buried solvent-accessible surface areas in 2Zn insulin and DTRI hexamers

	2Zn insulin	DTRI
Between monomers within dimers (Å^2):	3840	2940
Hydrophilic (Å^2)	1310	880
Hydrophobic (Å^2)	2530	2060
Between dimers (Å^2):	4200	2990
Hydrophilic (Å^2)	1250	860
Hydrophobic (Å^2)	2950	2130
Total (Å^2)	8040	5930

Surface areas are calculated per atom by X-PLOR as accessible surface areas with a detecting radius of 1.4 Å. The surface areas provided by N or O atoms are taken as hydrophilic and that provided by other atoms as hydrophobic. For DTRI the numbers given are averages over the various interfaces.

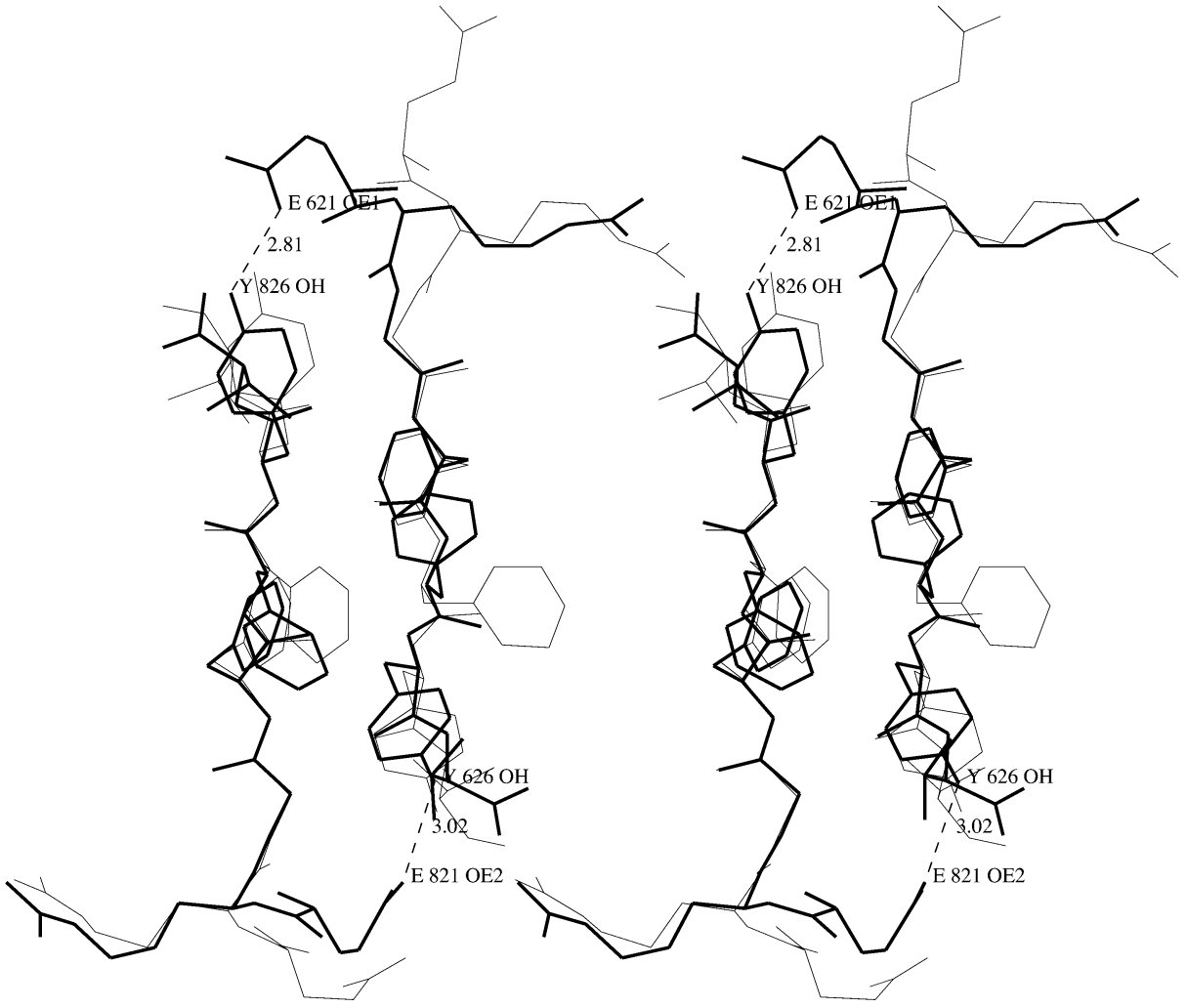

Fig. 4. New hydrogen bonds between B21 Glu and B26 Tyr from the other monomer within a dimer. Heavy and light lines represent DTRI and 2Zn insulin, respectively.

in the DTRI hexamer. The observations suggest that the B-chain C-terminal residues located in the monomer–monomer interface in a dimer are not only important for dimer formation but also influence the aggregation of the hexamer from dimers through their conformations and conformational changes.

Among the B28 Pro substituted insulin mutants, the crystal structures of $Lys^{B28}Pro^{B29}$ insulin [12] and B28 Pro→Asp insulin [13] have been determined. They were crystallized in the presence of phenolic derivatives. Until now there have been no reports on their crystallization with conditions similar to those for 2Zn insulin crystal growth. It is known that phenolic derivatives can substantially increase the stability of the insulin hexamer [14–16] because the induced R conformation results in better shielding of the zinc ions from bulk solvent and they introduce new interactions between dimers in the hexamer. However, DTRI can be crystallized as a hexamer in the absence of phenolic derivatives, indicating that the association ability of DTRI is a little stronger than that of $Lys^{B28}Pro^{B29}$ insulin or B28 Pro→Asp insulin. The reason may be that the replacement of B28 Pro in $Lys^{B28}Pro^{B29}$ insulin and B28 Pro→Asp insulin causes the interactions between B28 Pro and residues B20–B23 of the other monomer in the dimer to disappear. In addition, the new residue at B28 has a long side chain so that the steric hindrance probably prevents B20–B23 from moving as in DTRI and B21 Glu cannot

form a hydrogen bond with B26 Tyr of the other monomer in the dimer. Thus, the association ability of $Lys^{B28}Pro^{B29}$ insulin and B28 Pro → Asp insulin is weaker than DTRI.

3.3. Possible dissociation process of insulin hexamer

The C-terminal B-chain of insulin is a very flexible region in solution [7]. External random disturbances may weaken or even prevent the hydrophobic interactions between B28 Pro and the other molecule within the dimer so that the molecule can reach a conformation similar to DTRI. We hypothesize that the dissociation process of native insulin should be very similar to that of DTRI after it reaches an association state similar to that of DTRI. Therefore, the orthorhombic DTRI hexamer may be a transition state in the native insulin dissociation process.

We propose that the possible dissociation process of the zinc containing native insulin hexamer can be described as follows: in solution, the flexible B-chain C-terminus leaves its original position due to random disturbance and B28 Pro loses its hydrophobic interactions with B20–B23 of the other molecule within the dimer. Similar to DTRI, the interactions between the two monomers within the dimer undergo a series of changes and are gradually weakened, which changes the relative positions of the two monomers. The hexamer is 'distorted' as the dimers are 'distorted'. Three molecules (molecules I) of the hexamer almost maintain their original positions, but the other three (molecules II) rotate over 10°. This weakens the interactions between the dimers in the hexamer causing a coordinated zinc ion to be lost. The aggregation of the three molecules (molecules II) which are no longer coordinated to a zinc ion becomes loose and one end of the cylinder channel in the center of the three dimers opens. Non-equivalence of the two trimers in the insulin hexamer is common [26], although not as obvious as in DTRI. The movements of the solvent molecules in the cylinder become more active and the hexamer becomes unstable. Then the hydrophobic core within the dimer is further destroyed and the interactions between the two monomers are further weakened. Under the assault of the solvent molecules, the hexamer loses the remaining zinc ion and dissociates to more 'distorted' dimers. The interactions between the monomers within the 'distorted' dimer are very weak, probably not enough to keep the dimer stable. The dimer surrounded by solvent molecules will soon dissociate to monomers to perform their physiological function.

The crystal structure of the orthorhombic form of DTRI suggests that the insulin hexamer does not dissociate to dimers as in 2Zn insulin, then to monomers. In contrast, as the dissociation from hexamer to dimers occurs, the interactions within the dimer have already taken place a series of changes which weaken the dimer. Therefore, the dissociation from hexamer to dimers and the dissociation from dimer to monomers take place simultaneously. The 'dimer' dissociated from the hexamer, if it can be called a dimer, is already different from the dimer seen in the 2Zn insulin structure.

The association and dissociation of insulin is very complex. From the accurate structure of orthorhombic DTRI, we concluded that to change the association behavior of insulin, not only the interactions between molecules at a static state should be considered, but more importantly, the dynamic interactions during dissociation should be considered. Thus, we can further understand the association property of insulin to design more effective insulin agents which fit people's needs.

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