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Conformational change of adenosine deaminase during ligand-exchange in a crystal

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ABSTRACT

Adenosine deaminase (ADA) perpetuates chronic inflammation by degrading extracellular adenosine which is toxic for lymphocytes. ADA has two distinct conformations: open form and closed form. From the crystal structures with various ligands, the non-nucleoside type inhibitors bind to the active site occupying the critical water-binding-position and sustain the open form of apo-ADA. In contrast, substrate mimics do not occupy the critical position, and induce the large conformational change to the closed form. However, it is difficult to predict the binding of (+)-erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), as it possesses characteristic parts of both the substrate and the non-nucleoside inhibitors. The crystal structure shows that EHNA binds to the open form through a novel recognition of the adenine base accompanying conformational change from the closed form of the PR–ADA complex in crystalline state.

Adenosine deaminase (ADA) (EC 3.5.4.4) plays a significant role in purine metabolism and catalyzes the irreversible deamination of both adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. ADA is ubiquitous in all human tissues, and genetic ADA deficiency results in severe combined immunodeficiency disease by impairment of the differentiation and maturation of lymphoid cells [1]. ADA has been also found on the cell surface via binding to CD26, as well as in the cytosol [2]. Since CD26 is strongly up-regulated following T-cell activation, ADA can perpetuate chronic inflammation by degrading extracellular adenosine or 2′-deoxyadenosine which are toxic for lymphocytes [3]. Therefore, ADA inhibitors may have potential as anti-inflammatory drugs that work selectively at sites of inflammation.

A number of first generation ADA inhibitors that mimic the substrate have been reported, including purine riboside (PR), with a low-affinity of μM order, and (+)-erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) [4] and pentostatin [5], with higher affinities of nM order (Fig. 1). The structure–activity relationship of the EHNA derivatives have been intensively investigated [6–9], although the crystal structure of the EHNA–ADA complex has not yet been reported. More recently, second generation non-nucleoside type ADA inhibitors such as FR221647 (Fig. 1) and its derivatives, including the imidazole-4-carboxamide moiety, have been synthesized through structure-based drug design utilizing the X-ray crystal structures of the inhibitor-ADA complexes [10–14]. Some of the derivatives exhibited anti-inflammatory activity in an inflammatory model in mice [15]. Minute observation and understanding at atomic resolution regarding the interactions between ADA and diverse inhibitors would help to produce more effective ADA inhibitors.

During structure-based drug design cycles, it has become apparent that ADA has two distinct conformations of the active site: an open form and a closed form (Fig. 2). Several crystal structures of apo-ADA and ligated-ADA with various inhibitors led us to hypothesize that removal of a specific water molecule, referred to as a trigger water, binding at the bottom of the active site might be a trigger of conformational change from the open form to the closed from [16]. While the apo-ADA has the open form and the trigger water molecule exists at the end of the active site, substrate adenosine or substrate mimic compounds binding to the active site interfere with the water molecule. Consequently, the water molecule moves away and the side chain of Phe65 residing at the α-helix consisting of the active site lid moves slightly into the active site pocket to occupy the resulting space. As this movement is accompanied with a kink of the helix at Phe65, the substrate-binding ADA molecule has changed to the closed form and the interaction between substrate and ADA is increased. On the other hand, the bound imidazol-4-carboxamide derivatives, a type of non-nucleoside type inhibitor, occupy the critical position completely via

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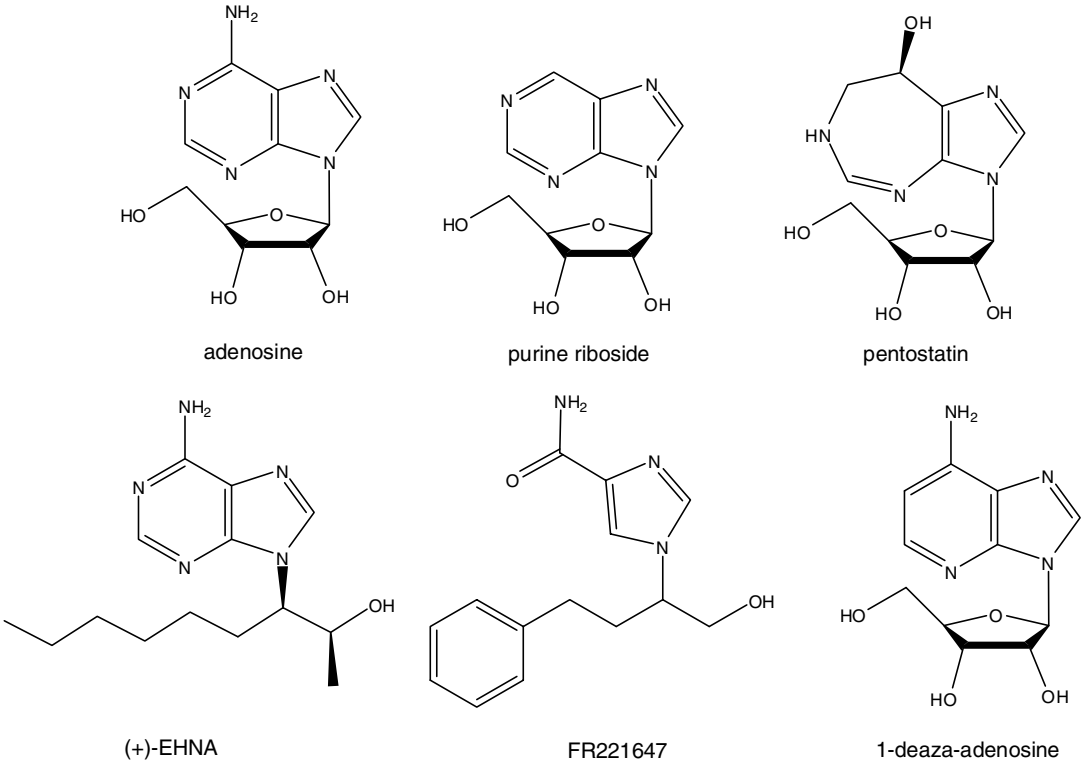

Fig. 1. Chemical structures of substrate adenosine and ADA-inhibitors.

the C2 atom of the imidazole framework instead of the water molecule, and they do not cause any significant conformational change of the protein. Similarly, the other non-nucleoside inhibitors would also retain the open form of ADA through occupation of the critical position.

However, it is unclear whether binding of the nucleoside framework or elimination of the trigger water molecule is predominant for the conformational change of ADA. In order to elucidate the binding mode of EHNA and confirm our hypothesis, we analyzed the crystal structure of the EHNA–ADA complex. The complex crystals suitable for X-ray analysis were ultimately prepared by a ligand-exchange operation, modified from the methods reported by Collins and Geremia [17,18], utilizing a significant affinity difference between PR and EHNA. To the best of our knowledge, this is the first report that an inhibitor molecule with a nucleoside framework binds to the open form of ADA and the first observation of conformational change of ADA in the crystalline state.

Materials and methods

Crystal preparation of the EHNA–ADA complex. ADA from bovine intestine suspended with 3.2 M ammonium sulphate was purchased from Roche Diagnostics Inc. After centrifuging, the pellet containing ADA was resolved in 25 mM Hepes buffer (pH 7.5). The concentration of ADA was adjusted to 20 mg/ml using the centrifuge, and the PR powder (a low-affinity ADA inhibitor; Sigma), was added to the protein solution up to 1 mM. The crystals of the PR–ADA complex were prepared using the sitting drop vapor diffusion method against solutions containing 2.0–2.2 M ammonium sulphate, 2–3% (v/v) 2-methyl-2, 4-pentanediol, and 0.1 M MES buffer (pH 6.0). The crystallization procedure has been previously described in detail [19]. The light brown colored crystals grew to maximum dimensions of approximately $0.3 \times 0.2 \times 0.2$ mm within two weeks. A small amount of the EHNA powder (Calbiochem) was directly added into the crystallization drop and was replaced with PR in crystal/solution phases. During the ligand-exchange operation of two days, the crystals gradually turned light yellow, the color derived from EHNA.

Data collection and structure analysis. A light yellow crystal treated with the (+)-EHNA powder was dipped into Paratone-N oil (Hampton Research Inc.) as a cryoprotectant, and was used for X-ray data collection. An intensity data set was collected at 100 K on a Rigaku R-AXIS IV^{++} imaging-plate system using a monochromatized Cu Kα radiation generated with MicroMax-007HF (Rigaku). The raw data was integrated and scaled with the Crystal Clear program (Rigaku). The structure of the complex was solved by the molecular replacement method using the MolRep program [20], and protein coordinates in the PR–ADA structure [21] as a search probe. Model building and structure refinement were performed with the DS Modeling and CNX programs, respectively (Accelrys). Data collection and refinement statistics of the EHNA–ADA complex are given in Table 1. Two amino acids from the N-terminal and five from the C-terminal were omitted from the final model because of ambiguous or discontinued electron density for the corresponding regions. Final coordinates and structure factors were deposited into the Protein Data Bank (entry code 2Z7G). Superposition studies of the EHNA–ADA complex against the other ADA complexes were performed with DS Modeling (Accelrys).

Results and discussion

Ligand-exchange operation

Co-crystallization trials of the EHNA–ADA complex were unsuccessful, although the ADA complexes with all the other inhibitors, even with the low-affinity PR, were co-crystallized easily. The reason for this is unknown, but the EHNA reagent as an HCl salt might adversely affect the protein stability in solution. From the crystal structures of apo-ADA, the active site of ADA is accessible using a pathway through the crystalline space, and its structural feature

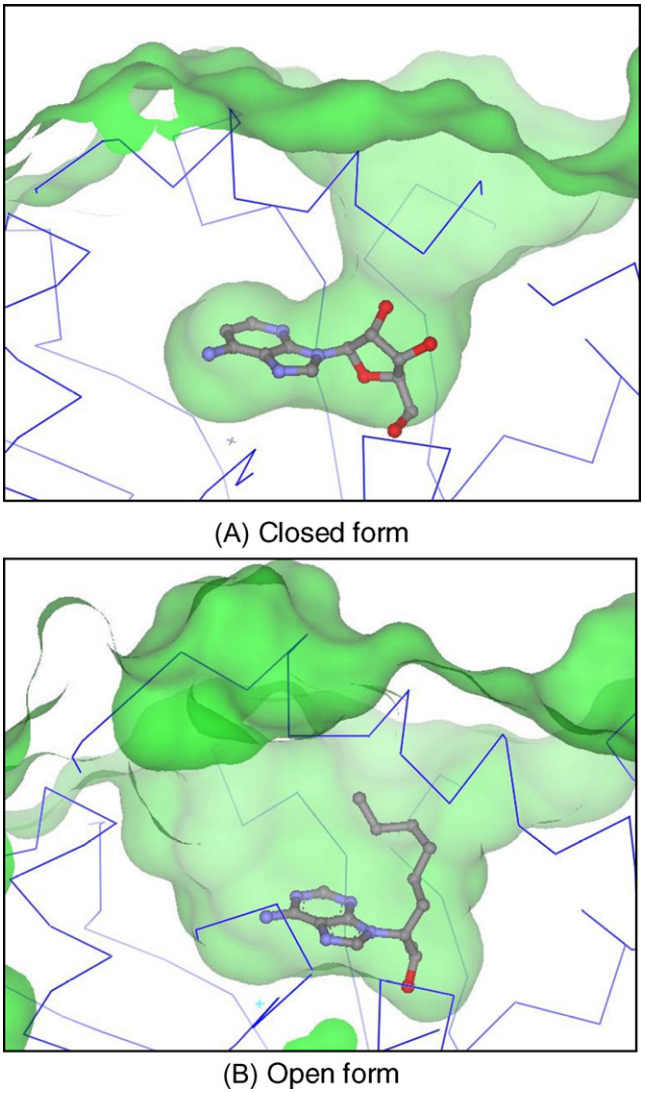

Fig. 2. ADA active sites of the closed and open forms. Accessible surface of the water molecules at the active sites are shown in green. The upper portion of each figure is the active site entrance (solvent region). The white regions with the blue Cα stick model are occupied by protein. (A) A closed form view based upon the 1-deazaadenosine–ADA complex. (B) An open form view based upon the EHNA–ADA complex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is considered favorable for a soaking operation. However, this method for preparation of the apo-ADA crystals requires special equipment usually unavailable. Thus, in the present study a ligand-exchange operation (LEO) was utilized instead of a soaking operation by the combination of a low-affinity-inhibitor PR diffusing and a high-affinity-inhibitor EHNA binding.

The ligand binding process can be regarded as a replacement of a group of water molecules in the ligand binding site with a ligand, as such a water cluster is also a kind of low-affinity ligand and can be replaced by a higher-affinity ligand during normal soaking operation (NSO). The diffusion speed of pre-bound ligands in LEO are rather lower than that of water cluster in NSO, varying from a few seconds to days, with often no rational basis for the exchange times [18]. In some cases, completeness of exchange can be monitored by additional experiments such as X-ray fluorescence, radioactivity counts of labeled inhibitors, and 3D laser scanning confocal microscopy [22–24]. Fortunately, in the present study, ligand-exchange could be confirmed by color-change of crystals from light brown (PR–ADA) to light yellow (EHNA–ADA) in the PR–EHNA–ADA system. Practically, the electron density map corresponding to the EHNA molecule was interpretable, while no residual density due to PR was observed. The LEO procedure in this system was completely successful without crystalline damage. This is an intriguing observation as ADA changed the active site shape largely during the ligand-exchange process. This point is discussed in detail below.

Table 1
Data collection and refinement statistics

Data collection	
Space group	$P4_32_12$
Unit cell (Å)	
a	77.10
c	135.66
Resolution (Å)	67.03–2.52 (2.75–2.52)
Observations	87461
Unique reflections	13675
Completeness (%)	94.7 (94.5)
R_{merge} (%)[a]	6.2 (16.7)
I/σ	13.4 (3.7)
Refinement statistics	
Resolution (Å)	67.03–2.52 (2.57–2.52)
Reflections	12956
Total atoms	2929
R-factor (%)	20.7 (25.1)
R_{free} (%)	24.7 (33.1)
Rms deviations	
Bond length (Å)	0.008
Bond angle (°)	1.6

Values in parentheses are for the highest resolution shell.
[a] $R_{merge} = \Sigma_h\Sigma_j|I_{hj} - \langle I_h\rangle|/\Sigma_h\Sigma_j|I_{hj}|$, where h represents a unique reflection and j represents symmetry-equivalent indices. I is the observed intensity and $\langle I\rangle$ is the mean value of I.

EHNA induces conformational change of ADA in the crystalline state

The crystal structure of the EHNA–ADA complex implies that the inhibitor prefers to bind to the open form, although the closed form is usually observed in the complexes with substrate analogues possessing the adenine framework [25–27]. EHNA could be expected to be bound in the closed conformation as EHNA has an adenine framework, the substructure of the substrate. In fact, the ADA protein co-crystallized with PR had the closed form before EHNA treatment [21]. Thus, during LEO the EHNA molecule induced the large conformational change to the open form in the crystalline state. We suggest that the following events have successively taken place during the LEO event. First, the active site of the ADA–PR complex opens, while simultaneously, the interaction between PR and ADA is weakened with the protein conformational change. Thus, the bound PR becomes easy to move and is released from the active site. Finally, EHNA with a higher-affinity binds to the active site through the open lid.

Judging from the PR–ADA complex crystal structure, at the beginning of this LEO event ADA must open the lid of the active site in order to dislodge the PR molecule as there is no pathway for PR to leave from the active site without changing the active site shape (Fig. 2A). Fortunately, the first motion involving opening the lid with a conformation change of the α-helix consisting of the lid is likely free from steric crush with the neighboring molecules in the crystal. The trigger of the active site opening motion is not clear from the static bound structures of both inhibitors. However, data suggest that the *n*-hexyl group of EHNA interacts with the narrow hydrophobic entrance of the active site of the PR–ADA complex, and it destabilizes the closed form. After PR diffusion, EHNA binds to the active site occupying the critical position by the C8 atom of the purine framework in the EHNA–ADA complex. A significant relationship between the occupancy at the trigger water-binding-

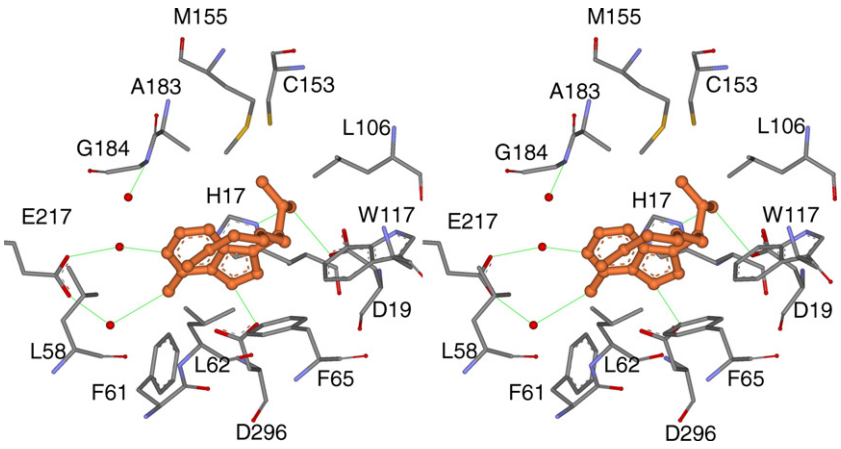

Fig. 3. A stereo view in the active site of the EHNA–ADA complex. Amino acid residues which interact with the inhibitor are shown by the stick model. Hydrogen-bonds are shown by green lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

position and the open conformation in the EHNA–ADA complex agrees with our previously reported hypothesis [16]. All together, access of EHNA to the active site induces a conformational change of ADA without any inter-atomic crash among the neighboring ADA molecules in the crystal. This also makes it possible to exchange the low-affinity inhibitor with the high-affinity inhibitor.

Structural dissection of the EHNA–ADA complex

EHNA tightly binds to the active site of ADA by several hydrogen bonds and van der Waals interactions (Fig. 3). The hydroxyl group of the inhibitor forms two hydrogen bonds with His17 and Asp19, whose side chains strongly recognize the 5′-hydroxyl group of the substrate adenosine or the hydroxyl group of the non-nucleoside inhibitors. The N9 atom of the purine framework makes a hydrogen bond with Asp296, which is probably protonated in the active site because the N9 atom is a proton acceptor. The 6-amino group and the N1 atom of the purine framework interact with two water molecules, mediating interaction through hydrogen bonds with the carboxyl group of Glu217. The N3 atom makes no hydrogen bond, although the nitrogen atom is a proton acceptor. This observation on the N3 atom agrees with the fact that substitution to the carbon atom keeping conjugated system in the EHNA structure does not affect the inhibitory activity against ADA [7]. Two aliphatic portions, the methyl and the *n*-hexyl groups, interact with the hydrophobic spaces by van der Waals interactions. The methyl group of EHNA, one of the structural features of high-affinity non-nucleoside inhibitors, is accommodated by a small hydrophobic pocket consisting of Cys153, Leu155, and Ala183. This small subsite contributes highly to increasing the affinity in the case of the imidazole-4-carboxamine derivatives [12]. The affinities of the EHNA isomers are also affected by the fit of the methyl group to this pocket [6]. The *n*-hexyl moiety of the inhibitor is also surrounded by hydrophobic amino acids consisting of Leu58, Phe61, Leu62, Phe65, Leu106, Trp117, Met155, and Gly184. The hydrophobic space referred to as the F1 subsite [11] is specific to the open form, and prefers the hydrophobic ligands [10–14]. Presumably, the F1 subsite forms favorable interactions with the additional aromatic portions of the several derivatives produced by modification of the *n*-hexyl group of EHNA, indicating higher affinities than EHNA [9]. In the EHNA–ADA complex, the side chain of Leu62 is slightly moved forward to the solvent region compared to the known open form such as apo-ADA, so that the subsite is widened to be able to accommodate the *n*-hexyl group.

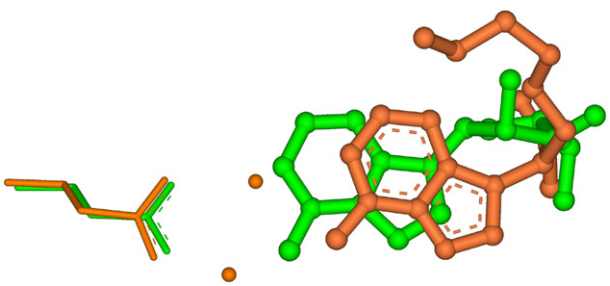

Fig. 4. Superimposed structures near the adenine moiety binding region in the EHNA- (orange) and 1-deazaadenosine- (green) ADA complexes. The side chains of Glu217, water molecules, and inhibitors are extracted from the superimposed complexes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Structural comparison between the EHNA–ADA complex and the 1-deaza-adenosine–ADA complex

The adenine component in the EHNA–ADA complex binds to the active site in a manner distinguishable from that of the complex with substrate mimic such as 1-deaza-adenosine (DAA) (Fig. 4). The adenine component of EHNA binds to Glu217 via two water molecules, and positions to the right side compared with that of DAA. Consequently, the C8 atom of the purine framework of EHNA just occupies the trigger water-binding-position. On the other hand, the 1-deaza-adenine component of DAA, or possibly the adenine of the substrate, directly interacts with Glu217, and positions the relatively to left side (Fig. 4). Thus, the trigger position becomes empty. Subsequently, the side chain of Phe65 slightly moves toward the space, and this motion causes conformation change of ADA from the open to the closed forms. In many crystal structures of the ADA complex with various inhibitors, hydrogen bonding interactions using the hydroxyl group in the inhibitor molecules are conserved. This implies that the hydroxyl group forming hydrogen bonds with His17 and Asp19 might be an anchor for ligand binding. The spacer bonds between this hydroxyl group and the adenine moiety are different between EHNA and DAA or PR. The number of spacer bonds is four for DAA and PR passing through the ribose moiety, which allows the adenine moiety to reach to the end of the active site, forming direct hydrogen bonds with Glu217. Therefore, in the case of the substrate, the amino leaving group on the adenine moiety is substituted by the water molecule ligated to and activated by the zinc atom. On the other hand, EHNA having only two spacer bonds, does not reach to Glu217, and interacts with Glu217 through two water molecules conserved in the

apo-structure (Fig. 3). One water molecule is located at the amino group binding-position of substrate. Consequently, the amino group of EHNA is far from the zinc-activated water molecule. Because the carboxylic acid moiety of Asp296 is allowed to be flexible in the active site, the purine frameworks from both EHNA and DAA (or the substrate) can make hydrogen bonds with these side chains in either appropriate orientation, and are finally stabilized in the respective manner. While many structural data have shown that ligands with the substrate-like framework induced the closed form, ADA does not recognize EHNA with the adenosine moiety as substrate-like. Most likely, interaction of the hydroxyl group rather than the nucleoside base with ADA is critical for determining the ligand binding mode. The hydroxyl group binding determines the positions of the nucleoside base of the individual ligands, thereby following retention or elimination of the trigger water molecule. Consequently, the conformational change of the active site would take place if the water molecule was removed. A four-atom spacer of the substrate is appropriate for direct binding of adenine to Glu217. On the other hand, a two-atom spacer is too short for the direct interaction with Glu217, and is therefore appropriate for indirect binding to Glu217. The imidazole-4-carboxamide derivatives, for instance, have a two-atom spacer and indirect-binding to Glu217. Terminally, the C2 atom of the imidazole moiety of the non-nucleoside derivatives occupies the trigger water position and their complexes have the open conformation.

In conclusion, the open form crystal structure of the EHNA–ADA complex supports our hypothesis that the occupancy at the trigger-water-position is critical for determining the open/closed conformational alternation, rather than the nucleoside framework binding. We believe that the structural penetration of the EHNA–ADA complex and structural comparison of the other inhibitor-ADA complexes will support the discovery of novel ADA inhibitors by structure-based drug design.

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