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Deduced catalytic mechanism of D-amino acid amidase from *Ochrobactrum anthropi* SV3

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D-Amino acid amidase (DAA) from *Ochrobactrum anthropi* SV3 catalyzes D-stereospecific hydrolysis of amino acid amides. DAA has attracted attention as a catalyst for the stereospecific production of D-amino acids, although the mechanism that drives the reaction has not been clear. Previously, the structure of DAA was classified into two types, a substrate-bound state with an ordered Ω loop, and a ground state with a disordered Ω loop. Because the binding of the substrate facilitates ordering, this transition was regarded to be induced fit motion. The angles and distances of hydrogen bonds at Tyr149 Oη, Ser60 Oγ and Lys63 Nζ revealed that Tyr149 Oη donates an H atom to a water molecule in the substrate-bound state, and that Tyr149 Oη donates an H atom to Ser60 Oγ or Lys63 Nζ in the ground state. Taking into consideration the locations of the H atoms of Tyr149 Oη, Ser60 Oγ and Lys63 Nζ, a catalytic mechanism of DAA activity is presented, wherein a shift of an H atom at Tyr149 Oη in the substrate-bound *versus* the ground state plays a significant role in the reaction. This mechanism explains well why acylation proceeds and deacylation does not proceed in the substrate-bound state.

Keywords: catalytic mechanisms; stereospecific hydrolysis; D-amino acid amidase.

1. Introduction

D-Amino acids are important intermediates in the production of a number of chemicals, including pharmaceuticals, agrochemicals and food additives (Asano & Lübbehüsen, 2000).

Currently, D-amino acids and D-amino acids containing peptides are produced by enzymatic transformation (Schulze & Wubbolts, 1999). Enzymatic transformation requires enzymes with high D-stereospecificity in order to approach the maximum theoretical yield (Schulze & Wubbolts, 1999). D-Amino acid amidase (DAA) from the soil bacterium *Ochrobactrum anthropi* SV3 is an enzyme with this type of high D-stereospecificity, and catalyzes D-stereospecific hydrolysis of amino acid amides *via* bulky hydrophobic side chains to yield D-amino acid and ammonia (Asano *et al.*, 1989; Komeda & Asano, 2000).

The crystal structures of DAA in the native form and in a complex with D-phenylalanine were determined and functionally characterized in our laboratory (Okazaki *et al.*, 2007). Several simulations suggested that Tyr149 Oη is an adequate candidate for a general acid in the acylation step (Okazaki *et al.*, 2008). However, an overview of the catalytic mechanism of DAA functions, which include both acylation and deacylation, has remained unclear.

Previously we reported that substrate binding occurred in concert with a conformation change in the Ω loop (residues 207–223; Okazaki *et al.*, 2007), and we regarded this motion as induced fit motion (Okazaki *et al.*, 2008). New concepts about pre-existing apo states, such as a conformational selection (Bosshard, 2001) or selected fit mechanism (Wang *et al.*, 2004), have been proposed. However, the concept of induced fit, *i.e.* that the location of active site residues around a substrate stabilize a transition state and thereby promote the reaction (Koshland Jr, 1958), remains very important.

In considering induced fit motion and geometries of hydrogen bonds at important residues in this work, we are able to speculate on the overall catalytic mechanism of DAA. The reaction mechanism that we propose provides a reasonable explanation of previously observed experimental results, including the fact that deacylation occurs at pH 6.8.

2. Speculated H-atom shift of Tyr149 Oη by induced fit motion

In the D-phenylalanine complex, subunits *A*–*E* belong to the substrate-bound state, and subunit *F* belongs to the ground state (Okazaki *et al.*, 2007). The distances of the shift between the substrate-bound state and the ground state in active site residues Ser60 Oγ, Lys63 Nζ and Tyr149 Oη were 0.2, 0.6 and 1.2 Å, respectively (Fig. 1). The r.m.s. deviation in atomic positions estimated *via* a Luzzati plot of the D-phenylalanine complex structures was 0.29 Å. This value suggests that the shifts of Lys63 Nζ and Tyr149 Oη were significant. The extent of the shift of Tyr149 Oη is the largest of the three; thus, it is plausible to consider that Tyr149 is important for induced fit motion. Tyr149 has been proposed to be a candidate for a general acid in acylation and a general base in deacylation (Okazaki

Table 1
Angles (°) and distances (Å) in the native enzyme and D-phenylalanine complex.

Values in parentheses indicate distances corresponding to angles. – indicates an unidentified water molecule.

		Tyr149			Ser60		Lys63			
Model	Subunit	149 Cζ—149 Oη—O_2H	149 Cζ—149 Oη—60 Oγ	149 Cζ—149 Oη—63 Nζ	60 Cβ—60 Oγ—63 Nζ	60 Cβ—60 Oγ—149 Oη	63 Cε—63 Nζ—149 Oη	63 Cε—63 Nζ—60 Oγ	63 Cε—63 Nζ—151 Oδ1	63 Cε—63 Nζ—242 O
Native	*A*	100.4 (2.96)	139.0 (2.67)	108.6 (3.14)	119.5 (2.68)	87.2 (2.67)	88.3 (3.14)	137.7 (2.68)	104.2 (2.67)	120.5 (2.72)
	B	97.6 (2.90)	138.4 (2.77)	111.2 (2.96)	111.4 (2.63)	83.2 (2.77)	87.3 (2.96)	142.0 (2.63)	107.4 (2.79)	121.5 (2.86)
	C	94.6 (3.13)	133.9 (2.82)	110.9 (3.22)	116.5 (2.70)	85.0 (2.82)	80.7 (3.22)	133.0 (2.70)	108.3 (2.72)	120.2 (2.67)
	D	90.4 (3.06)	137.8 (2.67)	112.0 (2.90)	120.6 (2.55)	89.2 (2.67)	91.4 (2.90)	143.1 (2.55)	106.4 (2.82)	109.6 (2.87)
	E	–	137.1 (2.86)	111.3 (2.86)	112.3 (2.62)	84.3 (2.86)	95.8 (2.86)	148.7 (2.62)	105.8 (2.75)	107.4 (2.87)
	F	–	124.4 (2.85)	105.8 (3.01)	109.9 (2.88)	76.1 (2.85)	84.8 (3.01)	141.3 (2.88)	101.1 (2.68)	114.9 (2.60)
D-Phe complex	*A*	91.1 (2.86)	140.0 (2.74)	111.8 (2.72)	105.3 (3.26)	87.3 (2.74)	96.8 (2.72)	141.0 (3.26)	116.9 (2.71)	121.5 (2.95)
	B	–	138.0 (2.70)	115.2 (2.68)	101.0 (2.80)	82.8 (2.70)	94.6 (2.68)	150.3 (2.80)	98.9 (2.84)	117.4 (2.77)
	C	83.0 (2.98)	137.1 (2.82)	109.9 (2.71)	105.4 (3.13)	83.4 (2.82)	98.9 (2.71)	146.1 (3.13)	116.6 (2.85)	115.3 (3.02)
	D	–	140.6 (2.85)	115.8 (2.73)	100.9 (3.16)	90.3 (2.85)	96.7 (2.73)	145.1 (3.16)	111.1 (2.83)	107.8 (2.88)
	E	–	131.9 (3.01)	105.2 (2.99)	100.7 (3.32)	78.7 (3.01)	91.5 (2.99)	140.9 (3.32)	118.6 (2.81)	117.8 (2.92)
	F	–	104.2 (3.09)	99.1 (3.25)	102.3 (3.13)	73.5 (3.09)	78.5 (3.25)	135.6 (3.13)	103.2 (2.88)	115.9 (2.50)

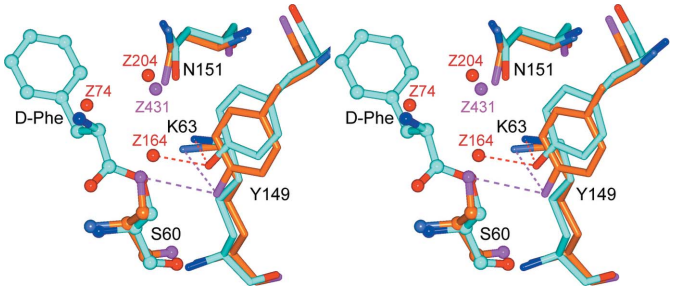

Figure 1
Induced fit motion of Tyr149 Oη in the ground (subunit *F*) *versus* the substrate-bound state (subunit *A*) D-phenylalanine complex. For subunit *A*, C atoms are shown in cyan, O atoms in red and N atoms in blue. For subunit *F*, C atoms are shown in orange, O atoms in magenta and N atoms in light blue. Possible hydrogen bonds around Tyr149 Oη based on the angles and distances for subunits *A* and *F* are shown as red or magenta broken lines, respectively. D-Phenylalanine and Ser60 are represented with a ball and stick model.

et al., 2008). The angles and distances of the hydrogen bonds related to Tyr149 Oη, Ser60 Oγ and Lys63 Nζ are summarized for all subunits in the native and D-phenylalanine complexes (Table 1). The data reveal that the Tyr149 Cζ—Tyr149 Oη—Ser60 Oγ angle (greater than 130°) is far from the regular tetrahedron angle of 109.5°, which is suitable for donating an H atom to a hydrogen bond. The data also reveal that the Tyr149 Cζ—Tyr149 Oη—H_2O (less than 100.4°) and Tyr149 Cζ—Tyr149 Oη—Lys63 Nζ angles (106–115°) are close to the regular tetrahedron angle. Taken together, these results suggested that the Tyr149 Oη H atom in the substrate-bound state is donated to a hydrogen bond with a water molecule (namely, Z164; see Fig. 1) or Lys63 Nζ. Additionally, the results of docking simulation using the MOE system (Version 2006.0801; Chemical Computing Group, Montreal, Canada) suggest that the leaving group NH_2 of the substrate D-phenylalanine amide locates to the same position as the water molecule (Z164 in Fig. 1) (Okazaki *et al.*, 2008). Together with the fact that acylation occurs in the substrate-bound state (Okazaki *et al.*, 2007), these findings lead us to propose that Tyr149 Oη donates an H atom to the water molecule rather than to Lys63 Nζ in the substrate-bound state.

However, the results summarized in Table 1 suggested that Tyr149 Oη forms hydrogen bonds to Ser60 Oγ or Lys63 Nζ in the ground state (subunit *F* in the D-phenylalanine complex). The Tyr149 Cζ—Tyr149 Oη—Ser60 Oγ (104.2°) and Tyr149 Cζ—Tyr149 Oη—Lys63 Nζ angles (99.1°) in subunit *F* in the D-phenylalanine complex seem to be suitable for hydrogen bonding. On the basis of these data alone, we cannot determine which residue interacts with the H atom of Tyr149 Oη in the ground state. Both locations of the H atom of Tyr149 Oη in the ground state are suitable for deacylation.

The hydrogen-bond geometry at Lys63 Nζ suggests that Lys63 Nζ may donate H atoms to Asn151 Oδ1 and Ala242 O (Table 1). Lys63 Nζ is thought to be protonated under the conditions of crystallization (pH 6.8) (Okazaki *et al.*, 2007). Thus, on the basis of the geometries, one additional H atom from Lys63 Nζ may be donated to Tyr149 Oη, rather than to Ser60 Oγ, in the substrate-bound state of the D-phenylalanine complex (Table 1). For the ground state of the D-phenylalanine complex, however, there are no suitable candidate residues for acceptance of an H atom from Lys63 Nζ (Table 1).

The angles and distances related to Ser60 Oγ suggest that Ser60 Oγ directs an H atom to Lys63 Nζ and not to Tyr149 Oη (Table 1). The effect is quite pronounced, particularly in the ground state.

3. Proposed catalytic mechanisms

Reflecting the observations described above, we are able to propose a mechanism for catalysis of DAA at the crystallization pH (6.8) (Fig. 2), wherein D-phenylalanine amide acts as a substrate.

In the unbound states, the proportion of molecules adopting the ground state is thought to be greater than the proportion adopting a substrate-bound state (Fig. 2*a*) (Okazaki *et al.*, 2007). In this state, and considering the geometries of the residues (Table 1), we propose that an H atom from Tyr149 Oη is donated to Ser60 Oγ (Fig. 2*a*). However, considering the angle and distances concerning Lys63 Nζ (Table 1), it seems that an H atom of Lys63 Nζ is free and located at an intermediate position between Tyr149 Oη and Ser60 Oγ (Fig. 2*a*).

There are at least two possible ways for the initial transition from ground state to occur in terms of the catalytic mechanism of DAA (Fig. 2*a*) *versus* the substrate-bound state (Fig. 2*b*). One is *via* the traditional induced fit mechanism; in this case, the substrate first binds at the ground state and the complex then switches to the substrate-bound state. The other is *via* the conformational selection mechanism (Eisenmesser *et al.*, 2005) or selected fit mechanism (Boehr *et al.*, 2006), in which the substrate binds to the minor substrate-bound state conformation already present in solution.

The presence of an incoming substrate in the binding pocket should shift the ratio of structures in favor of the substrate-bound state by ordering the Ω loop (Okazaki *et al.*, 2007) and bring about the relocation of Tyr149 Oη in the substrate-bound state as described

above (Fig. 2*b*). We consider that adoption of the substrate-bound state lowers the activation energy necessary for the proceeding acylation step by creating an environment in which the transition state of acylation is stabilized (Okazaki *et al.*, 2008). After a substrate is incorporated into the catalytic cleft in the substrate-bound state, the general base Lys63 Nζ may deprotonate Ser60 Oγ, and the nucleophile Ser60 Oγ may then attack the amide group of the substrate, generating tetrahedral intermediates [light-green arrows in Fig. 2(*b*)]. Subsequently, the leaving group of the tetrahedral intermediate should deprotonate the general acid Tyr149 Oη, leading to release of NH_3. In turn, Tyr149 Oη may deprotonate Lys63 Nζ, and finally the acylation is completed [cyan arrows in Fig. 2(*b*)]. Therefore, Lys63 and Tyr149 appear to be the most plausible candidates for the general base and acid, respectively, that act in acylation (Okazaki *et al.*, 2008).

In the substrate-bound state of the D-phenylalanine complex, acylation occurred but deacylation did not (Okazaki *et al.*, 2007). This may result from the specific position of the H atom of Tyr149 Oη in the substrate-bound state. The OH group of Tyr149 in the substrate-bound state could easily donate an H atom to either the leaving group of the substrate or a water molecule: that is, the location of Tyr149 OH is well situated to participate in acylation (Fig. 2*b*). In the next step, the positioning of Tyr149 OH in the substrate-bound state may prevent deprotonation of a nucleophilic water molecule and thus would not be well suited for deacylation [Fig. 2(*d*); note that the NH_3 group in Fig. 2(*d*) is replaced by H_2O in the D-phenylalanine complex]. After the release of NH_3, Tyr149 OH should become positioned in a way that is well suited for deacylation: that is to say, the H atom of Tyr149 Oη may be directed to either Ser60 Oγ or Lys63 Nζ (Fig. 2*e* or 2*f*).

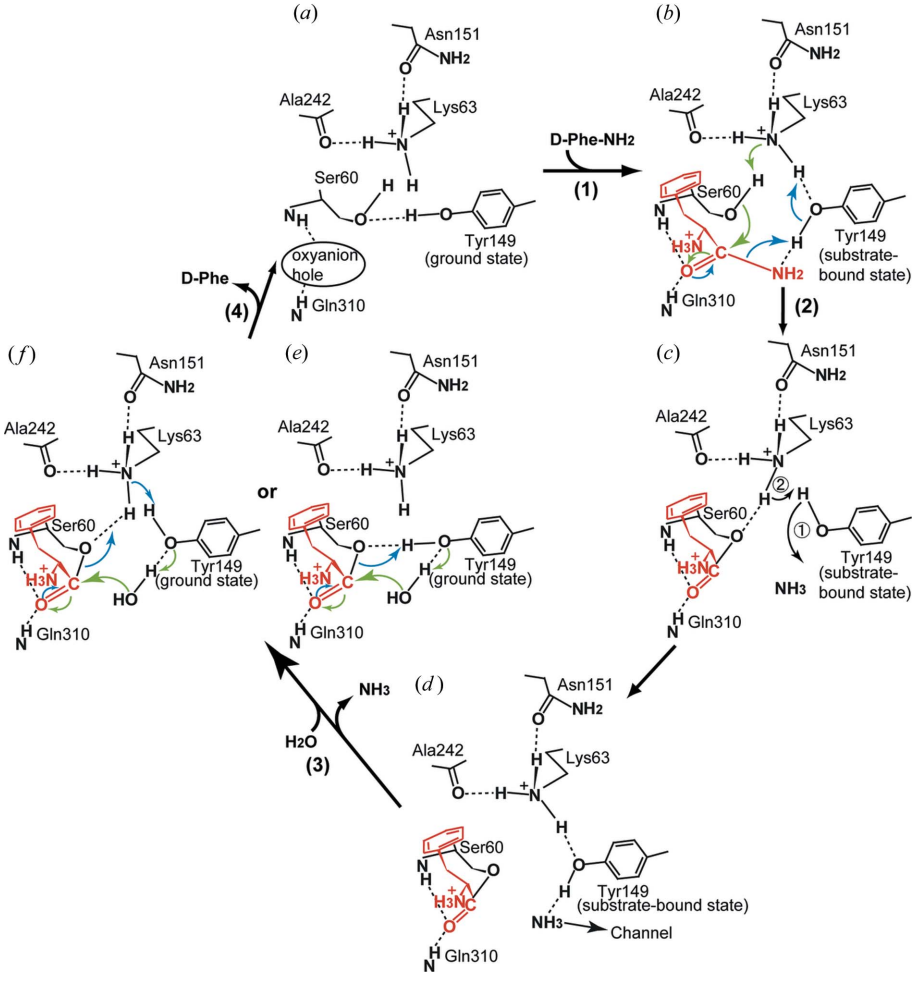

Figure 2
Proposed catalytic mechanism of DAA. The numerals in parentheses show the steps in the reaction. Proposed hydrogen bonds are represented as broken lines. The substrate D-phenylalanine is shown in red. In this model, enzyme catalysis proceeds according to the following steps. (1) By approaching the substrate D-Phe-NH_2 at the active site of DAA, Tyr149 Oη makes a transition to the location found in the substrate-bound state by induced fit motion. (2) Lys63 Nζ enhances the nucleophilic attack of Ser60 Oγ on D-Phe-NH_2 as general base, and a tetrahedral intermediate forms (light-green arrow). Finally, acyl enzyme is formed (cyan arrow). (3) NH_3 is released *via* a channel; Tyr149 Oη makes a transition to the location found in the ground state; and a nucleophilic water molecule enters. (4) Finally, deacylation occurs *via* formation of a tetrahedral intermediate (light-green arrow) and regeneration of the free enzyme (cyan arrow). (*a*) Arrangement of the active site residues in the free enzyme. (*b*) A noncovalent substrate complex is transferred to a tetrahedral intermediate in the substrate-bound state. (*c*) Acyl enzyme right after acylation. Black arrows indicate proton transfer facilitated by the location of Tyr149 in substrate-bound state, and circled numbers indicate the order of transition. (*d*) Acyl enzyme observed in the D-phenylalanine complex (Okazaki *et al.*, 2007), in which NH_3 is replaced by H_2O. (*e*) and (*f*) Acyl enzyme is transferred to a tetrahedral intermediate in the ground state. Either Tyr149 Oη (*e*) or Lys63 Nζ (*f*) may act as the general acid for deacylation.

The dynamics of transition from substrate-bound to ground state in DAA are still unclear; however, it is possible to speculate that the release of NH_3 through the proposed NH_3 channel (Okazaki *et al.*, 2008) can trigger a conformational transition from the substrate-bound to the ground state, and cause penetration of a nucleophilic water molecule in the catalytic center [Fig. 2; from (*d*) to (*e*) or (*f*)]. Interestingly, the transition from the substrate-bound to the ground state generates movement of the phenyl group of Phe282, producing a space that permits a water molecule to access the catalytic center from the exterior. In other penicillin-recognizing proteins, such as DD-peptidase, class C β-lactamase and EstB esterase, water molecules from the exterior are considered to be essential for deacylation (Wagner *et al.*, 2002). In fact, the catalytic clefts of DD-peptidase and extended spectrum class C β-lactamase are maintained in the open form after substrate binding, which permits a water molecule to access the acyl enzyme (Negoro *et al.*, 2007).

We previously proposed that deacylation in subunit *F* of the D-phenylalanine complex is facilitated by extraction of an H atom from Tyr149 Oη *via* a deprotonated His307 Nε2 (Okazaki *et al.*, 2007). The Tyr149 Cζ—Tyr149 Oη—His307 Nε2 angle (122.2°) and the distance between Tyr149 Oη and His307 Nε2 (2.9 Å) in subunit *F* in the D-phenylalanine complex suggests that Tyr149 Oη can donate an H atom to His307 Nε2 as well as Ser60 Oγ and Tyr149 Lys63 Nζ. Therefore we could not exclude the possibility that His307 Nε2 extracts the H atom of Tyr149 Oη, as in the case of either Fig. 2(*e*) or 2(*f*).

To bring about a transition from the acyl-enzyme state to a free (deacylated) state, initially the general base Tyr149 Oη must deprotonate a water molecule and the activated nucleophilic OH group must attack the carbonyl carbon of D-phenylalanine, generating a tetrahedral intermediate [light-green arrow in Fig. 2(*e*) or 2(*f*)]. Succes-

sively, a tetrahedral intermediate should deprotonate the general acid Tyr149 Oη, leading to the release of D-phenylalanine [cyan arrow in Fig. 2(*e*)]. Tyr149 Oη is the stronger candidate for the general acid in deacylation, on the basis of the geometries observed for Tyr149 Oη in the ground state (Table 1). In contrast, Lys63 Nζ may be able to donate an H atom to Ser60 Oγ as the general acid in deacylation and subsequently Lys63 Nζ extracts an H atom from Tyr149 Oη [cyan arrow in Fig. 2(*f*)]. This model is supported from the viewpoint that the location of the H atom of Ser60 Oγ is predominantly at Lys63 Nζ in the ground state, as shown in Table 1. Thus, the location of the H atom of Tyr149 Oη in the ground state is considered to be an essential step prior to deacylation (Fig. 2*e* or 2*f*).

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