Enzyme Mechanism

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A Single Residue Influences the Reaction Mechanism of Ammonia Lyases and Mutases**

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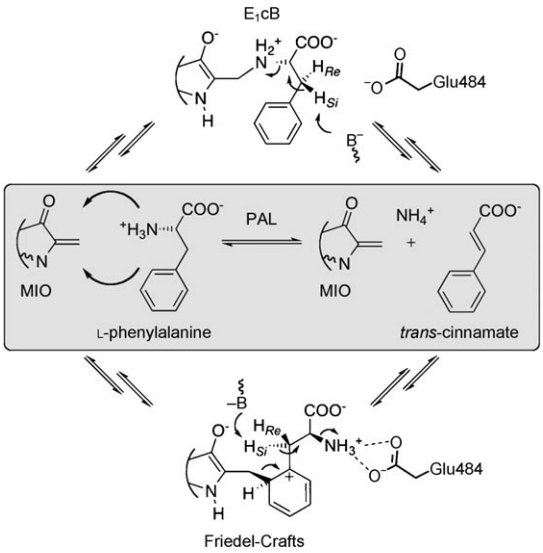
The mechanism for the non-oxidative deamination of aromatic amino acids by enzymes such as phenylalanine or tyrosine ammonia lyases (PAL/TAL), has been controversially discussed in literature for many years.[1] Two different pathways—elimination or Friedel–Crafts reaction—have been proposed and hence these enzymes show a type of catalytic promiscuity,[2] in which different ways of bond making or breaking are used. This situation opens the question why nature should use different approaches to reach the same goal. A possible explanation is addressed herein based on point mutations derived from computer modeling studies.

PALs convert phenylalanine (Phe) into *trans*-cinnamic acid and ammonia, whereas TALs convert tyrosine (Tyr) into *p*-coumaric acid (Scheme 1). All enzymes of this type contain the prosthetic 4-methylidene imidazole-5-one (MIO) in the active site, which is formed autocatalytically from the amino acids Ala-Ser-Gly.[3] The first reported mechanism was an elimination (E_1cB) with an attack of the electrophilic MIO group on the amino group of the substrate (Scheme 1).[4] The main drawback of the E_1cB mechanism is the difficult abstraction of the non-acidic benzylic proton with a pK_a of over 40[5] by an enzymatic base.[1] The mechanism is strongly supported by the recent crystal structure of a tyrosine ammonia mutase (TAM), showing a covalent adduct of the MIO and the amino group of the substrate.[6] This contradicts the finding that MIO-deficient, histidine ammonia lyases (HALs) react well with 5-nitrohistidine, which excludes the E_1cB mechanism, leading to an alternative reaction mechanism based on the electrophilic attack of the prosthetic group on the aromatic ring.[7] The latter mechanism seems to be less plausible for PAL than for HAL because of the less electron rich phenyl ring in the Phe substrate than in the His substrate, and the transient loss of aromaticity during the reaction



Scheme 1. Schematic presentation of the proposed reaction mechanisms of *pc*PAL: elimination reaction (E_1cB) as proposed by Hanson and Havir[4a] and Friedel–Crafts type as proposed by Schuster and Rétey.[9a]

(Scheme 1).[1] Nevertheless this was the first described biological Friedel–Crafts reaction[8] and was supported for PAL by different experiments.[9]

Former studies of TAL and PAL showed that a conserved Phe residue (Phe137 in *pc*PAL[10]) is replaced by a His in *rs*TAL[10] building hydrogen bonds with the *p*-OH-group of tyrosine leading to the difference in substrate specificity between these two enzymes.[11] In structure and sequence alignments, we now found glutamic acid as another conserved residue in PAL (Glu484 in *pc*PAL) and the corresponding amino mutase (PAM), but an asparagine in TAL (Asn432 in *rs*TAL) and TAM (Figure 1).

To reveal the function of Glu484, automated docking and molecular dynamics (MD) simulations were performed using the crystal structures of *rs*TAL[11a] (protein data base (pdb) code: 2o6y) and *pc*PAL[12] (pdb code: 1w27). The structure of *pc*PAL was supposed to represent an inactive enzyme because of an unfavored orientation of a loop containing the catalytically essential Tyr110.[13] A possibly active homology model with a correct conformation of the Tyr110-containing loop was generated, showing similar behavior with respect to substrate orientation and stabilization (see Supporting Information). During these MD simulations, we always observed a strong attraction of the substrate amino group and Glu484 with the consequence that the distance between the amino group of the substrate and the prosthetic MIO group is significantly too long for interactions (Figure 2a, squares).

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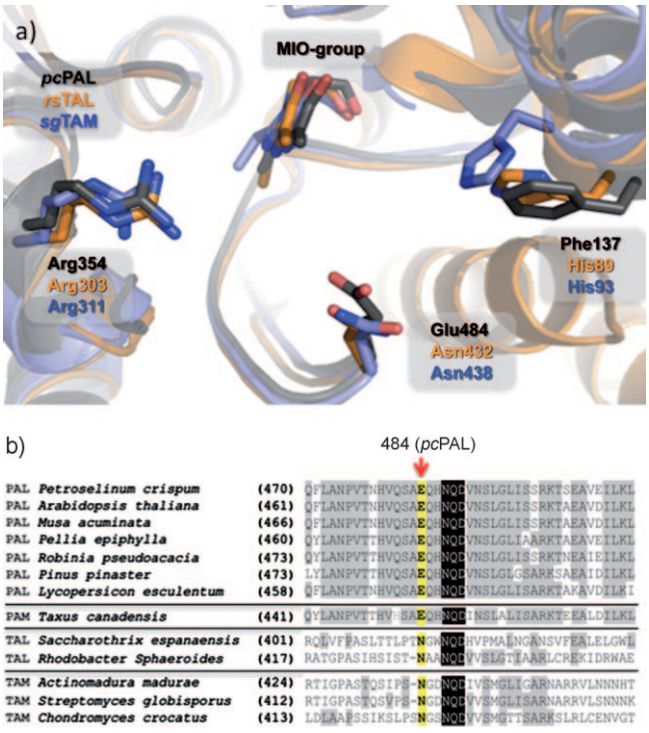

Figure 1. Structure and sequence alignments of different amino lyases and mutases. a) Structure alignment of *pc*PAL (black), *rs*TAL (orange), and *sg*TAM (blue) showing the most important residues for substrate binding. b) Sequence alignment of known PAL, PAM, TAL, and TAM enzymes. Black: Residues conserved among different proteins, gray: homologous and similar. Residue 484 (*pc*PAL) is highlighted yellow and indicated by an arrow.

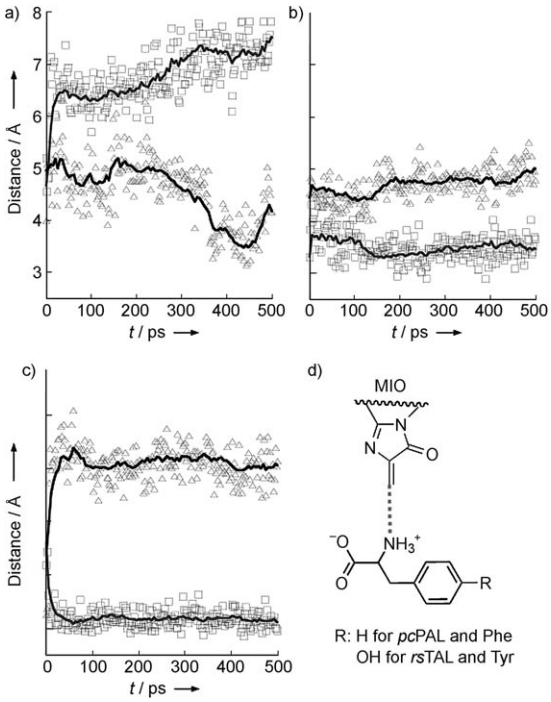

Figure 2. MD simulations of WT (*pc*PAL, *rs*TAL, *sg*TAM; squares) and the corresponding mutants (Glu484Asn *pc*PAL, Asn432Glu *rs*TAL, Asn438Glu *sg*TAM; triangles); a) *pc*PAL with Phe; b) *rs*TAL with Tyr; c) *sg*TAM with β-Tyrosine; d) Measured distance indicated by dotted line (β-Tyrosine not shown).

This finding supports a Friedel–Crafts mechanism for *pc*PAL and excludes the E_1cB mechanism, which is in contrast to the results from MD simulations on the *pc*PAL mutant Glu484Asn (Figure 2a, triangles), *rs*TAL wildtype (WT), or *sg*TAM WT[10] (Figure 2b,c). By the Glu→Asn substitution (Figure 2, triangles) in the active site of TAL-WT (Asn432 in *rs*TAL), TAM-WT (Asn438 in *sg*TAM) and the *pc*PAL mutant Glu484Asn active site, the amino group is not forced to face the Glu484 (*pc*PAL) during MD simulations and in all cases points towards the MIO group independently of the initial orientation. This result indicates the possibility of the E_1cB mechanism for TAL and TAM.

These findings led to the hypothesis that both mechanisms, the Friedel–Crafts and the E_1cB mechanism can occur in aromatic amino acid amino lyases and mutases depending on the nature of this newly discovered residue (Glu484 in *pc*PAL).

To verify this assumption experimentally, *m*-tyrosine (*m*-Tyr) was chosen as a substrate with which to study the likeliness of the Friedel–Crafts mechanism. It was reported that the free electron pair of the *m*-OH group stabilizes the intermediately occurring positive charge of the aromatic ring during the Friedel–Crafts reaction (Scheme 1).[9a] Other experiments, supporting the Friedel–Crafts reaction also reported similar electronic effects using pyrimidine alanines, halogenated or N-methylated phenylalanines.[14]

Kinetic constants measured for the *pc*PAL-WT and the Glu484Asn mutant showed significantly decreased activity towards *m*-Tyr for the Glu484Asn mutant compared to Phe (7.6-fold lower k_{cat} value), while the WT activities were similar but not higher for *m*-Tyr as expected (Table 1), perhaps *m*-Tyr is sterically more demanding than Phe. This result clearly indicates that the beneficial electronic effects of *m*-Tyr, which support the Friedel–Crafts reaction, were not observed for the Glu484Asn mutant, thus supporting our hypothesis.

Table 1: Comparison of the kinetic constants of *pc*PAL-WT and the Glu484Asn-mutant towards L-phenylalanine and *m*-tyrosine.

Enzyme	Substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}\,M^{-1}$]
*pc*PAL-WT	L-Phe	0.054 ± 0.01	31 ± 3	568 000
*pc*PAL-E484N		0.75 ± 0.08	13 ± 1	16 700
*pc*PAL-WT	L-*m*-Tyr	0.10 ± 0.01	24 ± 1	246 000
*pc*PAL-E484N		0.32 ± 0.03	1.7 ± 0.2	5410

This raises the question, why nature should enable two different reaction mechanisms to reach the same goal and why different residues are conserved at the same position depending on the preferentially converted substrate. To elucidate the molecular reasons for this, we focused on the need for the stabilization of the substrates in the active site: while the tyrosine is orientated in TAL/TAM by hydrogen bonds between the carboxy group of Tyr and Arg303 (in *rs*TAL)

and between the substrate *p*-OH group and His89 (*rs*TAL), the *p*-OH group is missing in PAL/PAM (Figure 3). This situation led us to propose that at least two independent hydrogen bonds are required for a correct substrate orientation and stabilization in the active site of PAL/PAM.

To identify and verify these hydrogen bonds, the corresponding phenylalaninol (Phe-ol) was used to avoid the strong hydrogen bond between Arg354 and the carboxylic group of the substrate (Figure 3 a/b). MD simulations using *pc*PAL indicated, that in contrast to Phe, Phe-ol is free to move in the active site because it is only bound by hydrogen bonds between the amino group and Glu484 (Figure 3 b/c), the hydrogen bonds of the carboxy group are missing and there is only a weak hydrophobic stabilization of the aromatic ring.

Kinetic measurements showed no detectable activity of *pc*PAL towards Phe-ol and supported the computational results. The supply of one additional hydrogen bond by using the *pc*PAL mutant Phe137His[11] and tyrosinol (Tyr-ol) as substrate (Figure 3 d) promised a stable orientation of the substrate in the active site during MD simulations. In fact, significant activity of 18.6 mU mg^{-1} was measured in contrast to the *pc*PAL WT that showed no detectable activity towards Tyr-ol. For comparison, *rs*TAL was used, which showed the expected higher activity (33.7 mU mg^{-1}) towards Tyr-ol (Table 2).

In the *pc*PAL mutant Glu484Asn, no strong hydrogen bonding between residue 484 and the amino group of the substrate is possible. Indeed, this mutant showed a significantly decreased activity and a dramatically increased K_M value towards Phe compared to the wide type (Table 2).

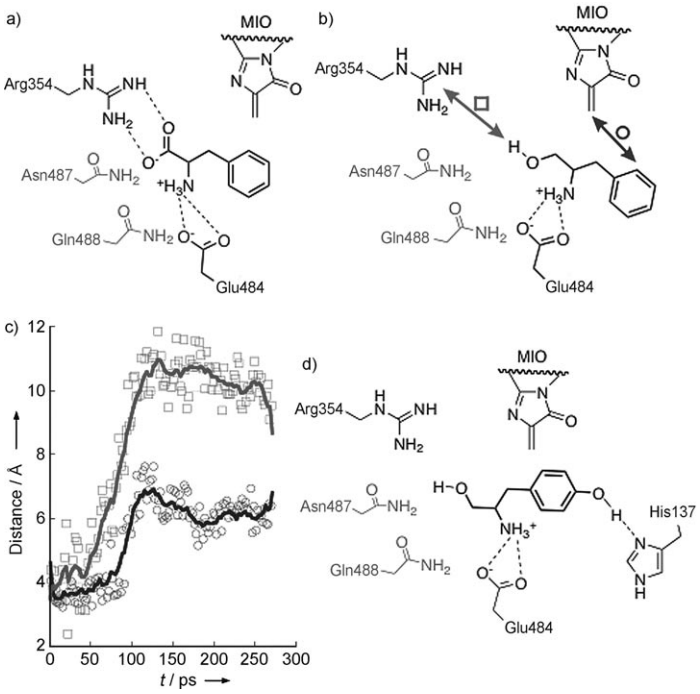

Figure 3. Active site of *pc*PAL with docked substrates. a) *pc*PAL WT with docked Phe; b) *pc*PAL WT with docked Phe-ol. The measured distances between Arg354 and the OH group (in (b) and (c) marked by squares) and between MIO group and aromatic ring (in (b) and (c) marked by circles) are plotted over time. d) A comparison to the active site of the *pc*PAL mutant Phe137His is shown with docked tyrosinol.

Table 2: Comparison of the kinetic constants of *pc*PAL-WT, mutants and *rs*TAL towards L-Phe and L-Tyr-ol.

Enzyme	Substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
*pc*PAL-WT	L-Phe	0.054 ± 0.01	31 ± 3	568 000
*pc*PAL-E484N		0.75 ± 0.08	13 ± 1	16 700
*pc*PAL-F137H		5.2 ± 0.6	21 ± 0.2	4000
*rs*TAL		2.7 ± 0.7	0.6 ± 0.02	226
*pc*PAL-WT	L-Tyr	2.0 ± 0.2	1.5 ± 0.1	754
*pc*PAL-E484N		0.20 ± 0.01	0.6 ± 0.02	3200
*pc*PAL-F137H		0.26 ± 0.02	1.0 ± 0.02	3600
*rs*TAL		0.10 ± 0.02	0.9 ± 0.1	10 000
*pc*PAL-WT	L-Tyr-ol	n.d.[b]	< 0.01[a]	n.d.[b]
*pc*PAL-F137H			0.11 ± 0.04[a]	
*rs*TAL			0.12 ± 0.04[a]	

[a] Activity measurement with 10 mM substrate assuming saturation conditions. [b] n.d. not detectable.

These results clearly indicate the weak binding of the substrate in the active site caused by the missing hydrogen bond between Phe and Glu484, and explains why in PAL/PAM the Glu at position 484 (*pc*PAL) is essential for an efficient conversion of Phe, but why it is not needed in TAL/TAM enzymes.

Furthermore, the Glu484Asn mutant also showed a significantly decreased K_M value for the conversion of Tyr resulting in a higher k_{cat}/K_M value, similar to the Phe137His mutant (Table 2).

These observations could not be explained by computer modeling, but indicate that there is a reason for an Asn residue at position 432 (*rs*TAL). Additionally, we expected the double mutant Phe137His-Glu484Asn to show even more TAL-like properties and also the Asn432Glu mutant of *rs*TAL to show more PAL-like properties. Unfortunately, these mutants showed only very low activity towards Phe and Tyr (data not shown), demonstrating the finely tuned and very sensitive active site of these enzymes. It also demonstrates that the differences of PAL and TAL are not exclusively based on two residues but also are influenced by other factors.

In conclusion, the mechanism of PAL/PAM and TAL/TAM enzymes, which for many years was controversially discussed, can now be explained by the observation that the Glu484 residue (*pc*PAL) prevents the MIO group from an attack on the amino group of the substrate, thus supporting a Friedel–Crafts mechanism in contrast to the TAL/TAM enzymes. This observation, which represents a "mechanistic promiscuity"—where different reaction mechanisms in enzymes can lead to the same product—answers the question why neither the E_1cB nor the Friedel–Crafts mechanism could be clearly favored to date. Thus, the differences in substrate specificities between PAL/PAM and TAL/TAM could be addressed by an investigation of substrate orientation and stabilization. The results of which indicate that in PAL/PAM at least two hydrogen bonds

are required to ensure the substrate is stabilized and correctly orientated. In addition, the observed activity of the Phe137His mutant of *pc*PAL and of *rs*TAL WT towards the amino alcohol opens the possibility of producing optically pure amino alcohols if the reverse reaction from the corresponding α/β-unsaturated alcohol is performed.

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[10] *pc*PAL, phenylalanine ammonia lyase from *Petroselinum crispum*; *rs*TAL, tyrosine ammonia lyase from *Rhodobacter sphaeroides*; *sg*TAM, tyrosine ammonia mutase from *Streptomyces globisporus.*
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