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BUDAPEST UNIVERSITY OF TECHNOLOGY AND ECONOMICS  
FACULTY OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY  
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## **Investigation of structure-function relationship in hydroxynitrile lyases and MIO-containing class I lyase-like enzymes**

Thesis booklet

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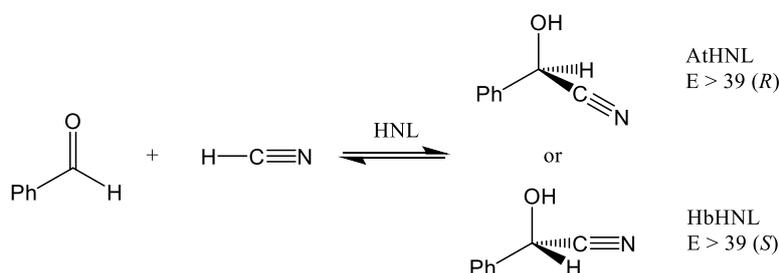
Budapest  
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# 1 Introduction

Scientific and technological advances have established biocatalysis as an alternative to traditional metallo- and organocatalysis in chemical synthesis, leading to the development of greener, more efficient and more sustainable synthetic processes <sup>1</sup>. In 2012, three waves of biocatalysis were discussed <sup>1</sup>, already today the fourth wave of biocatalysis is forecasted <sup>2,3</sup>. Results presented here constitute a small part of the effort to understand how protein structure defines substrate binding and catalyze chemical transformations by lowering the transition state energy barriers in hydroxynitrile lyases and MIO containing class-I lyase like enzymes.

## 1.1 Hydroxynitrile lyases

Hydroxynitrile lyases (HNL's, EC 4.1.2.X, X= 10, 11, 46, 47) are defense enzymes that catalyze the elimination of hydrogen cyanide from cyanohydrins. The released hydrogen cyanide kills or deters the predators. It is estimated, that 3,000 plant species, from a wide variety of families, utilize cyanogenic defense <sup>4</sup>. For synthetic applications, chemists carry out the reverse reaction, an enantioselective addition of hydrogen cyanide to carbonyl compounds, as shown on Figure 1.



**Figure 1. Reaction scheme of the HNL catalyzed reaction.** The natural reaction proceeds in the cyanide elimination direction while synthetic applications benefit from the enantioselective addition of hydrogen cyanide to carbonyl compounds such as benzaldehyde in the example reaction shown here.

HNLs have independently evolved at least in six different protein folds, suggesting that catalyzing the cyanohydrin cleavage reaction is a chemically easy task; only a catalytic base and a positive charge is required. The protein environment mostly serves as a scaffold for enantioselectivity. HNL activity developed multiple times within the  $\alpha/\beta$ -hydrolase fold that is most similar to esterases; rubber tree (*Hevea brasiliensis*, HbHNL) encodes an *S*-selective

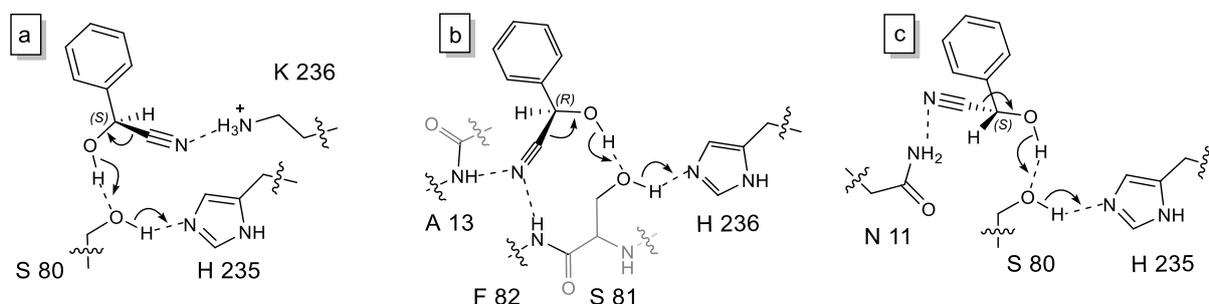
<sup>1</sup> Bornscheuer, U. T. et al. *Nature* **485**, 185–194 (2012).

<sup>2</sup> Bornscheuer, U. T. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* **376**, (2018).

<sup>3</sup> Poppe, L. & Vértessy, B. G. *ChemBioChem* **19**, 284–287 (2018).

<sup>4</sup> Dadashpour, M. & Asano, Y. *ACS Catal.* **1**, 1121–1149 (2011).

HNL<sup>5</sup>, while mouse-ear cress (*Arabidopsis thaliana* AtHNL) encodes an *R* selective HNL<sup>6</sup>. Distinct catalytic mechanisms enable opposite enantioselectivity of AtHNL and HbHNL as explained on Figure 2. High sequence similarity (73%) demonstrates that HbHNL and AtHNL are evolutionarily closely related, despite the opposite enantioselectivities and differing biological functions.



**Figure 2. Different catalytically competent orientations of mandelonitrile stabilize the leaving cyanide group differently and favor different enantiomers.** *a.* In the classical mechanism for HbHNL, the  $\epsilon$ -ammonium group of Lys236 stabilizes the leaving cyanide. This enzyme favors the (*S*)-enantiomer. *b.* In the mechanism for AtHNL, two main-chain *N*–*H* groups from the oxyanion hole stabilize the leaving cyanide. This enzyme favors the (*R*)-enantiomer. This enzyme cannot use the classical mechanism because it lacks Lys236. *c.* In the mechanism proposed here for the (*S*)-enantioselective composite active-site enzymes, derived from HbHNL, the  $\delta$ -amide *N*–*H* group of Asn11 stabilizes the cyanide leaving group. The other two mechanisms are not possible, because Lys236 is missing and because distant residues cause Phe81 to block the oxyanion hole region.

An ancestral enzyme reconstruction identified the last common ancestors of (*R*)- and (*S*)-HNLs and closely related plant esterases. On the whole, this group of plant esterases and hydroxynitrile lyases share >40% amino acid identity, which made it feasible to infer ancestral enzyme sequences. Four putative ancestral proteins; EST1, EST2, EST3 and HNL1 that are at the divergence points for different protein functions have been created, (Figure 6 a)<sup>7</sup>. HNL1 catalyzed mainly hydroxynitrile cleavage, but also showed low remaining esterase activity. More interesting, the older ancestor EST3mL<sup>8</sup> showed both esterase and HNL activity. EST3mL was 58% identical to HbHNL and 78% identical to AtHNL, including active site residues such as Met236 and Asn11. Similar to AtHNL, this ancestor was also (*R*)-selective, but only weakly (*E* = 6.7).

<sup>5</sup> Selmar, D., *et al. Physiol. Plant.* **75**, 97–101 (1989).

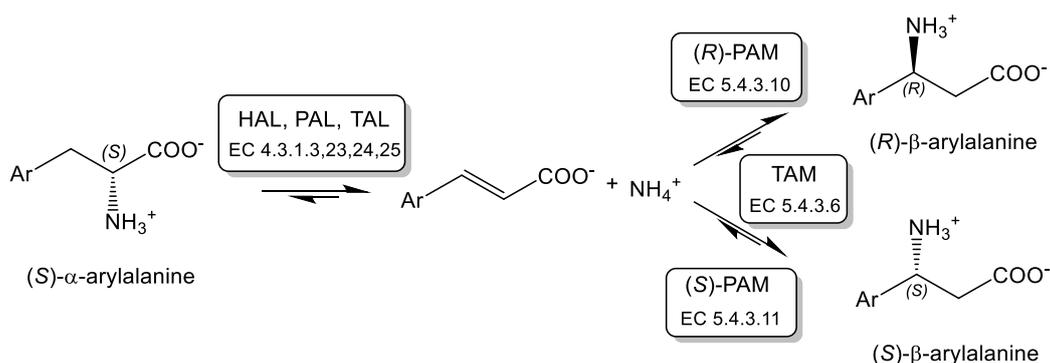
<sup>6</sup> Andexer, J. N. *et al. ChemBioChem* **13**, 1932–1939 (2012).

<sup>7</sup> Devamani, T. *et al. J. Am. Chem. Soc.* **138**, 1046–1056 (2016).

<sup>8</sup> EST3 have been reconstructed by more than one method. EST3mL refers to the reconstruction by the maximum likelihood method.

## 1.2 MIO-containing class I lyase-like enzyme family

The MIO-containing class I lyase-like enzyme family (MIO enzymes) constitute two functionally diverse but structurally related enzyme classes, the aromatic amino acid ammonia-lyases (ALs) and the aromatic amino acid 2,3-aminomutases (AMs), as shown on Figure 3<sup>9</sup>. ALs catalyze the reversible ammonia elimination from histidine (HAL, EC 4.3.1.3), phenylalanine (PAL, EC 4.3.1.24) or tyrosine (TAL, EC 4.3.1.23)<sup>10</sup>. AMs catalyze the interconversion between  $\alpha$ - and  $\beta$ -phenylalanine (PAM, EC 5.4.3.10, 11) or  $\alpha$ - and  $\beta$ -tyrosine (TAM EC 5.4.3.6). For their natural substrates AL and AM are highly selective for  $\alpha$ -arylalanines, those only cleave or produce L-aromatic amino acids ((*S*)-enantiomer)<sup>9</sup>. The PAM reaction for  $\beta$ -phenylalanine is also highly enantioselective, PAMs from eukaryotic origin usually produce (*R*)- $\beta$ -phenylalanine<sup>11</sup>, while PAMs from prokaryotic origin usually produce (*S*)- $\beta$ -phenylalanine<sup>12</sup>. The AL and AM reactions are catalyzed by a special electrophile 4-methylideneimidazole-5-one (MIO).



**Figure 3. Reactions catalyzed by MIO enzymes.** ALs catalyze the non-oxidative ammonia elimination from (*S*)- $\alpha$ -arylalanines; L-histidine, L-phenylalanine or L-tyrosine. AMs catalyze conversion of (*S*)- $\alpha$ -arylalanines; L-phenylalanine or L-tyrosine to (*R*) or, (*S*)- $\beta$ -arylalanines. Phenylalanine 2,3-aminomutases (PAM) produce (*R*) or (*S*)- $\beta$ -phenylalanine selectively, while Tyrosine 2,3-aminomutases (TAM) produce a mixture of (*R*) and (*S*)- $\beta$ -tyrosine.

Different reaction mechanisms for the AL reaction propose distinct roles for the MIO electrophile, Figure 4. The *N*-MIO mechanism suggests an *N*-MIO intermediate by covalent bond formation between the amino group of the substrate and the exocyclic methylene carbon of MIO electrophile<sup>13</sup>. The Friedel-Crafts (FC) mechanism hypothesizes an FC-intermediate resembling to the  $\sigma$ -complex involved in Friedel-Crafts reactions by covalent bond formation

<sup>9</sup> Parmeggiani, F., *et al. Chem. Rev.* **118**, 73–118 (2018).

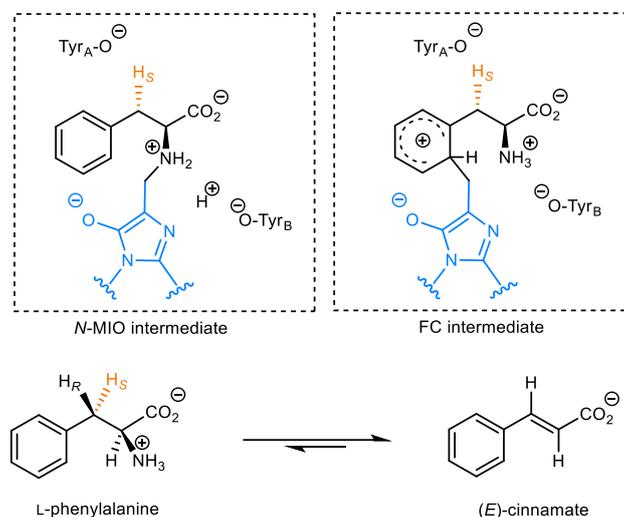
<sup>10</sup> A fourth aromatic ammonia lyase EC category 4.3.1.25 exists. Enzymes having both phenylalanine and tyrosine ammonia lyase activities belong to this family.

<sup>11</sup> Mutatu, W., *et al. Biochemistry* **46**, 9785–9794 (2007).

<sup>12</sup> Magarvey, N. A., *et al. ACS Chem. Biol.* **3**, 542–54 (2008).

<sup>13</sup> Hermes, J. D., *et al. Biochemistry* **24**, 2959–2967 (1985).

between the aromatic moiety of the substrate and the exocyclic methylene carbon of the MIO electrophile<sup>14</sup>. In both mechanisms hypothesizing a covalent intermediate, a tyrosine residue (TyrA on Figure 4) is functioning as the catalytic base which removes the *pro-S* proton ( $H_S$ ) from the  $\beta$  position during the elimination reaction.



**Figure 4. Suggested reaction mechanisms for AL reactions illustrated on phenylalanine.** In the N-MIO intermediate, a bond is formed between the amino group of the substrate and in the MIO electrophile. In the FC intermediate, a bond is formed between the phenyl ring of the substrate and in the MIO electrophile.

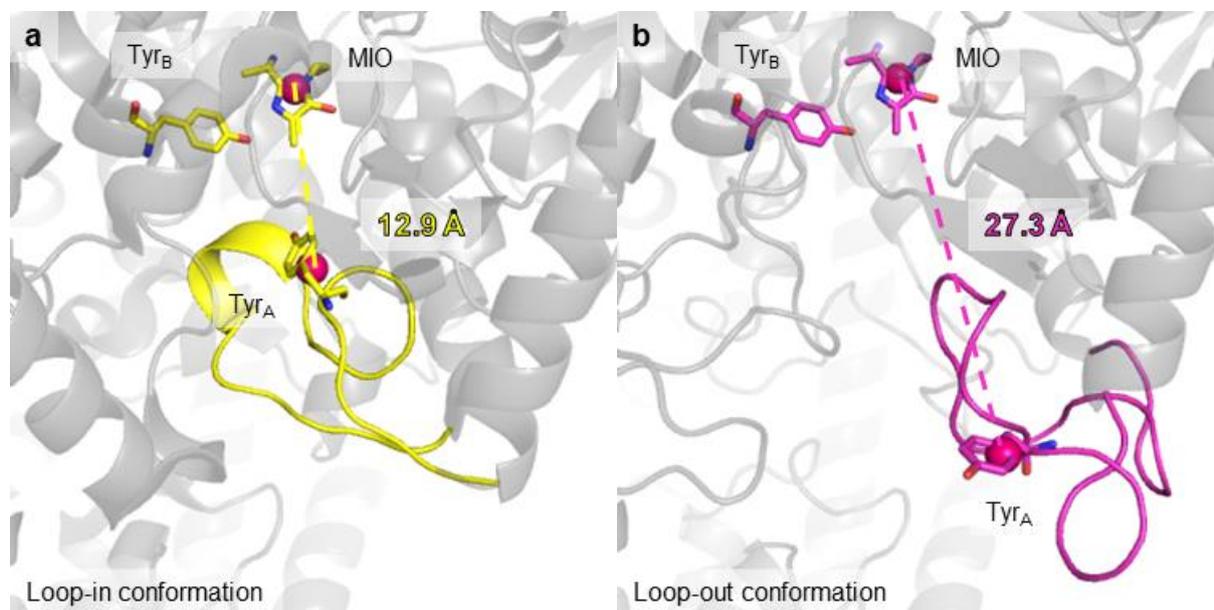
The active site of MIO enzymes is capped by two flexible loops named the inner and outer loop. Large crystallographic B factors suggested high flexibility of these two loops, and due to their spatial proximity these were implicated in substrate binding and catalysis. The flexibility of the inner loop was suggested to be the key element in differentiating ALs from AMs<sup>15</sup>. The hypothesis assumed the inner-loop to undergo large conformational changes and transition from the “loop-in conformation” to the “loop-out conformation” (similar to the one modeled for the crystal structure of *Petroselinum crispum* PAL (PcPAL)<sup>16</sup>, Figure 5) during the substrate binding and product release steps of the catalytic cycle. However, existence of the “loop-out conformation” in any MIO enzyme does not hold up under thorough scrutiny and suggested that the currently available crystal structure for PcPAL might contain modelling errors. Based on relevant interrogation of the PDB database, including electron density data, all selected representatives of each classes of MIO enzymes show well-ordered inner loop conformations in a “loop-in” state. Therefore, we aimed to experimentally determine the catalytically active conformation of PcPAL, and investigate if the addition of the strong binding

<sup>14</sup> Poppe, L. & Rétey, J. *Angew. Chemie - Int. Ed.* **44**, 3668–3688 (2005).

<sup>15</sup> Heberling, M. M. *et al. ACS Chem. Biol.* **10**, 989–997 (2015).

<sup>16</sup> Ritter, H., Schulz, G. E. *Plant Cell* **16**, 3426–3436 (2004).

inhibitor may enhance the crystallizability of PcPAL. Having strong binding inhibitors from both enantiomers we aimed to probe, how does the active site geometry of PcPAL influence ligand binding affinity and the enantioselectivity of the enzyme.



**Figure 5. Inner loop conformations in MIO enzymes. a.** Catalytically competent loop-in conformation of the inner loop in *Taxus canadensis* PAM (TcPAM) structure (3NZ4)<sup>17</sup>. **b.** Loop-out conformation of the inner loop in *Petroselinum crispum* PAL (PcPAL) structure (1W27)<sup>16</sup>. The catalytic base Tyr<sub>A</sub> points away from the active site, thus unable to fulfill its role.

## 2 Methods

The bacterial expression vectors of PcPAL and *Kangiella koreensis* PAL (KkPAL) and their mutants were produced by molecular cloning or site directed mutagenesis. Proteins were purified from cell extracts by Ni-NTA chromatography and protein quality was verified by SDS-PAGE and photometric methods.

Binding of aminophosphoric acid compounds to PcPAL, *Rhodospiridium toruloides* PAL (RtPAL) and *Anabaena variabilis* PAL (AvPAL) were characterized kinetically and by isothermal titration calorimetry. The tertiary structure of PcPAL was solved by X-ray protein crystallography in an apo form and in complex with (*R*)-APEP and (*S*)-APPA.

Ligand binding pathways and the dynamic behavior of the inner loop were investigated by random acceleration molecular dynamics simulations.

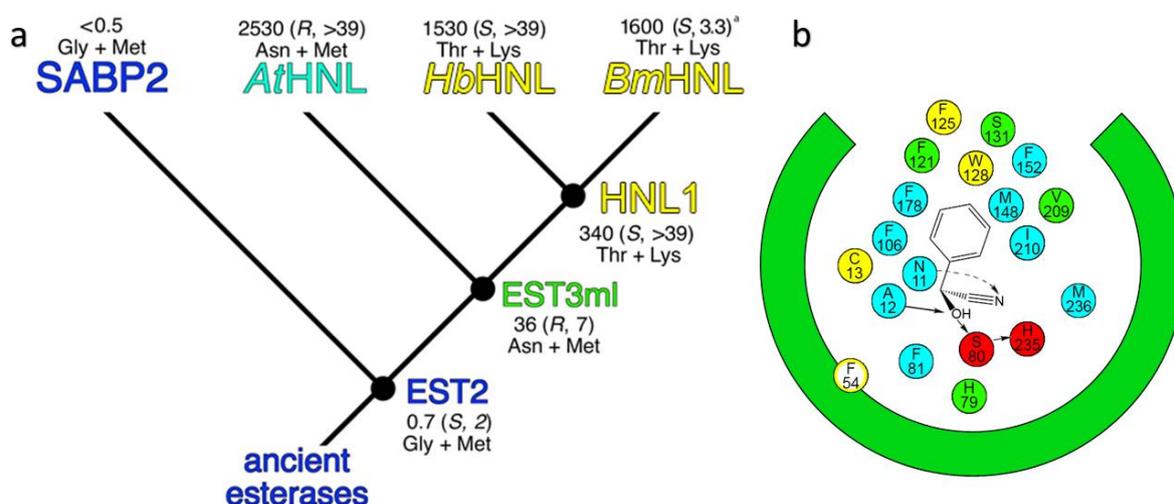
Molecular dynamics simulations modeled the structure and ligand binding behavior of the wild type (*S*)-selective AtHNL and the (*R*)-selective HbHNL, and of the composite active sites in the AtHNL and in the HbHNL scaffolds.

<sup>17</sup> Feng, L., *et al.* *Biochemistry* **50**, 2919–2930 (2011).

### 3 Results and Discussion

#### 3.1 Reactions mechanism in ancestral and modern HNLs

To understand the differences between the two types of HNL mechanisms in HbHNL and AtHNL, we attempted to exchange their catalytic abilities and thus their enantioselectivities by exchanging active-site residues. Exchanging all 16 non-homologous residues resulted in inactive or unfolded protein, hence we created consensus active sites that are made up partially of residues encoded by AtHNL and partially from those encoded by HbHNL. For example the consensus active site A9-H7 is formed by nine AtHNL residues and seven HbHNL residues. The choice of residues to exchange was guided by similarities to ancestral enzyme EST3mL.



**Figure 6. a.** Simplified phylogenetic tree including HbHNL (Lys mechanism, yellow text), AtHNL (oxyanion hole mechanism, cyan text), HNL from *Baliospermum montanum* (BmHNL), esterase from *Nicotiana tabacum* (SABP2, blue text), as well as several reconstructed ancestral enzymes (HNL1, EST2, EST3ml). The numbers in black are the HNL activity for cleavage of mandelonitrile (given in min<sup>-1</sup>), the favored enantiomer and the enantioselectivity are given in parentheses, and the active site residues associated with HNL activity (residues 11 and 236) are shown. [Superscripted “a” indicates that data for BmHNL are taken from <sup>18</sup>] **b.** The active site of EST3mL resembles the consensus active site A10-H6, and only 4 of the 16 investigated residues differ (these are shown in green). EST3mL contains phenylalanines at both positions 54 and 81, suggesting that the Phe81 may block the oxyanion hole, and thus require the Asn mechanism for catalysis.

Variant HbHNLs with composite active sites (Hb-A9-H7 and Hb-A10-H6) likely use a catalytic mechanism different from either wild-type protein. The nine substitutions that convert wild-type HbHNL to Hb-A9-H7 eliminate the possibility of the (S)-selective catalytic Lys mechanism of HbHNL <sup>19</sup>, because these substitutions remove the essential Thr11 and Lys236

<sup>18</sup> Dadashipour, M. *et al. J. Biotechnol.* **153**, 100–110 (2011).

<sup>19</sup> Gruber, K., *et al. J. Biol. Chem.* **279**, 20501–20510 (2004).

residues. While we expected that the addition of Asn11, Ala12, and Phe81 would enable the (*R*)-selective catalysis of AtHNL<sup>20</sup>, HbHNL-A9-H7 remained highly (*S*)-selective ( $E > 39$ ). The ability to maintain ~3% of wild-type activity without either catalytic residue suggests these composite enzymes catalyze (*S*)-selective mandelonitrile cleavage, using a novel mechanism.

The new asparagine mechanism proposed in the composite site enzymes may have been an evolutionary stepping stone to modern HNL mechanisms. Both HbHNL and AtHNL evolved from esterases, yet a direct transition between modern esterase and lysine mechanism HNL requires going through catalytically dead intermediates, because of the need to both block the oxyanion hole and to add a position for a charged lysine to bind the leaving cyanide<sup>21,22</sup>.

However, the reconstruction of the last common ancestor of HbHNL and AtHNL, called EST3mL (Figure 6)<sup>23</sup>, catalyzed both cyanohydrin cleavage and ester hydrolysis<sup>23,24</sup>. The active site of this ancestor resembles the composite active-site enzymes created here, including the critical Asn11. All active site residues in the ancestor either match A10-H6 (17 residues) or are residues not found in either modern enzyme (5 residues). EST3mL shows 2.3% of the HNL activity of HbHNL and shows low enantioselectivity (*R*, 7) similar to the activity (1.6% of HbHNL) and enantioselectivity (*S*, 3.3) of HbHNL-A10-H6. Similar to HbHNL-A10-H6, EST3mL has phenylalanine residues at both 54 and 81, which based on the modeling results could obstruct the oxyanion hole, thereby preventing HNL mechanism used by AtHNL. Since HbHNL-A10-H6 likely follows the Asn mechanism, we propose that ancestral enzyme EST3mL similarly follows the Asn mechanism. This suggests that finding the new asparagine mechanism in the composite enzymes may not be due to serendipity, but instead may be uncovering the ancestral stepping-stone mechanism between ancestral esterases and modern HNLs. A stepping stone mechanism would be compatible with both previous (esterase) and subsequent mechanisms (modern HNL mechanisms), allowing an evolutionary path from one to the other via a promiscuous enzyme, with a functional enzyme at every step. Since both the composite site enzymes and the ancestor EST3mL are significantly slower than modern, wild-type HNLs, evolutionary pressure could optimize HNL function by converting from an asparagine mechanism to the modern lysine or oxyanion hole mechanisms if a viable evolutionary path exists. A few mutants along a few possible trajectories were tested and found to be dead or insoluble, but these only represent a small sample of the 9! (>360 000) possible

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<sup>20</sup> Andexer, J. N. *et al. ChemBioChem* **13**, 1932–1939 (2012).

<sup>21</sup> Padhi, S. K. *et al. Chem. Biol.* **17**, 863–871 (2010).

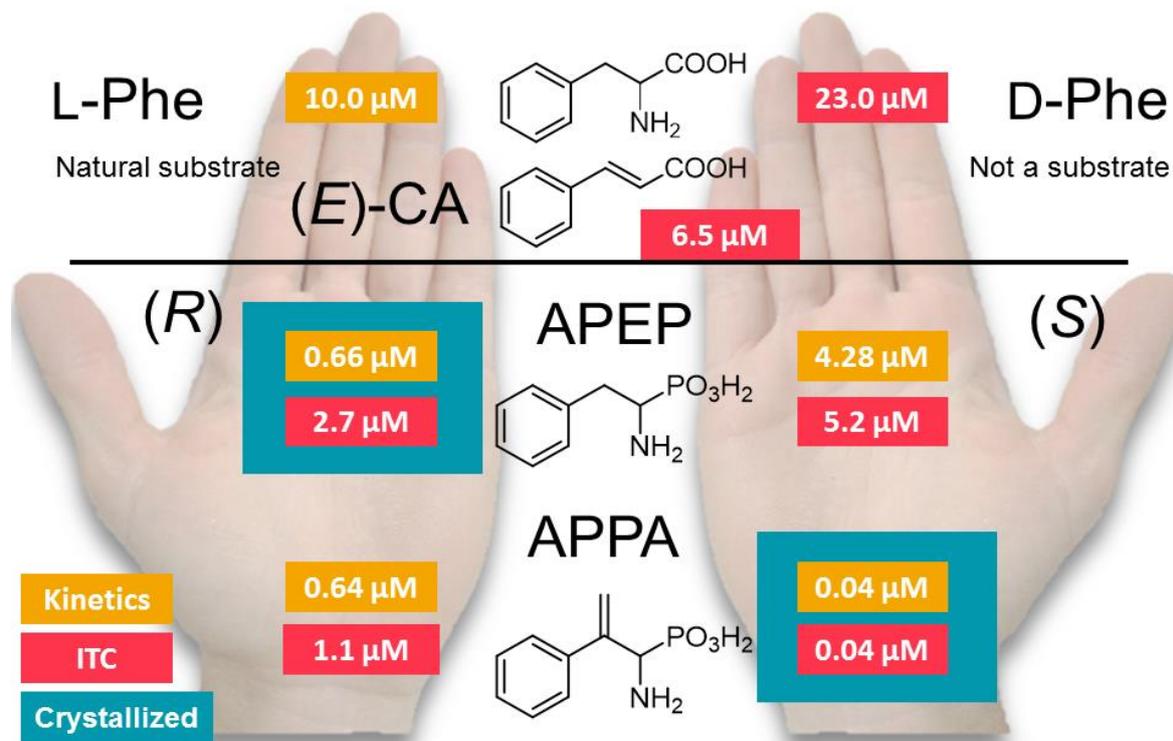
<sup>22</sup> Nedrud, D. M. *et al. Chem. Sci.* **5**, 4265–4277 (2014).

<sup>23</sup> Devamani, T. *et al. J. Am. Chem. Soc.* **138**, 1046–1056 (2016).

<sup>24</sup> Rauwerdink, A. *et al. Mol. Biol. Evol.* **33**, 971–979 (2016).

direct evolutionary routes to mutate the 9 residues that differ between Hb-A9-H6 and wild-type HbHNL.

### 3.2 Characterization of aminophosphonic acid inhibitors



**Figure 7. Kinetic and thermodynamic characterization of binding to PcPAL of various enantiomers of the natural substrates and aminophosphonic acids.** Yellow backgrounds show the Michaelis constant ( $K_m$ ) for the natural substrate L-Phe and the inhibition constants ( $K_i$ ) for the phosphonic acids. Red backgrounds show the equilibrium dissociation constants ( $K_d$ ) measured by isothermal titration calorimetry. Blue backgrounds highlight the two phosphonic acid enantiomers that were co-crystallized with PcPAL.

Characterization of novel inhibitors enables structural understanding of enzyme mechanism. We found that both enantiomers of the aminophosphonic acid analogue of the natural substrate phenylalanine ((1-amino-2-phenylethyl)phosphonic acid, APEP) and a novel derivative bearing a methylidene at the  $\beta$ -position ((1-amino-2-phenylallyl)phosphonic acid, APPA) inhibited PcPAL (Figure 7). X-ray crystal structure determination unambiguously confirmed the absolute configuration of all tested enantiomers during their synthesis. Enzyme kinetic measurements revealed the enantiomer of the methylidene-substituted substrate analogue as being a mirror image relation to the natural L-phenylalanine as the strongest, and slow binding inhibitor. Unexpectedly (S)-APPA and (R)-APEP proved to be slow binding inhibitors. Such behavior is characteristic of a two-step binding process, indicating that a structural change like conformation change or formation of a covalent bond, results in stronger

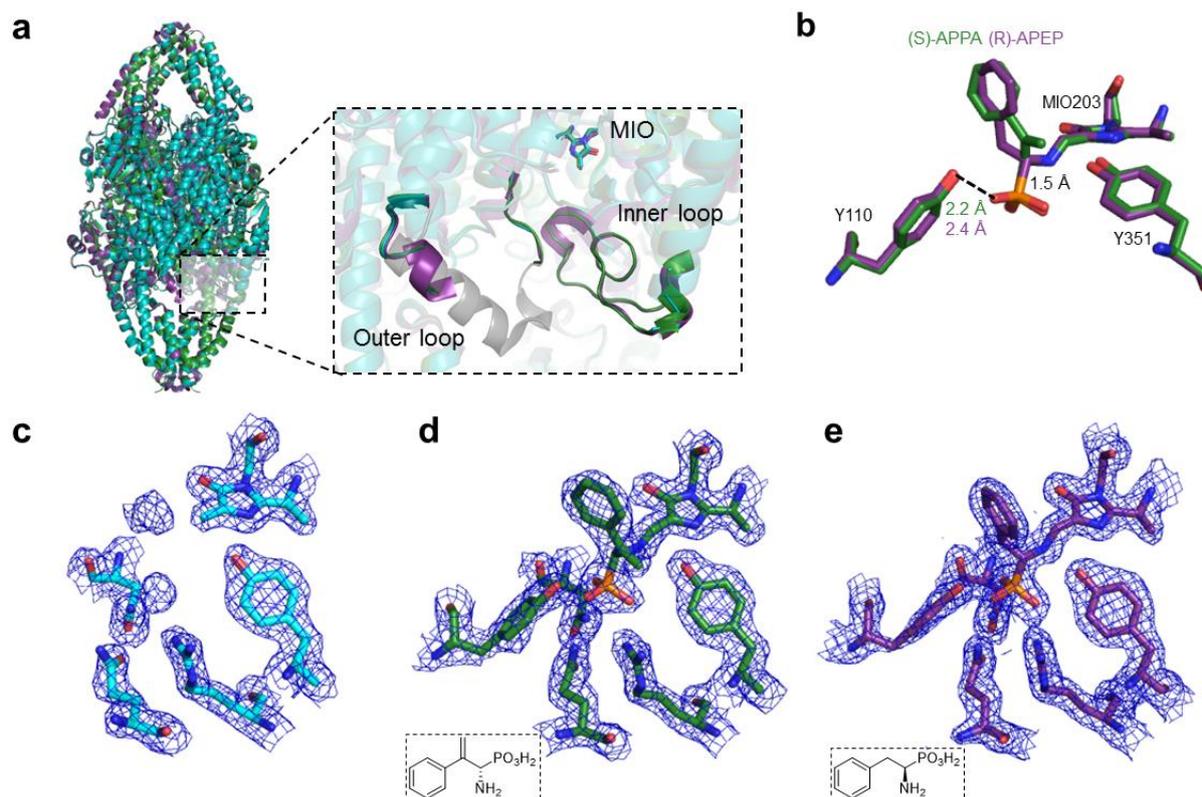
binding. Isothermal titration calorimetry (ITC) confirmed the binding constants, as shown on Figure 7, and provided a detailed analysis of the thermodynamic driving forces of ligand binding.

Kinetic measurements of the inhibitory properties of the phosphonic acid molecules by an additional eukaryotic PAL (RtPAL) and by an additional prokaryotic PAL (AvPAL) proved that the tested molecules were general PAL inhibitors, and thus we proposed those to be general MIO enzyme inhibitors.

### **3.3 Ligand binding in MIO enzymes**

The mirror image binding of (*S*)-APPA and (*R*)-APEP designed them to be ideal models for examining the reaction mechanism and the molecular basis of the enantioselectivity of the PAL reaction. Surprisingly, in the X-ray crystal structures of PcPAL, Figure 8, both inhibitors bound covalently to the MIO catalytic electrophile with their amino groups, despite only reversibly inhibiting the enzyme. This finding provided the first direct experimental evidence for the N-MIO mechanism for PAL. Position of the  $\beta$ -carbon atom differs the most between the two enantiomers, thus this difference is likely a general feature determining the enantioselectivity of the MIO enzymes, Figure 8 b.

Based on crystallographic and molecular modeling data four separate arguments disproved the biological relevance of the loop-out conformation in PcPAL and questioned the loop flexibility hypothesis in PAL and PAM. First we did not find any electron densities where the inner loop was modeled in the 1W27<sup>16</sup>. Second, no traces of the loop out conformation were seen in any of the novel PcPAL crystal structures. Third, RAMD simulations showed no transition to the loop-out conformation during ligand release. Fourth, RMSF values show identical dynamics for the inner loop residues in PcPAL and TcPAM during normal molecular dynamics simulations as well as during substrate release. As a result, it is quite probable that these conclusions about inner loop dynamics are valid for all MIO enzymes.



**Figure 8. Novel structures of PcPAL show the inner loop in a catalytically active conformation and suggest the origins of enantioselectivity of PALs.** **a.** Overlay of the cartoon representation of the three PcPAL structures (6H2O cyan, 6F6T green, 6HQF purple, with a close up view into the active site highlighting the inner and outer loops covering that. **b.** Overlay of the two inhibitor bound PcPAL active sites. **c.** Apo structure of PcPAL, 6H2O: cyan. **d.** (S)-APPA bound PcPAL structure, 6F6T: green. **e.** (R)-APEP bound PcPAL structure 6HQF: purple. Electron densities (blue mesh, 2Fo-Fc map, contoured at 1  $\sigma$  level) are displayed for the catalytically essential residues (Tyr110, Mio203, Tyr351) and for residues in direct interactions with the hydrophilic part of the ligands (Gln348, Arg354, Asn384).

Enhanced sampling simulations of the substrate access/ product release pathways in eukaryotic PAL and PAM indicated two main paths in both enzymes. The first path was previously suggested based on static crystallographic data<sup>25</sup>. The second yet unexplored one, coincided with a conserved water channel connecting the active site to the multi-helix domain in all known MIO enzymes, except for (S)-selective PAM, like *Pantoea agglomerans* PAM (PaPAM). All known such PAMs contain conserved mutations compared to the usual MIO enzymes, each closing one of the egress paths. We have selected, cloned and characterized KkPAL to be the model enzyme for testing the effects of these mutation on the enzymatic activity and attempt to engineer a *de novo* PAM activity for the first time.

<sup>25</sup> Christianson, C. V., *et al.* *Biochemistry* **46**, 7205–7214 (2007).

## 4 Theses

- I. We created identical active sites in the AtHNL and in the HbHNL scaffolds, and measured different enantioselectivities for these enzymes despite their theoretically identical active sites. Using molecular dynamics, I showed that catalytically active conformations could indicate the reaction mechanism in HNLs. Based on the catalytically active conformations during the molecular dynamics simulations of the composite active sites, I suggested the Asn mechanism for the composite active sites, which we hypothesized to be an ancestral HNL mechanism. [2]
- II. By analyzing the relative movement of the active site residues I identified seventeen of the forty-four residues occupy different positions rendering the theoretically identical active sites different in shape and function. I described two groups of residues whose relative positions correlated, and suggested that the second shell residues from these groups are the origins of the active site shape difference. [2]
- III. I characterized the binding of five aminophosphonic acid molecules to PcPAL using kinetic assays. Amongst those I characterized the binding of the most potent inhibitors; (1-amino-2-phenylethyl)phosphonic acid (APEP) and (1-amino-2-phenylallyl)phosphonic acid (APPA), in their enantiomerically pure form by kinetic analysis and isothermal titration calorimetry as well. I demonstrated that the presence of the methylidene group reverses the enantiopreference of PcPAL. I showed that (*R*)-APEP and (*S*)-APPA show slow binding kinetic characteristics. [1,3]
- IV. I measured the inhibition of another eukaryotic (RtPAL) and a prokaryotic (AvPAL) by enantiomerically pure APEP and APPA, thus showed those to be general PAL inhibitors and perhaps general MIO enzyme inhibitors. [3]
- V. I solved the crystal structure of PcPAL in an apo form and in complex with (*R*)-APEP and with (*S*)-APPA. I demonstrated that binding of the (*R*) and (*S*) enantiomers differs mostly in the position of the  $\beta$  carbon atom and suggested this to be the key element for the enantioselectivity of PAL enzymes.
- VI. I found independent arguments indicating that the loop-in conformation is the catalytically competent conformation of the inner loop, that does not open significantly during ligand binding. First, reevaluation of the experimental data of 1W27<sup>16</sup> showed no electron densities for the inner loop residues in the loop-out conformation. Second, I observed no indication for the loop out conformation in any of the novel PcPAL crystal structures, but found well defined electron densities in the

loop-in conformation in the inhibitor bound structures. Third, RAMD simulations showed no transition to the loop-out conformation during ligand release.

- VII. We identified and recombinantly expressed a novel MIO enzyme from *Kangiella koreensis*. Subsequent substrate analysis proved the enzyme to be a PAL. We showed that KkPAL has a high melting temperature, 81 °C. Thanks to the high  $T_m$  KkPAL may accommodate numerous potentially destabilizing mutations without hindering its expressibility making it an ideal candidate for enzyme engineering studies. [4]

## 5 Potential applications

Biocatalysis has great potential in developing sustainable environmental friendly chemical synthesis on laboratory and on industrial scale as well. Nature offers a great number of protein biocatalysts, enzymes. Those are already being used in many asymmetric synthetic and kinetic resolution reactions<sup>1,3</sup>. Cyanohydrins synthesized using hydroxynitrile lyases offer a broad range of possible follow-up reactions to give a wide spectrum of products, including hydroxy acids, primary and secondary  $\beta$ -hydroxy amines, azidonitriles, halogenonitriles, hydroxy aldehydes or ketones, hydroxyl carboxylic acids and esters, hydroxyl amino acids, acyl cyanides and aminonitriles. Application possibilities of MIO enzymes are manifold both in biotechnology and medicine, ranging from synthetic biotransformations to direct human therapy. MIO enzymes may be used for the synthesis of various active pharmaceutical ingredients, drugs candidates or precursors such as L-DOPA, taxol, melphalan or nateglinide<sup>9</sup>. Insights into the structure-function relationships of HNLs and MIO enzymes may enable, a deeper understanding of their catalytic mechanism and the molecular origins of their enantioselectivity, either of which may vary if the enzymes employed to synthesize their non-natural substrates in promiscuous reactions. This understanding will facilitate improvement of biocatalytic processes and thus contribute to the advent of the 4<sup>th</sup> wave of biocatalysts.

## 6 Publication list

### 6.1 List of my publications giving the foundation of the thesis points

1. Bata Zs., Belle E.:  
**Biokatalizátorok a zöldebb jövőért- enzimek a vegyiparban,**  
Élet és tudomány, 2018. 03. 23. LXXIII, 12. (BZs 55%)
2. Jones B.J., Bata Zs., Kazlauskas R.J:  
**Identical active sites in hydroxynitrile lyases show opposite enantioselectivity and reveal possible ancestral mechanism.**  
*ACS Catalysis*, 7, 4221–4229, **2017** (IF 10.614, BZs 100%, FI:1)
3. Bata Zs., Qian R., Roller A., Horak J., Bencze L. Cs., Paizs Cs., Hammerschmidt F., Vertessy B. G., Poppe L.:  
**Metylidene group on phosphonic acid analogue of phenylalanine reverses the enantiopreference of phenylalanine ammonia-lyases**  
*Advanced Synthesis and Catalysis*, 359, 2109-2120, **2017** (IF 5.646, BZs 100%, FI:2)
4. Varga A., Bata Zs., Csuka P, Bordea D., Vértessy B.G., Marcovici A., Irimie F. D., Poppe L., Bencze L. Cs.:  
**A novel phenylalanine ammonia-lyase from *Kangiella koreensis*,**  
*Studia Universitatis Babes-Bolyai Chemia*. LXII, 3, 293-308, **2017** (IF: 0.148, BZs 90%)

### 6.2 List of my other publications

1. Bata Zs., Vertessy B. G., Poppe L.,  
**Enzimmérsétség fenilalanin ammonia liázon**  
*Tavaszi szél konferencia kiadvány*, 2018.05.04-06., Győr, Hungary
2. Banoczi G., Szabo Cs., Bata Zs., Filip A., Hornyanszky G., Poppe L.:  
**Structural modeling of phenylalanine ammonia-lyases and related MIO-containing enzymes- and insight into thermostability and ionic interactions.**  
*Studia Universitatis Babes-Bolyai Chemia* 60(4), **2015** (IF: 0.148)
3. Tomin A, Weiser D, Hellner G, Bata Zs., Corici L, Péter F, Koczka B, Poppe L.:  
**Fine tuning the second generation sol-gel lipase immobilization with ternary alkoxysilane precursor systems.**  
*Process Biochemistry* 46, 52-58. **2011** (IF: 2.627)
4. Tomin A., Weiser D., Bata Zs., Corici L., Péter F., Poppe L.:  
**Entrapment of Lipases in Novel Sol-gel Systems.**  
*Studia Universitatis Babes-Bolyai Chemia*, 54(2), 77-82, **2009** (IF: 0.231)

## 6.3 List of conference presentations related to the thesis

### 6.3.1 Oral presentations at conferences

Bata Zs., Molnár B., Leveles I., Varga A., Paizs Cs., Poppe L., Vertessy B. G.:  
**Structural snapshots of multiple enzyme-ligand complexes pave the road for semi rational enzyme engineering.**

3. Debreceni Röntgendiffrakciós Kerekasztal Konferencia, 2019. 01. 24, Debrecen, Hungary

Bata Zs., Csuka P., Varga A., Bánóczy G., Madaras E., Bencze L. Cs., Paizs Cs., Vértessy B. G., Poppe L.:

**Expanding the MIO-enzyme toolbox –Novel enzymes, structures and mechanistic considerations**

Novel Enzymes, 2018. 10. 09-12, Darmstadt, Germany

Bata Zs., Molnár B., Leveles I., Varga A., Paizs Cs., Poppe L., Vertessy B. G.:

**Structural snapshots of multiple enzyme-ligand complexes pave the road for semi rational enzyme engineering**

31<sup>st</sup> European Crystallographer's Meeting, 2018. 08. 22-27, Oviedo, Spain

Bata Zs., Madaras E., Vertessy B. G., Poppe L.:

**MIO enzimeszaládban konzervált szubsztrát bekötődési útvonalak feltérképezése változóirányú gyorsítású molekuladinamikai szimulációkkal**

Kemometria és Molekula Modellezés (KeMoMo) szimpózium, 2018. 05. 24-25, Szeged, Hungary

Bata Zs., Qian R., Hammerschmidt F., Paizs Cs., Vertessy B. G., Poppe L.:

**Slow Binding Mechanism of High Affinity Mirror Image MIO Enzyme Inhibitor**

European Molecular Biology Organization (EMBO) Practical course: The application of kinetic methods to dynamic biological systems, 2017. 06. 25-30, Canterbury, England

Bata Zs., Qian R., Roller A., Hammerschmidt F., Paizs Cs., Vertessy B. G., Poppe L.:

**Mirror image inhibitor enhance crystallization of the industrially relevant PcPAL**

Hungarian Molecular Life Sciences 2017. 03. 31- 04.02, Eger, Hungary

Bata Zs., Jones J. B., Kazlauskas R. J.:

**Modeling explanation of altering the enantioselectivity of hydroxynitrilase**

BME Oláh György Doktoráns konferencia, 2017. 02. 02, Budapest, Hungary

Bata Zs., Paizs Cs., Vertessy B. G., Poppe L.:

**A fehérjekristályosítás módszerének bemutatása PcPAL példáján keresztül**

BME Oláh György Doktoráns konferencia, 2017. 02. 02. Budapest, Hungary

Bata Zs., Jones B.J., Kazlauskas R.J.:

**R or S, hogyan döntenek a hidroxinitrilázok?**

Kemometria és Molekula Modellezés (KeMoMo) szimpózium, 2016. 05. 12-13, Miskolc, Hungary

Bata Zs., Vida L, Bánóczy G, Hammerschmidt F., Vértessy B. G., Poppe L.:  
**Computational and experimental investigation of novel type MIO-enzyme inhibitors**  
Kemometria és Molekula Modellezés (KeMoMo) szimpózium, 2015 05. 14-15, Szeged, Hungary

### 6.3.2 Poster presentations at conferences

Bata Zs., Madaras E., Leveles I., Hammerschmidt F., Paizs Cs., Poppe L., Vértessy B. G.:  
**Bioactive 3D structure of phenylalanine ammonia-lyase reveal key insights into ligand binding dynamics**  
BME VBK 145, 2018. 06. 01, Budapest, Hungary

Bata Zs., Madaras E., Leveles I., Hammerschmidt F., Paizs Cs., Poppe L., Vértessy B. G.:  
**Bioactive 3D structure of phenylalanine ammonia-lyase reveal key insights into ligand binding dynamics**  
62<sup>nd</sup> Annual Meeting of the Biophysical society, 2018. 02. 17-21, San Francisco, California, USA. **Flash Talk, 90 s and poster presentation**

Bata Zs., Madaras E., Ibolya L., Hammerschmidt F., Paizs Cs., Poppe L., Vértessy B. G.:  
**Catalytically competent novel 3D structure of phenylalanine ammonia-lyase reveal key insights into ligand binding dynamics**  
Federation of European Biochemical Societies (FEBS) Youth Scientific Forum and Annual meeting, 2017. 09. 7-10 and 2017. 09. 10-14, Jerusalem, Israel

Bata Zs., Leveles I., Qian R., Hammerschmidt F., Paizs Cs., Poppe L., Vértessy B. G.:  
**Catalytically competent structure of eukaryotic phenylalanine ammonia-lyase**  
13<sup>th</sup> International Symposium on Biocatalysis and Biotransformations, 2017. 07. 09-13, Budapest, Hungary, **Short talk, 5 minutes and poster presentation**

Bata Zs., Qian R., Roller A., Hammerschmidt F., Paizs Cs., Vértessy B. G., Poppe L.:  
**Experimental and computational characterization of aminophosphonic acid MIO enzyme inhibitors**, Conference Poster prize  
BME Oláh György Doktoráns konferencia, 2017. 02. 02. Budapest, Hungary

Bata Zs., Qian R., Roller A., Hammerschmidt F., Paizs Cs., Vértessy B. G., Poppe L.:  
**Experimental and computational characterization of aminophosphonic acid MIO enzyme inhibitors**  
8<sup>th</sup> International meeting on biocatalysis, Biocat2016, 2016. 08. 28 – 09. 01. Hamburg, Germany

Bata Zs., Hammerschmidt F., Vértessy B. G., Poppe L.:  
**Experimental characterization of aminophosphonic acid MIO enzyme inhibitors**  
FEBS advances course: Advanced Methods in Macromolecular Crystallization VII.  
2016 06. 27- 07. 02. Nové Hradý, Czech Republic