

腈水解酶的催化混乱性研究进展

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摘要: 腈水解酶作为一种重要的生物催化剂广泛应用于重要医药中间体的合成, 它能高效地将腈基转化为酸和氨, 其反应具有温和、绿色环保等优点。腈水解酶不仅具有催化腈生成对应羧酸产物的水解活性, 即表现出催化专一性, 还兼具催化腈生成酰胺的水合活力, 即表现出催化混乱性。腈水解酶的催化混乱性具有两面性: 酰胺副产物的存在增加了后续羧酸产物分离纯化的难度和成本; 但若精准调控腈水解酶的催化反应路径实现酶功能的重塑, 可以拓宽腈水解酶生物催化的反应类型, 为高值酰胺类化合物的生物合成提供新思路和工艺, 这对人工酶的创制及生物催化均具有重要意义。本文结合近年来相关的研究成果, 综述了当前腈水解酶催化混乱性的研究进展, 并从腈水解酶的进化起源、催化结构域以及催化机理等方面, 探讨可能影响腈水解酶催化混乱性关键调控因子, 为腈水解酶在生物催化领域上的应用提供了借鉴和参考。

关键词: 腈水解酶; 催化混乱性; 酶催化机理; 生物催化

Advances in the catalytic promiscuity of nitrilases

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Abstract: As important biocatalysts, nitrilases can efficiently convert nitrile groups into acids and ammonia in a mild and eco-friendly manner, being widely used in the synthesis of important pharmaceutical intermediates. Early studies reported that nitrilases only had the hydrolysis activity of catalyzing the formation of corresponding carboxylic acid products from nitriles,

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showing catalytic specificity. However, recent studies have shown that some nitrilases exhibit the hydration activity for catalyzing the formation of amides from nitriles, showing catalytic promiscuity. The catalytic promiscuity of nitrilases has dual effects. On the one hand, the presence of amide by-products increases the difficulties and costs of subsequent separation and purification of carboxylic acid products. On the other hand, however, if the catalytic reaction pathways of nitrilases can be precisely regulated to reshape enzyme functions, the reactions catalyzed by nitrilases can be broadened to provide new ideas for the biosynthesis of high-value amides, which is crucial for the development of artificial enzymes and biocatalysis. This review summarized the research progress in the catalytic promiscuity of nitrilases and discussed the key regulatory factors that may affect the catalytic promiscuity of nitrilases from the evolutionary origin, catalytic domains, and catalytic mechanisms, hoping to provide reference and inspiration for the application of nitrilases in biocatalysis.

Keywords: nitrilase; catalytic promiscuity; catalytic mechanisms of enzymes; biocatalysis

传统观点认为, 酶作为生物催化剂高效专一催化一种反应, 即具有严格的催化特异性和专一性(enzyme specificity)。然而, 近年来研究发现一些酶还表现出混乱性(enzyme promiscuity)^[1-2], 也称为杂泛性、宽泛性或多功能性。酶的混乱性可以进一步细分为 3 类(图 1), 分别为条件混乱性(condition promiscuity)、底物混乱性(substrate promiscuity)、催化混乱性(catalytic promiscuity)^[3-5]。条件混乱性表现为酶能在不同于天然反应条件下(如无水介质、极端温度或 pH 值等)进行催化反应, 比如脂肪

酶在水溶液和有机溶剂中均能催化酯类底物发生反应^[6]。底物混乱性表现为酶具有较宽松的底物选择性, 酶可以催化非天然底物历经天然底物相同的催化路径发生反应, 比如甲烷单加氧酶除甲烷外还可羟基化 150 种底物^[7]。而酶的催化混乱性主要指酶在发挥其本身的催化功能外, 还能够催化不同于其“天然”的化学反应, 经历不同的过渡态或者中间体生成相对应的产物^[3-4,8-11]。

酶的催化混乱性被证实在自然界普遍存在, 比如烟草来源的马兜铃烯环化酶(5-epi-

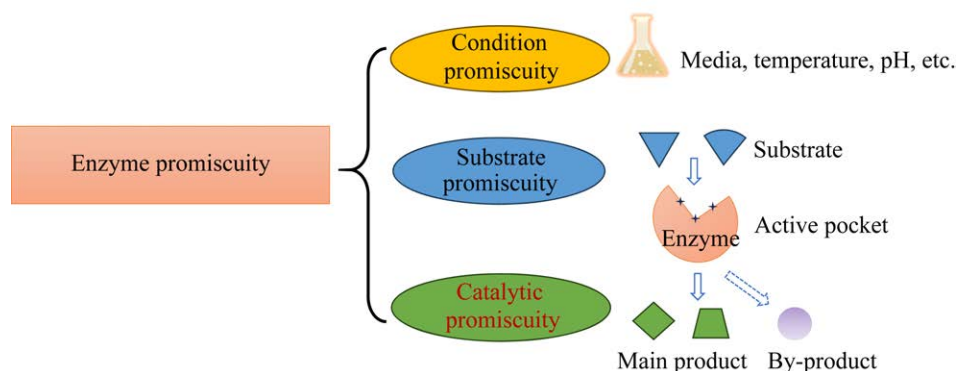


图 1 酶的条件混乱性、底物混乱性以及催化混乱性

Figure 1 Schematic diagram of conditional, substrate and catalytic promiscuity of enzymes.

aristolochene synthase, TEAS)可催化法尼基焦磷酸(farnesyl pyrophosphate, FPP)生成多种倍半萜天然产物^[12-13]。来自于酵母的丙酮酸脱羧酶不仅能催化丙酮酸发生脱羧反应,还能催化乙醛和苯甲醛形成 *R*-苯乙酰甲醇,表现出裂解酶活性^[14]。借助蛋白质工程等技术对具有催化混乱性的天然酶进行设计改造,可以拓宽其生物催化的反应类型^[3,15],实现一酶多用或者老酶新用,在酶工程及生物催化领域具有重要意义^[16-19]。比如, Yang 等^[20]报道了一种来自海洋细菌的脯氨酸二肽酶同时具有水解对氧磷的活性,通过定点饱和突变和组合突变将天然肽酶进化为对氧磷水解酶,为有机磷类污染物提供了微生物降解的高效催化剂。此外, Farwell 等^[21]和 Coelho 等^[22]通过定向进化策略实现了工程化 P450 氧化酶烯烃环氧化反应外的氮杂环丙烷化和环丙烷化等非天然反应类型。

腈水解酶(nitrilase, EC 3.5.5.1)可催化腈类化合物生成相应的羧酸和氨,其催化过程具有反应条件温和、催化速率高效、绿色环保且环境友好等优势,是生物催化领域中重要的催化剂之一,受到学术界和工业界广泛的关注^[23-24]。1964年, Thimann 等^[25]从大麦叶中首次发现了腈水解酶,该酶能够催化 3-吡啶乙腈产生吡啶-3-乙酸。迄今为止,腈水解酶在环保^[26]、医药^[27]和化工^[24]等领域均发挥着重要作用,比如腈水解酶催化烟酸和扁桃酸的生产,这充分体现了腈水解酶的应用潜力,因此对于腈水解酶的研究一直是科研和工业界的热点课题^[28-29]。

一般认为,腈水解酶催化腈类化合物仅能得到对应的羧酸产物,但研究发现一些腈水解酶(尤其是植物来源),同时表现出水合活力生成酰胺产物,即表现出催化混乱性(图 2)^[30-31]。腈水解酶的催化混乱性如一把双刃剑,一方面为高值酰胺类化合物的生物合成提供新思路和

工艺;另一方面水合途径的存在不仅会影响目标羧酸产物的收率,同时酰胺副产物的存在也增加了后续产物分离纯化的难度和成本。但由于缺乏对腈水解酶催化机制以及关键影响因子的认识,目前很难精准地调控腈水解酶催化反应方向,这无疑限制了腈水解酶的开发和工业应用。针对上述问题,本文对腈水解酶的催化混乱性潜在的进化起源、催化结构域以及催化机理等方面进行了综述,探讨可能影响腈水解酶催化混乱性关键调控因子,并结合近年来与其相关的研究实例,总结当前腈水解酶催化混乱性的研究进展,以期创制优良工业属性的腈水解酶提供借鉴和参考。

1 进化起源分析

腈水解酶在自然界中分布广泛,在植物、细菌和真菌等生物中都有发现。整理了文献报道的具有催化混乱性的腈水解酶(表 1),并列出了菌种来源、酶的命名、催化底物、产物中酰胺占比和参考文献出处,需要注意的是,酰胺与羧酸的比例可能会受到实验条件,如温度、pH 或其他环境因素影响。多数情况下,自然界存在的天然腈水解酶水合活力不高,其产物中酰胺占比较低,因此在之前研究中常常被忽视^[53]。直到近年来一些较高水合活力的腈水解酶被报道,研究人员开始重点关注腈水解酶的催化混乱性。Zhang 等^[31]筛选到一株来源于

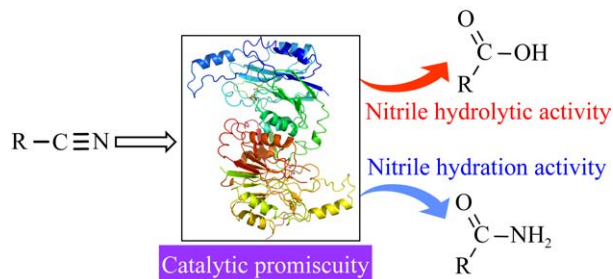


图 2 腈水解酶的催化杂泛性示意图

Figure 2 Schematic diagram of catalytic promiscuity of the nitrilase.

草根围副伯克霍尔德氏菌 (*Paraburkholderia graminis*) DSM 17151 的腈水解酶, 该酶能催化扁桃腈生成约 40% 的扁桃酰胺。Piotrowski 等^[30]发现来自植物拟南芥 (*Arabidopsis thaliana*) 的腈水解酶催化底物 β -氰基-L-丙氨酸分别生成了天冬氨酸及天冬酰胺, 且酰胺的占比高达 60% 以上。根据所催化的底物结构特征, 可以将腈水解酶大致分为^[54]芳香族腈水解酶 (aromatic nitrilase)、脂肪族腈水解酶 (aliphatic nitrilase)、芳基乙腈酶 (arylacetonitrilases)。不过也有一些腈水解酶具有较广的底物谱, 如来源于 *A. thaliana* 的腈水解酶 AtNit1 可催化脂肪族和芳香族腈类。

为了进一步挖掘不同来源的催化混乱性腈水解酶之间的进化关系, 从 UniPort 蛋白数据库中下载了表 1 中腈水解酶的蛋白序列信息,

进行多序列比对和进化关系分析, 分别借助 MOE 软件^[55]进行序列一致性比较并使用 MEGA 11 软件^[56]进行系统进化树构建。从酶的蛋白序列之间一致性分析结果可以看出 (图 3), 植物来源的腈水解酶之间相似度大于 70%, 具有高同源性 (深蓝色区域), 其他物种之间的腈水解酶同源性相对较低 (红色区域)。令人感兴趣的是, 来源于大豆根瘤菌 (*Bradyrhizobium japonicum*) USDA110 和假单胞菌 (*Pseudomonas* sp.) UW4 的腈水解酶与植物来源的腈水解酶之间的序列相似度较高 (浅蓝色区域), 推测其进化关系上也是如此。进一步的进化关系分析发现 (图 4), 细菌来源的 BrjNIT 和 PsNIT 腈水解酶表现出特殊的进化起源, 并不是和其他细菌腈水解酶位于同一进化分支, 推测是和植物来源的腈水解酶由同一祖先酶共同进化而来。

表 1 文献中报道的催化混乱性腈水解酶

Table 1 The catalytic promiscuity of the nitrilases reported in the literature

Class	Organism	Substrate	Amide ratio (%)	References	
Plant	<i>Arabidopsis thaliana</i> NIT1 (<i>AtNIT1</i>)	2-fluorobenzyl cyanide	70–85	[32–33]	
		2-fluoro-2-(3-methylphenyl) ethanenitrile			
		2-fluoro-2-(4-methylphenyl) ethanenitrile			
		2-fluoro-2-(4-nitrophenyl) ethanenitrile			
		2-fluoro-2-(3-methoxyphenyl) ethanenitrile			
		Fumaronitrile	93–95		[33]
	<i>Arabidopsis thaliana</i> NIT4 (<i>AtNIT4</i>)	<i>Arabis alpina</i> (<i>AaNIT</i>)	3-nitroacrylonitrile		[33–35]
			2-butenitrile	≤5	
			3-methoxyacrylonitrile		
			α -fluorobutyronitrile		
			2-phenylacetoneitrile		
			2-isobutyl-succinonitrile		
			<i>Brassica rapa</i> (<i>BrNIT</i>)	β -cyano-L-alanine	
<i>Brassica oleracea</i> (<i>BoNIT</i>)	<i>Brassica oleracea</i> (<i>BoNIT</i>)	Isobutylsuccinonitrile	About 3	[34–35]	
		Phenylacetoneitrile			
		Isobutylsuccinonitrile	10–15		
		Phenylacetoneitrile			
		Mandelonitrile	62		
<i>Brassica oleracea</i> (<i>BoNIT</i>)	<i>Brassica oleracea</i> (<i>BoNIT</i>)	Mandelonitrile	45	[34]	
		Phenylacetoneitrile	3		

(待续)

(续表 1)

Class	Organism	Substrate	Amide ratio (%)	References
	<i>Camelina sativa</i> (CsNIT)	Mandelonitrile	61	
		Phenylacetoneitrile	17	
	<i>Capsella rubella</i> (CrNIT)	Mandelonitrile	61	
		Phenylacetoneitrile	18	
	<i>Eutrema salsugineum</i> (EsNIT)	Mandelonitrile	13	
		Phenylacetoneitrile	1	
	<i>Oryza sativa</i> (OsNIT)	Mandelonitrile	72	
		Phenylacetoneitrile	49	
	<i>Nicotiana tabacum</i> TNIT4 (NtNIT4)	β -cyano-L-alanine	About 50	[30]
	<i>Zea mays</i> NIT2 (ZmNIT2)	Benzyl cyanide	≤ 10	[36]
		1,4-dicyanobutane		
		Mandelonitrile		
		<i>n</i> -butyronitrile	19–40	
		Valeronitrile		
		Hexanenitrile		
		Heptanenitrile		
		β -hydroxynitrile series	63–88	
Bacteria	<i>Alcaligenes faecalis</i> ATCC 8750 (AlfNIT)	Mandelonitrile	<1	[37]
		2-phenylpropionitrile		
	<i>Acidovorax facilis</i> 72W (AcfNIT-72W)	2-cyanopyridine	<1	[38]
	<i>Acidovorax facilis</i> (AcfNIT)	2-chloronicotinonitrile	54	[39]
	<i>Bradyrhizobium japonicum</i> strain USDA110 (BrjNIT)	3-aminopropionitrile	23	[40]
	<i>Pseudomonas fluorescens</i> EBC191 (PfNIT)	2-(methoxy)-mandelonitrile	<5	[37,41-46]
		2-phenylacetoneitrile		
		2-phenylpropionitrile		
		(<i>R</i>)-phenylglycinenitrile		
		(<i>S</i>)-mandelonitrile	10–50	[41-42]
		(<i>R</i>)-mandelonitrile		
		(<i>S</i>)-phenylglycinenitrile		
		mandelonitrile		
		(<i>R</i>)-O-acetylmandelonitrile		
		(<i>R</i>)-2-acetoxy-2-phenylacetoneitrile		
		O-acetylmandelonitrile		
		(<i>S</i>)-2-acetoxy-2-phenylacetoneitrile	60–90	[42]
		2-chloro-2-phenylacetoneitrile		
	<i>Paraburkholderia graminis</i> DSM 17151 (PgNIT-17151)	Mandelonitrile	About 40	[31]
	<i>Paraburkholderia graminis</i> (PgNIT)	2-chloronicotinonitrile	About 30	[39,47]
	<i>Pseudomonas</i> sp. UW4 (PsNIT)	Indole-3-acetonitrile	About 81	[48]
	<i>Rhodococcus</i> ATCC 39484 (RhNIT-39484)	Phenylacetoneitrile	2	[49]
	<i>Rhodococcus zopfii</i> (RhznIT)	2-chloronicotinonitrile	88	[39]
	<i>Synechocystis</i> sp. PCC 6803 (SsNIT)	2-cyanopyridine	2	[50]
Fungi	<i>Gibberella intermedia</i> CA3-1 (GiNIT)	3-cyanopyridine	<5	[51]
	<i>Neurospora crassa</i> OR74A (NcNIT)	Mandelonitrile	40	[52]

Identity (%)	<i>At</i> NIT4	<i>At</i> NIT1	<i>Aa</i> NIT	<i>Br</i> NIT	<i>Cr</i> NIT	<i>Cs</i> NIT	<i>Es</i> NIT	<i>Os</i> NIT	<i>Ni</i> NIT4	<i>Zm</i> NIT2	<i>Ac</i> fNIT	<i>Al</i> fNIT	<i>Br</i> jNIT	<i>Pf</i> NIT	<i>Ps</i> NIT	<i>Pg</i> NIT	<i>Rhz</i> NIT	<i>Ss</i> NIT	<i>Nc</i> NIT
<i>At</i> NIT4	100.0	74.4	75.6	74.4	73.7	74.4	74.8	77.9	83.2	77.9	40.8	42.7	63.4	43.1	59.2	42.7	43.5	43.9	39.3
<i>At</i> NIT1	74.4	100.0	91.0	85.8	91.3	89.6	88.7	75.7	75.1	75.4	45.1	43.9	59.5	44.2	55.5	38.7	44.2	43.6	41.9
<i>Aa</i> NIT	75.6	91.0	100.0	88.7	90.5	89.3	90.8	74.3	76.6	73.7	44.5	43.4	61.0	44.2	55.2	39.0	45.4	42.2	42.8
<i>Br</i> NIT	74.4	85.8	88.7	100.0	86.6	86.0	87.8	73.5	75.0	73.5	45.1	44.5	59.0	44.2	54.7	39.5	45.1	43.9	41.3
<i>Cr</i> NIT	73.7	91.3	90.5	86.6	100.0	94.2	85.6	72.7	72.4	72.4	42.5	41.7	56.9	42.3	54.1	37.6	41.4	40.6	38.4
<i>Cs</i> NIT	74.4	89.6	89.3	86.0	94.2	100.0	84.4	73.5	73.5	72.7	43.5	42.6	57.4	43.7	54.3	38.4	43.5	42.6	39.6
<i>Es</i> NIT	74.8	88.7	90.8	87.8	85.6	84.4	100.0	73.9	74.8	73.7	43.6	42.8	60.3	43.9	55.5	39.1	43.3	41.1	40.2
<i>Os</i> NIT	77.9	75.7	74.3	73.5	72.7	73.5	73.9	100.0	84.8	92.5	42.5	39.2	60.5	43.1	54.4	39.5	42.3	42.0	37.0
<i>Ni</i> NIT4	83.2	75.1	76.6	75.0	72.4	73.5	74.8	84.8	100.0	85.7	44.7	40.7	61.3	45.3	56.4	40.1	44.4	43.6	38.7
<i>Zm</i> NIT2	77.9	75.4	73.7	73.5	72.4	72.7	73.7	92.5	85.7	100.0	41.6	40.7	60.1	43.8	55.1	40.2	42.4	42.9	37.7
<i>Ac</i> fNIT	40.8	45.1	44.5	45.1	42.5	43.5	43.6	42.5	44.7	41.6	100.0	53.1	37.7	59.1	38.5	41.2	66.4	45.3	50.7
<i>Al</i> fNIT	42.7	43.9	43.4	44.5	41.7	42.6	42.8	39.2	40.7	40.7	53.1	100.0	36.8	61.0	39.0	46.6	56.2	48.0	47.2
<i>Br</i> jNIT	63.4	59.5	61.0	59.0	56.9	57.4	60.3	60.5	61.3	60.1	37.7	36.8	100.0	43.0	60.7	40.5	42.4	45.5	38.9
<i>Pf</i> NIT	43.1	44.2	44.2	44.2	42.3	43.7	43.9	43.1	45.3	43.8	59.1	61.0	43.0	100.0	40.0	46.9	62.3	50.6	50.6
<i>Ps</i> NIT	59.2	55.5	55.2	54.7	54.1	54.3	55.5	54.4	56.4	55.1	38.5	39.0	60.7	40.0	100.0	41.7	44.6	45.6	41.0
<i>Pg</i> NIT	42.7	38.7	39.0	39.5	37.6	38.4	39.1	39.5	40.1	40.2	41.2	46.6	40.5	46.9	41.7	100.0	48.2	67.6	43.2
<i>Rhz</i> NIT	43.5	44.2	45.4	45.1	41.4	43.5	43.3	42.3	44.4	42.4	66.4	56.2	42.4	62.3	44.6	48.2	100.0	50.3	50.3
<i>Ss</i> NIT	43.9	43.6	42.2	43.9	40.6	42.6	41.1	42.0	43.6	42.9	45.3	48.0	45.5	50.6	45.6	67.6	50.3	100.0	43.8
<i>Nc</i> NIT	39.3	41.9	42.8	41.3	38.4	39.6	40.2	37.0	38.7	37.7	50.7	47.2	38.9	50.6	41.0	43.2	50.3	43.8	100.0

图 3 不同来源腈水解酶之间的蛋白序列一致性对比结果 图酶名称缩写的全称见表 1。数值代表蛋白序列的相似度。

Figure 3 Comparative results of protein sequence identity between nitrilases from different sources. Full name of the enzyme name abbreviations in the figure is shown in Table 1. Numerical value represents the similarity of protein sequence.

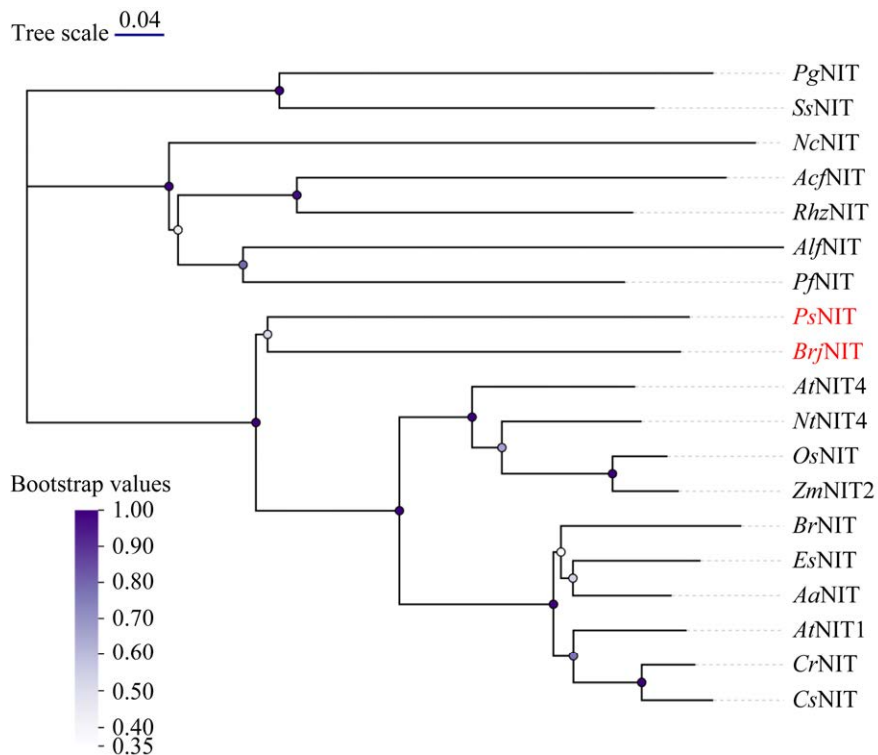


图 4 植物、细菌和真菌来源的腈水解酶的系统进化树

Figure 4 Phylogenetic tree for nitrilases in bacteria, fungi and plant.

2 催化结构域分析

为了进一步研究脲水解酶功能和结构之间的构效关系,需要对脲水解酶的结构进行解析。研究人员通过 X 射线晶体学和核磁共振等结构生物学方法解析了酶的三维结构^[57-58],收集了目前 RCSB PDB 数据库已收录的脲水解酶晶体的 PDB ID 号、来源、基因、分辨率等信息(表 2)。脲水解酶一般以同源二聚体或者多聚体的形式存在,以拟南芥脲水解酶的晶体结构(图 5)为例,其存在形式为同源十二聚体,最小重复单元为四聚体,并且每个单体具有典型的 $\alpha\beta\alpha$ 三明治结构特征。催化活性中心具有高度保守的催化三联体半胱氨酸-谷氨酸-赖氨酸(Cys-Glu-Lys),其在脲水解酶发挥催化功能中扮演重要的角色^[37],Cys 作为亲核试剂,Lys 及 Glu 一般被认为起到稳定反应中间体的作用,此外 Glu 也作为广义碱,夺取半胱氨酸的质子以活化半胱氨酸。另外,研究表明多聚体相互作用的界面残基(“A” “C” surface)也会影响酶的活性和催化特性^[34]。Park 等^[59]将来源于敏捷食酸菌(*Acidovorax facilis*) ZJB09122 脲水解酶的“A”界面上第 201 位点苏氨酸替换为苯丙氨酸,可增强底物在蛋白口袋中的稳定性;同时发现将“C”界面的第 339 和 343 位点的谷氨酸替换为正电性残基赖氨酸,有利于增强蛋白-蛋白的相互作用;最终获得了 45 °C 下的半衰期延长了约 14 倍的最佳突变体 T201F/Q339K/Q343K。

相对于已报道的脲水解酶序列,目前解析的脲水解酶晶体数量仍很少,且脲水解酶与底物的共晶复合物结构尚未解析出来。随着计算生物学技术的快速发展,可借助计算工具构建蛋白质三维结构,分子对接和分子动力学模拟等^[34,38-39,47,50,57,60]计算手段获得底物和酶的复合物结构模型。基于蛋白质三维结构信息,锚定要改造的候选区域及关键氨基酸位点,通过

定点突变等手段获得催化性能更优的人工改造酶。前期本课题组^[34]对水稻(*Oryza sativa*)来源脲水解酶(*OsNIT*)的催化混乱性进行了深入研究,通过获得合理的复合物模型指导天然 *OsNIT* 酶的分子改造,实现对催化反应路径的双向调控。*OsNIT* 酶的晶体结构尚未被解析出来,借助 AlphaFold2 工具构建了 *OsNIT* 酶的空蛋白结构。然后采用 MOE 软件将底物苯乙脲对接到蛋白活性空腔,获得多个候选的复合物初始模型。由于分子对接得到的是静态结构,为了模拟蛋白在生理状态下动态变化过程,借助分子动力学模拟软件 AMBER 对初始对接模型进行 30 ns 时长的动态弛豫。通过对 MD 模拟轨迹进行聚类分析获得了代表性结构(图 6),并标注了影响酶的催化混乱性的区域如活性口袋、底物进出通道以及“A/C”界面(图 6A),通过对底物的结合特征分析(图 6B),可以看到底物的氰基 C 原子与催化三联体的 Cys196 的 S 原子之间的距离为 3.2 Å,Thr220 通过氢键作用进一步固定 Cys196,此时复合物处于可发生催化反应的“预反应态”(near-attack state)。催化三联体中的 Glu71 可发挥催化碱的作用,接受碳正中间体的质子氢从而终止催化反应。催化三联体中的 Lys163 与底物的氰基 N 原子形成氢键作用从而固定底物的结合姿势。此外,在催化三联体相邻的区域存在 1 个带负电的 Glu169,其负电性可以维持 Lys163 处于质子化状态。Trp197 的芳香侧链与底物苯乙脲形成 π - π 堆积作用力,对于维持反应构象具有重要的意义。在催化三联体空间相邻区域的 Ile136 和 Asn246,通过空间立体位阻分别固定 Cys196 和 Glu71。对 246、136 位残基突变调控关键距离从而改变产物中酰胺的占比,最终获得了 2 株选择性催化生成主产物羧酸(98.49%)的三突变体和选择性催化生成酰胺(96.36%)的六突变体。

表 2 RCSB PDB 数据库收录的脲水解酶基本结构信息

Table 2 Basic structure information of nitrilases in the RCSB PDB database

PDB entry	Organism	Gene names	Resolution (Å)	Chains	Sequence length (bp)	Released (year)
6ZBY	<i>Pseudomonas fluorescens</i>	<i>nitA</i>	3.10	Homo 12-mer	350	2021
6I00	<i>Arabidopsis thaliana</i>	<i>NIT4</i>	3.40	Homo 12-mer	361	2019
6I5T	<i>Arabidopsis thaliana</i>	<i>NIT4</i>	3.90	Homo 6-mer	361	2019
6I5U	<i>Arabidopsis thaliana</i>	<i>NIT4</i>	3.90	Homo 6-mer	361	2019
3WUY	<i>Syechocystis</i> sp. PCC 6803	<i>merR</i>	3.10	Homo 2-mer	349	2014
3IVZ	<i>Pyrococcus abyssi</i> GE5	<i>PAB1449</i>	1.57	Homo 2-mer	262	2010
3K18	<i>Pyrococcus abyssi</i> GE5	<i>PAB1449</i>	1.60	Homo 2-mer	262	2010
3IW3	<i>Pyrococcus abyssi</i> GE5	<i>PAB1449</i>	1.80	Homo 2-mer	262	2010
3KLC	<i>Pyrococcus abyssi</i> GE5	<i>PAB1449</i>	1.76	Homo 2-mer	262	2010

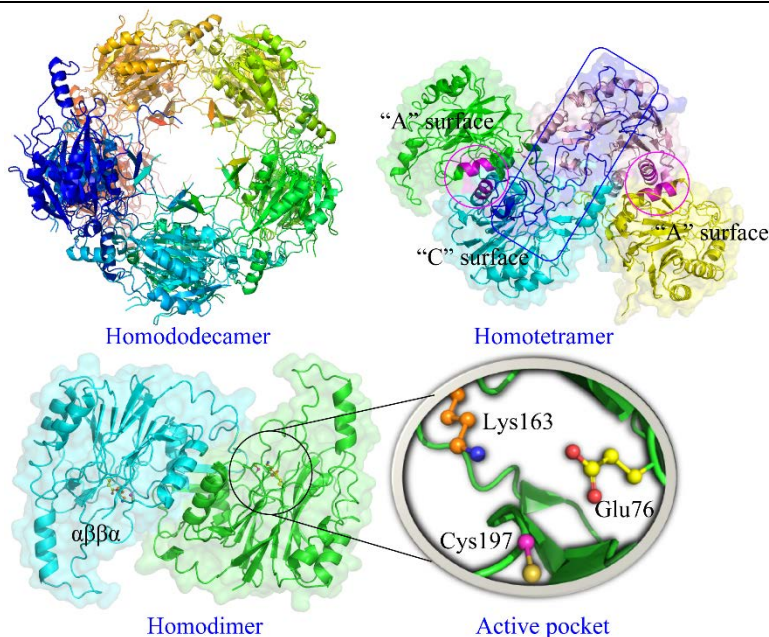


图 5 拟南芥脲水解酶(PDB ID: 6I00)的多聚体结构和催化三联体示意图

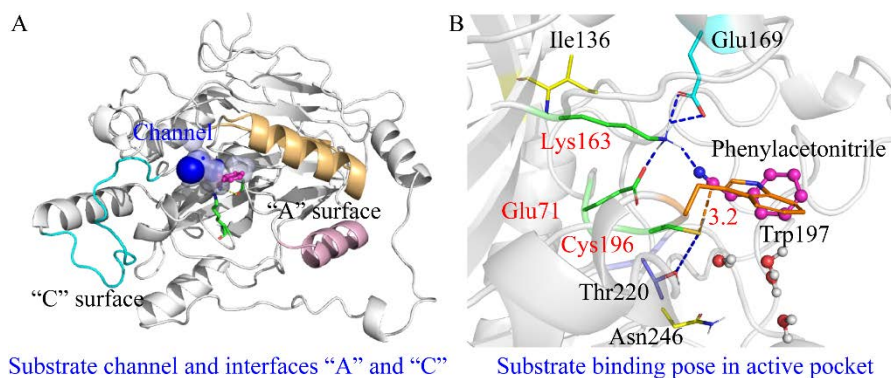
Figure 5 Schematic diagram of the multimeric structure and catalytic triad of *Arabidopsis thaliana* nitrilase (PDB ID: 6I00).

图 6 苯乙腈-OsNIT 复合物结构中的关键催化域(A)及底物在蛋白活性口袋中的结合特征分析(B)

Figure 6 The key catalytic domains in the phenylacetone nitrile-OsNIT complex structure (A) and analysis of substrate binding pose in the protein active pocket (B).

3 脲水解酶催化混乱性的机理研究

脲水解酶的催化活性位点含有高度保守的催化三联体半胱氨酸-谷氨酸-赖氨酸(Cys-Glu-Lys)^[23,61], 其中 Cys 和 Glu 起关键作用, 直接参与催化反应, 虽然 Lys 不直接参与催化过程, 但在维持底物在活性口袋中的结合模式以及稳定催化过程中形成的四面体中间体起到了非常重要的作用^[62]。脲水解酶虽然具有水解酶超家族经典的催化三联体结构特征, 但目前尚未在脲水解酶中发现特定氧阴离子口袋(oxyanion pocket)的存在。其他水解酶的催化三联体中充当亲核试剂的残基多为 Ser 或者 Thr, 而脲水解酶是由半胱氨酸 Cys 作为亲核试剂。已知 Cys 的 pKa 数值约为 8, Ser 和 Thr 的 pKa 数值约为 13, 因此生理状态下(pH 7), Ser 和 Thr 在酶发生催化反应前均处于质子化状态, 相反 Cys 能够以质子化和去质子化状态交替存在, 这说明在催化反应前脲水解酶催化三联体中 Cys 的活化可能会自发进行, 表明脲水解酶的催化机制可能与其他水解酶存在差异。

脲水解酶同时产生羧酸和酰胺(催化混乱)的催化机理虽然是研究者的关注重点之一, 但由于目前脲水解酶的晶体结构解析较为匮乏, 且天然底物脲化合物与脲水解酶结合的复合物晶体尚未见报道, 所以脲水解酶的催化机制仍处于研究初期^[34,38,50,62-66]。目前相对被认可的一种脲水解酶催化机理假说如图 7 所示, 底物进入活性位点后, 催化三联体之一的 Cys 残基上的巯基(-SH)作为亲核试剂进攻底物氰基(-CN) α -碳原子, 形成硫代亚胺-酶中间体(中间体 I), 接着 1 分子水加成到中间体 I 得到 1 个四面体中间体(中间体 II); 从中间体 II 开始发生分歧, 通过 2 种路径反应得到产物: 在路径 A 中, 通过水解途径(实线箭头)质子化末端的-NH₂ 基团, 得到羧酸产物和氨; 而路径 B

中, 中间体 II 发生硫醇消除形成酰胺产物, 这步反应称为水合反应途径(虚线箭头)。

根据上述所推测脲水解酶催化混乱性的机制, 底物氰基 N 原子与催化三联体 Glu 羧基 O 原子之间的距离(氮氧距离 D_{NO})和催化三联体 Cys 巯基 S 原子与 Glu 羧基 O 原子之间的距离(硫氧距离 D_{SO}), 可能会影响脲水解酶催化混乱性的程度。当 D_{NO} 比 D_{SO} 小, 即 $D_{SO}-D_{NO}>0 \text{ \AA}$, 四面体中间体 II 的氨基更容易质子化, 此时路径 A (水解途径)为主路径, 羧酸为主产物; 反之即 $D_{SO}-D_{NO}<0 \text{ \AA}$, 则水合途径占主导, 产物中酰胺的占比更高。Jiang 等^[50]通过计算模拟构建了中间体 II 并监测了 MD 模拟过程中 D_{SO} 和 D_{NO} 的差值($D_{SO}-D_{NO}$), 发现野生型脲水解酶的差值约 1.1 \AA , 仅产生少量的酰胺(占比 2.1%); 突变体 F193A、F193D 和 F193N 差值为 -0.4 \AA 、 -0.6 \AA 和 -1.1 \AA , 分别产生了 42%、66.2%和 73.4%的酰胺; 突变体 F193Y 和 F193K 的差值都大于 0 (分别是 0.6 \AA 和 1.4 \AA), 其产物分布类似于野生型酶, 都只产生少量酰胺。A. facilis 72W 来源的野生型脲水解酶催化 2-氰基吡啶产生的酰胺/羧酸为 0.4, 突变体 W188M 产物中酰胺/羧酸高达 78.3。Wang 等^[38]分别比较了野生型酶和突变体中 $D_{SO}-D_{NO}$ 差值, 在野生型酶中 $D_{SO}-D_{NO}=0.3 \text{ \AA}$, 突变体 W188M 中 $D_{SO}-D_{NO}=-1.4 \text{ \AA}$, 因此突变体 W188M 中酰胺为主产物。Tang 等^[34]的研究也有类似的结果, 水稻来源的脲水解酶催化苯乙脲底物时(图 8), Asn246 和 Ile136 通过空间立体位阻固定 Cys196 和 Glu71, 突变 246、136 残基可以调控催化三联体与底物氰基间的关键距离, 进而影响产物的形成。Asn246 突变为侧链较小的 Val, 使得 Cys196 活动的空间变大并使其与 Glu71 的距离变大。同时第 2 个水分子及时传递至反应位点, 通过路径 A 生成主产

物苯乙酸；Ile136 突变为 Gln，空间位阻效应使 Glu71 向蛋白口袋下方移动，缩短了其与 Cys196 间的距离，经路径 B 生成主产物苯乙酰胺。

4 腈水解酶催化混乱性的影响因素研究

酶催化反应条件变化如 pH、温度等会影响

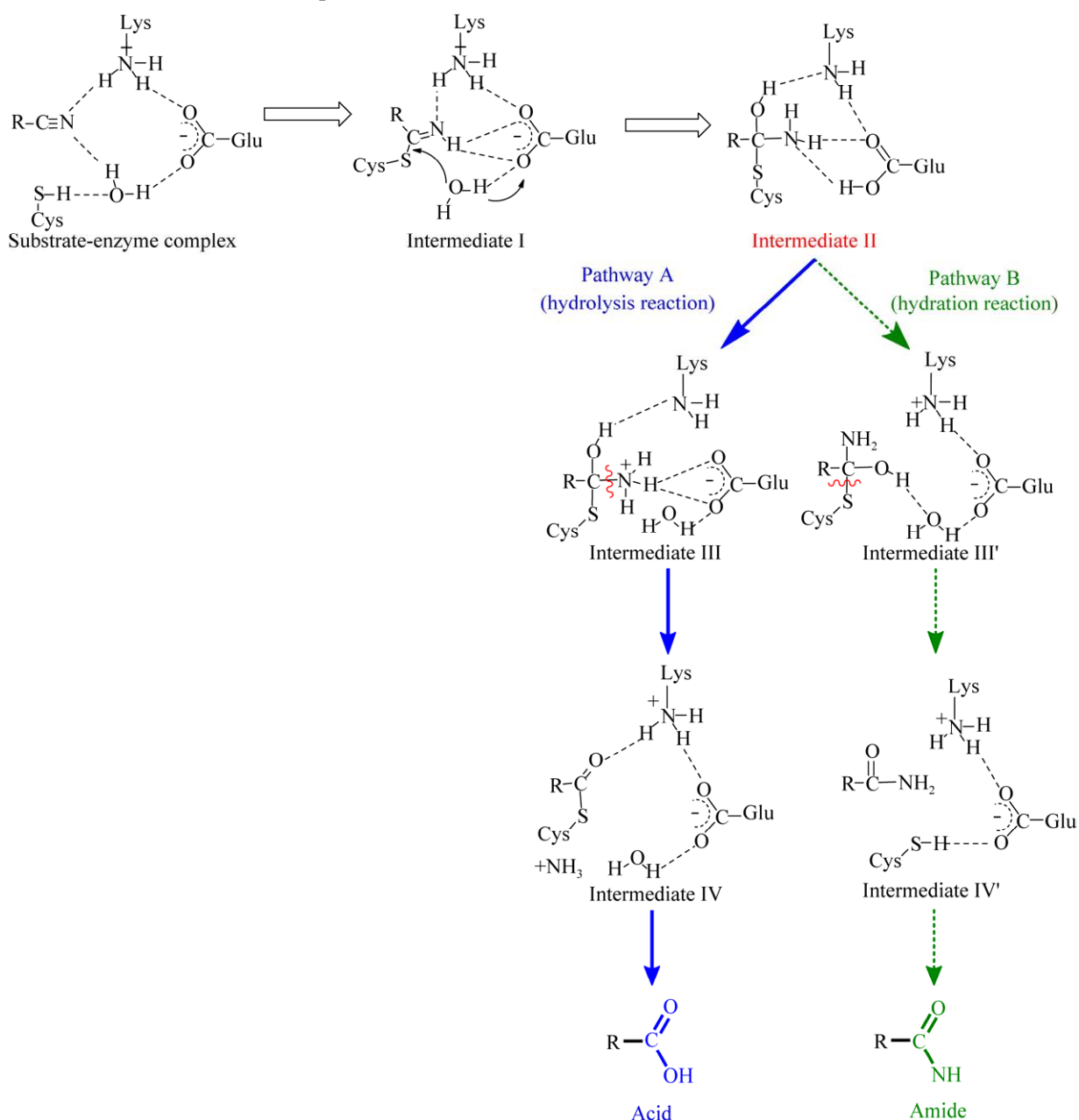


图 7 腈水解酶催化混乱性的催化机理假说

Figure 7 Catalytic mechanism hypothesis of nitrilase catalytic promiscuity.

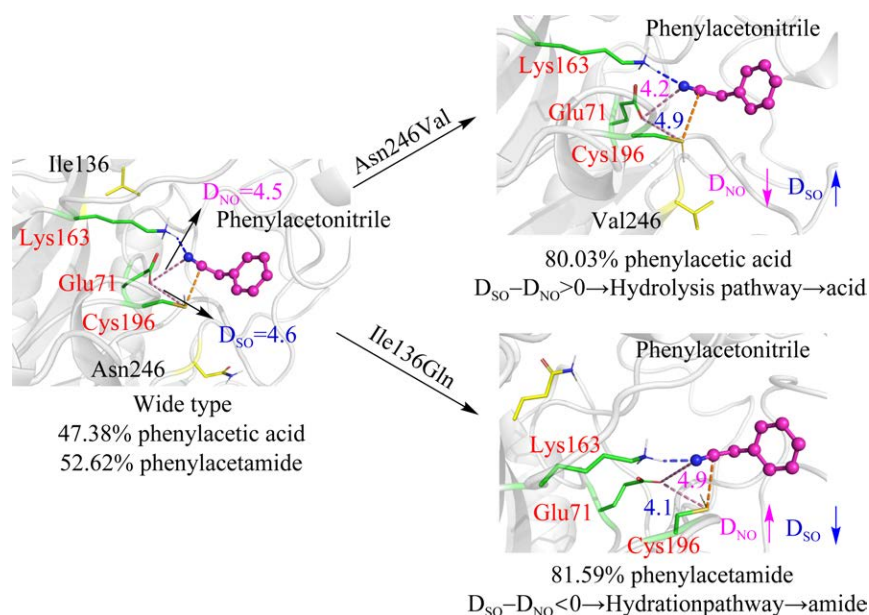


图 8 水稻来源的腈水解酶催化混乱性调控机制

Figure 8 The catalytic promiscuity mechanism of nitrilase derived from *Oryza sativa*.

pH 9 时, 产物中酰胺占比达到 84%, 而温度升到 30 °C 时酰胺占比只有 71%。Fernandes 等^[42]以 *P. fluorescens* EBC191 来源的腈水解酶催化 2-苯乙腈, 发现 pH 在 5–9 范围内对产物酰胺的形成影响较小, 而温度对酶的催化混乱性影响较大, 在 5–40 °C 范围内, 随温度的升高, 酰胺的比例明显下降, 相对于 5 °C 时酰胺的占比为 89%, 40 °C 时降低至 62%。从上述结果可以看到, 较高的 pH 值及较低的温度使腈水解酶的催化方向偏向酰胺产物。不过也有研究^[30,36]发现一些腈水解酶对于外界环境因子不是很敏感。

研究表明催化底物也会对腈水解酶的催化混乱性产生一定影响, 如底物的结构特征、取代基的电子效应和空间位阻效应等。Zhang 等^[31]研究了 *P. graminis* DSM 17151 腈水解酶 NitPG 催化多类底物所呈现的水合活力差异, 发现催化苯乙腈类底物相对苯甲腈和 3-苯基丙腈产生更多的酰胺产物, 证明其类乙腈结构对于酰胺

的形成比较重要; 此外, 实验结果显示 NitPG 催化 4-甲基苯乙腈、苯乙腈、4-硝基苯乙腈分别产生 11%、16% 和 58% 的酰胺, 酰胺占比逐渐增加。Dai 等^[39]以佐式红球菌(*Rhodococcus zopfii*)来源的腈水解酶 R_zNIT 为催化剂, 当催化烟腈底物几乎不产生酰胺, 但引入卤素取代时即催化底物为 2-氯烟腈, 反应生成约 87.5% 的 2-氯烟酰胺产物。Osswald 等^[33]发现 *A. thaliana* 来源的腈水解酶催化不同 3-取代丙烯腈所产生的酰胺比例有着明显的差异, 当取代基团为氰基(-CN)和硝基(-NO₂)时, 酰胺占比高达 93% 和 95%, 相反, 取代基为甲基(-CH₃)和甲氧基(-OCH₃)时酰胺占比不超过 5%。以上研究结果表明, 取代基的电子效应会影响产物中羧酸和酰胺的比例, 增强取代基的电负性有利于酰胺的形成。当取代基为电负性强的原子如 -NO₂、-CN、-CF₃ 等, 其表现出的吸电子效应使催化区域的氰基 N 原子不易被质子化, 反应路径倾向于 pathway B, 导致酰胺变成主产物。Mukherjee

等^[36]研究来源于玉米(*Zea mays*)的脲水解酶 ZmNIT2 催化脂肪族脲类底物时,发现该酶催化丁脲、戊脲、己脲和庚脲所产生的酰胺占比分别为 19%、26%、36%和 40%,随着碳链延长,其空间位阻可能会影响关键中间体的水解,造成产物中酰胺的比例升高。此外,*P. fluorescens* EBC191 来源的脲水解酶在催化外消旋底物时所产生的酸和酰胺比例也会不同^[53,41-42,67],该酶催化 *R*-扁桃脲、*R*-2-乙酰氧基-2-苯乙脲和 *R*-苯胺基乙脲分别产生约 10%、33%和不足 1%的酰胺,而催化该 3 种 *S* 构型底物所产生的酰胺占比分别提升至约 50%、62%和 10%。粗糙链孢菌(*Neurospora crassa*) OR74A 来源^[52]的脲水解酶也有类似的结果,该酶催化 *R*-扁桃脲几乎不产生酰胺,而催化 *S*-扁桃脲产生了约 84%的 *S*-扁桃酰胺。从上述结果可以看出,底物的手性也会对脲水解酶的催化混乱性造成一定的影响,但其影响因素尚不明晰。

另外,脲水解酶催化混乱性还受到酶本身结构的影响,尤其是活性位点中催化三联体相邻的氨基酸残基,主要是通过突变相关残基调控底物的结合姿势和相互作用特征。*P. fluorescens* EBC191 来源^[37]的脲水解酶催化扁桃脲底物产生约 19%的酰胺,而将与催化三联体 Cys164 直接相邻的 Ala165 突变为 Arg 后,产物酰胺比例提高至 41%;粪产碱杆菌(*Alcaligenes faecalis*) ATCC 8750 来源的脲水解酶催化扁桃脲仅产生 0.7%的酰胺,但将催化域中 Trp164 突变为 Ala 时,酰胺比例提高至 70%。同样是 *P. fluorescens* EBC191 来源的脲水解酶,与催化三联体 Cys164 直接相邻的另一个位点 Cys163 的突变也提高了酰胺占比^[44]。同时,处于催化三联体附近的 188 和 206 位点,其突变体 Trp188Leu 和 Asn206Lys 均以酰胺为主产物(80%和 60%以上)^[68],并且 188 位

点突变为 Arg、Lys 和 Pro,可催化生成 90%以上的扁桃酰胺^[43];此外,一些突变体的 C 末端缺失也表现出更强的酰胺形成能力^[45]。黑曲霉(*Aspergillus niger*)来源的脲水解酶催化扁桃脲的产物中未检测到扁桃酰胺,当对催化三联体附近的位点改造,发现 W163A 突变体产生了 33%的酰胺;*N. crassa* 来源的脲水解酶突变体 W168A 的产物酰胺占比也从 40%提高到 85%^[52]。

5 脲水解酶催化混乱性的应用案例

催化混乱性给脲水解酶的应用带来“混乱”的影响:一方面它使得目标羧酸产物中含有一定比例的酰胺,降低了羧酸产物的收率,同时增加了产物后续分离纯化的成本,给脲水解酶的工业应用带来挑战;而另一方面,脲水解酶的催化混乱性也是一个机遇,相对于常规的脲水合酶通常表现出对脂肪族底物的偏好且对氰类化合物敏感^[43],而脲水解酶不依赖于金属离子,可耐受高浓度的氰离子,且重组表达更简单,有利于大规模的工业应用。如果能使脲水解酶的反应偏向于生成酰胺,那将为高附加值酰胺类化合物的生物合成提供新工艺。

脲水解酶催化混乱性的两面性,引起了众多学者的关注,其相关应用案例如表 3 所示。当羧酸为目标产物,需要消除酰胺副产物。一些小组通过采用双酶一锅两步级联反应策略来消除酰胺副产物,如 Tao 等^[40]将脲水解酶与酰胺酶“串联”,消除脲水解酶产生的酰胺副产物,将 β -丙氨酸的产量提高到 90%,收率达到 15.02 g/(L·h)。Zhang 等^[35]将高山南芥(*Arabis alpina*)来源的脲水解酶突变体 N258D 与泛菌属(*Pantoea* sp.)来源的酰胺酶“串联”,消除了酰胺副产物(*S*)-3-氰基-5-甲基己酰胺,催化异丁基琥珀脲转化(*S*)-3-氰基-5-甲基己

酸, 转化率达到 45%, ee_p 高达 99.3%。另一种策略即蛋白质分子改造工程, 抑制催化混乱腈水解酶的水合活力, 专一地生产目标羧酸产物, 如 *R. zopfii* 来源^[39]的野生型腈水解酶催化 2-氯烟腈生成 88%的酰胺, 目标产物 2-氯烟酸仅占 12%, 而其突变体 W167G 完全消除了水合活力并将水解活性提高了 20 倍。Lu 等^[60]将嵌合型腈水解酶 *BaNIT* 所产生的酰胺副产物 (*S*)-3-氰基-5-甲基己酰胺从 15.8%降低到了 1.9%, 同时将酶活力提高了 5.4 倍。当酰胺作为目标产物时, 需要尽可能消除羧酸产物的生成。如 Zhang 等^[69]从蛋白数据库中鉴定出来源于恶疫霉 (*Phytophthora cactorum*) 的腈水解

酶, 该酶催化扁桃腈转化生成 89.7%的扁桃酰胺, 其突变体 W167A 将产物中扁桃酰胺的占比提升至 99.8%。野生型的集胞藻(*Syechocystis* sp.) PCC 6803 腈水解酶转化 2-氰基吡啶生成 2.1%的相应酰胺, 其突变体 F193N 将酰胺占比提升至 73.4%, Sun 等^[70]在单突变体 F193N 上进一步迭代突变, 获得的突变体 F193N/G101K/Q192H/I201M 将酰胺的占比提升至 98.5%。如前所述, 通过蛋白质工程可将酰胺从副产物转变成主产物(表3)。此外, 也有一些天然腈水解酶(表 1)表现出高的水合活力, 显示出腈水解酶具有成为新型“腈水合酶”的潜力, 这在生物合成领域中具有重大的意义。

表 3 关于腈水解酶催化混乱性的应用案例

Table 3 Application cases regarding the catalytic promiscuity of nitrilases

Objective	Organism	Substrate & Product	Mutation	Result	References
Eliminate amide products	<i>Arabidopsis thaliana</i>	Isobutylsuccinonitrile & (<i>S</i>)-3-cyano-5-methylhexanoic acid	No mutation	The amide byproduct was eliminated and acid was obtained with a conversion of 45.0% and ee_p of 99.3%	[35]
	<i>Arabidopsis thaliana</i> & <i>Brassica rapa</i> (chimeric nitrilase)	Isobutylsuccinonitrile & (<i>S</i>)-3-cyano-5-methylhexanoic acid	V82L/M127I/C237S (<i>BaNIT</i> _{M2})	1.5 g/L <i>Escherichia coli</i> cells harboring <i>BaNIT</i> _{M2} as biocatalyst converted 150 g/L Isobutylsuccinonitrile afforded (<i>S</i>)-3-cyano-5-methylhexanoic acid with >99.0% <i>e.e.</i> and 45.9% conversion	[60]
	<i>Bradyrhizobium japonicum</i> strain USDA110	3-aminopropionitrile & β -alanine	No mutation	The isolated yield of β -alanine was 90%, the space-time-yield was 15.02 g/(L·h)	[40]
Enhance amide products	<i>Rhodococcus zopfii</i>	2-chloronicotinonitrile & 2-chloronicotinic acid	W167G, W167A, N165C, N165A, N165G	W167G converted 100 mmol/L 2-chloronicotinonitrile exclusively into 2-chloronicotinic acid within 16 h	[39]
	<i>Alcaligenes faecalis</i> ATCC 8750	Mandelonitrile & (<i>S</i>)-mandeloamide	W164A	W164A variant formed significantly more (<i>S</i>)-mandeloamide than wide type	[37]
	<i>Acidovorax facilis</i> 72 W	2-cyanopyridine & 2-picolinamide	W188G, W188A, W188C, W188L, W188M, W188S, W188V	W188M mutant converted 250 mmol/L 2-cyanopyridine to more than 98% 2-picolinamide in 12 h with a specific activity of 90 U/mg	[38]
	<i>Neurospora crassa</i> OR74A	2-phenylpropionitrile & 2-phenylpropionamide	W168A	W168A mutant formed significantly increased amounts of 2-phenylpropionamide	[52]

(待续)

(续表 3)

Objective	Organism	Substrate & Product	Mutation	Result	References
	<i>Oryza sativa</i>	Phenylacetonitrile & phenylacetamide	A87M/I91P/I136 Q/M164V/R224S /V226R	The phenylacetamide was increased from 49.1% to 96.4% by the hexamutant	[34]
	<i>Phytophthora cactorum</i>	Mandelonitrile & mandeloamide	W167A, W167V, W190C, W190R	These four variants reduced the acid by-product to less than 1% yield	[69]
	<i>Pseudomonas fluorescens</i> EBC191	Mandelonitrile & mandeloamide	C-terminal deletions, C163A, C163N, C-terminal deletions, W188L, W188K, W188P, W188R, N206K	Nit(DelC-60)-Cys163Asn nitrilase variant, which produces about 70% of amide from mandelonitrile These mutants formed increased amounts of mandeloamide from mandelonitrile	[44] [43,45,68]
	<i>Syechocystis</i> sp. PCC 6803	2-cyanopyridine & 2-picolinamide	F193A, F193D, F193N, F193E, F193Q F193N/G101K/ Q192H/I201M	F193N improved amide product up to 73%, which was about 35-fold that of wild type This mutant retained only 1.5% of the carboxylic acid ratio	[50] [70]

ee_p refers to enantiomeric excess of the product.

6 展望

腈水解酶在生物催化中具有广泛的应用前景,其催化混乱性已成为当前研究的热点之一。然而,目前对于腈水解酶催化混乱性的调控机制和结构基础的认识还相对有限,其具体的催化机制尚未完全阐明,需要进一步深入研究。尽管通过酶工程等手段已获得了一些具有特定催化性能的腈水解酶突变体,但对于如何更精准地设计和优化腈水解酶仍需要更多的努力。未来,随着对腈水解酶催化机制的深入研究,有望发现和设计更多具有特定催化性能的腈水解酶,为工业生产中的废物处理、有机合成和医药行业等领域提供更多的可能性。此外,对腈水解酶混乱性的深入理解还将促进对生物催化的发展,为生物工程领域提供更多的新思路和方法,从而为环境保护和可持续发展作出更大的贡献。

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