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Chorismatase Mechanisms Reveal Fundamentally Different Types of Reaction in a Single Conserved Protein Fold

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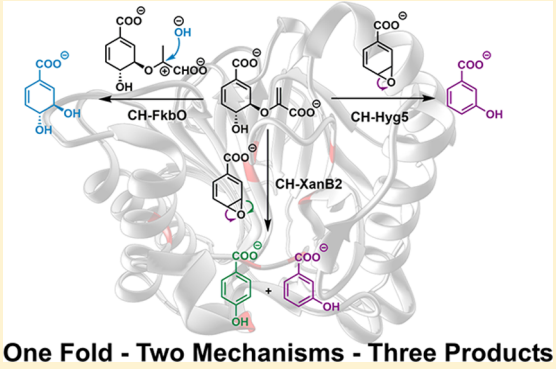
Supporting Information

ABSTRACT: Chorismatases are a class of chorismate-converting enzymes involved in the biosynthetic pathways of different natural products, many of them with interesting pharmaceutical characteristics. So far, three subfamilies of chorismatases are described that convert chorismate into different (dihydro-)benzoate derivatives (CH-FkbO, CH-Hyg5, and CH-XanB2). Until now, the detailed enzyme mechanism and the molecular basis for the different reaction products were unknown. Here we show that the CH-FkbO and CH-Hyg5 subfamilies share the same protein fold, but employ fundamentally different reaction mechanisms. While the FkbO reaction is a typical hydrolysis, the Hyg5 reaction proceeds intramolecularly, most likely via an arene oxide intermediate. Two nonconserved active site residues were identified that are responsible for the different reaction mechanisms in CH-FkbO and CH-Hyg5. Further, we propose an additional amino acid residue to be responsible for the discrimination of the CH-XanB2 subfamily, which catalyzes the formation of two different hydroxybenzoate regioisomers, likely in a single active site. A multiple sequence alignment shows that these three crucial amino acid positions are located in conserved motifs and can therefore be used to assign unknown chorismatases to the corresponding subfamily.



INTRODUCTION

Chorismate (**1**) is a central branching point between primary and secondary metabolism in most organisms except animals. Compounds derived from chorismate (**1**) are important metabolites or part of complex natural products such as siderophores, polyketides and terpenoids, some of which are used as pharmaceuticals.[1,2] Additionally, products of chorismate-converting enzymes are often chiral and contain multiple functional groups suitable for follow-up chemistry.[3−5]

In previous work, we described a new class of bacterial chorismate-converting enzymes named chorismatases (CH).[6] They catalyze the cleavage of chorismate (**1**) to pyruvate and (dihydro-)benzoate derivatives (Figure 1); as yet, their mechanism has not been elucidated in detail.[7,8] Depending on the products produced CH can be grouped in three distinct subfamilies: CH-FkbO (chorismatases/chorismate hydrolases; EC 3.3.2.13), such as FkbO from *Streptomyces hygroscopicus* subsp. *ascomyceticus* (accession code AAF86394.1, UniProt Q9KID9), produce 3,4-*trans*-dihydroxy-cyclohexa-1,5-dienecarboxylate (3,4-*trans*-CHD, **2**). 3,4-*trans*-CHD (**2**) serves as starter unit in the biosynthesis of important polyketidic immunosuppressants such as rapamycin or tacrolimus.[6] In contrast, CH-Hyg5 (chorismatases/3-hydroxybenzoate synthases; EC 4.1.3.45), such as Hyg5 from *S. hygroscopicus* (accession code AAC38060.1, UniProt O30478) produce 3-hydroxybenzoate (3-HBA, **3**).[6,9] CH-Hyg5 have been identified in the biosynthetic gene clusters of compounds such as cuevaene A and the immunosuppressant brasilicardin.[6,9,10] 3-HBA (**3**) has also been described for CH-XanB2 (chorismatases/hydroxybenzoate synthases), such as XanB2 from *Xanthomonas campestris* pv *campestris*, but along with a chorismate lyase-like (EC 4.1.3.40) activity resulting in 4-

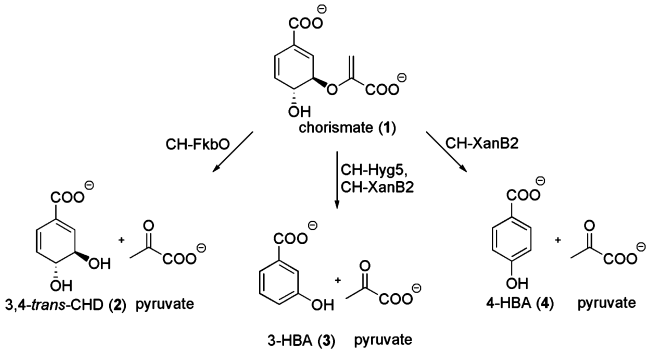


Figure 1. Reaction products of the different chorismatase subfamilies.

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hydroxybenzoate (4-HBA, **4**).[8,11] CH-XanB2 are found in different phytopathogenic *Xanthomonas* strains, where they are involved in the biosynthesis of xanthomonadins.[6,8,12,13]

Despite catalyzing reactions with different outcomes, the different subfamilies of chorismatases show high sequence identity (26.3−40.4% on the protein level), notably around the active site that is located at the C-terminus of the enzymes (Figure S1). Regarding the reaction catalyzed, CH-FkbO are very similar to isochorismatases, both performing a typical hydrolysis reaction. However, their sequences and structures are not related.[6,14,15] Consequently, we suggested an isochorismatase-like hydrolysis mechanism for the CH-FkbO subfamily. This postulate has been supported by mutagenesis experiments.[7] CH-Hyg5 were assumed to perform a hydrolysis-elimination mechanism with either **2** or enoyl benzoate as an intermediate during 3-HBA (**3**) formation. Due to the trans configuration of the vicinal diol of chorismate (**1**) and 3,4-*trans*-CHD (**2**), the elimination reaction would have to proceed in a syn fashion, which is chemically unfavored (Figure S2). Nevertheless, syn elimination was described for the chorismate-converting MqnA leading to enoyl benzoate.[16]

RESULTS AND DISCUSSION

For the CH-FkbO subfamily an isochorismatase-like hydrolysis mechanism was suggested where the acidic active site residue $E338^{FkbO}$ initially protonates the C3′ of chorismate (**1**, methylene group at the enol ether moiety). This resulting carbocation intermediate is nucleophilically attacked by (activated) water leading to the formation of an unstable tetrahedral intermediate (hemiketal), which decomposes spontaneously into the products 3,4-*trans*-CHD (**2**) and pyruvate (Figure 2).[7]

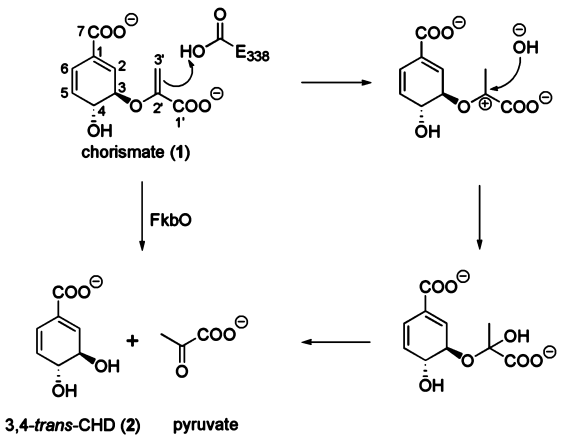

Figure 2. Hydrolysis mechanism of the CH-FkbO subfamily.

The importance of the postulated catalytic glutamate ($E338^{FkbO}$ and $E334^{Hyg5}$) was demonstrated previously for FkbO using site-directed mutagenesis,[7] and the corresponding Hyg5 variant ($E334Q^{Hyg5}$) also shows a complete loss of activity (Figure S3). This observation supports that an initial protonation step of the methylene group at C3′ is essential for the reaction in both subfamilies.

Subsequently, the postulated hydrolysis mechanism was investigated by performing assays in ^{18}O-labeled water ($H_2{}^{18}O$) followed by LC-MS analysis. As expected, the FkbO reaction in the presence of $H_2{}^{18}O$ resulted in incorporation of labeled oxygen into the pyruvate moiety (Figures 3 and S4).

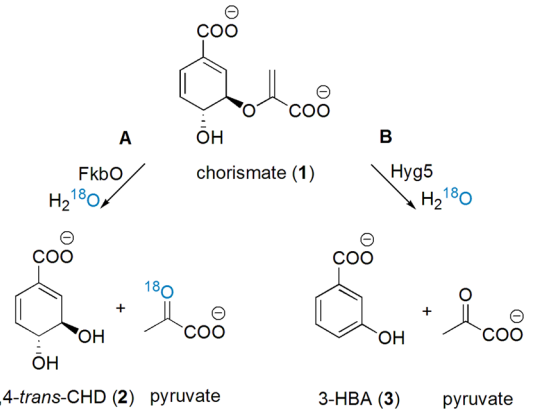

Figure 3. Outcome of the chorismatase reactions performed by FkbO and Hyg5 in ^{18}O-labeled water to test the expected and postulated mechanisms.[19] (A) Hydrolysis reaction of FkbO and (B) intramolecular reaction of Hyg5.

Surprisingly, no incorporation of ^{18}O could be detected in either product [pyruvate and 3-HBA (**3**)] of the Hyg5 reaction under these conditions (Figures 3 and S4). This result clearly contradicts a hydrolysis−elimination mechanism for CH-Hyg5. Additionally, the two potential intermediates, 3,4-*trans*-CHD (**2**) and enoyl benzoate, were tested as substrates; neither of them was converted by Hyg5 (Figure S2). The observation that no external oxygen was incorporated into the Hyg5 products indicated an intramolecular mechanism for the Hyg5 reaction. A pericyclic mechanism, as described for chorismate-converting enzymes producing the 2- and 4-regioisomeric hydroxybenzoates,[17,18] can also be ruled out due to the position of the hydroxyl group in 3-HBA (**3**). This suggested that CH-Hyg5 employ an alternative catalytic strategy to form the products.

In order to elucidate this strategy, we decided to perform further assays with isotopically labeled chorismate (**1**).[20] We were specifically interested in gaining information about the localization and fate of the chorismate C3 and C4 oxygen atoms in the Hyg5 products 3-HBA (**3**) and pyruvate. Therefore, we selectively introduced an ^{18}O-label at the C4-hydroxyl group of chorismate. The method was based on the observation that FkbO and Hyg5 are strictly specific for chorismate (**1**).[21] This allows for a convenient assay of these enzymes with in situ produced 4-^{18}O-labeled chorismate using the isochorismate synthase EntC from *E. coli*. EntC catalyzes the introduction of a hydroxyl group at the chorismate C4 at the expense of the isochorismate hydroxyl at C2 (and vice versa for the reverse reaction), reaching a chorismate/isochorismate equilibrium (3:2). Starting from pure nonlabeled isochorismate in $H_2{}^{18}O$, EntC produces a mixture of 2-^{18}O-labeled isochorismate and 4-^{18}O-labeled chorismate. When added to this reaction mixture, the chorismatases FkbO and Hyg5 entirely convert the present 4-^{18}O-labeled chorismate derived from isochorismate into the chorismatase reaction products pyruvate and 3,4-*trans*-CHD (**2**) or 3-HBA (**3**), respectively (Figure 4).

The assay confirmed that FkbO catalyzes the hydrolysis reaction as proposed: the labeled oxygen from 4-^{18}O-labeled chorismate is retained in 3,4-*trans*-CHD (**2**), as well as the labeled oxygen from the solvent $H_2{}^{18}O$ is incorporated into the second product pyruvate (Figures 4, S4). However, when FkbO was replaced by Hyg5 in otherwise identical conditions, the 4-^{18}O-label was found in the 3-hydroxyl of 3-HBA (**3**), while pyruvate was unlabeled, as seen before (Figures 4, S4).

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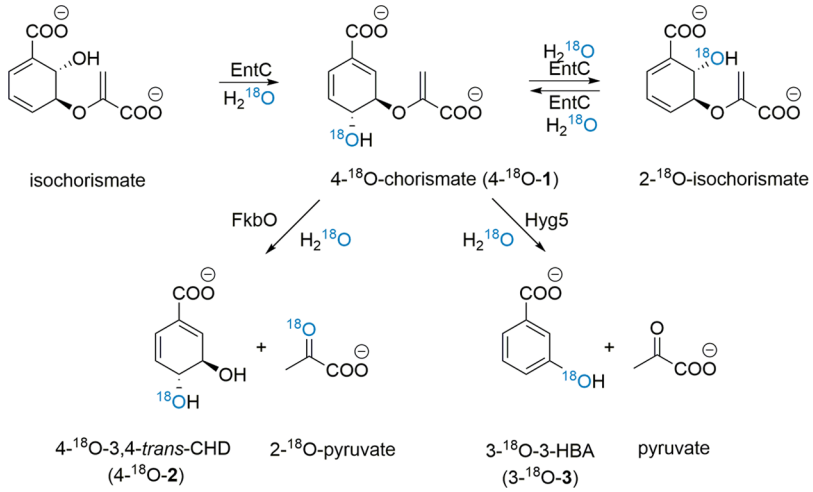

Figure 4. In situ synthesis of selectively labeled 4-^{18}O-chorismate (4-^{18}O-**1**) and formation of 4-^{18}O-3,4-*trans*-CHD (4-^{18}O-**2**), as well as 2-^{18}O-pyruvate (FkbO) or 3-^{18}O-3-HBA (3-^{18}O-**3**) and pyruvate (Hyg5).

The complete migration of labeled oxygen from C4 in the substrate chorismate (**1**) to C3 in the product 3-HBA (**3**) supports an intramolecular mechanism, most probably featuring an arene oxide as intermediate. The rearrangement of the arene oxide to 3-HBA (**3**) could proceed via an NIH shift (Figure 5).[22] Based on these results, other potential mechanistic

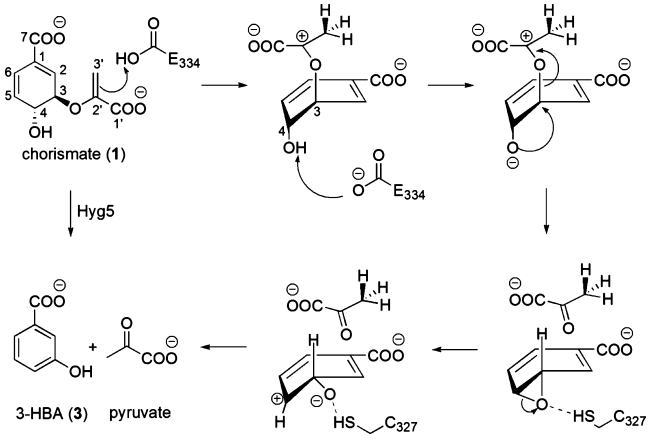

Figure 5. Postulated arene oxide mechanism of CH-Hyg5: initial protonation of the enolpyruvyl moiety, intramolecular nucleophilic attack of the activated 4-hydroxyl group at the C3 position of the cyclohexadiene ring, formation of an arene oxide intermediate, and a hydrogen-shift during aromatization leads to the products 3-HBA (**3**) and pyruvate.

alternatives such as syn elimination of water or a reaction via an acetonide five-membered cyclic acetal intermediate could be clearly ruled out (Figure S2). Examples for the occurrence of arene oxides in other enzymatic reactions are given in ref 23.

In order to support this theory with structural data, the three-dimensional structure of Hyg5 from *S. hygroscopicus* was solved at 1.9 Å resolution (PDB-ID 5AG3, Figure S5 and Table S1). Comparison with the structure of FkbO from *S. hygroscopicus* subsp. *ascomyceticus*[7] allowed an assessment of the molecular basis for the different reactions. The overall structure of Hyg5 was almost identical to the one published for FkbO[7] [root-mean-square deviation (r.m.s.d.) of atomic positions = 1.18 Å with bound inhibitor 3-(2-carboxyethyl) benzoate, r.m.s.d. = 1.40 Å without inhibitor], showing the same partition of the protein in three domains. The location and orientation of the cocrystallized competitive inhibitor 3-(2-carboxyethyl) benzoate[24] in the active site clearly corresponds to the situation in FkbO (Figure S5). Only two amino acids in close proximity to the inhibitor differ in the two enzymes: a cysteine (C327^{Hyg5}) in Hyg5 corresponding to an alanine (A331^{FkbO}) in FkbO, and a glycine (G240^{Hyg5}) in Hyg5 corresponding to another alanine (A244^{FkbO}) in FkbO (Figure 6).

How can these differences be responsible for the fundamentally different molecular mechanisms of the two chorismatase subfamilies? The active site geometry of Hyg5 suggests that the absence of the methyl group in G240^{Hyg5} (A244^{FkbO}) causes a small but effective change in the orientation of the catalytic glutamate side chain by abolishing sterical hindrance (Figure 6). Due to this orientational change, E334^{Hyg5} can activate the 4-hydroxyl moiety of chorismate for a nucleophilic attack in Hyg5 by deprotonating it. The cysteine in Hyg5 is likely involved in selective product formation by directing the putative arene oxide opening to the C3 position [as opposed to the C4 position leading to 4-HBA (**4**)] of the ring via electrostatic and/or steric interaction (Figure 5).

To prove this structure–function relationship, sets of single and double variants of the two enzymes were constructed to investigate their influence on the CH-Hyg5 and CH-FkbO activities (Figure 7). Both Hyg5 single variants, C327A^{Hyg5} and G240A^{Hyg5}, showed decreased activity compared to the wildtype (6% and 55%, respectively), and resulted in a complete loss of product selectivity. The FkbO product 3,4-*trans*-CHD (**2**) as well as the XanB2 product 4-HBA (**4**) were produced (Figures 7 and S3). The double variant C327A/G240A^{Hyg5} showed the same behavior, but along with a more dramatic loss of activity [only 5% of chorismate (**1**) was converted after 20 h]. This agrees with our hypothesis that C327 and G240 are responsible for the selectivity of the CH-Hyg5 reaction. In the case of the cysteine variant, loss of electrostatic stabilization by the missing polar moiety and reduced steric requirement of the side chain leads to the additional production of 4-HBA (**4**), through an unselective opening of the postulated arene oxide intermediate. The involvement of C327 in directing the selectivity of product formation was underlined by the crystal structure of the variant C327S^{Hyg5} with the product 3-HBA (**3**) (PDB-ID 5A3K, Figure S5, Table S1). The variant showed comparable characteristics

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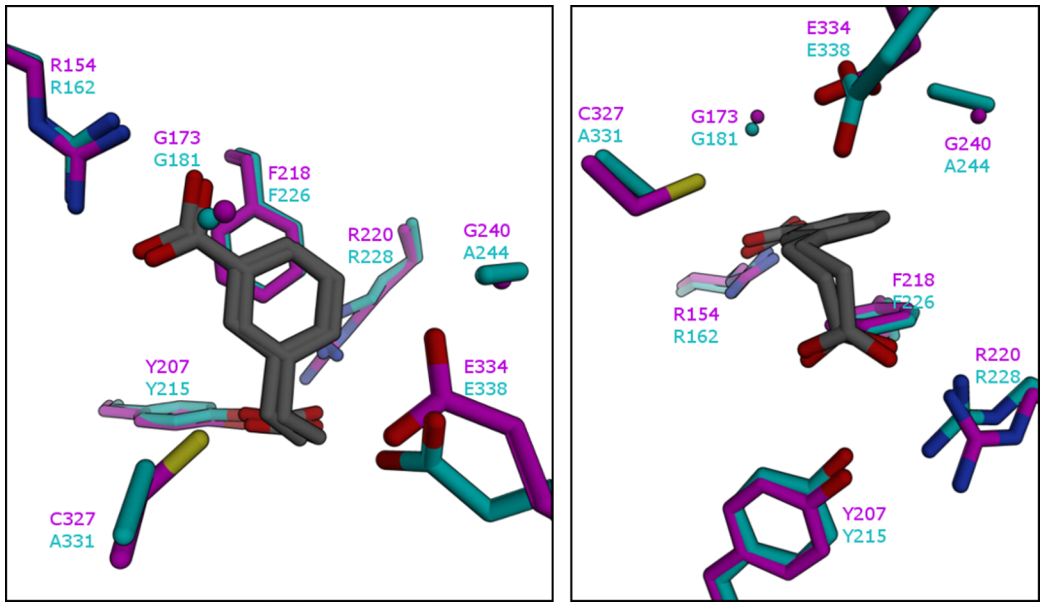

Figure 6. Overlay of the active sites of FkbO (cyan) and Hyg5 (magenta) with the inhibitor 3-(2-carboxyethyl) benzoate in two different views. Left: face-on view; right: edge-on view.

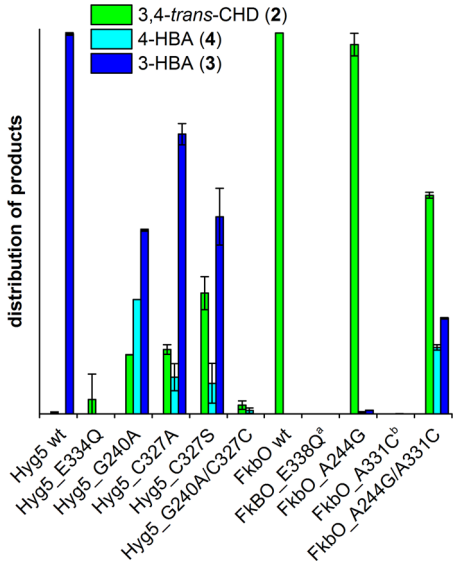

Figure 7. Product range of chorismatases and chorismatase variants. The relative amounts of products [3,4-*trans*-CHD (**2**), 3-HBA (**3**), and 4-HBA (**4**)] are plotted in relation to all compounds (remaining substrate and products) detected in the HPLC assay after 20 h reaction time. All variants show a loss of product selectivity. Residual activities of the variants in comparison to the corresponding wildtype enzymes are given in the main text. [a]Data from ref 7. [b]FkbO_A331C was inactive and not properly folded (see also Figure S3).

as observed for C327A^{Hyg5}, while the crystal structure revealed coordination of the 3-HBA (**3**) hydroxyl group by the introduced serine side chain (Figure S5). The formation of the CH-FkbO product 3,4-*trans*-CHD (**2**) derived from the competing hydrolysis reaction can be explained by both the decreased velocity of the Hyg5 variants and the larger steric requirements of the Hyg5 variant G240A^{Hyg5} resulting in a change of orientation of the catalytic glutamate (Figure 6). In FkbO, the situation is similar: both the single variant A244G^{FkbO} (38% of wildtype activity) and the double variant A244G/A331C^{FkbO} (15% of wildtype activity) showed an extended product range that was more pronounced for the double variant.

The loss of selectivity experienced in the FkbO and Hyg5 variants suggests that product formation in the CH-XanB2 subfamily is unselective. In this case the opening of the arene oxide intermediate is not exclusively directed to the C3 position, which results in a mixture of 3-HBA (**3**) and 4-HBA (**4**) as products. Consequently, the formation of 3-HBA (**3**) and 4-HBA (**4**) takes place in one single active site. This is in contrast to previous postulations: based on sequence alignments and data received from experiments with full-length and truncated XanB2 variants, Zhou et al. proposed that the CH-XanB2 subfamily possesses two different active sites that convert chorismate (**1**) into 3-HBA (**3**) and 4-HBA (**4**), respectively.[8]

In the next step, we attempted to transfer the knowledge gained for CH-FkbO and CH-Hyg5 to a general sequential basis and to get insight in the discrimination between the three chorismatase subfamilies (CH-FkbO, CH-Hyg5, CH-XanB2). A Blastp search with an E-value < e^{-90},[25] followed by a multiple sequence alignment, revealed that all amino acid residues contributing to the formation of the active site are embedded in conserved sequence motifs (Figure S1). The only two amino acids that differ between the CH-FkbO and CH-Hyg5 subfamilies are those described above. G240^{Hyg5} (A244^{FkbO}) is part of a conserved S-(**A**/**G**)-T-A motif, while C327^{Hyg5} (A331^{FkbO}) is part of a conserved I-(**A**/**C**)-R motif (Figure S1). As shown in this study, these two residues are solely responsible for the selectivity of product formation in the CH-FkbO and CH-Hyg5 subfamilies. Surprisingly, the CH-XanB2 subfamily shows the same characteristics as the CH-Hyg5 subfamily.

Nevertheless, based on further analysis of the crystal structures and the multiple sequence alignment we identified another subtle difference between CH-FkbO/CH-Hyg5 and CH-XanB2 in a conserved motif close to the active site. XanB2^{A173} (corresponding to G173^{Hyg5}/G181^{FkbO}) deviates from an otherwise conserved sequence motif: P-A-A-T-(**G**/**A**)-I-G (Figure S1). The higher steric requirement of the alanine

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side chain could be responsible for a slightly different positioning of the substrate in the CH-XanB2 subfamily leading to an unselective opening of the postulated arene oxide intermediate. Additionally, sequence comparison and phylogenetic studies on 134 chorismatases based on these conserved active site motifs enabled us to assign them as members of the CH-FkbO, CH-Hyg5 and CH-XanB2 subfamilies (Figure S6).

CONCLUSION

In conclusion, we showed that the chorismatase subfamilies CH-FkbO and CH-Hyg5 catalyze two fundamentally different molecular mechanisms despite their conserved protein structure and almost identical active site architectures. The mechanism proposed for the CH-Hyg5 subfamily can directly be used to explain the broader product range of the CH-XanB2 subfamily, which likely converts chorismate via the same arene oxide intermediate to 3-HBA (**3**) and 4-HBA (**4**) in one single active site. We propose that in total only three amino acid residues in conserved motifs are responsible for the discrimination between the CH-FkbO, CH-Hyg5 and CH-XanB2 subfamilies. Phylogenetic analysis also shows three distinct clades in the phylogenetic tree (Figure S6); all of the sequences analyzed contain the expected amino acid residues that correspond to the three subfamilies. The active site patterns described in this study complement and confirm the phylogenetic method by providing a molecular explanation. With this combined approach it is now possible to clearly assign a new chorismatase to one of the subfamilies. The existence of highly conserved motifs will greatly facilitate the detection of further chorismatases and subsequently novel natural products. Especially the CH-Hyg5 and CH-XanB2 subfamilies that provide building blocks with meta-only substitutions will be promising targets for the identification of new biosynthetic gene clusters for such compounds.

ASSOCIATED CONTENT

Supporting Information

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Experimental details, analytical data, and structural data (PDF)

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Notes

The authors declare no competing financial interest.

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