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Rational Active-Site Redesign Converts a Decarboxylase into a C=C Hydratase: 'Tethered Acetate' Supports Enantioselective Hydration of 4-Hydroxystyrenes

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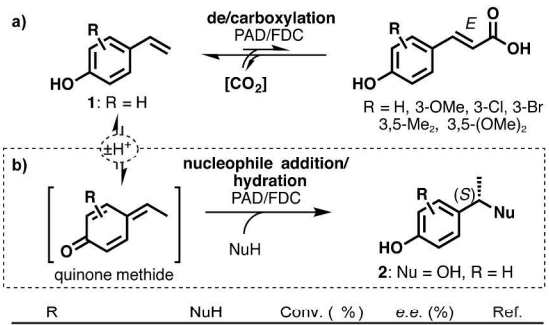
ABSTRACT: The promiscuous regio- and stereoselective hydration of 4-hydroxystyrenes catalyzed by ferulic acid decarboxylase from *Enterobacter* sp. (FDC_*Es*) depends on bicarbonate bound in the active site, which serves as proton relay activating a water molecule for nucleophilic attack onto a quinone methide electrophile. This 'co-factor' is crucial to achieve improved conversions and high stereoselectivities for (*S*)-configured benzylic alcohol products. Similar effects were observed with simple aliphatic carboxylic acids as additives. Rational redesign of the active site by replacing the bicarbonate or acetate 'co-factor' by a newly introduced side-chain carboxylate from an adjacent amino acid yielded mutants which efficiently acted as C=C hydratase. A single point mutation of valine 46 to glutamate or aspartate improved the hydration activity by 40% and boosted the stereoselectivity 39-fold in the absence of bicarbonate or acetate.

***Keywords**: Biocatalysis, Hydration, Enzyme Engineering, Decarboxylase, Hydratase*

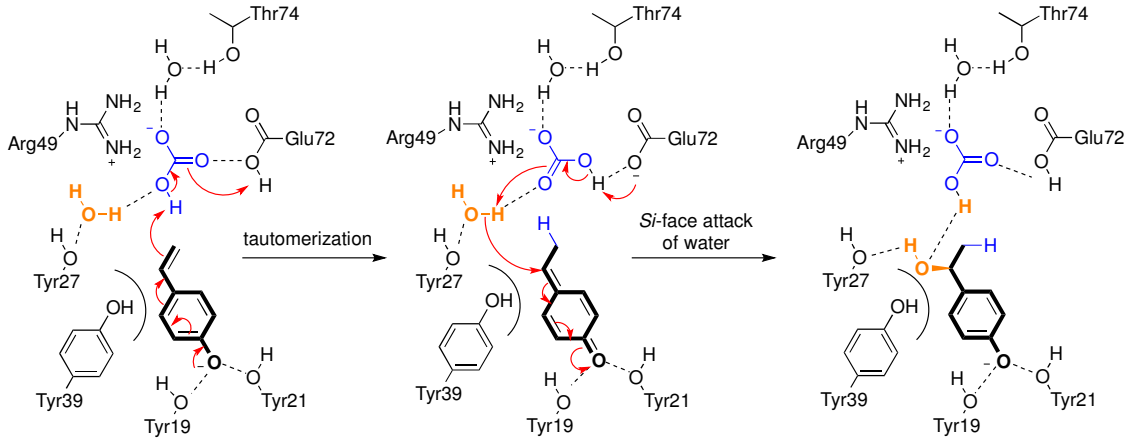
The ability of an enzyme to catalyze a reaction other than its annotated 'natural' activity is known as catalytic promiscuity[1-3] and is the result of evolutionary processes upon the encounter of 'non-natural' substrates and the organism's strive for survival.[4-6] This phenomenon is an important criterium for the selection of enzymes for the development of biocatalysts for the selective transformation of synthetic compounds, using rationally guided or random based directed evolution protocols.[7] Coumaric acids and their derivatives constitute monomeric units of lignin in plant cell walls[8] and are a major waste product of palm oil manufacture.[9-10] In nature, their degradation is achieved by ferulic- and phenolic acid decarboxylases (FDCs and PADs, respectively). These enzymes cleave their substrates into CO_2 and the respective 4-hydroxystyrenes (**1**, Scheme 1a), which constitute undesired off-flavor components in beer and wine[11] but have also found application as renewable building blocks for polymers with interesting dielectric properties.[12-14] By supplying an excess of CO_2 in the form of bicarbonate, the process can be run in the reverse β-carboxylation for the production of substituted coumaric acid derivatives (Scheme 1a).[15-17] During studies on the regioselective carboxylation, the promiscuous hydration of 4-hydroxystyrene derivatives by FDCs and PADs in the presence of bicarbonate was discovered (Scheme 1b), best results were obtained with a ferulic acid decarboxylase from *Enterobacter* sp. (FDC_*Es*).[18] The nucleophile scope apart from water was extended to methoxyamine, cyanide and propanethiol, which add via an analogous mechanism to furnish (*S*)-configured benzylic amines, nitriles and thioethers without the need for bicarbonate (Scheme 1b).[19]

The biocatalytic hydration of 4-hydroxystyrenes opens an easy access to the (*S*)-1-(4-hydroxyphenyl)ethanol structural motif (**2**) from renewable resources in a stereoselective fashion without the need for protecting groups. The product hydrate is a substructure of bioactive molecules exerting herbicidal,[20] insecticidal,[21-22] anti-proliferative[23] and (hepatitis C-) protease inhibition activities.[24-25] Though little is known about the active enantiomeric forms of these congeners, an improvement of the biocatalytic styrene hydration would be of practical interest to facilitate the exploitation of the benzylic alcohols as chiral building blocks.



R	NuH	Conv. (%)	*e.e.* (%)	Ref.
H, 3-OMe 3-OEt, 3-Cl 3-Me, 3,5-(OMe)$_2$	H_2O	≤ 82	≤ 71	[15]
H	$MeONH_2$	71	22	[19]
	HCN	71	85	
	*n*PrSH	56	81	

Scheme 1. a) Reversible (de)carboxylation of coumaric acids and b) promiscuous nucleophile addition to 4-hydroxystyrenes catalyzed by phenolic- and ferulic acid decarboxylases.



Scheme 2. Mechanism of the FDC_*E*s catalyzed hydration of 4-hydroxystyrene with bicarbonate acting as 'co-factor' for proton-transfer.

Experimental data[18] and quantum mechanical calculations[26] suggest, that the promiscuous hydration of 4-hydroxystyrene requires a bicarbonate anion as 'cofactor', which is bound onto Arg49 in the active site of the decarboxylase to achieve good conversion and high *e.e.* of the benzylic alcohol product (*S*)-**2** (Scheme 2). Potassium bicarbonate (0.5 M, pH 8.5) promotes the hydration of 4-vinylphenol (**1**) with ~65% conversion and ~80% *e.e.* using FDC_*E*s as biocatalyst.[18] In the absence of bicarbonate, however, a water molecule occupies its position, resulting in (almost complete) loss of stereoselectivity.[26] Consequently, supplementation of bicarbonate (>0.5 M) is required to maintain stereoselectivity, which renders this process economically and operationally less attractive.

In initial studies, the compensability of bicarbonate as 'cofactor' was investigated by replacing it with different (anionic) additives (Figure 1). Potassium phosphate buffer (50 mM) was used as reaction buffer and the pH was adjusted to 8.0 after dissolving the additive salts to avoid bicarbonate decomposition (pK_{aH} = 7.7) and to allow for direct comparison of additive effects. Control reactions in neat phosphate buffer gave moderate conversion (~60%) but completely lack stereoselectivity. Addition of Cl^-, HPO_4^{2-} or SO_4^{2-} improved the conversion slighty, while imidazole had adverse effects, again without stereoinduction. Borate gave low *e.e.* (15%), while conversion and *e.e.* were significantly improved by bicarbonate. Remarkably, best values for conversion and *e.e.* were obtained in the presence of acetate. Next, the concentration of bicarbonate and acetate was varied within a range of 10 and 500 mM (Figure S3). While the conversion profiles for both ions showed a similar behavior at increasing ion concentration (leveling off at ~90–95% conv. and 250 and 300 mM), the product *e.e.* profile with bicarbonate differs from that with acetate in a shallow maximum of 33% *e.e.* (at ~100 mM), while the *e.e.* rises linear with increased acetate concentration, reaching an *e.e.* of 45% at 500 mM. Since the carboxylate moiety appears to be an ideal promoter for stereoselective hydration, a set of carboxylic acid sodium salts with varying chain lengths, substituents and branching patterns was compared at neutral pH (7.0) (Figure 1b). Significant positive effects on the stereoselectivity were detected with all aliphatic carboxylates, depending on the side chain size (Et > ^{i}Pr > ^{t}Bu ≈ Me ≈ H) with a maximum for propanoate (74 % *e.e.*). Trifluoroacetate neither enhanced conversion nor the *e.e.*, due to its low pK_a, which disables it to act as catalytic acid in proton transfer (*c.f.* Scheme 2). Due to the formation of an unidentified side product, only 60% of the starting material was recovered in presence of formate.

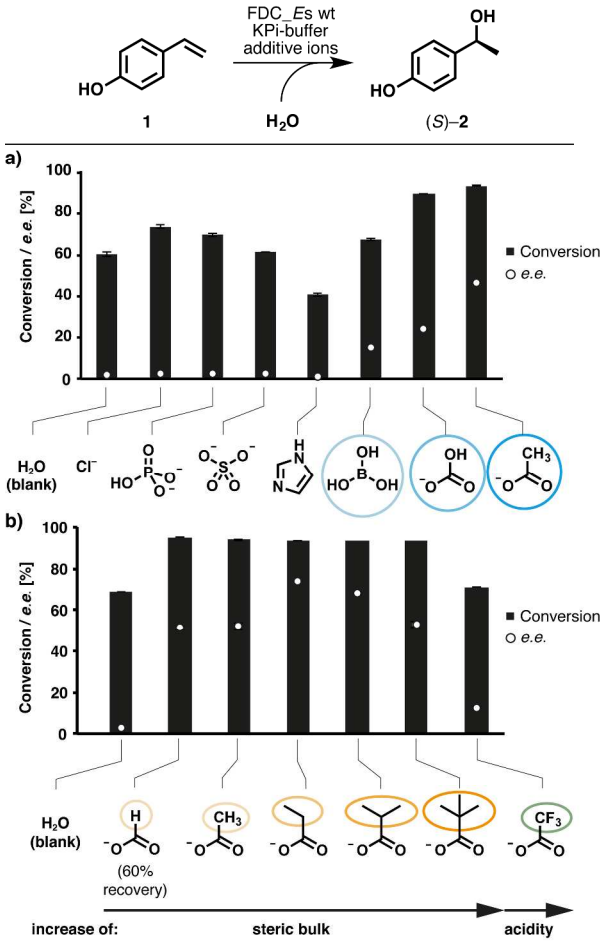


Figure 1. Asymmetric hydration in presence of (anionic) additives. Conditions: substrate **1** (10 mM, from 10 % w/w propylene glycol solution) in potassium phosphate buffer (50 mM), lyophilized *E. coli* cells containing wild type FDC_*Es* (20 mg/mL, 32 U). Effect of (a) various anions (0.5 M, pH 8.0) and (b) different carboxylates (0.5 M, pH 7.0) on the enzymatic hydration (additives are shown in the respective protonation state at the corresponding pH).

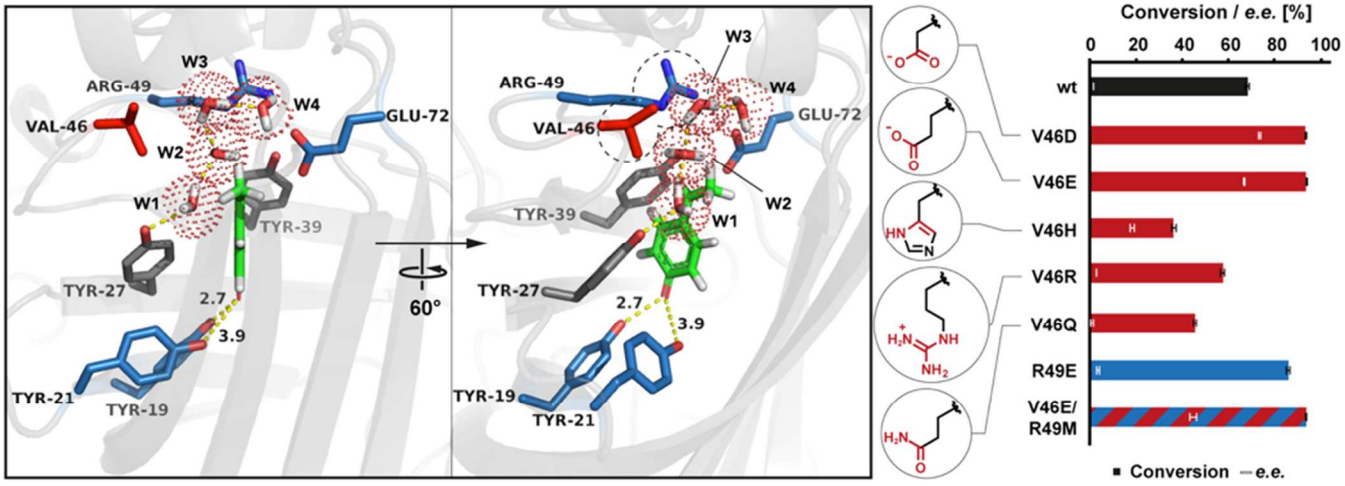


Figure 2. FDC_*Es* variants with tethered bicarbonate/acetate surrogates in positions Val46 and Arg49 (indicated by dashed circles). The quinone-methide form of substrate 4-vinylphenol (green) was docked into the active site of FDC_*Es* (PDB ID 4UU3) using the UCSF Chimera AutoDock Vina plugin. Catalytic key residues are shown in blue, Val46 in red and others in gray. Dotted red spheres indicate water molecules in the superimposed apo-structure. Surrogate residues at positions 46 and 49 are shown in their protonation state at pH 7.0. Conditions: substrate **1** (10 mM) in potassium phosphate buffer (50 mM, pH 7.0), lyophilized *E. coli* whole cells containing FDC_*Es* variants (20 mg/mL).

Based on these promising results, we envisioned the design of mutants bearing a 'tethered carboxylate' acting as catalytic residue in the active site to mediate the proton transfer,[27] thereby waiving the need for an external bicarbonate/carboxylate 'co-factor'. The amino acid residues introduced should meet two crucial requirements: (i) the spatial arrangement in the active site needs to mimic bound bicarbonate, suggesting residues near the bicarbonate binding-site as targets for mutagenesis, and (ii) residues must be chemically competent surrogates of bicarbonate/carboxylate to shuttle protons between the substrate C_β and Glu72.

After detailed inspection of active-site residues of FDC_*Es*, Val46 located on a mobile loop in vicinity to the bicarbonate binding-site was selected for mutations. Based on their ability to act as three-atom proton shuttle, Val46 was exchanged to Glu, Gln, Asp, His and Arg (Figure 2). The validity of this strategy was proven by the fact that the introduction of a carboxylate group in position 46 by aspartate (Val46Asp) or glutamate (Val46Glu) enhanced the stereoselectivity 39-fold and boosted the conversion by ~40%. The slightly higher stereoselectivity (ca. 6%) for the Asp versus the Glu mutant may be explained by the one CH_2-unit shorter tether, which is pulling the water nucleophile further out of the plane of symmetry towards the *Si*-face of the substrate (Figure 3). Apparently, only a carboxylate moiety is an efficient bicarbonate/acetate mimic, since mutations to histidine, glutamine and arginine were not beneficial (max. 57% conv. for Val46Arg). However, despite its lower activity, the Val46His mutant showed slight stereoselectivity (up to 17% *e.e.*).

A homology model of the Val46Asp/Glu mutants reveals that the additional carboxylate groups are well positioned in proximity to a water molecule (W1) to activate it as catalytic base within the hydrogen bond network of Tyr27, Glu72 and Arg49 (Figure 3). Changing the latter into a glutamate residue (thereby inverting the positive charge at this position into a negative one) could gain some additional hydration activity (86 % conv.) but yielded racemic product. Tyr39 was suggested to dictate the (*S*)-stereoselectivity through steric repulsion between its aromatic ring and C_β of the quinone methide intermediate, which consequently presents its *Si*-face to the water nucleophile located above the plane (Scheme 2).[26] A double variant having both the beneficial Val46Glu and a neutral Arg49Met mutation did not affect the conversion but showed a decrease in stereoselectivity compared to the single mutant. Removal of the hydrogen bond donor Arg49 weakens the bonding network and renders Tyr39 less rigid, which is responsible for the lower *e.e.* of the product. The Val46Glu mutant was also tested with other nucleophiles (methoxyamine, cyanide),[19] which gave improved stereoselectivities compared to the wild-type enzyme (Table S3).

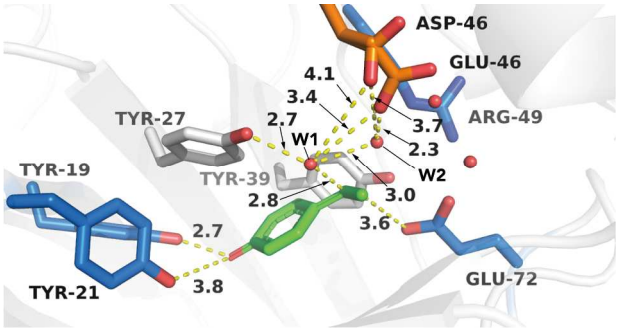


Figure 3. Overlay of the active site structures of the FDC_*Es* Val46Glu and Val46Asp variant (Swissmodel webserver). Mutated residues Asp/Glu46 are highlighted in

orange and the docked quinone methide form of the substrate 4-vinylphenol is shown in green (Autodock Vina plugin, UCSF Chimera). Water molecules shown as red spheres are derived from the superimposed wild-type FDC_*E*s crystal structure (PDB ID: 4UU3). Distances are given in Å.

The reaction conditions for the rationally designed 'hydratases' were optimized in terms of pH and effects of various organic co-solvents were examined (Figure S5). Furthermore, the stereoselectivity of the process was found to increase with decreasing temperature (Figure S4), indicating a major contribution of the enthalpy difference ($\Delta\Delta H^{\neq}$) to the free energy difference between the enantiomeric transition states ($\Delta\Delta G^{\neq}$).[28-29]

Finally, hydration of **1** was achieved with both the Val46Glu and Val46Asp variants with a maximum TON of ~220 and a product *e.e.* of ≤91% with 50 mM substrate loading at pH 6.0 and 25 °C (Tables S1 and S2). The process was subsequently performed on 100 mg scale with 0.8 mol% of FDC_*E*s V46E to afford 68 mg of (*S*)-hydrate product (60% yield) with high enantiomer purity (96% *e.e.* after recrystallization) underpinning the usability of this reaction on preparative scale.

In conclusion, prompted by the observation of bicarbonate- and acetate-assisted asymmetric hydration of hydroxystyrenes catalysed by ferulic acid decarboxylase we rationally designed a hydratase from this decarboxylase through mutation of an Asp- or Glu-carboxylate moiety into the active site, which efficiently functions as proton-shuttle. The mutants showed 40% higher activity and 39-fold improved stereoselectivity, which allowed preparative-scale transformations with turnover numbers of up to 220.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Experimental details, preparation of variants, docking and details on structural biology as well as supporting screening results (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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