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# Conversion of Pyruvate Decarboxylase into an Enantioselective Carboligase with Biosynthetic Potential

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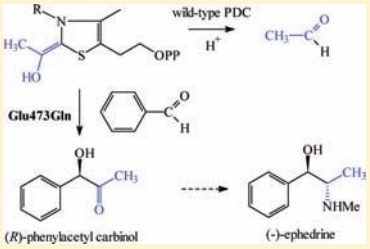
Supporting Information

**ABSTRACT:** Pyruvate decarboxylase (PDC) catalyzes the decarboxylation of pyruvate into acetaldehyde and $CO_2$ and requires the cofactors thiamin diphosphate and $Mg^{2+}$ for activity. Owing to its catalytic promiscuity and relaxed substrate specificity, PDC catalyzes carboligation side reactions and is exploited for the asymmetric synthesis of 2-hydroxy ketones such as (*R*)-phenylacetyl carbinol, the precursor of (−)-ephedrine. Although PDC variants with enhanced carboligation efficiency were generated in the past, the native reaction, i.e., formation of aldehydes, is heavily favored over carboligation side reactions in all these biocatalysts. We characterized an active site variant (Glu473Gln) in which partitioning between aldehyde release versus carboligation is inverted with an up to 100-fold preference for the latter pathway. Due to a defective protonation of the central carbanion/enamine intermediate, substrate turnover stalls at this catalytic stage and addition of external aldehydes leads to quantitative and enantioselective formation of 2-hydroxy ketones as shown for (*R*)-phenylacetyl carbinol, which is afforded with unmatched yields, rates, and purity. This protein variant thus constitutes an example for the rational design of biocatalysts with greatly enhanced accidental catalytic promiscuity by selective blockage of the native reaction and accumulation of reactive intermediates under steady-state turnover conditions.




Over billions of years, evolution has generated a multitude of mechanistically diverse enzymatic activities from a relatively small number of progenitor activities and ancestral scaffolds. Although the constant evolutionary pressure for optimized and efficient turnover mostly resulted in enzymes with tight substrate and reaction specificities, some enzymes exhibit a broad substrate specificity (for which they evolved), whereas almost every enzyme catalyzes off-pathway side reactions, features that have been termed substrate and catalytic promiscuity.[1−3]

Exploitation of enzyme promiscuity has become very popular in terms of redesigning and optimizing enzymatic activities for industrial applications.[4,5] Whereas substrate promiscuity has been studied for a long time, biocatalytic applications relying on catalytic promiscuity of enzymes have come to the fore relatively lately. A huge challenge of the latter approach is to make an existing (accidental) low promiscuous activity become higher than the original activity or to newly induce a desired activity without crippling the enzyme's ability to bind and turn over substrate. The redesign of enzyme specificities is difficult to achieve because of the inherent robustness of the original activity as manifested in the effect of mutations acquired in directed evolution.[6]

A well-characterized enzyme that is exploited for biocatalytic applications owing to its inherent substrate and catalytic promiscuity is pyruvate decarboxylase (PDC). PDC catalyzes the nonoxidative decarboxylation of pyruvate into acetaldehyde and $CO_2$ as part of the alcoholic fermentation in yeast and some bacteria and requires the cofactors thiamin diphosphate (ThDP) and $Mg^{2+}$ for catalytic activity.[7] The structure, function, and chemical mechanism of PDCs from different organisms have been studied in great detail and are well understood.[7,8] Conversion of the substrate pyruvate involves a series of substrate−cofactor conjugates as reaction intermediates (Scheme 1). Initially, carbonyl addition of pyruvate to the reactive C2 atom of the cofactor thiazolium portion yields the predecarboxylation intermediate 2-lactyl-ThDP (LThDP). Subsequent elimination of $CO_2$ produces the resonating α-carbanion and enamine forms of 2-hydroxyethyl-ThDP ($HEThDP^-$), a central and highly reactive intermediate state that is formed in any enzyme turning over pyruvate. In PDC, the carbanion/enamine is protonated at C2α, yielding its conjugate acid (HEThDP), before eventual liberation of acetaldehyde completes the catalytic cycle. A carboligation side reaction of the carbanion/enamine with the native substrate pyruvate (product acetolactate) is not observed for the wild-type enzyme but for some mutant proteins. Mutagenesis studies suggest an active site Asp residue (Glu in other

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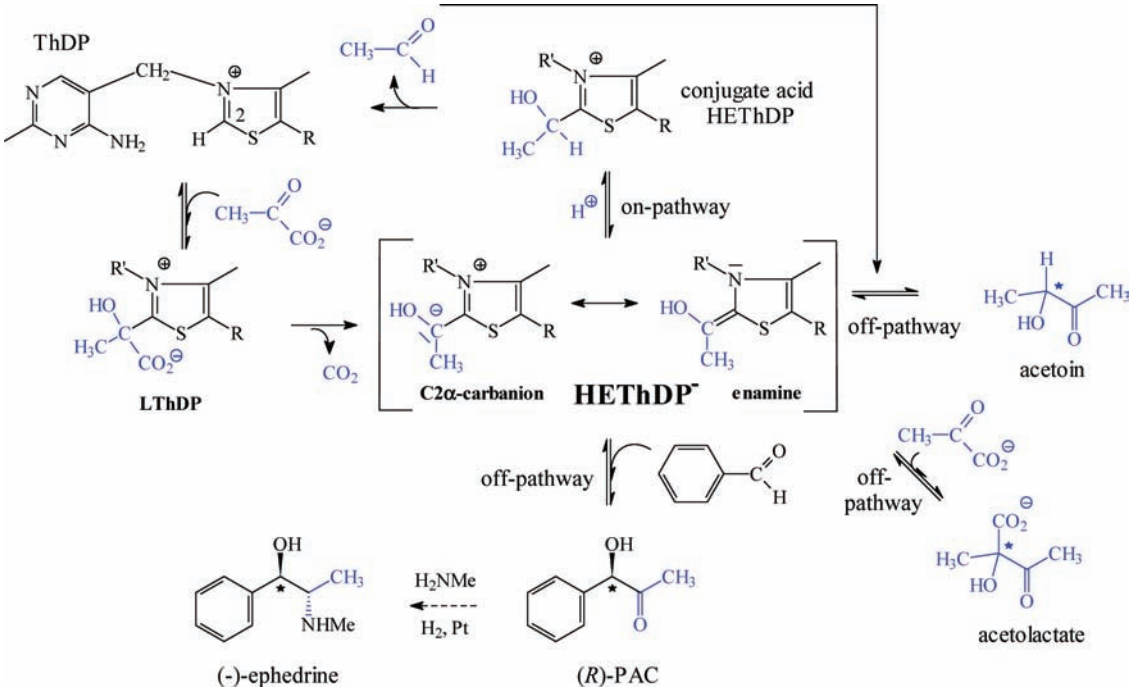
**Scheme 1. Catalytic Cycle and Side Reactions of PDC with Intermediates Involved**




ThDP enzymes) to prevent pyruvate from binding by electrostatic repulsion once the carbanion/enamine intermediate has been formed.[9−11] However, when large amounts of product acetaldehyde are accumulated or other aldehydes are externally added, carboligation side reactions of the carbanion/enamine with these acceptors take place with ≤1% rates relative to on-pathway protonation and acetaldehyde formation. The 2-hydroxy ketone products that arise from this promiscuous activity supply two versatile, exchangeable chemical groups in the immediate vicinity of a chiral center and thus constitute valuable building blocks for the synthesis of numerous compounds with pharmacological importance as, for instance, the tranquilizer and smoking cessation drug bupropion,[12] the antiallergic drug (−) cytoxazon,[13] or the multidrug-pump inhibitor 5-methoxyhydnocarpin.[14]

In this context, the PDC-catalyzed carboligation of pyruvate (or acetaldehyde) and benzaldehyde has been successfully used for the enantioselective synthesis of (*R*)-phenylacetyl carbinol (*R*-PAC) starting as early as in the 1930s.[15] This compound is the chemical precursor of several congestants and antiasthmatics like ephedrine, pseudoephedrine, and norephedrine.[16] The biosynthetic potential of PDCs from yeast (different *Candida* and *Saccharomyces* strains) or bacteria (*Zymomonas mobilis*) for (*R*)-PAC synthesis has been extensively explored for the purified protein as well as PDC-containing cells and cell lysates.[17,18] Despite the general suitability of PDC for chemoenzymatic (*R*)-PAC synthesis, formation of several byproducts (acetoin, benzyl alcohol, and 1-phenylpropane-1,2-diol), limited solubility, as well as toxicity of substrates/products constitute severe problems. Therefore, substantial efforts were made to increase the stability of the protein and strains, to optimize fermentation parameters, and to minimize formation of byproduct.[16,19−21]

In particular, PDC from *Z. mobilis* (*Zm*PDC) has found widespread application for chemoenzymatic synthesis, since it is relatively stable and constitutively active as opposed to PDCs from yeast, which are subject to homotropic substrate activation. Mutagenesis studies on *Zm*PDC revealed that substitutions of residue Trp392 located at the entrance to the active site resulted in an increase in yield of (*R*)-PAC formation, presumably reflecting an improved affinity for benzaldehyde or an easier release of (*R*)-PAC.[22] However, the main decarboxylase activity in these and other "carboligation variants" is barely affected such that on-pathway production of acetaldehyde exceeds that of off-pathway PAC synthesis by several orders of magnitude.

Analysis of the molecular reaction mechanism of *Zm*PDC and of related enzymes that act on pyruvate shed light on the stereochemical course of substrate processing, identified individual contributions of active site residues for discrete steps of catalysis, and allowed estimating the lifetimes of key intermediates under turnover conditions (see Scheme 1).[23] These studies revealed that the side chain of Glu473 in *Zm*PDC has a dual catalytic role: it serves to orient the substrate carboxylate for facile decarboxylation of LThDP and additionally acts as a general acid that protonates the incipient carbanion/enamine formed upon decarboxylation (Figure 1).[24] Quantitative analysis of the intermediate distribution by NMR and optical spectroscopy showed that the predecarboxylation intermediate LThDP and the conjugate acid of the carbanion/enamine are largely accumulated in wild-type *Zm*PDC at steady state, whereas the fraction of the carbanion/enamine is negligible, prompting us to propose that decarboxylation and protonation of the incipient carbanion may occur in the same transition state.[24] Comparative characterization of active site variants could identify variant Glu473Gln, in which protonation of the carbanion/enamine is crippled and takes place 2000-fold slower than in wild-type *Zm*PDC. Since all preceding catalytic steps (binding of pyruvate, decarboxylation of LThDP) are fairly catalyzed by the Glu473Gln variant with rates exceeding that of protonation by

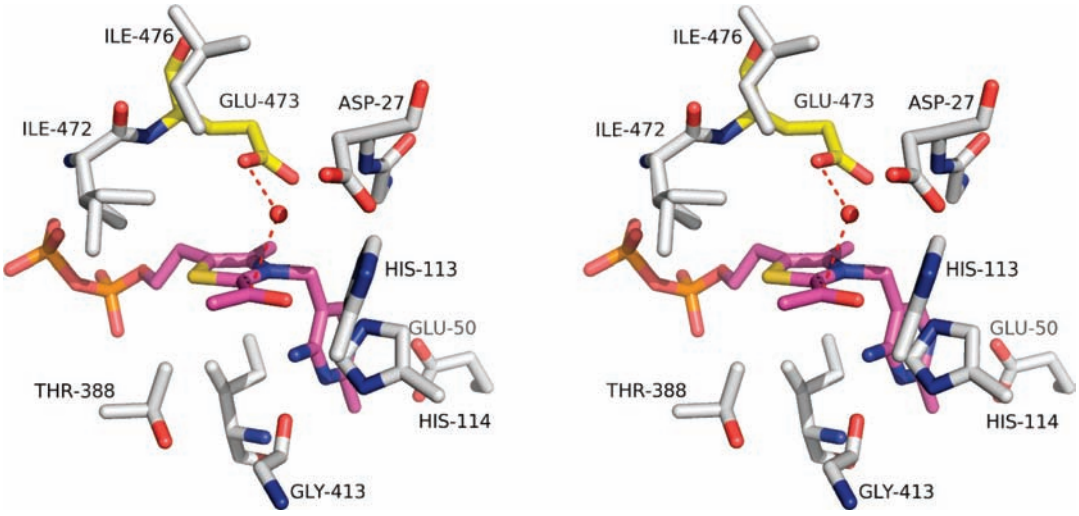



**Figure 1.** Active site of *Zm*PDC in stereoview with modeled HEThDP carbanion/enamine intermediate as trapped in pyruvate oxidase from *L. plantarum* (pdb code 2EZT).[34] Residue Glu473 is highlighted in yellow. A bridging water molecule observed as a place holder in the resting state of *Zm*PDC is likely to be involved in protonation of the carbanion/enamine with Glu473 acting as the general acid.

at least 1 order of magnitude, the catalytic cycle stalls at the carbanion/enamine intermediate state, which is accumulated to almost full occupancy. Earlier studies on the yeast ortholog also indicated a role of the conserved glutamate in postdecarboxylation steps.[10] The aforementioned properties make *Zm*PDC variant Glu473Gln a promising candidate for carboligation applications including (*R*)-PAC synthesis as partitioning of the carbanion/enamine between on-pathway protonation and off-pathway carboligation is expected to be significantly changed in favor of carboligation. Here, we demonstrate that a substitution of Glu473 by glutamine indeed converts *Zm*PDC into an efficient and enantioselective carboligase with negligible background of the original decarboxylase activity.

## MATERIAL AND METHODS

**Gene Expression and Protein Purification.** Plasmid pZY134b containing the gene of wild-type *Zm*PDC was used for expression in *E. coli* strain SG13009. Expression was induced by addition of 1 mM IPTG at an $OD_{600}$ of 0.5, and cells were grown for another 15 h at 30 °C. *Zm*PDC variant Glu473Gln was expressed in *E. coli* JM109 cells using plasmid pPLZM as vector that contains a heat-inducible promoter. Cells were initially cultivated at 30 °C, and expression was induced by rapidly shifting the temperature to 42 °C. Cells were then grown for 4 h at 42 °C and subsequently harvested by centrifugation (20 min, 4.000*g*, 4 °C).

Cells were gently solubilized in low salt buffer (10 mM MES, 100 $\mu$M ThDP, 1 mM $MgSO_4$, pH 6.5) on ice. After cell disruption and removal of cell debris by centrifugation (20 min, 59.000*g*, 4 °C) DNA was digested using DNaseI. *Zm*PDC was initially enriched by a two-step fractional ammonium sulfate precipitation (20% and 43% (w/v)). The conductivity of the protein solution was adjusted to <0.7 mS/cm$^2$ by exhaustive dialysis against low salt buffer. The protein solution was applied to an anion exchange column (Fractogel EMD TMAE (S), Merck KGaA Germany) using a linear gradient from 0 to 40% high salt buffer (10 mM MES, 100 $\mu$M ThDP, 1 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, pH 6.5) over a total volume of 150 mL. Fractions containing PDC were combined, concentrated, and further separated by gel fitration (Superdex 200 16/60, GE Healthcare, equilibrated with 50 mM MES, 100 $\mu$M ThDP, 1 mM $MgSO_4$, pH 6.0). The purity of the fractions was judged by SDS-PAGE, and concentrations were determined by the method of Bradford using BSA as standard.[25]

**Circular Dichroism-Based Detection of Carboligase Activity.** Near-UV circular dichroism (CD) spectroscopy was used as a tool for detection of chiral carboligation products (acetoin, acetolactate, PAC, and PAC derivatives). All CD experiments were carried out on a Jasco J-810 spectropolarimeter equipped with a thermostatted cell holder and using a 1 mm cuvette. In a typical experiment, 190 $\mu$L of the reaction mixture (50 mM potassium phosphate buffer pH 6.0, 100 $\mu$M ThDP, 1 mM $MgSO_4$, plus varied concentrations of pyruvate and benzaldehyde) was preincubated at 30 °C for 5 min, and substrate turnover was initiated by addition of 10 $\mu$L of enzyme (1.5 mg/mL after mixing). CD spectra were recorded in the 260−340 nm range. Due to the pronounced absorption of benzaldehyde at 280 nm, the absorption maximum of product PAC, and the corresponding unfavorable signal-to-noise ratio of the CD signal at high benzaldehyde concentrations, formation of chiral products was kinetically analyzed at 300 nm.

**Product Analysis by $^1$H NMR Spectroscopy.** Assay conditions were identical as given for the CD experiments (see above), except that the reaction mixture contained 10% (v/v) $D_2O$. 1D $^1$H NMR spectra were recorded at 300 K before and at distinct time points during substrate turnover (pyruvate as sole substrate and pyruvate + benzaldehyde) using an ARX 400 MHz Avance FT-NMR spectrometer from Bruker. Chemical shifts of standards ($\delta$ ppm vs *d*-TSP) were used for unambiguous identification of substrates and products formed during turnover.

Pyruvate (keto form): 2.38 (s, 3H). Pyruvate (hydrate): 1.49 (s, 3H). Benzaldehyde: 8.0−7.6 (m, 5H), 9.96 (s, 1H). Acetaldehyde (aldo form): 2.25 (d, 3H), 9.68 (q, 1H). Acetaldehyde (hydrate): 1.34 (d, 3H), 5.26 (q, 1H). Acetoin: 1.38 (d, 3H), 2.23 (s, 3H), 4.43 (q, 1H). PAC: 2.12 (s, 3H), 5.4 (s, 1H), 7.55−7.40 (m, 5H). Acetolactate: 1.46 (s, 3H), 2.26 (s, 3H). Acetate: 1.93 (s, 3H).

**Determination of Enantiomeric Excess of Chiral Products.** *Reaction Conditions.* Aliphatic substrates and enzyme (3 mg/mL) were dissolved in reaction buffer (50 mM potassium phosphate pH 6.0, 100 $\mu$M ThDP, 2.5 mM $MgSO_4$,). Stock solutions of aromatic substrates were prepared in DMSO. Substrate and enzyme solutions were mixed to give final concentrations of 18 mM substrate(s) and 0.3 mg/mL enzyme in 40 mM potassium phosphate, 80 $\mu$M ThDP, 2 mM $MgSO_4$, 20% (v/v) DMSO in a total volume of 1.5 mL. The reactions were carried out for 20−40 h at 30 °C, and products were subsequently extracted using 300 $\mu$L of ethylacetate. After vortexing and centrifugation, the organic phase was used for determination of the products' enantiomeric excess (ee).

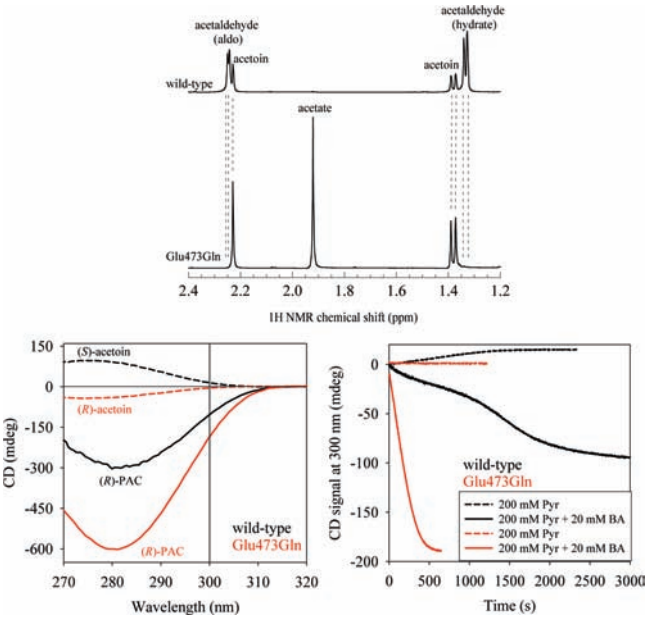



**Figure 2.** Analysis of carboligation side reactions in *Zm*PDC wild type and variant Glu473Gln by $^1$H NMR and CD spectroscopy. (Top) Section of NMR spectra showing resonances of products after complete conversion of substrate pyruvate (wild type 50 mM, variant 10 mM). (Bottom) CD spectroscopic analysis of chiral reaction products acetoin (pyruvate as sole substrate) and PAC (pyruvate and benzaldehyde) showing both near-UV CD spectra of the product mixture and corresponding kinetics monitored at 300 nm. Experimental details are given in the Material and Methods section.

*Pyruvate as Sole Substrate.* Separation of (*R*)- and (*S*)-acetoin was performed by chiral GC as described elsewhere.[26]

*Pyruvate + Benzaldehyde.* Analysis of (*R*)- and (*S*)-PAC and of halogenated derivatives was performed by chiral HPLC. For analysis of PAC, a Chiralcel OB (Daicel) column (250 × 4.6 mm) was used with the following parameters: mobile phase, 90% hexane:10% 2-propanol; flow, 0.75 mL/min; pressure, 25 bar; temperature, 20 °C; detection at 210 nm; $t_R$ (*S*)-product = 13.1 min; $t_R$ (*R*)-product = 14.6 min.

*Pyruvate + 2-Chlorobenzaldehyde.* A Chiral OM (CS Chromatography Service) column (250 × 4.6 mm) was employed using the following parameters: mobile phase, 90% hexane:10% 2-propanol; flow, 0.75 mL/min; pressure, 25 bar; temperature, 25 °C; detection at 210 nm; $t_R$ (*S*)-product = 13.1 min, $t_R$ (*R*)-product = 15.6 min.

*Pyruvate + 3,5-Dichlorobenzaldehyde.* A Chiral OM column (250 × 4.6 mm) was employed using the following parameters: mobile phase, 99% hexane:1% 2-propanol; flow, 0.75 mL/min; pressure, 25 bar; temperature, 25 °C; detection at 210 nm; $t_R$ (*S*)-product = 59.7 min; $t_R$ (*R*)-product = 53.7 min.

*2-Ketobutyrate + Benzaldehyde.* A Chiral OM column (250 × 4.6 mm) was employed using the following parameters: mobile phase, 95% hexane:5% 2-propanol; flow, 0.5 mL/min; pressure, 25 bar; temperature, 40 °C; detection at 210 nm; $t_R$ (*S*)-product = 20.8 min; $t_R$ (*R*)-product = 24.2 min.

**Analysis of the Thermal Stability of ZmPDC.** The thermal stability of *Zm*PDC wild type and variant Glu473Gln was comparatively analyzed in the 338−353 K (65−80 °C) range. The protein (0.1 mg/mL final concentration in 25 mM MES pH 6.0, 50 $\mu$M ThDP, 0.5 mM $MgSO_4$) was incubated at defined temperatures, and unfolding was monitored at 208 nm using a Jasco J-810 spectropolarimeter equipped with a thermostatted cell holder and a 1 mm cuvette. The obtained first-order rate constants of unfolding where evaluated according to Eyring's equation

$$\ln\left(\frac{k_{obs}\cdot h}{k_B\cdot T}\right) = \frac{-\Delta H^{\ddagger}}{R\cdot T} + \frac{\Delta S^{\ddagger}}{R} \quad (1)$$

## RESULTS

**Decarboxylase Activity and Intrinsic Carboligation Reactions of PDC Wild Type versus Variant Glu473Gln.** The nonoxidative conversion of pyruvate into acetaldehyde and $CO_2$ is catalyzed by *Zm*PDC wild type with a catalytic constant of 150 ± 5 $s^{-1}$ and a Michaelis constant of 0.31 ± 0.03 mM for substrate pyruvate (pH 6.0, 30 °C). Variant Glu473Gln exhibits a residual decarboxylase activity of only 0.1% ($k_{cat}$ = 0.15 ± 0.004 $s^{-1}$), while substrate affinity is barely affected ($K_M$ = 0.40 ± 0.05 mM) as previously reported.[24] Quantitative analysis of the intermediate distribution revealed that the tetrahedral intermediates LThDP and HEThDP (conjugate acid of carbanion/enamine) are both accumulated to high occupancy in the wild-type enzyme under steady-state turnover conditions, whereas the fraction of the carbanion/enamine intermediate is marginal. In contrast, variant Glu473Gln accumulates the HEThDP carbanion/enamine (HEThDP$^-$) to almost full occupancy (>95%).[24]

Both wild-type *Zm*PDC as well as variant Glu473Gln catalyze the formation of acetaldehyde and byproduct acetoin, the latter resulting from a carboligation side reaction of the HEThDP carbanion/enamine and acetaldehyde, as shown by NMR and CD spectroscopy (Figure 2). After complete conversion of pyruvate, large amounts of acetoin and native product acetaldehyde as well as traces of acetate (~1%) can be detected in the case of the wild-type enzyme. Acetoin is predominantly formed as the (*S*)-enantiomer (ee 23%). Variant Glu473Gln exhibits a different enantioselectivity as it produces (*R*)-acetoin in excess (ee 33%) and large amounts of acetate, which originates from an oxygenase side reaction of the HEThDP carbanion/enamine with dioxygen (the thereby formed peracetic acid is in turn converted to acetate in the presence of pyruvate).[27] For neither protein, formation of acetolactate (carbanion/enamine + pyruvate) could be detected. The observation that *Zm*PDC variant Glu473Gln produces acetoin with higher yields than the wild-type enzyme and further exhibits a markedly increased oxygenase side reaction supports the notion that it kinetically stabilizes the highly reactive HEThDP carbanion/enamine intermediate. In addition, residue Glu473 appears to be a determinant for controlling enantioselectivity as different enantiomers are preferentially formed for wild-type enzyme ((*S*)-acetoin) and variant ((*R*)-acetoin).

**Biosynthetic Potential of Variant Glu473Gln for Phenylacetyl Carbinol Synthesis.** In view of the aforementioned enhanced intrinsic carboligation (product acetoin) and oxygenase (product acetate) side reactions of *Zm*PDC variant Glu473Gln compared to the wild-type enzyme, we studied if the variant is an effective catalyst for the enantioselective synthesis of PAC that is formed when PDC is simultaneously reacted with either pyruvate *or* acetaldehyde as donor and benzaldehyde as acceptor. Initial trials showed that acetaldehyde is a poor substrate for PAC synthesis in the case of the variant (data not shown). This finding comes not unexpected since Glu473 was shown to be vitally involved in protonation of the carbanion/enamine, and by invoking the rule of microscopic reversibility, a substitution of this residue impairs the reverse reaction, that is, formation of the carbanion/enamine by deprotonation of

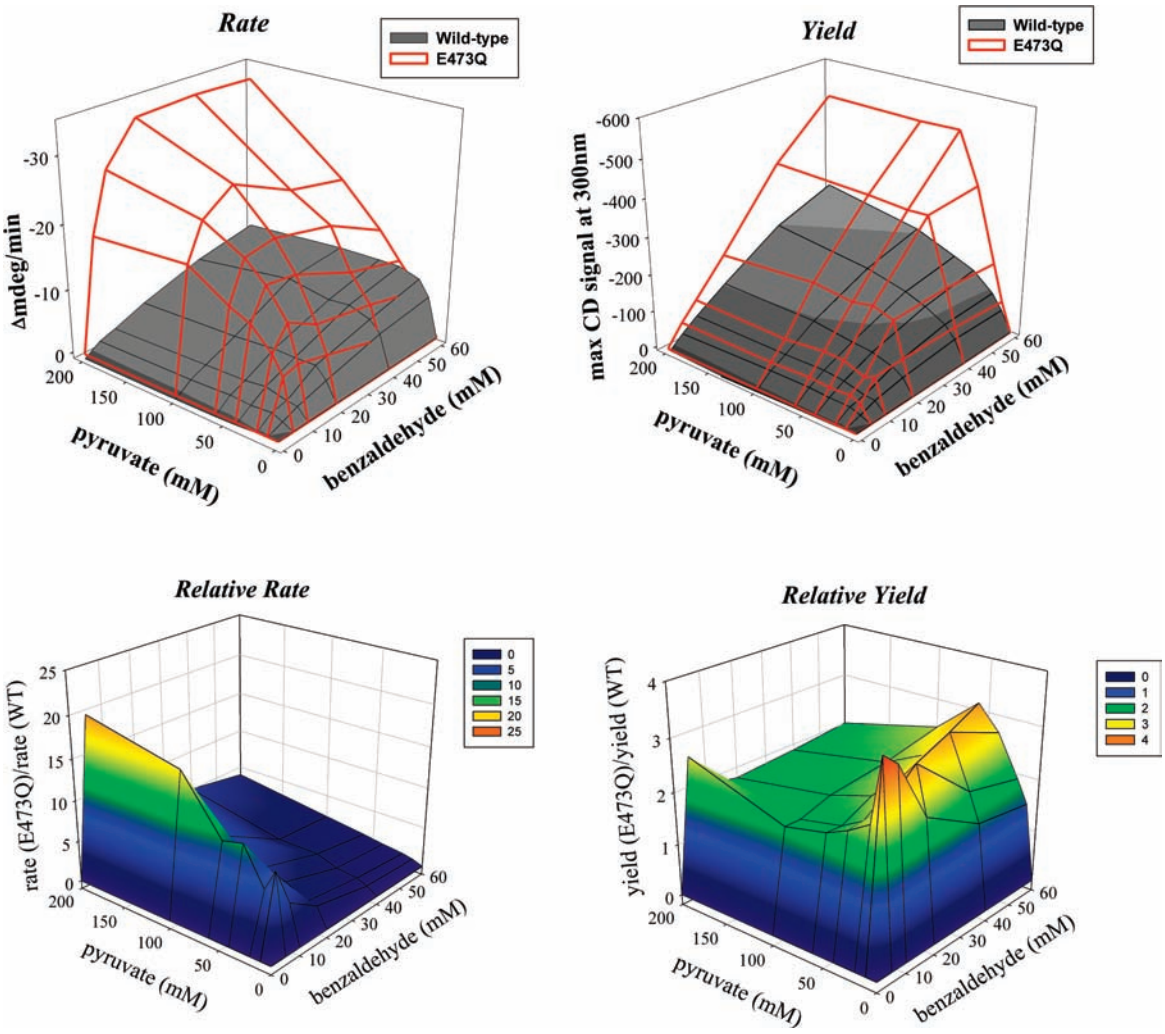



**Figure 3.** Concentration dependence of PAC formation rates and yields (top), and corresponding ratio between variant Glu473Gln and wild type (bottom) as detected by CD spectroscopy. Experimental details are given in the Material and Methods section.

HEThDP after covalent addition of acetaldehyde to enzyme-bound ThDP.[24] Similar conclusions were reported for a PDC variant from yeast, in which the homologous replacement of glutamate by glutamine significantly enhanced the acetoin synthase (pyruvate as donor and acetaldehyde as acceptor) activity.[9] When pyruvate is used along with benzaldehyde, the *Zm*PDC variant produces PAC with rates and yields that clearly exceed these of the wild-type enzyme when relying on CD spectroscopic analysis of chiral reaction products (Figures 2 and 3). Whereas wild-type *Zm*PDC typically exhibits multiphasic progress curves, which under the conditions deployed may take as long as a few thousand seconds until completion, a rapid linear decrease of the CD signal is observed in the case of the variant with half-times of ~150 s under identical reaction conditions (Figure 2). Also, the total CD signal amplitude is much higher for the variant. Given that wild-type *Zm*PDC produces (*R*)-PAC with an almost perfect ee of >98%, this finding indicates that the variant synthesizes more chiral product(s) than the wild-type enzyme. A systematic analysis of the product yields and reaction rates of variant Glu473Gln versus wild-type enzyme shows that under all concentration conditions deployed the variant is a better catalyst for carboligation of pyruvate and benzaldehyde. The rates of product formation are up to 20-fold higher for the variant at high pyruvate and low benzaldehyde concentrations (Figure 3). The total product yield is 2−4-fold higher in the case of the variant and is particularly prononouced at low benzaldehyde concentrations, which are commonly used for biocatalytic applications due to enzyme inactivation at high (>50 mM) benzaldehyde concentrations. In addition to CD spectroscopic analysis of chiral reaction products, the product pattern was further characterized by 1D $^{1}$H NMR spectroscopy (Figure 4), which is a suitable tool to quantitatively and simultaneously analyze all products that may derive from conversion of pyruvate and benzaldehyde. When *Zm*PDC wild type is reacted with equimolar amounts of both substrates, a product mixture including native product acetaldehyde, acetoin, PAC, and acetate is detected after complete conversion of pyruvate. Also, more than 50% of the initially applied substrate benzaldehyde can be detected. The yield of PAC relative to substrate pyruvate amounts to ~30%. In contrast, variant Glu473Gln almost quantitatively and stoichiometrically converts both substrates to PAC (up to 98% yield) along with some traces of acetate and benzaldehyde. Analysis of the enantioselectivity by chiral HPLC reveals that both the wild-type enzyme (ee 98.2%) as well as variant Glu473Gln (ee 98.4%) preferentially catalyze the formation of (*R*)-PAC. For neither protein, potential carboligation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products benzoin (benzaldehyde + benzaldehyde) or 2-hydroxypropiophenon (benzaldehyde as donor plus acetaldehyde as acceptor) could be detected.

Given that *Zm*PDC Glu473Gln exclusively catalyzes formation of (*R*)-PAC with an ee of >98% and negligible background of the original decarboxylase or other carboligase activities, the signals obtained in the CD-based assay can be directly translated into absolute concentrations. Accordingly, formation of 1 mM (*R*)-PAC gives a change of −8.6 mdeg of the CD signal at 300 nm (−8600 deg cm$^2$ dmol$^{-1}$). Using this relation, the maximal specific activity of *Zm*PDC Glu473Gln for PAC synthesis can be estimated as 2.5 U/mg (200 mM pyruvate, 40 mM benzaldehyde). Remarkably, this value exceeds that of the decarboxylase activity of this variant even in the absence of benzaldehyde (0.15 U/mg) by a factor of ~17. When the variant is reacted with both pyruvate and benzaldehyde, carboligation of the carbanion/enamine with benzaldehyde to yield PAC is >100-fold favored over protonation and aldehyde release as evidenced by the predominant formation of PAC (judged by NMR). In fact, we could detect only traces of acetaldehyde or acetoin as products (<1% yield). Relying on the same premises as outlined for the variant, we estimated the carboligase activity (PAC formation) of *Zm*PDC wild type. The maximal activity for PAC synthesis was estimated to ~0.7 U/mg (40 mM pyruvate, 60 mM benzaldehyde), which accounts for ~0.5% of the main decarboxylase activity (Figure 5).

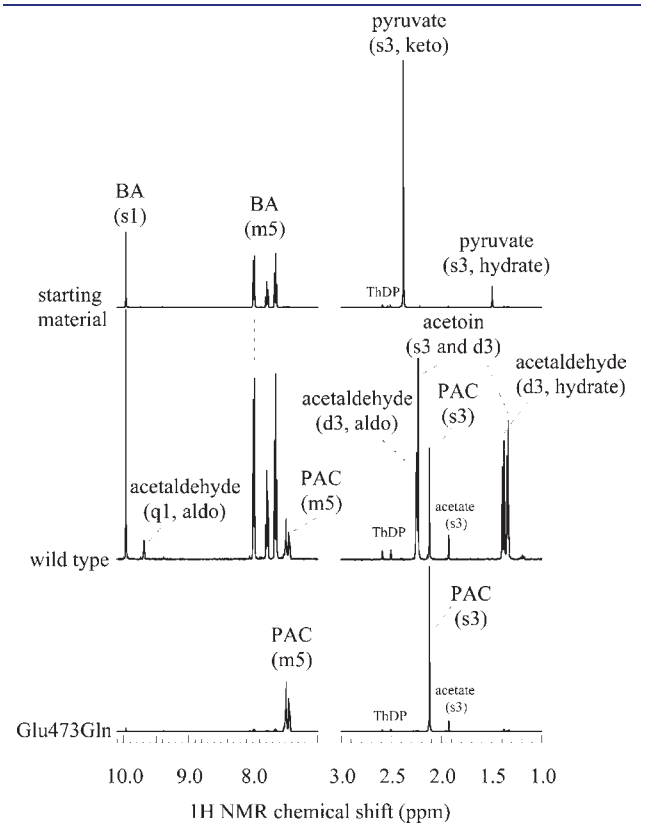


**Figure 4.** 1D $^1$H NMR spectroscopic analysis of product pattern after turnover of pyruvate and benzaldehyde (8 mM each) by *Zm*PDC wild type and variant Glu473Gln under equilibrium conditions (complete conversion of pyruvate, no further change of near-UV CD spectra). Assignment of the resonances is given in the spectra. Further experimental details are given in the Material and Methods section.

**Chemoenzymatic Synthesis of PAC Derivatives.** In view of the ability of variant Glu473Gln to highly favor carboligation reactions versus aldehyde release, we investigated the substrate range and characterized potential carboligation reactions using pyruvate and benzaldehyde derivatives. In an initial screen, we relied on a colorimetric assay that allows one to rapidly identify promising substrates for carboligation (data not shown).[28] Two different approaches were undertaken. First, we tested whether 2-ketobutyrate could effectively replace pyruvate as a donor. Recent studies have demonstrated that the binding pockets of ThDP enzymes that act on pyruvate are perfectly shaped to accommodate the methyl substituent of pyruvate and that larger substrates are bound with largely decreased affinities.[29] Second, we used halogenated benzaldehyde analogs (2-chlorobenzaldehyde, 3,5-dichlorobenzaldehyde) as substrates. In particular, halogenated PAC derivatives appear to be useful building blocks as they may be used for further derivatization. Carboligation products were analyzed by GC-MS and chiral HPLC. When 2-ketobutyrate was applied as a substrate instead of pyruvate,

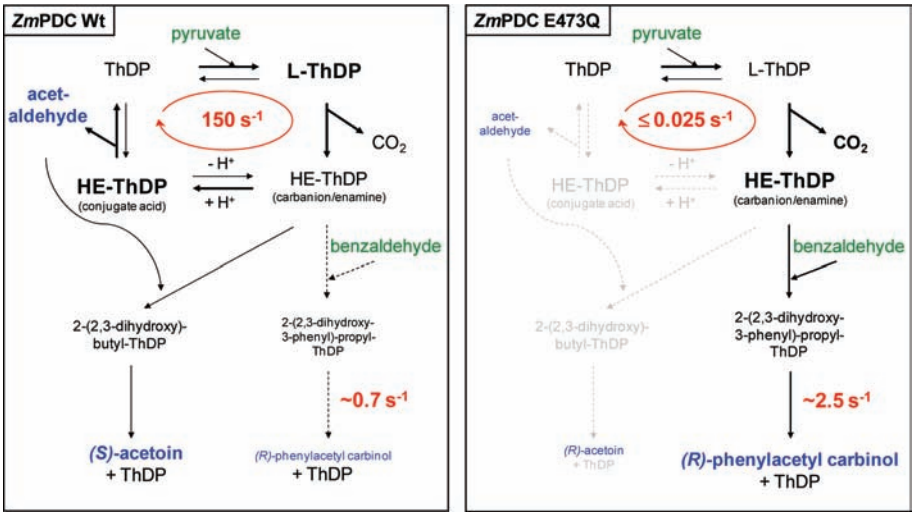


**Figure 5.** On-pathway and off-pathway reactions in *Zm*PDC wild type and variant Glu473Gln for turnover of substrates pyruvate and benzaldehyde with intermediates involved. Intermediates that are accumulated to high occupancy at steady state are shown in bold. Catalytic constants for the decarboxylase (product acetaldehyde) and carboligation (product PAC) activity are highlighted.

both wild type as well as the variant converted substantially less substrates to the corresponding (*R*)-phenylpropionyl carbinol product relative to PAC (pyruvate plus benzaldehyde), although a high enantioselectivity was observed (ee > 98%). The product yield, however, was approximately 2-fold higher for the wild-type enzyme compared to the variant. In contrast, the variant showed either 6-fold (3,5-dichlorobenzaldehyde) or 2-fold (2-chlorobenzaldehyde) higher yields (carboligation product) relative to the wild-type enzyme, clearly demonstrating that variant Glu473Gln could be a good starting point for the design of carboligation variants with altered (tailormade) substrate pockets. Both proteins preferentially produce the (*R*)-enantiomer for the two halogenated PAC derivatives, although enantioselectivity is not as high as in case of PAC formation (2-chloro-PAC: wild-type ee 90%, variant ee 92.5%; 3,5-dichloro-PAC: wild-type ee 37%, variant ee 17%).

**Thermal Stability of ZmPDC Variant Glu473Gln.** PDC from *Z. mobilis* exhibits a higher stability than the yeast or plant orthologs and is thus suited for long-term biocatalytic applications such as fermentation. In order to test whether or not a substitution of Glu473 by Gln affects the thermal stability, we compared the kinetics of thermal unfolding of wild-type *Zm*PDC and variant Glu473Gln in the range of 338−353 K (Supporting Information, Figure 1). Surprisingly, the variant showed an increased thermal stability relative to the wild type as, e.g., at 343 K (70 °C), the half-life for unfolding increased from 50 (wild type) to 500 s (variant). Analysis of the unfolding kinetics according to Eyring revealed a difference in $\Delta\Delta H^{\ddagger}$ of about +100 kJ/mol. It is interesting to note in this regard that most mutations that alter enzyme functions tend to be destabilizing; however, it was shown that substitution of key residues that result in a loss of function tend to be stabilizing.[30] Even though the molecular origins for this increased stability of the Glu473Gln variant remain to be elucidated, this observation highlights an additional advantage of the Glu473Gln variant for biocatalytic applications.

## CONCLUSIONS

The redesign of enzyme activities for biocatalytic applications constitutes a great challenge that has been approached relying on different strategies including directed evolution, domain swapping, iterative saturation mutagenesis, or mutagenesis of residues at the active site.[5,31,32] Here, we demonstrated that the detailed analysis of an enzymatic reaction mechanism can provide essential information to divert central reaction intermediates down promiscous pathways by blocking the native reaction. This principle was applied for the ThDP-dependent enzyme PDC that is exploited for synthesis of drug precursor (*R*)-PAC. This compound is formed in a side reaction when the central HEThDP carbanion/enamine intermediate undergoes carboligation with externally added benzaldehyde in competition to on-pathway protonation of the intermediate and acetaldehyde release. However, the yields for the promiscous activity are rather low since the protein favors protonation kinetically by a factor of 100 or more. Our studies on *Zm*PDC revealed that Glu473 participates in catalysis at different stages. Specifically, when Glu473 is substituted by Gln, the resulting variant is deficient in protonating the HEThDP carbanion/enamine and accumulates this intermediate to full occupancy. Addition of pyruvate and benzaldehyde to variant Glu473Gln leads to quantitative formation of (*R*)-PAC with a negligible background of <1−2% of the original decarboxylase activity (product acetaldehyde) or other side reactions (product acetoin) commonly observed for PDCs (Figure 5). Hence, PAC can be afforded with great purity in the case where equimolar amounts of pyruvate and benzaldehyde are reacted. This constitutes a great advantage, since in all cases studied so far, including wild-type *Zm*PDC and related variants of other PDCs,[33] acetaldehyde and acetoin are largely accumulated as byproduct in the course of chemoenzymatic PAC synthesis, which requires laborious product workup. In the case of PDC from yeast, substitution of the homologous glutamate residue (Glu477) to glutamine did result in a variant with enhanced carboligation potency, although the product yields (∼60% starting from 4 mM benzaldehyde and 4 mM pyruvate) were not as high as that observed for *Zm*PDC.[33] Over the whole pyruvate and benzaldehyde concentration range tested, *Zm*PDC variant Glu473Gln produces (*R*)-PAC with higher yields and rates compared to wild-type *Zm*PDC. While the absolute yields are 2−4-fold higher, the rate of PAC formation is up to 20-fold increased at low benzaldehyde concentrations (Figure 3). The high enantioselectivity of the wild-type enzyme (ee 98.2%) is retained in the variant (ee 98.4%). A preliminary analysis of carboligation reactions using different substrates indicates that the variant is a good starting point for the rational design of enantioselective biocatalysts with additional mutations that may bring about new substrate specificities. In addition to the multifold enhanced carboligation potency that affords (*R*)-PAC with unparalleled yields, rates, and purity, variant Glu473Gln further exhibits a markedly increased thermal stability and thus appears ideally suited to be exploited for large-scale applications.

## ASSOCIATED CONTENT

**Supporting Information.** Thermal unfolding of *Zm*PDC wild type and variant Glu473Gln. This material is available free of charge via the Internet at http://pubs.acs.org.

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