Transketolase catalyzes asymmetric C–C bond formation of two highly polar compounds. Over the last 30 years, the reaction has unanimously been described in literature as irreversible because of the concomitant release of CO$_2$ if using lithium hydroxypyruvate (LiHPA) as a substrate. Following the reaction over a longer period of time however, we have now found it to be initially kinetically controlled. Contrary to previous suggestions, for the non-natural conversion of synthetically more interesting apolar substrates, the complete change of active-site polarity is therefore not necessary. From docking studies it was revealed that water and hydrogen-bond networks are essential for substrate binding, thus allowing aliphatic aldehydes to be converted in the charged active site of transketolase.

Introduction

Transketolase (TK, E.C. 2.2.1.1) is a Mg$^{2+}$ and thiamine diphosphate (ThDP) dependent enzyme that naturally catalyzes the conversion of glycolysis-derived metabolites into carbohydrates utilized for nucleotide synthesis and the production of essential aromatic amino acids by the Shikimate pathway\cite{1}. The overall reaction comprises the reversible transfer of a C$_2$ ketol group and an asymmetric C–C bond formation (Scheme 1). This makes the reaction interesting for synthetic applications. A multitude of enzymatic strategies have been developed to address the substantial importance of asymmetric C–C bond formation in organic synthesis, many of which rely on the decarboxylation as driving force for the C$_2$ ketol transfer\cite{2–5}.

To obtain an improved understanding of the TK-catalyzed reaction, two points will be addressed herein: first, the impact of decarboxylation on the reversibility/irreversibility of the reaction and, second, the effective conversion of aliphatic substrates in TK-catalyzed reactions although they are not the natural substrates. With regard to the first point, hydroxypyruvate (HPA) has been utilized as the ketol donor of choice because the liberation of CO$_2$ results in an equilibrium constant entirely in favor of the product (Scheme 2). With this large change in Gibbs free energy, the TK-catalyzed reaction with lithium hydroxypyruvate (LiHPA) is described as irreversible\cite{2–10}. The first Saccharomyces cerevisiae TK-catalyzed synthesis of L-erythulose was performed with LiHPA to ensure it to be irreversible\cite{11–13}. However, in 2004, the TK-catalyzed coupling of two molecules of glycolaldehyde to L-erythulose was reported\cite{14}. As the natural TK-catalyzed reactions are reversible, irreversible product formation seems to be unlikely here. In recognition of the extensive use of decarboxylation reactions in contemporary C–C bond formation strategies, a better understanding of the actual impact of decarboxylation on the thermodynamics of TK-catalyzed reactions is thus of great importance. In particular, the synthetically very powerful decar-

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**Scheme 1.** Natural TK-catalyzed reaction.

**Scheme 2.** Use of LiHPA as a ketol donor in TK catalyzed synthetic applications.
boxylation strategy has the disadvantage of a poor atom
economy.

TKs have phosphorylated polyols as typical substrates and
are naturally not disposed towards aliphatic substrates. Howev-
er, as aliphatic substrates were successfully converted, it re-
 mains yet to be fully understood how this is possible. Escheri-
chia coli TK has been engineered by single-point mutations to
convert a variety of aromatic and aliphatic aldehydes.[6,7] This
catalytic activity is surprising because the mutations intro-
duced in E. coli TK do not render the active site highly lipophilic.[6]

S. cerevisiae TK shares 47% sequence identity with E. coli TK,
and the aligned crystal structures (1QGD and 1TRK) have a root mean square deviation of 0.81 indicating extensive
structural homology. Owing to its facile heterologous overex-
pression in E. coli, S. cerevisiae TK was chosen as model enzyme
to investigate both the actual impact of decarboxylation in asymmetric C–C bond synthesis and the cause of enhanced ac-
tivity towards aliphatic aldehydes previously observed for single-point mutations.[6,7]

Results and Discussion

The E. coli TK mutants D469E and D469T have demonstrated
that highly polar or even charged amino acids improve
enzyme activity towards aliphatic aldehydes.[6] This is in con-
trast to our results that showed that nonphosphorylated sub-
strates are better converted by TK mutants of reduced polarity
(R528K, R528Q, R528K/S527T, and R528Q/S527T).[15,16] There-
fore, the equivalent mutations D477E and D477T were created
in S. cerevisiae TK to allow for direct comparison. The results of
the reactions with the different mutants for substrates 1–3 a
(Figure 1, Table 1) were in line with those reported for E. coli TK mutants.[6,8] Again, mutant D477E was identified as the best
catalyst for the conversion of aliphatic aldehydes. These data,
however, do not allow the evaluation of the catalytic activity
of the separate mutants for synthetic application.

Analysis of the Michaelis–Menten parameters confirmed these
conversion results. Mutant D477E performed best in the conversion
of aliphatic aldehydes 1a and 2a showing an enhanced activi-
ty of 50- to 100-fold compared to the WT (Table 2). Although
mutations at position R528, which natively binds to the
phosphate group of phosphorylated substrates,[15,16] and the
incorporation of a group mutation strategy[17] did enhance
enzyme activity, the improvements were only minor compared to the
effect of mutation D477E.

In silico docking studies

With an observed improvement of 50- to 100-fold in kcat/Km–1
for the conversion of substrates 1a and 2a with D477E by
only a single-point mutation, mutation D477E was introduce-
d in silico into the corresponding crystal structure 1GPU[18] to
investigate the resulting changes in the active site. The obtained
model was energy-minimized before docking of substrates
1a-4a into the active site using YASARA program.[19] The
model showed that the extension of the carbon chain by mut-
ating aspartate to glutamate newly enabled hydrogen-bond
interactions between the glutamate carboxylate and the sub-

![Figure 1. Overview of substrates (a), products (b), and derivatized products (c) required for chiral analysis. Products 1–3 (b) and (c) were obtained in the 3-(S) configuration with TK. Products 4b/c were not accessible enzymatically.](image)

| Table 1. Isolated product yields and enantiomeric excess (% ee) of the (S)-configured enantiomer. |
|----------|----------|----------|----------|----------|----------|
|          | WT (%)   | D477E (%) | D477T (%) | R528K (%) | R528Q (%) | R528K/S527T (%) | R528Q/S527T (%) |
| 1b        | 11 ± 8 (84) | 34 ± 15 (94) | 8 (n.d.)[9] | 10 ± 8 (81) | 8 ± 2 (77) | 8 ± 3 (73) | 6 ± 4 (66) |
| 2b        | 7 (91) | 61 ± 13 (90) | 12 ± 4 (84) | 6 ± 4 (82) | 5 ± 1 (87) | 6 ± 1 (68) | 5 ± 1 (82) |
| 3b        | 0 (n.d.)[9] | 41 ± 20 (n.d.)[9] | 3 ± 1 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] |
| 4b        | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] | 0 (n.d.)[9] |

[a] Reaction conditions: 20 U of S. cerevisiae TK, 5 mM THDP, 18 mM LHPA, 1 mM aldehyde, 10 mL final volume in 50 mM sodium phosphate buffer, pH 7.0, 25 °C, 200 rpm, 18 h. Enantiomeric excess in % [b] Not determined.
strate carbonyl groups bridged by a molecule of coordinated water at 1.7 Å each. In this manner, the substrate is correctly aligned towards the cofactor and the forming oxanion is stabilized by charge delocalization during the nucleophilic attack. This interaction was correctly predicted by the model for the converted substrates 1a–3a and not predicted for the unconverted substrate 4a (Figure 2 and Figure S5–S8, Supporting Information). In combination with preparative and kinetic data, these mechanistic reflections consequently suggest that TK-catalyzed synthesis reactions are irreversible for the mechanism of the one-substrate reaction, splitting the product back into one molecule of the respective acceptor aldehyde and one molecule of glycoaldehyde. The thermodynamic contribution of decarboxylation, therefore, should not affect the position of the overall equilibrium (Scheme 3) and thus makes irreversible product formation unlikely. In conclusion, it should thus be possible to avoid the release of CO₂ and improve the atom economy of the reaction even on a preparative scale.

Mechanistic reflections

For the synthesis of L-erythrulose from glycoaldehyde and LiHPA as substrates in aqueous solution under standard conditions, the total change in Gibbs free energy ΔG°eq amounts to −264.5 kJ mol⁻¹ (L-erythrulose, S18 in the Supporting Information), largely owing to the contribution of decarboxylation. Overall, this would correspond to an equilibrium constant of Keq = 10²⁰ in favor of the product. In 2004, the one-substrate TK-catalyzed reaction coupling two molecules of glycoaldehyde to L-erythrulose was reported,[10] and in strong contrast to the decarboxylation-driven reaction, an equilibrium constant of Keq = 5.0 was calculated from the change in Gibbs free energy (ΔG°eq = 4.0 kJ mol⁻¹) L-erythrulose in aqueous solution under standard conditions, S18, Supporting Information). In agreement with the natural reversible reactions, the one-substrate reaction should, therefore, be a true equilibrium reaction. In the proposed mechanism for TK-catalyzed reactions with LiHPA, the thermodynamically irreversible decarboxylation of LiHPA effects the direct formation of the carbonyl on the activated ketol. For the one-substrate reaction, however, the activated carbanion must be formed by catalytic deprotonation from residue His481 as an alternative to decarboxylation, generating the activated intermediate at a lower rate in comparison to its generation by decarboxylation. At the stage of the activated ketol bearing the carbanion, the enzyme can no longer distinguish whether the carbanion was formed by a reaction pathway involving decarboxylation or by catalytic deprotonation. The information about the thermodynamically driving force of decarboxylation is therefore already lost prior to the actual product formation. These mechanistic reflections to the decarboxylation-driven reaction, an equilibrium constant of Keq = 5.0 was calculated from the change in Gibbs free energy (ΔG°eq = 4.0 kJ mol⁻¹) L-erythrulose in aqueous solution under standard conditions, S18, Supporting Information). In agreement with the natural reversible reactions, the one-substrate reaction should, therefore, be a true equilibrium reaction. In the proposed mechanism for TK-catalyzed reactions with LiHPA, the thermodynamically irreversible decarboxylation of LiHPA effects the direct formation of the carbonyl on the activated ketol. For the one-substrate reaction, however, the activated carbanion must be formed by catalytic deprotonation from residue His481 as an alternative to decarboxylation, generating the activated intermediate at a lower rate in comparison to its generation by decarboxylation. At the stage of the activated ketol bearing the carbanion, the enzyme can no longer distinguish whether the carbanion was formed by a reaction pathway involving decarboxylation or by catalytic deprotonation. The information about the thermodynamically driving force of decarboxylation is therefore already lost prior to the actual product formation. These mechanistic reflections consequently suggest that TK-catalyzed synthesis reactions are irreversible for the mechanism of the one-substrate reaction, splitting the product back into one molecule of the respective acceptor aldehyde and one molecule of glycoaldehyde. The thermodynamic contribution of decarboxylation, therefore, should not affect the position of the overall equilibrium (Scheme 3) and thus makes irreversible product formation unlikely. In conclusion, it should thus be possible to avoid the release of CO₂ and improve the atom economy of the reaction even on a preparative scale.

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Table 2. Michaelis–Menten parameters.[b]

|     | WT         | D477E      | D477T      | R528K      | R528Q      | R528K/S527T | R528Q/S527T |
|-----|------------|------------|------------|------------|------------|-------------|-------------|
| 1b  | kcat       | 1.2        | 42         | 0.5        | 0.8        | 1.5         | 1.9         | 0.8         |
|     | kM         | 272        | 163        | 48         | 181        | 239         | 260         | 106         |
|     | kcat/KM⁻¹  | 4.2        | 260        | 10         | 4.4        | 6.1         | 7.4         | 7.5         |
| 2b  | kcat       | 0.8        | 9.3        | 0.4        | 0.1        | 2.1         | 0.3         | 0.4         |
|     | kM         | 327        | 40         | 43         | 16         | 611         | 67          | 42          |
|     | kcat/KM⁻¹  | 2.4        | 233        | 9.9        | 6.9        | 3.5         | 4.2         | 8.2         |
| 3b  | kcat       | 0.4        | 0.6        | n.d.       | n.d.       | 0.3         | 0.6         | n.d.        |
|     | kM         | 150        | 66         | n.d.       | 99         | 86          | 96          | n.d.        |
|     | kcat/KM⁻¹  | 2.9        | 8.3        |            |            | 2.5         | 3.7         |             |

[a] k_cat in s⁻¹, k_M in mm, k_cat/K_M⁻¹ in mm⁻¹ s⁻¹. For error bars, see Supporting Information Figures S10–S12. Reaction conditions: 50 µg purified S. cerevisiae TK, 1 mM ThDP, 4 mM Mg₂⁺, 100 mM LiHPA, S=150 mM aldehyde, 5 mM sodium phosphate buffer, pH 7.0, 25 °C, 500 rpm. [b] Not determined.

Equilibrium analysis

To confirm the theoretically suggested reversibility of the TK-catalyzed product formation experimentally, L-erythrulose was synthesized by both the one-substrate reaction coupling two molecules of glycoaldehyde and the conversion of glycoaldehyde with LiHPA to afford the product L-erythrulose in 100 mM concentration for complete conversion using wild-type (WT) S. cerevisiae TK (Scheme 4). The reactions were performed in sealed NMR tubes allowing for direct measurements of the product formation of erythrulose[21] (Figure S40). The substrates...
were not followed because consumption was completed within 30 min for LiHPA and because of the issue of oligomerization and hydration of glycolaldehyde in aqueous solution.[22] Both reactions were followed over an extended period of time. In line with the results earlier published,[14] l-erythrulose formation was observed. The one-substrate reaction proceeded relatively rapidly (Figure 3A) but was limited to less than 30% yield by the thermodynamic equilibrium of the reaction (Figure 3B).

If LiHPA was used as ketol donor, fast and complete conversion was observed as expected[3–10, 15,16, 20] (Figure 3A). If this reaction was thermodynamically controlled by the release of CO₂ it should stop at complete conversion. However, in line with a reversible reaction, a slow decline of l-erythrulose concentration was subsequently observed ultimately coinciding with the equilibrium concentration of the one-substrate reaction at $K_{eq} = 29.1 \pm 0.6 \text{ mm}$.

The synthesis reaction was thus shown to benefit from a kinetic effect enabling high yields at the beginning of the reaction. The reverse reaction causing thermodynamic equilibration to occur over a time course of several weeks then shifted the product distribution; in line with the outcome of the one-substrate reaction (Figure 3B). To confirm that the observed equilibration was indeed enzyme-catalyzed, another portion of LiHPA was added at the end. Retained enzymatic activity was observed (Figure 3B, inset), whereas control reactions without enzyme showed no conversion.

The representative formation of l-erythrulose from glycolaldehyde and LiHPA was thus shown to be initially kinetically controlled contrary to all earlier assumptions about the thermodynamic driving force of CO₂ release. The proposed reaction mechanisms depicted in Scheme 3 suggest these findings to generally hold true for all TK-catalyzed reactions with HPA. Following the example of the pyruvate decarboxylase catalyzed...

**Scheme 3.** Proposed mechanism for the formation of the activated ketol bearing the carbanion by either decarboxylation (top) or catalytic deprotonation (bottom).
lyzed synthesis of (R)-phenylacetylcarbinol with acetaldehyde replacing the traditional donor substrate pyruvate,\(^\text{[22]}\) the development of novel strategies which do not rely on decarboxylation is of commercial relevance. To do so, a correct understanding of decarboxylation is of utmost importance. In syntheses in which aldehydes other than glycolaldehyde are used as acceptors, formation of the desired product will be competing with the one-substrate reaction. Active-site engineering as pioneered by Pohl for a range of ThDP-dependent enzymes could ensure that glycolaldehyde will be the donor molecule in mixed carbo-ligation reactions.\(^\text{[24]}\)

Conclusions
Creating novel interactions between an active-site residue and a desired substrate should include a network of hydrogen bonds.\(^\text{[25–29]}\) As was shown, this is an effective strategy to increase the substrate’s affinity towards the active site, although a polarity-based analysis would suggest the opposite. This alternative approach for the rational mutagenesis of transketolases towards hydrophobic substrates was demonstrated. As decarboxylation-driven C–C bond formation reactions traditionally are misinterpreted in literature as irreversible, mechanistic reflections and experimental evidence unambiguously showed the reaction to be initially under kinetic control. In the context of man-made climate change, people thus have to extensively re-evaluate the choice of donor substrates and the utilization of decarboxylation strategies in synthetic applications.

Experimental Section
Materials
Chemicals and solvents were obtained as reagent grade from Sigma–Aldrich. Aldehydes were freshly distilled and their purity confirmed by \(^1\text{H} \text{NMR before usage. Petroleum ether (bp 40–60 °C)} \) was freshly distilled before usage. Lithium hydroxypyruvate was obtained both commercially and synthesized as previously described.\(^\text{[19]}\)

Methods
Reaction progress was monitored by TLC (TLC Silica gel 60 \(\text{F}_{254}\)\text{-}Merck) using UV light and a potassium permanganate stain for visualization. NMR spectra were recorded on an Agilent 400 MHz \((\text{H}, 9.4 \text{ Tesla}) \) spectrometer operating at 399.67 MHz for \(^1\text{H} \) at 298 K and were subsequently interpreted using MNOVA. A benzene-D\(_6\) NMR insert capillary (Sigma–Aldrich) was used for external locking during water suppression experiments using the PRESAT-PURGE pulse sequence in sealed Wilmad screw-cap NMR tubes (Sigma Aldrich). Spectra were recorded by using a recycle delay of 2 s and 64 repetitions. Preparative-scale biocatalyses were performed in an Excella E24 Incubator Shaker (New Brunswick Scientific).

Preparation of cell free extract: The cell pellet containing the respective mutant TK was resuspended in sodium phosphate buffer (5 mM, pH 7.0, 10 mM g\(^{-1}\) cell pellet). A protease inhibitor (PMSF, 200 \(\mu\)M, 0.1 mM in EtOH) was added to each sample. Lysozyme was added at 20 mg g\(^{-1}\) cell pellet and a spatula tip of DNAse was added to each sample and incubated on ice for 30 min. The cells were broken using a sonifier 250 (Branson) and the cell debris removed by centrifugation.

Enzyme purification: The cell pellet was resuspended in binding buffer (5 mM sodium phosphate, pH 7.4, 20 mM imidazole) and incubated with PMSF, lysozyme and DNAse as previously described. The cells were subsequently broken using a cell disrupter (Constant Systems Ltd, 1.8 kbar), the cell debris removed by centrifugation and the cell free extract filtered (0.45 \(\mu\)m). Affinity chromatography was performed on a NGC Quest 10 system (Biorad) using XK16/20 columns (GE Healthcare Life Sciences) packed with 10 mL Ni-sepharose 6 FF resin (GE Healthcare Life Sciences). For full details see Supporting Information.

Synthesis of racemic standards: Racemic standards were synthesized according to a method previously described.\(^\text{[15]}\) \(N\)-methylmorpholine (330 \(\mu\)L, 3.0 mmol, 1.0 equiv.) was dissolved in water (40 mL) and the pH was adjusted to 8.0 using 10% HCl. LiHPA (330 mg, 3.0 mmol, 1.0 equiv) and the corresponding aldehyde (3.0 mmol, 1.0 equiv) were added and the reaction was stirred overnight at room temperature. Conversion was monitored by TLC (n-pentane/EtOAc 1:1). Silica powder was added, the water removed in vacuo and the crude product purified by flash chromatography (n-pentane/EtOAc 1:1). For full details see ESL.

Dibenzylation of enantiomers: Dihydroxyketone (1.0 equiv.) was dissolved in dry dichloromethane (10 mL) under \(\text{N}_2 \) atmosphere in a flame dried round bottomed flask. Dry triethylamine (10.0 equiv.) and benzoyl chloride (5.0 equiv per hydroxyl) were added and the reaction mixture was stirred for 2 h at room temperature. It was quenched by addition of saturated NaHCO\(_3\) (30 mL), the phases separated, and the organic phase was washed (sat. NaHCO\(_3\), 2 × 50 mL, then saturated \(\text{NH}_2\text{Cl}_2\) 1 × 50 mL, then brine, 1 × 30 mL). The organic phase was dried over Na\(_2\text{SO}_4\), the solvent was removed in vacuo and the crude product was purified by flash chromatography for the racemic standards (petroleum ether/EtOAc 10:1). Purification by flash chromatography was omitted in the determination of the enantiomeric excess. For full details see Supporting Information.

Glycolaldehyde activity assay:\(^\text{[15]}\) The volumetric activity of cell free extracts was determined by incubating 50 \(\mu\)L with the cofactors (25 °C, 800 rpm, 20 min, ThDP: 5 \(\mu\)M, Mg\(^{2+}: 18 \mu\)M). LiHPA and glycolaldehyde were added to achieve final concentrations of 50 mM in 300 \(\mu\)L total reaction volume, 5 mM sodium phosphate buffer, pH 7.0). The reaction mixture was shaken (25 °C, 800 rpm, 15 min), quenched by addition of TFA (300 \(\mu\)L, 0.2% v/v), the enzyme precipitated by centrifugation and analyzed by RP HPLC (R\(^2\) = 0.998) to determine the volumetric activity. Owing to considerably varying volumetric activities of cell free extracts the enzyme content was normalized to 20 U of activity based on a glycolaldehyde activity assay previously reported.\(^\text{[15]}\)

Computational docking of glycolaldehyde into the corresponding mutant active sites with YASARA predicted comparable binding energies for all mutants. It was thus concluded that none of the mutations are likely to have introduced a major bias to an activity-based analysis using glycolaldehyde as reference. For full details, see Supporting Information.

Preparative-scale biocatalyses. Cell-free extract (20 U based on the glycolaldehyde activity assay) was incubated with its cofactors (20 min, room temperature, 5 mM sodium phosphate buffer, pH 7.0, 18 mM ThDP, and 5 mM Mg\(^{2+}\)). LiHPA (110 mg, 1.0 mmol,
and purification. Financial support from STW (grant 11142) to L. G. is gratefully acknowledged.

Conflict of interest

The authors declare no conflict of interest.

Keywords: aldehydes • C–C coupling • enzyme catalysis • kinetics • thermodynamics

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