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Synthesis with good enantiomeric excess of both enantiomers of α-ketols and acetolactates by two thiamin diphosphate-dependent decarboxylases

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Frank Jordan dedicates this paper to the memory of a valued collaborator and friend Miriam Hasson.

Abstract

In addition to the decarboxylation of 2-oxo acids, thiamin diphosphate (ThDP)-dependent decarboxylases/dehydrogenases can also carry out so-called carboligation reactions, where the central ThDP-bound enamine intermediate reacts with electrophilic substrates. For example, the enzyme yeast pyruvate decarboxylase (YPDC, from *Saccharomyces cerevisiae*) or the E1 subunit of the *Escherichia coli* pyruvate dehydrogenase complex (PDHc-E1) can produce acetoin and acetolactate, resulting from the reaction of the central thiamin diphosphate-bound enamine with acetaldehyde and pyruvate, respectively. Earlier, we had shown that some active center variants indeed prefer such a carboligase pathway to the usual one [Sergienko, Jordan, Biochemistry 40 (2001) 7369–7381; Nemeria et al., J. Biol. Chem. 280 (2005) 21,473–21,482]. Herein is reported detailed analysis of the stereoselectivity for forming the carboligase products acetoin, acetolactate, and phenylacetylcarbinol by the E477Q and D28A YPDC, and the E636A and E636Q PDHc-E1 active-center variants. Both pyruvate and β-hydroxypyruvate were used as substrates and the enantiomeric excess was analyzed by a combination of NMR, circular dichroism and chiral-column gas chromatographic methods. Remarkably, the two enzymes produced a high enantiomeric excess of the opposite enantiomer of both acetoin-derived and acetolactate-derived products, strongly suggesting that the facial selectivity for the electrophile in the carboligation is different in the two enzymes. The different

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stereoselectivities exhibited by the two enzymes could be utilized in the chiral synthesis of important intermediates.

Keywords: Thiamin diphosphate; Yeast pyruvate decarboxylase; *Escherichia coli* pyruvate dehydrogenase E1 subunit; Carboligase side reactions; β-Hydroxypyruvate; Acetoin; Acetolactate; Enzymatic chiral synthesis; Enantiomeric excess; Circular dichroism

1. Introduction

The use of thiamin diphosphate-dependent (ThDP) enzymes in synthesis leading to carboligation is a well-exploited idea that has been reviewed recently [1–4]. It takes advantage of the multiple catalytic potentials of the central enamine intermediate [5,6] as depicted with the reaction of yeast pyruvate decarboxylase (YPDC) (Fig. 1). With some enzymes, this carboligation, or its reverse, is the natural course of the pathway while for virtually all 2-oxoacid decarboxylases it is a side reaction. Earlier, we had reported that certain active-center substitutions of YPDC could convert it to an effective acetoin synthase [7] (the E477Q variant; resulting from the reaction of the enamine with acetaldehyde the product of the reaction), or acetolactate synthase [7] (the D28A or D28N variants; resulting from the reaction of the enamine with a second molecule of pyruvate). More recently [8], we realized that the E636A (or E636Q) substitution at the active center of the pyruvate dehydrogenase complex E1 subunit from *Escherichia coli* (PDHc-E1) also converted it to an effective carboligase, giving the same two products. More remarkably, the two ThDP enzymes, both of which carry out decarboxylation of pyruvic acid as a first step, gave a preponderance of the opposite enantiomer of both carboligase products.

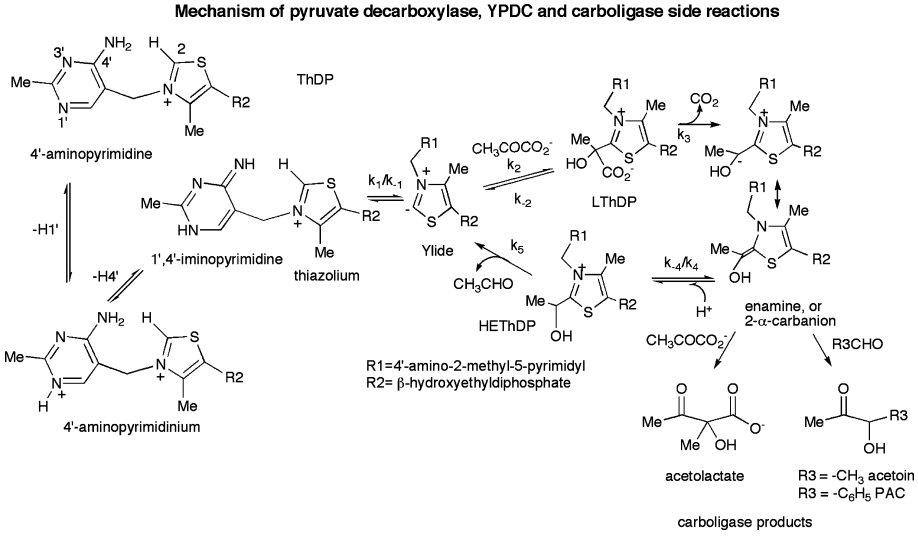

Fig. 1. Mechanism of yeast pyruvate decarboxylase and of the carboligase reactions.

It is important to note that each of these wild-type enzymes only give acetoin and at <1% yield. In an extension of those studies, we report amplification of our qualitative reports by determining the enantiomeric excess (*ee*) of each reaction. We also report studies on a frequently used pyruvate derivative β-hydroxypyruvate, which is accepted by both enzymes and also produces the carboligase products. Finally, we also produce (*R*)-phenylacetylcarbinol [(*R*)-PAC] using the YPDC variants with outstanding *ee*. The studies are very much assisted by the use of circular dichroism experiments. The ability to produce the opposite enantiomer of the same chiral compound by two closely related enzymes makes the findings of potential synthetic significance.

2. Materials and methods

2.1. Materials

The chemicals acetoin, sodium pyruvate, (*R*)-(+)-α-[trimethylsilyl)oxy]-benzeneacetonitrile, and methylmagnesium iodide were bought from Sigma–Aldrich and were used without any further purification.

2.2. Methods

2.2.1. Reactions with the enzymes

Acetoin, acetolactate and PAC formation with YPDC variants were carried out in 10 mM KH_2PO_4 buffer (pH 6.0, the pH optimum), containing 2 mM $MgCl_2$, 0.1 mM ThDP [9]. Acetoin and acetolactate formation with *E. coli* PDHc-E1 were carried out in 10 mM KH_2PO_4(pH 7.0, the pH optimum for the E1 subunit) containing 2 mM $MgCl_2$, 0.1 mM ThDP [8].

2.2.2. (*R*)-Phenylacetylcarbinol formation with the E477Q and D28A variants of YPDC

The E477Q and D28A variants were dissolved (0.24 and 3.35 mg/mL, respectively) in 10 mM KH_2PO_4 (pH 6.0) with 2 mM $MgCl_2$, 0.1 mM ThDP, then 4 mM benzaldehyde was added at 30 °C. The reaction was initiated by the addition of 4 mM pyruvate.

2.2.3. Acetolactate formation with the E636A and E636Q PDHc-E1 variants and the D28A YPDC variant

The D28A YPDC variant was dissolved (3.35 mg/mL) in 10 mM KH_2PO_4 (pH 6.0) with 2 mM $MgCl_2$ and 0.1 mM ThDP added at 30 °C. The E636Q and E636A PDHc-E1 variants were dissolved (both at 1 mg/mL) in 10 mM KH_2PO_4 (pH 7.0) with 2 mM $MgCl_2$, 0.1 mM ThDP added at 25 °C. Acetolactate formation was started by addition of 20 mM pyruvate.

2.2.4. Acetoin formation by the E636A and E636Q PDHc-E1 variants and the E477Q and D28A YPDC variants

The E477Q and D28A YPDC variants were dissolved (0.24 and 3.35 mg/mL, respectively) in 10 mM KH_2PO_4 (pH 6.0) with 2 mM $MgCl_2$, 0.1 mM ThDP, and 100 mM acetaldehyde was added at 30 °C. The E636Q and E636A PDHc-E1 variants

were dissolved (both at 1 mg/mL) in 10 mM KH_2PO_4 at pH 7.0 with 2 mM $MgCl_2$, 0.1 mM ThDP added at 25 °C. Acetoin formation was started by addition of 20 mM pyruvate.

2.2.5. 1,3,4-Trihydroxy-2-butanone formation by parental PDHc-E1 and its E636A and E636Q variants and the E477Q YPDC variant

PDHc-E1 and its E636Q and E636A variants were dissolved (1 mg/mL) in 10 mM KH_2PO_4(pH 7.0) with 2 mM $MgCl_2$ and 0.1 mM ThDP added at 25 °C. The E477Q YPDC variant was dissolved (0.24 mg/mL) in 10 mM KH_2PO_4 (pH 6.0) with 2 mM $MgCl_2$, 0.1 mM ThDP added at 25 °C. To initiate formation of 1,3,4-trihydroxy-2-butanone, the reaction was started with simultaneous addition of 40 mM β-hydroxypyruvate and 100 mM glycolaldehyde. Parental PDHc-E1 did not need glycolaldehyde to produce 1,3,4-trihydroxy-2-butanone.

2.2.6. 2,4-Dihydroxy-2-hydroxymethyl-3-oxo-butanoic acid formation by the PDHc-E1 E636A and E636Q variants

The E636Q and E636A variants were dissolved (1 mg/mL) in 10 mM KH_2PO_4 (pH 7.0) with 2 mM $MgCl_2$ and 0.1 mM ThDP added at 25 °C. The reaction was initiated by addition of 40 mm β-hydroxypyruvate.

2.2.7. Chemical synthesis

To a solution of 2 mL of 3.0 M methylmagnesium iodide (6 mmol) in 7 mL ether was added dropwise 1 mL of (*R*)-(+)-α-[(trimethylsilyl)oxy]-benzeneacetonitrile (4.61 mmol) in 8 mL of ether. After the addition was completed the reaction mixture was left at room temperature for 2 h. During the first five minutes of the reaction the mixture turned cloudy yellow and after 10 min precipitation was observed. After 2 h of reaction time the mixture was placed on ice for 15 min. Next, the mixture was poured on 50 g of ice containing 2 mL of concentrated sulfuric acid and was left overnight to hydrolyze the trimethylsilyl group. The ether layer was first rinsed with 20 mL of 10% HCl then with brine. The organic layer was dried over magnesium sulfate and the volatiles were removed in vacuo. NMR chemical shifts and GC–MS fragmentation patterns confirmed the product. NMR (DMSO-d_6/TMS): δ (ppm) 2.05 (s, 3H), 4.25 (s, 1H), 5.05 (s, 1H), 7.24 (m, 5H). GC–MS, [M^+] 150, 107, 79, 51 [10].

2.2.8. Overexpression and purification of the YPDC, PDHc-E1, and their active center variants

Overexpression and purification of the PDHc-E1 and its E636A and E636Q variants was carried out following the protocol described previously for *E. coli* PDHc-E1 [8]. Overexpression of the E477Q and D28A active site variants of YPDC was carried out following the protocol described previously [11]. The E477Q variant has a His_6-tag attached to its C-terminal residue hence it was purified on a Talon column [11]. The D28A variant was purified following the protocol reported previously [11].

2.2.9. Circular dichroism (CD)

Experiments were carried out on an AVIV 202 and an Applied Photophysics Chirascan spectrometer. Spectra were recorded in the 250–350 nm range. For each sample, the baseline was first recorded with the buffer and then appropriate amounts of $MgCl_2$ and ThDP

was added and the spectrum was re-recorded to make certain that there is no signal in the wavelength range studied. Product formation was monitored at the ellipticity maximum of the particular compound. After termination of the reaction, the enzyme was removed with a Centricon microcentriprep unit (1.5 mL and the supernatant was scanned with CD. For acetoin and (*R*)-PAC samples a 0.1 cm, and for acetolactate a 1.0 cm path length cell was used.

2.2.10. Chiral GC analysis

Analysis was carried out on a HP 5950 gas chromatograph equipped with a Chiraldex B-DM chiral column (Astec, Advanced Separation Technologies, Inc.) and a flame ionization detector at a flow rate of 0.7 mL/min. Acetoin was analyzed at 50 °C, while phenylacetylcarbinol was analyzed at 100 °C. A 20 μL volume of sample was injected with a 100:1 split ratio. Acetoin and phenylacetylcarbinol were extracted from the reaction mixture with chloroform and injected onto the Chiraldex B-DM chiral column. Since this column interacts more favorably with the (*S*)-enantiomer thus delaying its retention time, we could readily assign the enantiomers. We could not analyze acetolactate samples with the chiral GC method since it would be decarboxylated under GC conditions. Therefore, acetolactate samples were scanned with CD and the enantiomeric excess was calculated with the reported molar ellipticity [12].

2.2.11. Determination of steady-state kinetic parameters for β-hydroxypyruvate with YPDC

Activity was assayed with the NADH/alcohol dehydrogenase (ADH) coupled assay [13], converting the glycolaldehyde to ethylene glycol and monitoring the depletion of NADH at 340 nm. One unit of YPDC activity is defined as the amount of protein required to convert 1.0 μmol of β-hydroxypyruvate to glycolaldehyde per minute at 25 °C. Kinetic studies were carried out on a Varian Cary 300 Bio UV–visible spectrophotometer. The kinetic parameters were calculated by curve fitting using the Sigma-plot program (Systat). The total sample solution was 1 mL. YPDC (5 μL of 6.88 mg/mL, containing 34.4 μg enzyme) was added to assay buffer containing 20 mM KH_2PO_4 (pH 6.0), 1 mM ThDP, 2 mM $MgCl_2$, 20 U of yeast ADH (360 U/mg), and 0.15 mg NADH (both from Sigma, St. Louis, MO) was added to the mixture. The enzyme was assayed with 0.5–100 mM of β-hydroxypyruvate and activity was measured by monitoring the NADH depletion at 25 °C using the coupled enzyme assay.

2.2.12. Determination of steady-state kinetic parameters for β-hydroxypyruvate with PDHc-E1

The PDHc-E1 activity was measured by reconstituting PDHc activity with saturating amount of E2-E3 subcomplex (the mass ratio of E1 to E2–E3 sub-complex was 1:5) using a Varian Cary 300 Bio UV–visible spectrophotometer, monitoring the β-hydroxypyruvate-dependent reduction of NAD^+ at 340 nm [14]. One unit of activity is defined as the amount of NADH produced (micromoles per minute per milligram of E1). The kinetic parameters were calculated by curve fitting using the Sigma-plot program (Systat). The reaction medium contained in 1 mL: 0.1 M Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM sodium pyruvate, 2.5 mM NAD^+, 0.2 mM ThDP, and 2.8 mM DTT at 30 °C [8]. The reaction was initiated by adding 0.1 mM CoA. Activity was measured with 2, 5, 10, 20, 30, 40, and 100 mM of β-hydroxypyruvate at 25 °C.

3. Results

3.1. Phenylacetylcarbinol formation with YPDC variants

Phenylacetylcarbinol is the major product formed when pyruvate and benzaldehyde are added to a phosphate buffer (pH 6.0) containing the E477Q or D28A variants of YPDC, along with the required cofactors ThDP and Mg^{2+}. The CD spectrum of this reaction develops a distinct band with a maximum at 283 nm and the band persists even after protein removal with Micro Centriprep and heat treatment (Fig. 2). The negative CD band corresponds to the (*R*)-enantiomer. Phenylacetylcarbinol is a stable compound and can be characterized by GC–MS showing the expected parent peak, fragmentation pattern, and the expected ^{1}H NMR spectrum.

After protein removal, phenylacetylcarbinol was extracted into chloroform and was analyzed with the chiral column for estimation of the enantiomeric excess (*ee*), using a chiral B-DM column from Chiraldex. An approximately 20 μL sample was injected with a 1:100 split ratio at a flow rate of 0.7 mL/min and at 100 °C. The retention time for the (*R*) and (*S*) enantiomers under these GC conditions is 23.5 and 26.1 min, respectively. To confirm that the column preferentially binds the (*S*) enantiomer, thus delaying its retention time as suggested by the manufacturer's specifications, authentic (*R*)-phenylacetylcarbinol was synthesized and analyzed under these GC conditions. Indeed, we confirmed that the first peak to emerge corresponds to the (*R*)-enantiomer, and the second one to the (*S*)-enantiomer (Fig. 3).

The results of phenylacetylcarbinol production with the E477Q and D28A YPDC indicated that both enzyme variants form only of (*R*)-PAC according to the CD spectrum (figure not shown). The chiral GC chromatograms confirm formation solely of the (*R*)-enantiomer and no detectable (*S*)-enantiomer (Fig. 3). The molar ellipticity for (*R*)-PAC was calculated to be 30736 deg cm^2 dmol^{-1} at 283 nm.

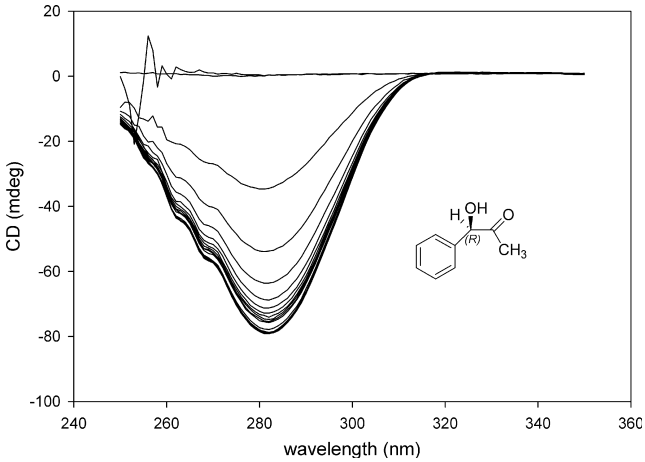

Fig. 2. CD spectra of (*R*)-phenylacetylcarbinol formation with the E477Q YPDC variant. Conditions are described under Section 2.

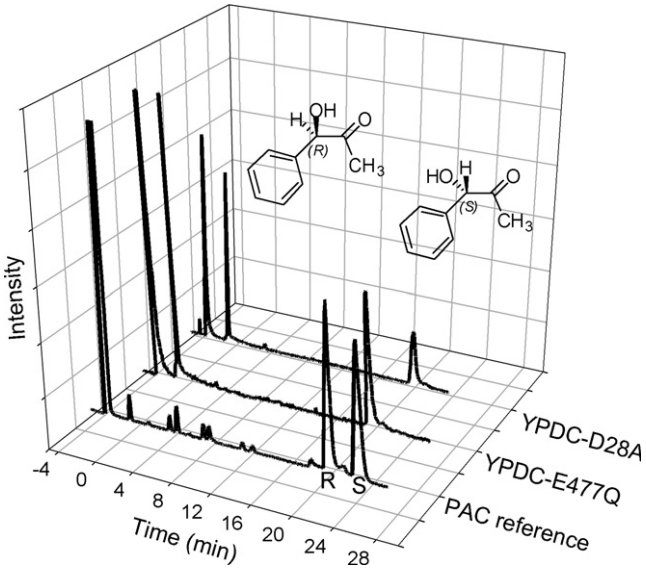

Fig. 3. Chiral GC analysis of PAC produced by the E477Q and D28A YPDC variants vs. authentic (*R*)-PAC as reference.

Unlike the E477Q variant, the D28A variant could also catalyze the condensation of two pyruvate molecules yielding acetolactate (Fig. 4), but formation of acetolactate is pyruvate concentration dependent and with 4 mM pyruvate the rate of acetolactate formation is very slow. The reaction to form (*R*)-PAC was examined with equimolar concentrations of pyruvate and benzaldehyde (4 mM). Under these conditions no acetolactate formation was observed, as also confirmed with ^{1}H NMR (data not shown).

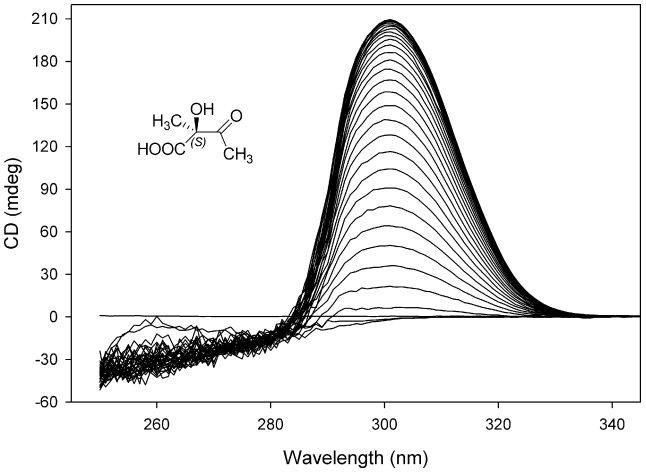

Fig. 4. Time development of the CD spectra of acetolactate formed with the D28A YPDC variant. Time delay between each scan was approximately 4 min. Conditions are described under Section 2.

3.2. Acetoin formation with YPDC and PDHc-E1 variants

Acetoin (3-hydroxy-2-butanone) is the major product formed when pyruvate and acetaldehyde are used as substrates with the YPDC and PDHc-E1 variants. CD spectra of the reaction mixtures with these variants show a major CD band with a maximum at 278 nm. The negative and the positive band correspond to the (*R*)- and (*S*)-enantiomers, respectively. The structure of the product formed was confirmed with GC–MS and ^{1}H NMR.

The yield and the stereochemistry of acetoin formed with each variant are different. The YPDC variants and the PDHc-E1 variants catalyze the formation of opposite enantiomers. It can be clearly seen in the CD spectrum that the YPDC variants preferentially catalyze formation of (*R*)-acetoin and the PDHc-E1 variants catalyze formation of (*S*)-acetoin (Fig. 5).

Assignment of the absolute configurations was again achieved with chiral gas chromatography (Fig. 6). The product formed was extracted into chloroform and injected into the GC at 0.7 mL/min and at 50°. The E477Q variant produced 98% yield of acetoin (4.1 mg) with 94% *ee*, while the D28A variant produced 68% yield (2.9 mg) with 88% *ee* (Table 1).

Apparently, both YPDC variants preferentially catalyze formation of the (*R*) enantiomer. Both variants can achieve high enantiomeric excess showing that the facial selectivity is very good in both cases. The reason for the lower yield of acetoin produced with the D28A variant is that with 20 mM pyruvate and 100 mM acetaldehyde there is acetolactate being produced as well. The molar ratio of acetolactate to acetoin formed was 30:70 according to ^{1}H NMR.

The PDHc-E1 variants will produce acetolactate instead of acetoin in the absence of exogenouos acetaldehyde, but with saturating concentration of acetaldehyde they will produce acetoin as the major product and only negligible amounts of acetolactate. Unlike the YPDC variants, the PDHc-E1 variants catalyze the formation of (*S*)-acetoin. Percent yield for acetoin production was calculated based on the starting pyruvate concentration and

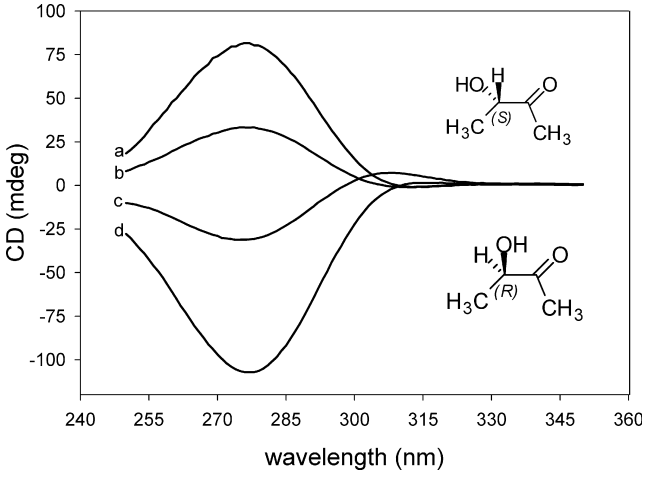

Fig. 5. CD spectra of acetoin produced by the E636A and E636Q PDHc-E1 variants and the E477Q and D28A YPDC variants. (a) PDHc-E1 E636Q, (b) PDHc-E1 E636A, (c) YPDC D28A, and (d) YPDC E477Q. ($\lambda_{max} = 278$ nm). Conditions are described under Section 2.

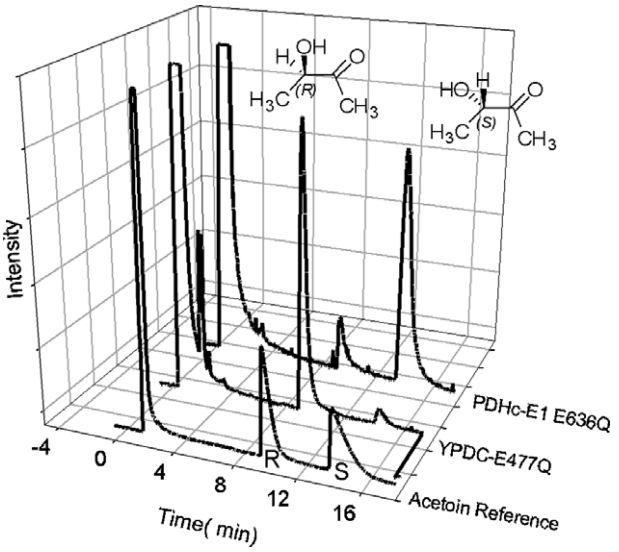

Fig. 6. Chiral GC analysis of the acetoin produced by E477Q YPDC and E636Q PDHc-E1 vs. racemic acetoin reference.

Table 1
Enantiomeric excess of acetoin, phenylacetylcarbinol, and acetolactate formed by the various enzymes used and percent yields for the products formed under the specified conditions

Enzyme variant	Enantiomeric excess and yield of product[a]		
	PAC	Acetoin	Acetolactate
YPDC-E477Q	>99% (*R*)[b](62)	94% (*R*)[b](98)	Not produced
YPDC-D28A	>99% (*R*)[b](60)	88% (*R*)[b](68)	40% (*S*)[c](92)
PDHc-E1 E636A	Not produced	24% (*S*)[b](92)	60% (*R*)[c](93)
PDHc-E1 E636Q	Not produced	70% (*S*)[b](93)	>99% (*R*)[c](95)

[a] Yield in parenthesis in %.
[b] *ee* was calculated by Chiral-GC.
[c] *ee* was calculated from CD data.

the amount of product formed. The percent yield of acetoin was 92% (3.8 mg) with the E636A, and 93% (3.9 mg) with the E636Q variant.

Interestingly, the enantiomeric excess achieved with PDHc-E1 variants varied significantly with the substituent: the E636Q variant formed (*S*)-acetoin with 70% *ee* but the E636A variant gave (*S*)-acetoin with only 24% *ee* (Table 1). This shows the significant effect of the amino acid side chain on the stereochemical outcome of the reaction. A clear comparison of the enantiomers formed according to the chiral-GC analysis of the YPDC and PDHc-E1 variants vs. racemic acetoin mixture is presented in Fig. 5.

3.3. Acetolactate formation with YPDC and PDHc-E1 variants

Acetolactate is the major product formed when pyruvate is used as the sole substrate with the D28A YPDC variant and with the E636A or E636Q PDHc-E1 variants.

Acetolactate is the result of the condensation of a second pyruvate molecule onto the enamine. The D28A YPDC variant produces (*S*)-acetolactate while PDHc-E1 variants produce (*R*)-acetolactate (Fig. 7). The negative and the positive CD bands correspond to the (*R*)- and (*S*)-enantiomers, respectively.

The assignment of absolute configuration of the acetolactate enantiomers was based on literature values. The enzyme acetohydroxyacid synthase catalyzes the formation (*S*)-acetolactate corresponding to a positive CD band according to Chipman and coworkers [12]. The E477Q YPDC variant doesn't catalyze acetolactate formation. The percent yield of acetolactate formed with the D28A YPDC and the E636A and E636Q PDHc-E1 variants is 92% (5.8 mg), 93% (5.9 mg), and 95% (6.0 mg), respectively.

Acetolactate is a thermally unstable compound, which is converted to racemic acetoin on decarboxylation irrespective of the enantiomer of acetolactate undergoing decarboxylation, hence it was not possible to analyze it under GC conditions. We therefore used the reported molar ellipticity for acetolactate [12]. The samples were scanned with CD after protein removal and based on the starting concentration of pyruvate and the molar ellipticity, the following *ee* were calculated: D28A YPDC, 40%; E636A PDHc-E1, 60%; and E636Q PDHc-E1, >99%.

3.4. β-Hydroxypyruvate as substrate for YPDC and PDHc-E1

First, it was demonstrated that β-hydroxypyruvate is indeed a substrate for the two enzymes. With YPDC, normal decarboxylation results in glycolaldehyde formation. Data for the β-hydroxypyruvate-dependent glycolaldehyde production by YPDC were fitted to the Hill equation with substrate inhibition [9]; $v = V_{\max}[S]^{n_H}/(S_{0.5}^{n_H} + [S]^{n_H}(1 + [S]/K_i))$, which yielded, $V_{\max} = 0.379 \pm 0.007$ U/mg, $n_H = 1.71 \pm 0.09$, $S_{0.5} = 2.01 \pm 0.063$ mM, $K_{i\,app} = 1.156 \pm 0.387$ M; $k_{cat} = 1.46\ s^{-1}$, $k_{cat}/S_{0.5} = 0.726\ s^{-1}\ mM^{-1}$. With PDHc-E1,

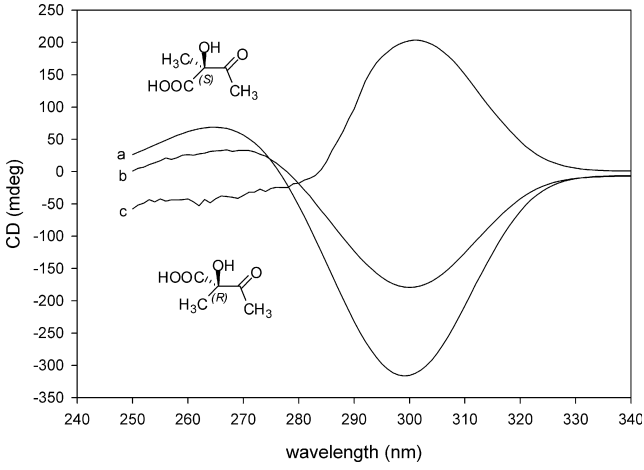

Fig. 7. CD spectra of acetolactate produced by the E636A and E636Q PDHc-E1 variants and the D28A YPDC variants. (a) PDHc-E1 E636Q, (b) PDHc-E1 E636A, and (c) YPDC D28A. ($\lambda_{max} = 300$ nm). Conditions are described under Section 2.

we had to reconstitute the entire complex (by adding E2–E3 sub-complex) to demonstrate activity and the data for the β-hydroxypyruvate-dependent 1,3,4-trihydroxy-2-butanone production by PDHc-E1 were fitted to the Michaelis–Menten equation; $v = V_{\max}[S]^{n_H}/(K_m^{n_H} + [S]^{n_H})$ which yielded, $V_{\max} = 3.342 \pm 0.305$ U/mg, $K_m = 7.768 \pm 1.706$ mM, $k_{cat} = 11.1\ s^{-1}$, $k_{cat}/K_m = 1.43\ s^{-1}\ mM^{-1}$.

3.5. 1,3,4-Trihydroxy-2-butanone formation with YPDC and PDHc-E1 variants

When β-hydroxypyruvate was used as substrate along with glycolaldehyde as co-substrate, 1,3,4-trihydroxy-2-butanone was produced with both the YPDC and PDHc-E1 variants. Except with parental PDHc-E1, both YPDC variants and PDHc-E1 variants need saturating concentrations of glycolaldehyde as the second substrate. Again, the YPDC variants produce the *R*-enantiomer in excess and the PDHc-E1 variants produce the *S*-enantiomer in excess (Fig. 8); we have not determined the *ee* of the reactions.

3.6. 2,4-Dihydroxymethyl-3-oxo-butanoic acid formation with PDHc-E1 and YPDC variants

2,4-Dihydroxymethyl-3-oxo-butanoic is the sole product formed from the reaction of the E636A, and E636Q PDHc-E1 variants with β-hydroxypyruvate as the sole substrate. The CD spectrum shows two bands corresponding to the same species with a maximum at 301 nm (Fig. 9; apparently with β-hydroxypyruvate, there are two CD bands in this carboligase product). The product formed is the (*S*)-enantiomer. The D28A YPDC variant produces the (*R*)-enantiomer (data not shown).

The λ_{max} values of the CD bands for the compounds studied are: acetoin, 278 nm; acetolactate, 300 nm; phenylacetylcarbinol, 283 nm; 1,3,4-trihydroxy-2-butanone, 279 nm; and 2,4-dihydroxymethyl-3-oxo-butanoic acid, 301 nm.

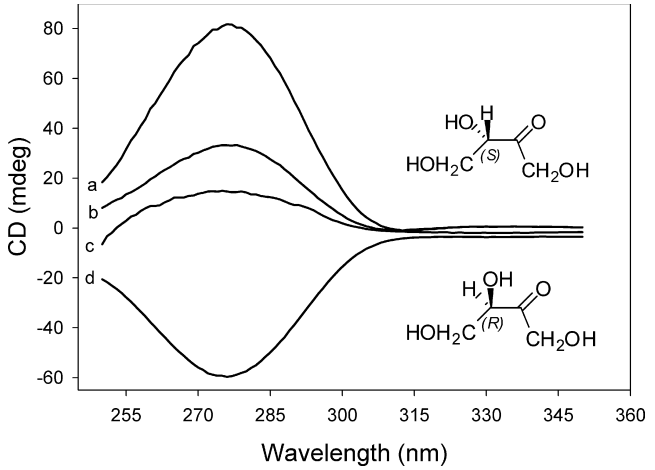

Fig. 8. CD spectra of 1,3,4-trihydroxy-2-butanone produced by parental PDHc-E1 and its E636A and E636Q variants and the E477Q YPDC variant. (a) PDHc-E1 E636Q + β-hydroxypyruvate and glycolaldehyde, (b) PDHc-E1 E636A + β-hydroxypyruvate and glycolaldehyde, (c) PDHc-E1 + β-hydroxypyruvate, and (d) YPDC E477Q + β-hydroxypyruvate and glycolaldehyde. ($\lambda_{max} = 278$ nm). Conditions are described under Section 2.

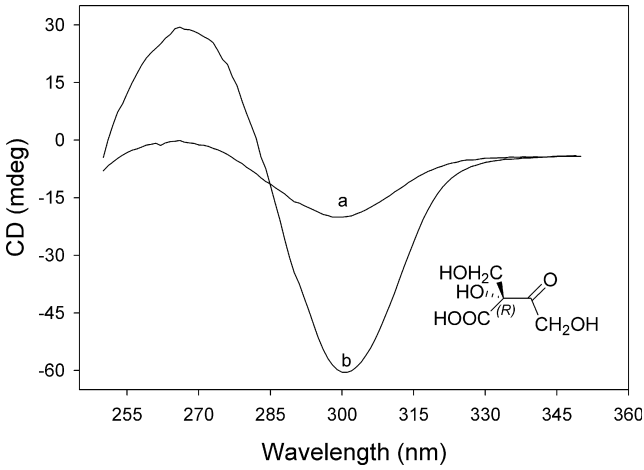

Fig. 9. CD spectra of 2,4-dihydroxy-2-hydroxymethyl-3-oxo-butanoic acid produced by the PDHc-E1 E636A and E636Q variants. (a) PDHc-E1 E636A + β-hydroxypyruvate and (b) PDHc-E1 E636Q + β-hydroxypyruvate. ($\lambda_{max} = 301$ nm). Conditions are described under Section 2.

4. Discussion

This report quantifies the yield and stereochemical outcome of the carboligase reactions of two ThDP-dependent enzymes which share a common pathway through decarboxylation and convert pyruvate to different enantiomers of acetoin and acetolactate. The active center variants of YPDC and PDHc-E1 were shown to produce high yields and high *ee*, depending on the conditions used. Further, we showed that the YPDC active center variants produce essentially a single enantiomer of phenylacetylcarbinol in good yield. We note that Crout and coworkers had published extensively on the use of wild-type YPDC and pyruvate decarboxylase from *Zymomonas mobilis*, to produce α-ketols such as (*R*)-PAC and derivatives as well as aliphatic acyloins [15–17]. We also report that β-hydroxypyruvate a commonly used substrate analog can also undergo carboligase reactions catalyzed by YPDC variants and PDHc-E1 from *E. coli* leading to the substituted derivatives of acetoin and acetolactate, again providing excess of the same enantiomer as produced with pyruvate by the same enzyme. This is important to show since β-hydroxypyruvate is often used with the enzyme transketolase as it produces the 1,2-dihydroxyethylidine ThDP or enamine on decarboxylation that is sufficiently stable for high-resolution X-ray studies [18]. The λ_{max} values of the CD bands for the acetoin and acetolactate derivatives produced from β-hydroxypyruvate are very similar to those from acetoin and acetolactate itself. This raises a significant complication in the interpretation of UV–vis and CD data on ThDP enzymes. As we had reported, the enamine-derived from aliphatic 2-oxo acids has a λ_{max} near 295 nm [5,6], the 1′,4′-imino tautomer of ThDP when enzyme-bound has a λ_{max} between 300 and 310 nm with a positive CD band [19–22], and, as is here shown, acetolactate (or derivative derived from β-hydroxypyruvate), also gives rise to a positive or negative CD band with λ_{max} of 300–302 nm (sign depending on the particular enantiomer) and a corresponding UV signal.

A comparison of the stereochemical outcome of the carboligase reaction is instructive. To explain why the two enzymes prefer to form different enantiomers of the same

carboligase product, we need to consider the facial selectivity of the approach of the carbonyl compound to the enamine intermediate (Fig. 10). Apparently, the two enzymes preferentially recognize the opposite faces (*re* or *si*) of the incoming carbonyl compound (aldehyde or 2-oxo acid). One can then ask whether electrostatic or size effects control the recognition. Superimposition of the carbonyl groups of the two products, suggests that the relative sizes of the two other (non-carbonyl) groups, rather than the charge at the carboxylate, influence the preferred approach leading to the predominant enantiomer. This conclusion is reached since PDHc-E1 preferentially synthesized (*S*)-acetoin and (*R*)-acetolactate, while YPDC formed (*R*)-acetoin and (*S*)-acetolactate. That YPDC and pyruvate decarboxylase from *Zymomonas mobilis* produce opposite enantiomers of acetoin had been noted earlier, and Lobell and Crout carried out molecular modeling using the 3-dimensional X-ray structure of YPDC to provide an explanation for its selectivity in acetoin formation [23].

Our results suggest that engineering of the bacterial pyruvate dehydrogenase complex E1 subunit could be exploited to produce the opposite enantiomer of many compounds than the one readily produced by pyruvate decarboxylases from several sources and by acetohydroxyacid synthetases.

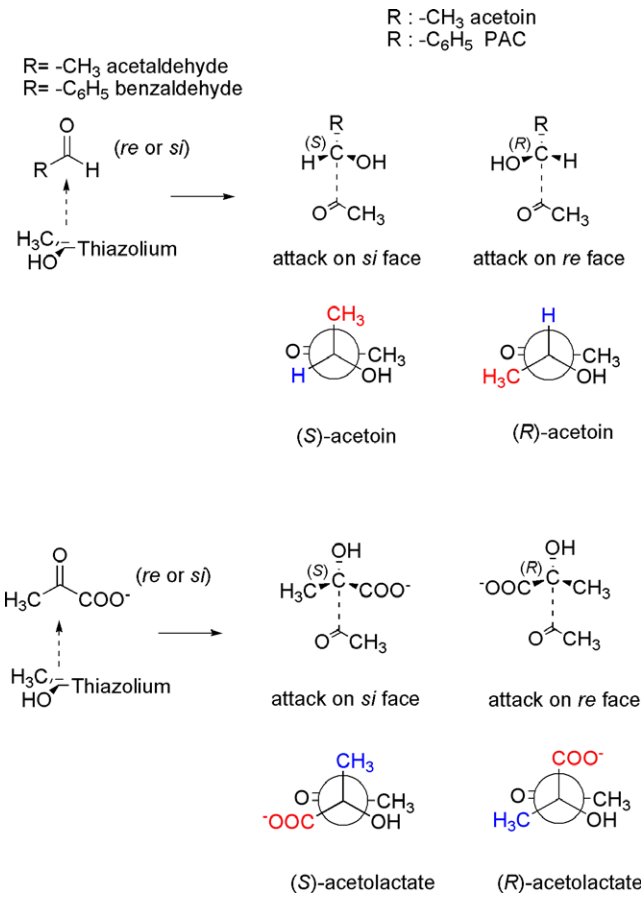

Fig. 10. Proposed mechanism for the formation of the *R*- and *S*-enantiomers by the two enzymes.

Acknowledgments

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