

Glyoxylate Carboligase: A Unique Thiamin Diphosphate-Dependent Enzyme That Can Cycle between the 4'-Aminopyrimidinium and 1',4'-Iminopyrimidine Tautomeric Forms in the Absence of the Conserved Glutamate

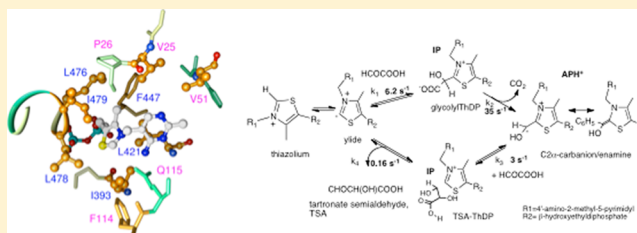
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S Supporting Information

ABSTRACT: Glyoxylate carboligase (GCL) is a thiamin diphosphate (ThDP)-dependent enzyme, which catalyzes the decarboxylation of glyoxylate and ligation to a second molecule of glyoxylate to form tartronate semialdehyde (TSA). This enzyme is unique among ThDP enzymes in that it lacks a conserved glutamate near the N1' atom of ThDP (replaced by Val51) or any other potential acid–base side chains near ThDP. The V51D substitution shifts the pH optimum to 6.0–6.2 (pK_a of 6.2) for TSA formation from pH 7.0–7.7 in wild-type GCL. This pK_a is similar to the pK_a of 6.1 for the 1',4'-iminopyrimidine (IP)–4'-aminopyrimidinium (APH⁺) protonic equilibrium, suggesting that the same groups control both ThDP protonation and TSA formation. The key covalent ThDP-bound intermediates were identified on V51D GCL by a combination of steady-state and stopped-flow circular dichroism methods, yielding rate constants for their formation and decomposition. It was demonstrated that active center variants with substitution at I393 could synthesize (*S*)-acetolactate from pyruvate solely, and acetylglucolate derived from pyruvate as the acetyl donor and glyoxylate as the acceptor, implying that this substituent favored pyruvate as the donor in carboligase reactions. Consistent with these observations, the I393A GCL variants could stabilize the predecarboxylation intermediate analogues derived from acetylphosphinate, propionylphosphinate, and methyl acetylphosphonate in their IP tautomeric forms notwithstanding the absence of the conserved glutamate. The role of the residue at the position occupied typically by the conserved Glu controls the pH dependence of kinetic parameters, while the entire reaction sequence could be catalyzed by ThDP itself, once the APH⁺ form is accessible.



The enzyme glyoxylate carboligase (GCL) catalyzes the thiamin diphosphate (ThDP)-assisted ligation of two molecules of glyoxylate to form (*R*)-tartronate semialdehyde (TSA) and CO_2 .^{1–7} GCL is a member of the homologous family of ThDP-dependent enzymes catalyzing reactions that begin with the decarboxylation of a 2-oxoacid substrate.² It is a homotetramer and requires FAD in addition to ThDP and Mg^{2+} for catalysis.^{1–3} Despite its structural similarity to enzymes whose physiological substrate is pyruvate (pyruvate decarboxylases, pyruvate oxidases, and especially aceto-hydroxyl synthases), the sole substrate of GCL is the 2-aldooxalate glyoxylate and the enzyme is unreactive with 2-ketoacids (Scheme 1A). It is important to note that GCL lacks both the conserved glutamate residue that is found in all other known ThDP enzymes, where its carboxylate group is within hydrogen bonding distance of N1' of the 4'-aminopyrimidine ring of ThDP^{5,6} and any other potential acid–base side chain near ThDP. This unique property among ThDP enzymes makes GCL particularly useful for mechanistic studies where interpretation of pH-dependent behavior is sought. In GCL,

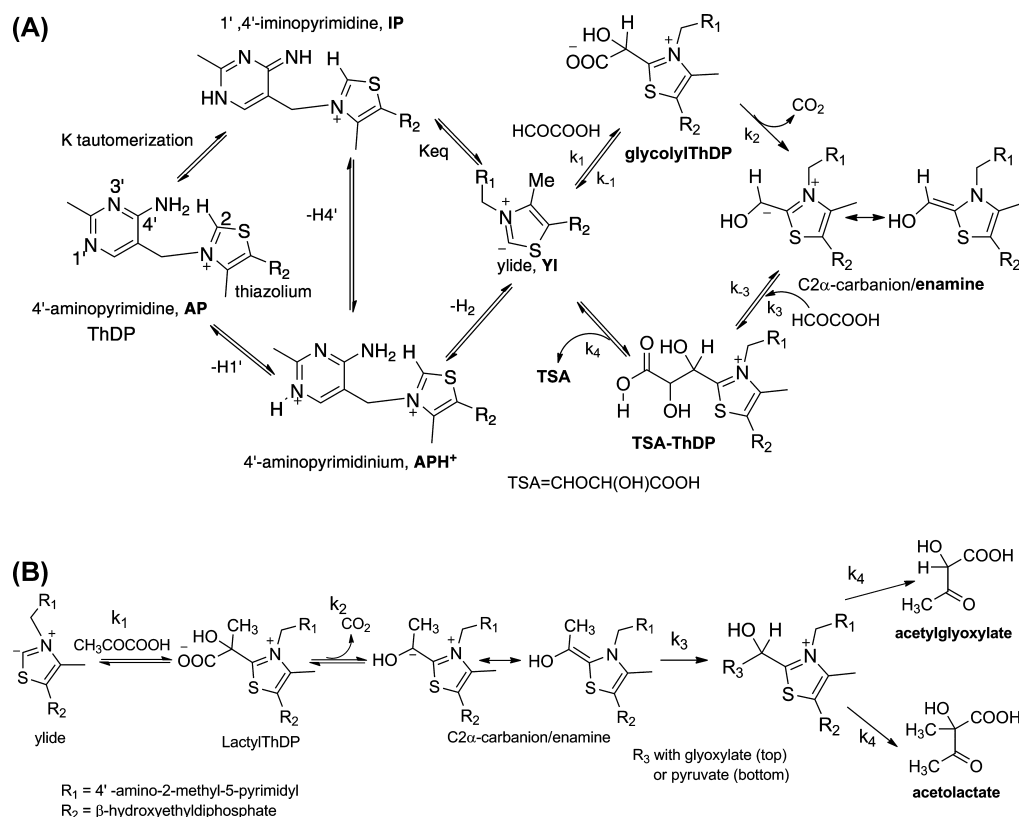
valine (residue V51 in the wild-type *Escherichia coli* enzyme) appears in the position expected for this Glu residue. It had been assumed that the conserved glutamate is essential for activation of the coenzyme in ThDP-dependent enzymes by facilitating the tautomerization of the weakly basic 4'-aminopyrimidine (AP) to the more basic 1',4'-iminopyrimidine (IP) tautomer (Scheme 1A). Surprisingly, steady-state kinetic analyses revealed that the V51D and V51E variants are less active than GCL despite having higher rates of activation of the coenzyme (deprotonation at C2 of the thiazolium moiety). The turnover rates are 2 orders of magnitude lower for the V51D variant and 7-fold lower for the V51E variant.⁵ However, as the coenzyme analogue N3'-pyridyl-ThDP (in which N1' is replaced by CH) leads to an inactive GCL holoenzyme, the 1',4'-iminopyrimidine tautomer of ThDP must have a role in

Received: July 3, 2012

Revised: August 17, 2012

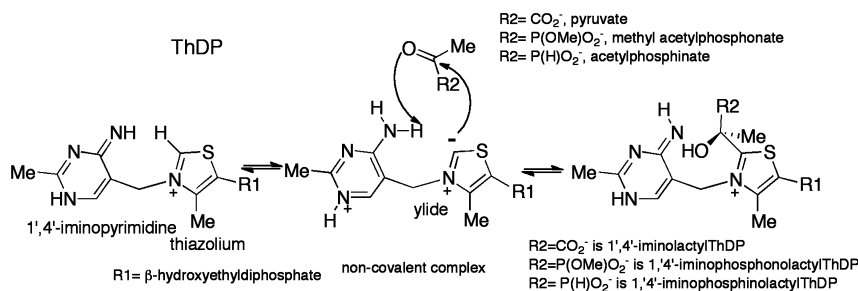
Published: September 12, 2012



Scheme 1^a


^a(A) Mechanism of the GCL reaction, including tautomerization/ionization states of thiamin. Possible reactions of a decarboxylase carboligase with glyoxylate. (B) Possible reactions of a decarboxylase carboligase with pyruvate as the sole substrate, in which acetolactate is produced. With I393A GCL, in the presence of pyruvate and glyoxylate the mixed product acetylglglycolic acid is the expected product.

Scheme 2. Formation of Covalent Adducts of ThDP with Pyruvate and with Pyruvate Analogues MAP and AcPhi



catalysis despite the absence of the conserved Glu.⁵ Circular dichroism (CD) evidence suggested that the V51D variant can in fact stabilize the IP tautomer of ThDP.⁵ To understand the mechanism of GCL in more detail, it is important to identify the intermediate species on the reaction pathway or analogues for such species (Scheme 1A). Covalent adducts of ThDP with the pyruvate analogues (Scheme 2) methyl acetylphosphonate (MAP) and acetylphosphinate (AcPhi) are stable analogues of the first predecarboxylation intermediate formed between pyruvate and ThDP, C2α-lactyl-ThDP (LThDP). These adducts, as well as LThDP itself, have provided many insights into mechanisms of ThDP-dependent enzymes that act on pyruvate.^{8–12} Importantly, it has been demonstrated for several ThDP enzymes that incremental addition of MAP and AcPhi produced a positive CD band near 300–310 nm, a band assigned to the 1',4'-iminophosphonolactyl-ThDP (PLThDP) and 1',4'-iminophosphinolactyl-ThDP, respectively.^{12–14} Un-

fortunately, no synthesis of phosphonyl or phosphinyl analogues of glyoxylate (e.g., formylphosphinate) has yet been published. To gain further understanding of substrate binding and specificity in GCL, we used the I393A GCL and V51D/I393A GCL, predicted by modeling based on the crystal structure of GCL,⁵ to allow GCL to accept pyruvate or its analogues as donor substrates. Modeling indicates that the side chain of Ile393 creates steric hindrance for substrates larger than glyoxylate, like the molecular determinants for substrate specificity identified on acetohydroxyacid synthase II from *E. coli*.³⁶

In this study, we employ kinetic studies using steady-state and stopped-flow circular dichroism (CD) spectroscopy to further investigate the mechanistic ability of GCL to function despite the absence of the “conserved” glutamate, as well as the determinants of its specificity for glyoxylate as the substrate. By using V51D GCL, we demonstrated that the pH optimum of

TSA formation and the pK_a for the IP–APH⁺ equilibrium for this variant are both significantly shifted to the acid side by the same magnitude compared with those of GCL. Using the unfavorable pH of 7.6 and a low temperature in the CD experiments allowed us to identify the key ThDP-bound intermediates on V51D GCL. By using MAP, AcPhi, and propionylphosphinate (PrPhi), we now present CD evidence that GCL variants with Ile393 substituted with Ala can stabilize the predecarboxylation intermediates derived from pyruvate and 2-oxobutanoate analogues even in the absence of the V51D substitution.

EXPERIMENTAL PROCEDURES

Materials. Sodium glyoxylate, pyruvate, FAD, ThDP, ampicillin, tetracycline, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Coenzyme Q₀), DTT, and NADH were obtained from Sigma (St. Louis, MO). Ammonium sulfate and SDS were obtained from BDH Chemicals Ltd. (Poole, U.K.). Yeast extract, peptone, and agar were from DIFCO (Detroit, MI). Restriction enzymes were from New England BioLabs Inc. (Beverly, MA), Novagen (Darmstadt, Germany), and Promega (Madison, WI). All reagents were of analytical grade.

Synthesis of AcPhi. was conducted according to the procedure reported by Baillie et al.¹⁵ PrPhi was prepared as described previously.¹⁶ Methyl acetylphosphonate (MAP) was synthesized using trimethylphosphite and acetylchloride as reagents according to the method of Kluger et al.¹⁷ Purity and correct synthesis of all compounds were confirmed by NMR spectroscopy and mass spectrometry.

Expression, Purification, and Site-Directed Mutagenesis of GCL. *E. coli* gene *gcl* encoding GCL was cloned as previously described.¹⁸ GCL and its variants were expressed from the pQE60-GCL plasmid and purified by affinity chromatography using a HiTrap Chelating HP Ni column (5 mL, Pharmacia) and the Pharmacia ÄKTA prime FPLC system.⁵ The single substitutions were introduced into pQE60-GCL using the overlap extension site-directed mutagenesis method with the FailSafe kit (EPICENTRE Biotechnologies). The primers used were designed to introduce a modification into the restriction site as an aid in screening for clones with mutated plasmids. The mutations were verified by sequencing of the coding region of each plasmid used for expression of variant proteins.

Enzyme Assays. TSA Activity Measurements. The rate of steady-state formation of TSA by GCL was measured in a coupled enzyme assay that follows the disappearance of NADH with tartronate semialdehyde reductase (TSAR).¹⁹ TSAR was cloned, expressed, and purified as previously described.⁵ The TSA activity was also measured by direct detection of TSA formation using a Chirascan CD spectrometer at 290 nm (Applied Photophysics, Leatherhead, U.K.).¹⁸ The reaction medium in a volume of 2.4 mL contained 50 mM KH₂PO₄ (pH 7.7), 0.06 M KCl, 0.1 mM ThDP, 5 mM MgCl₂, and 50 μM FAD. The glyoxylate was added to a final concentration of 10 mM, and the reaction was started by addition of GCL (25 μg) or V51D GCL (30–50 μg) at 37 °C and monitored for 300 s. The linear part of the progress curves was used to calculate the initial velocity (slope per second) using the Pro-Data Viewer program supplied by the manufacturer.

Acetolactate Determination. The modified Westerfeld creatine-naphthol colorimetric method was used for assaying acetolactate (AL) formation^{20,21} with absorbance measurements taken with a Beckman DU640 spectrophotometer.

Reaction of I393A GCL with Pyruvate Monitored by Reduction of 2,6-Dichlorophenolindophenol. The 2,6-dichlorophenolindophenol (DCPIP) reaction was conducted at 5 °C by monitoring its reduction at 600 nm in a 1 mL reaction mixture containing 0.5 mM ThDP, 5 mM MgCl₂, 80 μM DCPIP, and 20 μg of protein in 50 mM KH₂PO₄ (pH 7.0). The reaction was initiated by addition of pyruvate (0.025–5 mM) after equilibration of I393A in the reaction mixture for 5 min because of the trace amounts of DTT present in the protein. The linear part of the progress curves was used to calculate the initial velocity (slope per minute).

CD Spectroscopy. CD experiments were conducted on a Chirascan CD spectrometer from Applied Photophysics in a 1 cm path length cell in the near-UV (250–450 nm) wavelength region. For each experiment, conditions are presented in the figure legend.

pH Dependence of CD Bands. To study the pH dependence of the CD bands pertinent to the IP or AP forms, the pH of the protein solution was adjusted using a sympHony pH electrode (VWR, Batavia, IL) and spectra were recorded after each adjustment.²² The pK_a was determined from the fit of the log CD_{302–304} versus pH according to eq 1 for a single ionizing group or according to eq 2 for two ionizing groups using SigmaPlot version 10.0

$$\log(\text{CD}) = \log(\text{CD}_{\text{max}}) - \log(1 + 10^{pK_1 - x}) \quad (1)$$

$$\log(\text{CD}) = \log(\text{CD}_{\text{max}}) - \log(1 + 10^{pK_1 - x} + 10^{x - pK_2}) \quad (2)$$

where x is pH.

The pH dependence of the TSA activity catalyzed by V51D GCL and GCL as measured by direct CD detection of TSA was determined in 50 mM KH₂PO₄, adjusted to the desired pH (pH 5.9–7.5), containing 0.06 mM KCl, 0.1 mM ThDP, 5 mM MgCl₂, and 50 μM FAD. Glyoxylate was added to a final concentration of 10 mM, and the reaction was initiated by addition of GCL (25 μg) or V51D GCL (30–50 μg). The CD₂₉₀ per second slope was calculated at different pH values, and a plot of log CD₂₉₀ per second versus pH was prepared. Data were fit using eq 1 or 2.

Titration of GCL with AcPhi, PrPhi, and MAP. CD spectra of GCL and its variants in the absence and presence of pyruvate analogues and PrPhi were recorded in the near-UV region at 290–550 nm and 25 °C. Conditions of each experiment are described in the figure legends. A plot of ellipticity at 302 nm versus substrate analogue concentration was constructed by subtracting the ellipticity of the enzyme at the same wavelength, but recorded in the absence of a substrate analogue, as reported previously.²³ The K_d values were calculated using the Hill equation (eq 3):

$$\text{CD}_{302} = (\text{CD}_{302}^{\text{max}} [\text{ligand}]^{n_H}) / (S_{0.5}^{n_H} + [\text{ligand}]^{n_H}) \quad (3)$$

where n_H is a Hill coefficient. When $n_H = 1.0$, $S_{0.5} = K_d$.

CD Titration of I393A GCL with Pyruvate. I393A GCL (2.5 mg/mL, active center concentration of 38.6 μM) in 0.10 M KH₂PO₄ (pH 7.6) containing 5 mM MgCl₂ and 0.50 mM ThDP was titrated with pyruvate (0.02–4.0 mM) at 4 °C. After 15 h at 4 °C, the protein was removed from the reaction mixture with an Ultracel-30K Centrifugal Filter Unit (Millipore) and CD spectra were recorded at 250–400 nm for (S)-acetolactate detection.

Stopped-Flow CD Spectroscopy. Kinetic traces were recorded on a Pi*-180 stopped-flow CD spectrometer (Applied

Photophysics) using a 10 mm path length cell at the specified wavelengths. The temperature was maintained at 6 or 15 °C as stated in the figure legends.

Pre-Steady-State Formation of Covalent 1',4'-IminopyrimidylThDP-Bound Intermediates on V51D GCL. A solution of V51D GCL (3.6 mg/mL, active center concentration of 55.6 μ M) in 0.1 M KH_2PO_4 (pH 7.6) containing 0.5 mM ThDP, 2.5 mM MgCl_2 , 1.0 mM DTT, 10 μ M FAD, and 1% glycerol in one syringe was mixed rapidly with an equal volume of glyoxylate (2.0 mM) in the same buffer placed in the second syringe at 6 °C. The data points were collected at 302 nm over a period of 50 s. Data from four or five repetitive shots were averaged and were fit to a double-exponential (eq 4), single-exponential (eq 5), or triple-exponential (eq 6) model using SigmaPlot version 10.0:

$$\text{CD}_{302-304}(t) = \text{CD}_1 e^{-k_1 t} + \text{CD}_2 e^{-k_2 t} + c \quad (4)$$

$$\text{CD}_{302-304}(t) = \text{CD}_1 e^{-k_1 t} + c \quad (5)$$

$$\text{CD}_{302-304}(t) = \text{CD}_1 e^{-k_1 t} + \text{CD}_2 e^{-k_2 t} - \text{CD}_3 e^{-k_3 t} + c \quad (6)$$

where k_1 , k_2 , and k_3 are the apparent rate constants and c is $\text{CD}_{302}^{\text{max}}$ in the exponential rise to maximum model or $\text{CD}_{302}^{\text{min}}$ in the exponential decay model.

RESULTS

Rationale for the Selection of Active Center Residues V51 and I393 for Substitution. According to the X-ray structure of GCL [Figure 1, Protein Data Bank (PDB) entry

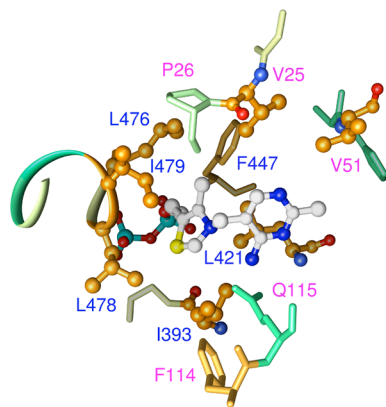


Figure 1. Structure of the active center of GCL demonstrating the hydrophobic environment of the thiazolium ring of ThDP. This figure was created using PyMOL based on the published structure of GCL (PDB entry 2PAN).⁵

2PAN], residues I393, L478, and I479 are located close to the thiazolium ring of ThDP and apparently form a pocket proximate to C2 that can accommodate a glyoxylate moiety but cannot easily accommodate a pyruvate molecule, with an extra methyl group.⁵ To understand the structure–function relationship, these residues were substituted with alanine or the amino acids conserved at the homologous positions in the AHAS (Table 1). The single I393A, I393V, L478A, and I479V substitutions led to a lower catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) of GCL in TSA formation [approximately 10–36-fold lower compared with that of GCL, as detected, in a coupled enzyme assay with TSA reductase (TSAR)¹⁹] (Table 1). As a result of

substitution I393A or double substitutions V51D and I393A, GCL is converted to an acetolactate synthase that can use pyruvate as a substrate with a catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) ~ 20 times higher than that of wild-type GCL (Table 1). Formation of acetolactate was detected by a colorimetric measurement^{20,21} and by direct CD detection of (S)-acetolactate (see below).¹⁸ The I393V, L478A, and I479V variants did not exhibit marked increases in the level of acetolactate synthesis compared to that of the GCL.

The Val51 residue in GCL is critical for TSA synthesis. Although the V51D variant catalyzes proton exchange at C2 of the thiazolium ring of ThDP ~ 6 -fold more rapidly than GCL, the TSA–ThDP complex accumulates in this enzyme and its turnover rate is lower by 2 orders of magnitude.⁵ The X-ray structure of GCL suggested that aspartate can be easily accommodated in place of the V51 position in the active center of GCL with its carboxylate group positioned within hydrogen bonding distance of N1' of the 4'-aminopyrimidine ring. On the other hand, we suggested that the carboxylate in the V51E variant can make such a hydrogen bond only if the structure is distorted.⁵ Hence, the V51D substitution appeared to be more fruitful for our investigation.

The doubly substituted V51D/I393A GCL was created to address whether the combination of a carboxylate group, which can accelerate the activation of ThDP, with relief of the steric hindrance to the larger substrate pyruvate by replacement of Ile393 with Ala might allow the variant to effectively stabilize the 1',4'-iminopyrimidine tautomeric intermediates derived from pyruvate and to effectively catalyze the synthesis of acetolactate. As shown in Table 1, the catalytic efficiency for formation of acetolactate by V51D/I393A GCL is similar to that of the singly substituted I393A GCL despite the presence of the Asp residue at position 51. The formation of TSA by this doubly substituted variant cannot be detected in a coupled enzyme assay. The observation that the presence or absence of an aspartate residue at position 51 has little effect on the activity of a V51D/I393A variant in the formation of acetolactate (compared to the result with the single I393A substitution) implies that the effect of Asp51 on TSA formation is specifically related to the chemistry of the natural substrate glyoxylate.

Identification of ThDP-Bound Intermediates and Their Tautomeric Forms on V51D GCL by CD. It was suggested that ThDP enzymes with a variety of active center environments can stabilize the IP form of ThDP, and that the presence of a glutamate residue within hydrogen bonding distance of the N1' atom of the 4'-aminopyrimidine ring is important for catalyzing the tautomeric equilibration and stabilizing the IP form.^{12,13,24} It was further suggested that all C2 α -tetrahedral ThDP adducts on the pathway for an enzymatic reaction will likewise exist as their 1',4'-iminopyrimidine tautomers at pH values above the pK_{a} of the APH^+ ionization state. With these findings in mind, CD has now been applied to GCL and its variants.

The 1',4'-Iminopyrimidine Tautomeric Form of ThDP Is Stabilized on V51D GCL. It was demonstrated earlier that V51D GCL displayed a positive CD band at 302 nm in the resting state, suggested to correspond to the IP form of ThDP bound to this variant, not displayed on the V51E variant.⁵ The following experimental evidence is presented here to confirm this suggestion. (1) The CD band at 302 nm was partially reduced on replacement of ThDP by thiamin 2-thiothiazolone diphosphate (ThTTDP), an analogue of ThDP with the C2–H bond in the thiazolium ring substituted with a C2=S bond

Table 1. Kinetic Properties of GCL and Its Active Center Variants

enzyme	tartronate semialdehyde formation ^a		acetolactate formation ^b	
	specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) (%)	k_{cat}/K_m ($\text{s}^{-1} \text{mM}^{-1}$)	specific activity ($\text{nmol min}^{-1} \text{mg}^{-1}$)	k_{cat}/K_m ($\text{s}^{-1} \text{M}^{-1}$)
wild type	17.5 \pm 2.5 (100%)	21.0	8.2 \pm 0.9	0.05
I393A	0.68 \pm 0.02 (3.9%)	2.06	82.0 \pm 3.4	0.86
I393V	0.93 ^c (5.3%)	0.65	3.0 \pm 0.5	0.11
L478A	0.06 ^c (0.34%)	1.79	3.0 \pm 0.14	0.37
I479V	0.84 \pm 0.05 (4.8%)	0.58	4.0 \pm 0.2	0.30
V51D	0.2 \pm 0.02 ^d (1.2%)	1.3 ^d	—	—
V51D/I393A	nd ^e	nd ^e	90.2 \pm 3.0	0.94

^aThe rate of TSA formation was measured in a coupled enzyme assay with TSAR.¹⁹ ^bThe modified colorimetric method by Westerfeld was used for measurement of acetolactate activity.^{20,21} ^cFor these two variants, the activity was over a range of substrate levels and was measured once, so that the errors cannot be estimated. ^dData from ref 5 were used. ^eNo activity could be detected.

(not shown). (2) The amplitude of the CD band at 302 nm was reduced upon addition of AcPhi, an analogue of pyruvate; however, no signature for formation of 1',4'-iminophosphino-lactylThDP was in evidence. (3) The positive CD band at 302 nm is replaced by a negative one at 290 nm upon addition of glyoxylate, because of formation of the (R)-TSA product. (4) The intensity of the positive CD band at 302 nm depends on pH, in a manner similar to that reported previously by the Jordan group for other ThDP-dependent enzymes.²²

Effect of the V51D Substitution on Equilibria of Tautomeric and Ionization States of ThDP. The AP form of ThDP on benzaldehyde lyase and benzoylformate decarboxylase, and both the AP and IP forms on pyruvate oxidase from *Lactobacillus plantarum*, as well as on the human pyruvate dehydrogenase E1 component all exhibited pH-dependent behavior.^{22,25} Here, we conduct a pH titration to determine the pK_a for the IP–APH⁺ equilibrium (there is no evidence of the presence of a detectable concentration of the AP form on GCL so far) on V51D GCL. When the pH is reduced from 7.95 to 5.72, the amplitude of the positive CD band at 302–304 nm observed on V51D GCL was reduced (Figure 2), indicating that the IP form of ThDP is dominant at pH 7.95, while the APH⁺ form is likely dominant at pH 5.72, with no CD band detected for this form so far. The pH titration data fit to a single proton titrating with a pK_a of 6.1 for the IP–APH⁺ equilibrium (Figure 2B). On several ThDP enzymes studied so far, the pK_a for the IP–APH⁺ equilibrium is near the pH optimum of enzyme activity,²² which is pH 7.7 for GCL. The data thus suggest that the V51D substitution significantly shifted the pH optimum of enzyme activity to the acid side.

To test this hypothesis, the rate of formation of (R)-TSA by GCL and V51D GCL was estimated directly by using CD₂₉₀, as reported previously.¹⁸ Both GCL and V51D GCL displayed a pH dependence for TSA formation. A plot of the log of the reaction slope detected from the progress curves (Figure 3A) [the molar ellipticity for (R)-TSA is not available in the literature] versus pH revealed a maximal activity at pH 7.0–7.7 for the GCL (Figure 3B), with the bell-shaped plot being described by two acid–base groups with an apparent pK_1 of 6.1 and a pK_2 of >8.0. For V51D GCL, the pH optimum of activity was shifted to pH 6.0–6.2 (Figure 4). The apparent pK_a of 6.2 calculated from activity measurements correlates well with the pK_a of 6.1 determined for the IP–APH⁺ equilibrium from the previous CD experiment, suggesting that the same groups control both events, probably the D51COO[−]–APH⁺ dyad (kinetically equivalent to, and indistinguishable from, the D51COOH–IP dyad), perhaps assisting with release of product from the TSA–ThDP covalent complex (see below).

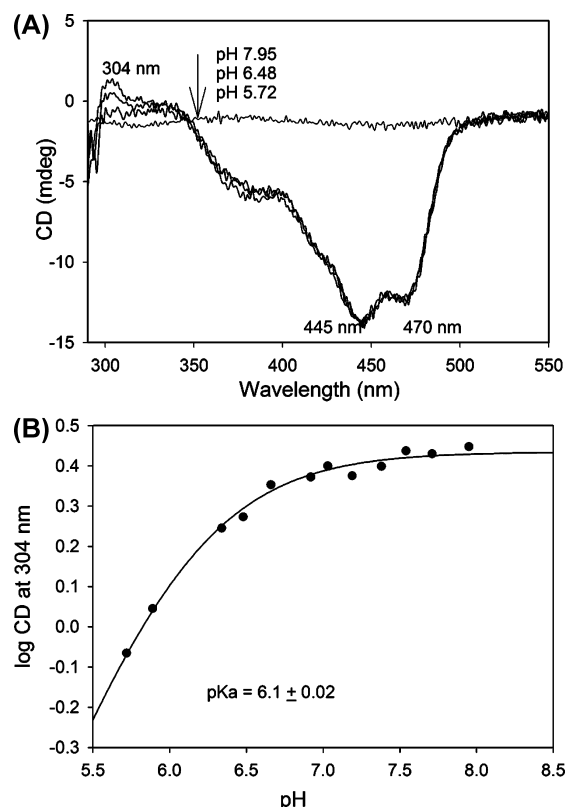


Figure 2. Effect of pH on CD spectra of V51D GCL. (A) Near-UV CD spectra of V51D GCL at different pH values. The V51D GCL from stock was diluted to a concentration of 2.6 mg/mL (active center concentration of 40 μM) in 0.1 M KH_2PO_4 containing 0.5 mM ThDP and 2.5 mM MgCl_2 . CD spectra were recorded after the pH had been adjusted to the desired value (pH 5.72–7.95). (B) Dependence of log CD₃₀₄ on pH. Data were fit to a single ionizing group (eq 1).

Stabilization of the 1',4'-Iminopyrimidine Form of ThDP-Bound Intermediates Derived from Glyoxylate on V51D GCL. Because V51D GCL is a slow variant [0.20 $\mu\text{mol min}^{-1}$ (mg of protein)^{−1}] compared to GCL [17.5 $\mu\text{mol min}^{-1}$ (mg of protein)^{−1}], the ThDP-bound intermediates could be detected by steady-state CD at a low temperature (5 °C). Upon titration of V51D GCL by glyoxylate using concentrations in excess of the concentration of active centers (1, 5, and 8 mM as compared with the active center concentration of 9.5 μM), the magnitude of the positive CD band at 302 nm assigned to the IP form of ThDP on V51D GCL was reduced and was finally replaced by a negative band when the temperature was

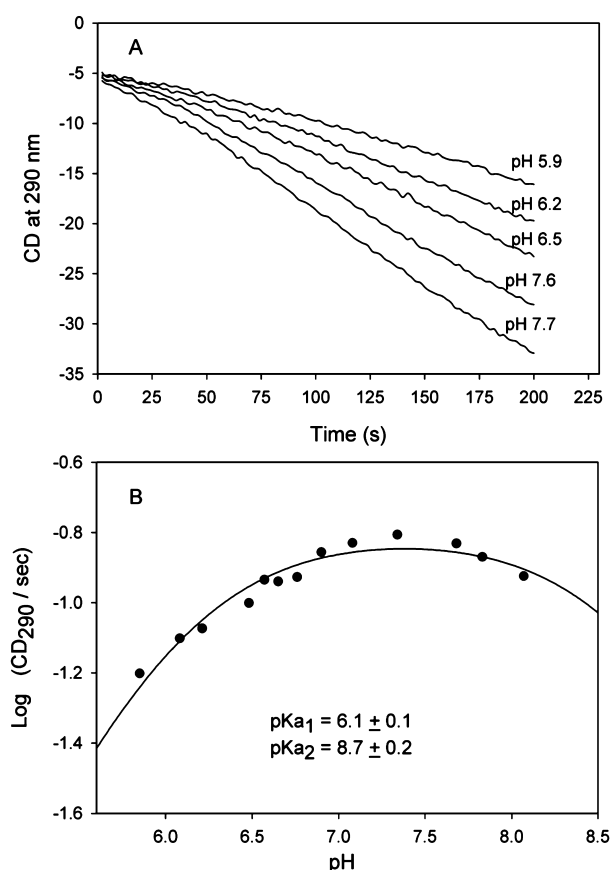


Figure 3. Effect of pH on the TSA activity of GCL. (A) Progress curves of TSA formation were recorded at different pH values using CD at 290 nm. Conditions of the experiment are presented in Experimental Procedures. The linear part of the progress curves was used to calculate the initial velocity (CD₂₉₀ per second) using the Pro-Data Viewer program. (B) Dependence of log(CD₂₉₀ per second) on pH. The log(CD₂₉₀ per second) was plotted vs pH, and data were fit to eq 2 for two ionizing groups.

increased to 20 °C (Figure 5). This is largely due to the formation of (R)-TSA, which has a strong negative CD band at 290 nm.¹⁸ Formation of this product was confirmed by the CD spectrum of the reaction mixture after removal of protein: the negative CD band detected at 290 nm is what was expected for (R)-TSA (not shown). A similar decrease in the amplitude of the positive CD band at 302 nm was observed when the experiment was conducted at pH 7.6 or 6.5 (Figure 5 presents data at pH 6.5), giving clear evidence of (R)-TSA (product) formation.

Time-Resolved CD Experiments with ThDP-Bound Intermediates on V51D GCL. Next, in a series of experiments, the reaction of V51D GCL with glyoxylate was monitored by stopped-flow CD at 302 nm and pH 7.6. In the first experiment conducted at 6 °C, the V51D GCL (active center concentration of 56 μM) in one syringe was mixed with 2 mM glyoxylate in the second syringe, resulting in a time-dependent accumulation of a species with a positive ellipticity at 302 nm (Figure 6A). The reaction reached steady state within 5 s with a k_1 of 6.2 s⁻¹ and a k_1' of 0.58 s⁻¹ (the data points were analyzed as a double exponential resulting in two rate constants, k_1 and k_1' , for the fast and slow phases of the reaction, respectively), suggesting formation of a covalent glycolyl-ThDP predecarboxylation

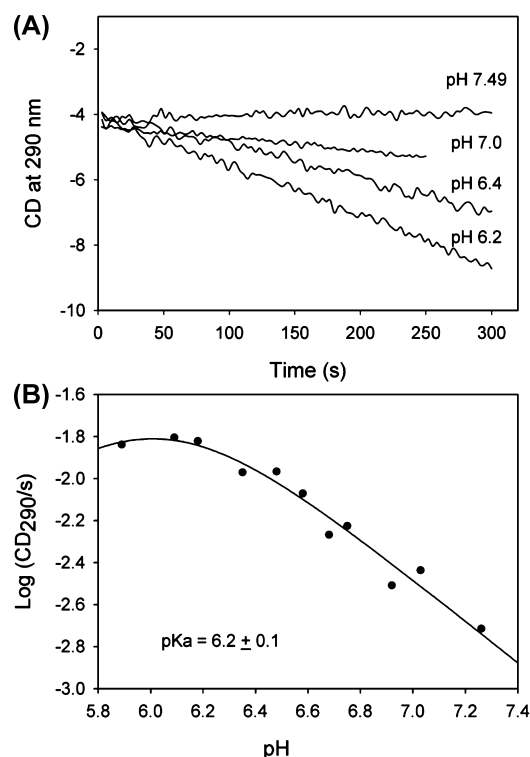


Figure 4. Effect of pH on the TSA activity of V51D GCL. (A) Progress curves of TSA formation at different pH values using CD at 290 nm. Conditions of the experiment are presented in Experimental Procedures. The linear part of the progress curves was used to calculate the initial velocity (CD₂₉₀ per second) using the Pro-Data Viewer program. (B) pH dependence of log(CD₂₉₀ per second). Data were fit to eq 1 for a single ionizing group.

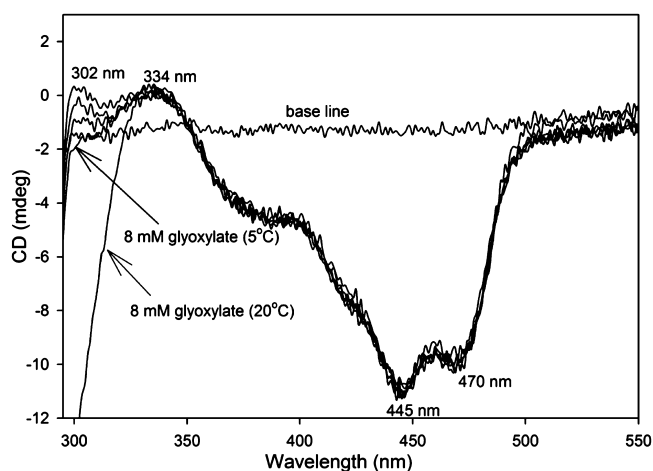


Figure 5. CD spectra of V51D GCL titrated by glyoxylate. The V51D GCL (1.23 mg/mL, active center concentration of 19.0 μM) in 0.1 M KH₂PO₄ (pH 6.5) containing 0.5 mM ThDP, 2.5 mM MgCl₂, 1.0 mM DTT, 10 μM FAD, and 1% glycerol was titrated by glyoxylate (0.010–8 mM) at 5 °C. CD spectra were recorded in the near-UV region after each addition of glyoxylate. Observable changes in the intensity of the CD band at 302 nm were detected with 1, 5, and 8 mM glyoxylate, but not with 0.01–0.5 mM glyoxylate. When the temperature was increased to 20 °C, formation of (R)-TSA was evident.

intermediate (Schemes 1A and 3 for rate constant assignment and Table 2 for values).

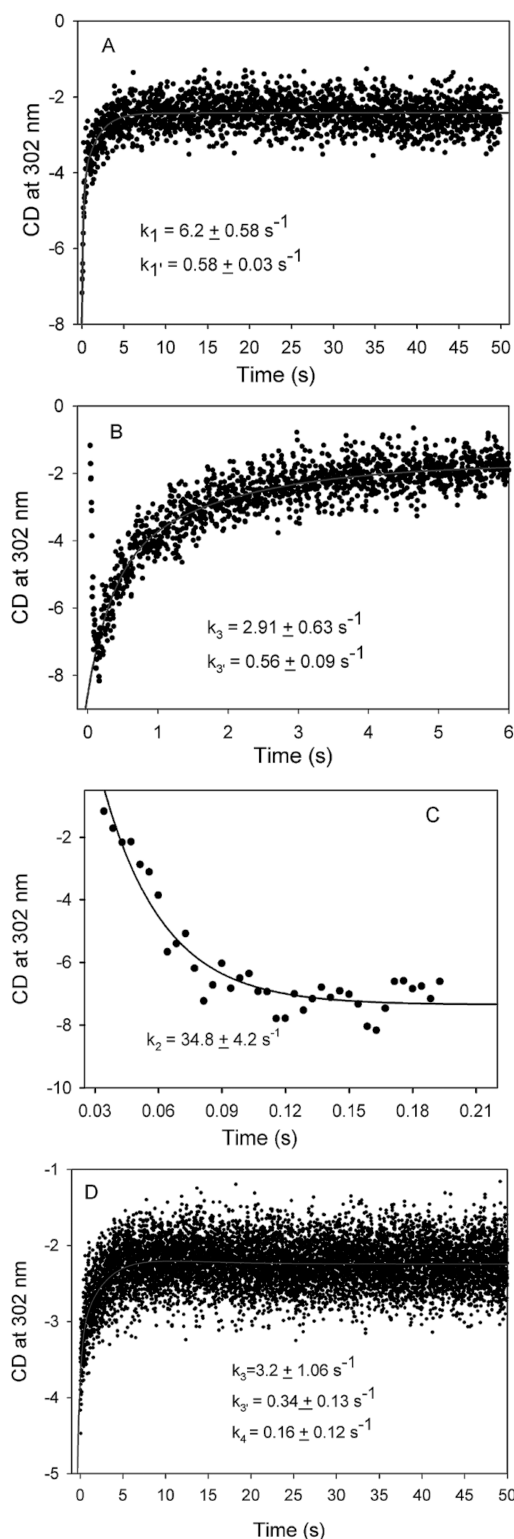


Figure 6. Time course of the reaction of VS1D GCL with glyoxylate monitored by stopped-flow CD. (A) Formation of 1',4'-iminoglycolylThDP at pH 7.6 and 6 °C. VS1D GCL (3.6 mg/mL, active center concentration of 55.6 μ M) in 0.1 M KH_2PO_4 (pH 7.6) containing 0.5 mM ThDP, 2.5 mM MgCl_2 , 1.0 mM DTT, and 10 μ M FAD in one syringe was mixed with an equal volume of 2.0 mM glyoxylate in the same buffer in the second syringe. Data were fit to a double-exponential equation (eq 4). (B) Decarboxylation of 1',4'-iminoglycolyl-ThDP and formation of the TSA-ThDP complex at pH 7.6 and 15 °C. VS1D GCL (2.3 mg/mL, active center concentration of 35.5 μ M) in one syringe was mixed with 2.0 mM

Figure 6. continued

glyoxylate in the second syringe at 15 °C. (C) Time-dependent decarboxylation of 1',4'-iminoglycolylThDP with data from Figure 6B expanded. Data were fit to a single exponential (eq 5). (D) Formation of the TSA-ThDP complex and release of TSA by VS1D GCL at pH 7.6. The VS1D GCL (1.7 mg/mL, active center concentration of 26 μ M) was preincubated with 0.5 mM glyoxylate for 15 min in one syringe and then mixed at 6 °C with 16 mM glyoxylate in the second syringe. The data were fit to a triple exponential (eq 6).

The second experiment was conducted under similar conditions but at 15 °C. Initially, the intensity of the CD signal at 302 nm decreased for at least the first 0.2 s (Figure 6B for early times and Figure 6C for details), followed by an increase in CD_{302} with an exponential rise to maximum. We assign the final positive CD signal developed in this experiment to the TSA-ThDP covalent adduct, identified by a quench-NMR method.⁵ We assign the rate constant in Figure 6C (expansion of Figure 6B, the initial decrease) to decarboxylation of the glycolyl-ThDP predecarboxylation intermediate ($k_2 = 35 \text{ s}^{-1}$ in Figure 6C; see Schemes 1A and 3 and Table 2) and $k_3 = 2.9 \text{ s}^{-1}$ and $k_{3'} = 0.56 \text{ s}^{-1}$ to the formation of the TSA-ThDP complex on GCL (Figure 6B). The formation of the glycolyl-ThDP predecarboxylation complex was too fast to be detected at 15 °C, but it most likely was detected at a low concentration of glyoxylate at 6 °C in Figure 6A.

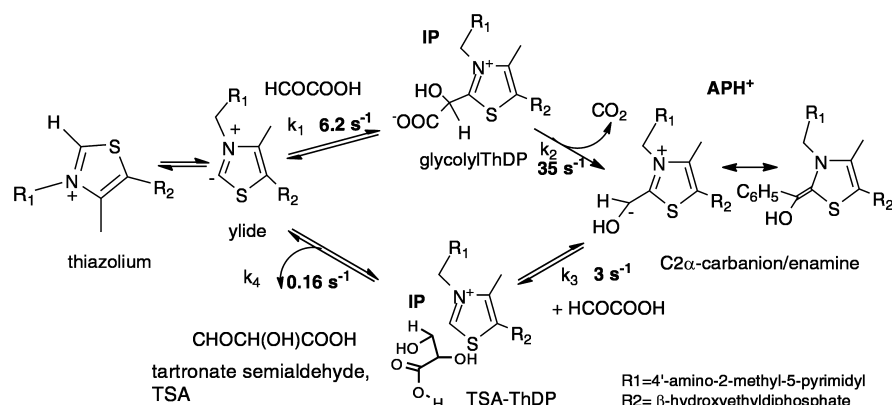
In the third experiment, the VS1D GCL was preincubated with 0.5 mM glyoxylate in one syringe to preform the covalent glycolyl-ThDP predecarboxylation intermediate and then mixed with 16 mM glyoxylate in the second syringe at 6 °C. An initial fast rise was observed [providing the rate constants for formation of the (R)-TSA-ThDP complex with a k_3 of 3.2 s^{-1} and a $k_{3'}$ of 0.34 s^{-1}] followed by a much slower decrease associated with a rate constant k_4 of 0.16 s^{-1} for the release of (R)-TSA (product) from the enzyme (Figure 6D).

This sequence of experiments could be realized only because pH 7.6 is unfavorable for the VS1D GCL variant. At the same time, it allowed us to detect all key ThDP-bound intermediates on the reaction path and to calculate rate constants for formation of glycolyl-ThDP, its decarboxylation to the enamine, carbonylation of the enamine with the second molecule of glyoxylate to provide the TSA-ThDP complex, and finally the release of product from GCL. Under these experimental conditions, product release is clearly the rate-limiting step.

Finally, when the experiment was conducted at pH 6.5 (more favorable pH for VS1D GCL) and 6 °C, only the TSA-ThDP product accumulated, and its release was also detected (Figure 7); the positive ellipticity at 302 nm was developed within 5 s with rate constants k_3 of 3.4 s^{-1} and $k_{3'}$ of 0.55 s^{-1} , followed by a slow decrease in the ellipticity with a rate constant k_4 of 0.04 s^{-1} , again confirming TSA release as the rate-limiting step in agreement with NMR data.⁵

CD Titration of VS1D GCL with Analogues of Pyruvate. Upon addition of AcPhi (0.001–5 mM) or MAP (0.001–2.6 mM) to VS1D GCL (active center concentrations of 15.2 and 38 μ M, respectively), the amplitude of the CD band at 302 nm assigned to the IP form of ThDP was reduced by approximately 57% in both cases but no 1',4'-iminopyrimidine tautomeric forms of the predecarboxylation adduct resulting from addition of AcPhi and MAP were detected (not shown). The $S_{0.5, \text{AcPhi}}$ calculated for half of the reduction of the CD band at 302 nm

Scheme 3. Minimal Mechanism and Microscopic Rate Constants for V51D GCL^a



^a k_1 represents the formation of glycolyl-ThDP, the predecarboxylation intermediate, positive CD band at 302 nm. k_2 represents the decarboxylation to enamine. k_3 represents carbonylation with glyoxylate to provide the TSA–ThDP complex, the postdecarboxylation intermediate, positive CD band near 300 nm. The values represent the average from three independent experiments. k_4 represents the release of TSA product from ThDP·GCL (identified by NMR⁵). k_1 , k_3 , and k_4 were determined at 6 °C, and k_2 was determined at 15 °C.

Table 2. Kinetic Analysis of the Mechanism of V51D GCL by Stopped-Flow CD under Different Experimental Conditions

expt ^a	conditions (T, pH, [glyoxylate])	glycolyl-ThDP formation, k_1 (s ^{−1})	glycolyl-ThDP decarboxylation, k_2 (s ^{−1})	TSA–ThDP complex, k_3 (s ^{−1})	TSA release, k_4 (s ^{−1})
1	6 °C, pH 7.6, 1 mM	6.2 ± 0.6 ^b	nd ^c	nd ^c	nd ^c
2	15 °C, pH 7.6, 1 mM	0.58 ± 0.03	35 ± 4	2.9 ± 0.6 ^b	nd ^c
3	6 °C, pH 7.6, 8 mM	nd ^c	nd ^c	0.56 ± 0.09	0.16 ± 0.12
4	6 °C, pH 6.5, ^d 1 mM	nd ^c	nd ^c	3.2 ± 1.1 ^b	0.04 ± 0.01
				0.34 ± 0.13	
				3.4 ± 0.4 ^b	
				0.55 ± 0.04	

^aExperiments are numbered according to the text in Results. The experimental data are presented in Figure 6A–D. ^bThe experimental curves were treated as a double exponential, leading to two rate constants (fast and slow). ^cNot detected. ^dExperiment conducted at pH 6.5, favorable for V51D GCL as demonstrated in this work.

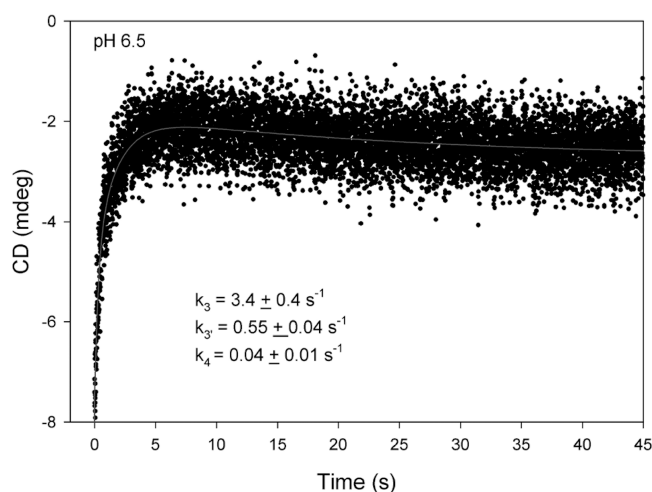


Figure 7. Reaction of V51D GCL with glyoxylate monitored by stopped-flow CD at pH 6.5. The V51D GCL (6.45 mg/mL, active center concentration of 99.6 μM) in 0.10 M KH₂PO₄ (pH 6.5) containing 0.5 mM ThDP, 2.5 mM MgCl₂, 1.0 mM DTT, and 10 μM FAD in one syringe was mixed with an equal volume of 2 mM glyoxylate in the second syringe. The reaction was monitored over 45 s at 6 °C. Data were fit to a triple exponential (eq 6).

was 0.18 ± 0.11 mM, indicating that AcPhi is likely bound in the active centers of V51D GCL (Table 3). A similar

Table 3. Kinetic Parameters for Equilibrium Binding of Pyruvate and 2-Oxobutanoate Analogues to GCL Variants^a

GCL substitution	K_d ($S_{0.5}$) for AcPhi (mM)	K_d ($S_{0.5}$) for PrPhi (mM)	K_d for MAP (mM)
I393A	0.32 ± 0.02 ($n_H = 2.23$)	0.46 ± 0.03 ($n_H = 1.7$)	1.4 ± 0.09
V51D	0.18 ± 0.11 ^b	–	0.11 ± 0.09 ^b
V51D/I393A	0.37 ± 0.09 ($n_H = 1.29$)	0.28 ± 0.04 ($n_H = 1.1$)	0.11 ± 0.1

^aKinetic parameters were determined in steady-state CD experiments. ^bFor V51D GCL, no 1',4'-iminopyrimidine tautomers from addition of AcPhi and MAP were detected. Instead, the intensity of the CD₃₀₂ band was reduced by AcPhi and MAP with the values of $S_{0.5}$ listed here.

experiment with MAP revealed an $S_{0.5,MAP}$ of 0.11 ± 0.09 mM for the CD band at 302 nm, indicating similar behavior with MAP (Table 3). Hence, the evidence for covalent complex formation with these substrate analogues is tentative.

Interestingly, with the doubly substituted V51D/I393A GCL, we did observe changes in the CD spectrum upon binding of these pyruvate analogues (Table 3). We must emphasize, however, that while we have assigned CD spectroscopic signatures for the IP and AP forms of ThDP on the enzymes, we do not have such a characteristic signature for the APH⁺ form. It is therefore possible that addition of the pyruvate analogues to the V51D variant produces this “CD silent” form

(now identified on three ThDP enzymes by solid-state NMR methods^{26,27}). Alternatively, an adduct whose thiazolium ring is sufficiently distorted to eliminate or radically shift the CD band may be formed, which is a less likely scenario.

Stabilization of the 1',4'-Iminopyrimidine Form of ThDP-Bound Intermediates Derived from Glyoxylate on GCL. Upon titration of GCL with glyoxylate in a steady-state CD experiment, formation of positive CD bands at 302 and 337 nm was observed. The maximal intensity of the CD band at 302 nm was reached at concentrations of glyoxylate equivalent or slightly higher than the concentration of active centers. Increasing the concentration of glyoxylate produced (R)-TSA (Figure S1 of the Supporting Information, top).

In a single-turnover experiment (where $[GCL \text{ subunits}] > [\text{glyoxylate}]$) using stopped-flow CD at 302 nm, the reaction reached steady state within approximately 5 s with rate constants k_1 of 1.13 s^{-1} and k_1' of 0.28 s^{-1} (Figure S1 of the Supporting Information, bottom), at least 5 times slower than with V51D GCL (Figure 6A). The CD band at 302 nm can be assigned to the first covalent predecarboxylation intermediate, 1',4'-iminopyrimidinylglycolylThDP. Support for this assignment comes from a comparison of the rate constants determined for the individual steps in GCL by NMR methods.⁵ It was demonstrated that formation of the first covalent predecarboxylation intermediate from glyoxylate was the rate-limiting step for GCL in TSA (product) formation.⁵ The CD band at 337 nm has not yet been assigned. It developed simultaneously with that at 302 nm upon addition of low concentrations of glyoxylate, but it did not respond to increasing concentrations of glyoxylate: the CD band at 302 nm was replaced by a negative CD band at 290 nm due to TSA release (Figure S1 of the Supporting Information, top).

Identification of ThDP-Bound Intermediates on GCL Variants with the I393A Substitution. *Stabilization of 1',4'-iminopyrimidinylThDP Predecarboxylation Intermediates from Pyruvate on I393A GCL and V51D/I393A GCL.* It is evident from kinetic studies (Table 1) that with the I393A substitution the GCL becomes an acetolactate synthase, accepting pyruvate as a substrate. In this study, by using pyruvate and 2-oxobutanoic acid analogues, we were able to demonstrate that I393A GCL can stabilize the 1',4'-iminopyrimidine ThDP predecarboxylation intermediates even in the absence of aspartate at position 51. V51D/I393A GCL behaves like I393A GCL.

A steady-state CD titration of I393A GCL by pyruvate at 4 °C revealed two positive CD bands, one at 302 nm and the second at 335 nm (Figure 8A). The CD band at 335 nm was not assigned but was similar to the CD band at 330 nm observed for V51D GCL (so far unassigned, but also seen at 337 nm on GCL). It was suggested⁵ that the weak CD band at 330 nm possibly originated from FAD.

The CD band at 302 nm approached its maximal intensity at concentrations of pyruvate equivalent to the concentration of active centers (Figure 8A). A plot of ellipticity at 302 nm versus pyruvate concentration clearly shows a biphasic curve with saturation at concentrations of pyruvate of $<1 \text{ mM}$ ($K_{d,\text{pyruvate}} = 8.2 \text{ } \mu\text{M}$) (Figure 8B). At pyruvate concentrations of $>1 \text{ mM}$, the plot was not saturated; rather, the release of (S)-acetolactate was detected and was confirmed by a CD spectrum of the reaction mixture after the protein had been removed (Figure S2 of the Supporting Information). Similar data were obtained for V51D/I393A GCL; a plot of ellipticity at 302 nm versus pyruvate concentration displayed saturation at pyruvate

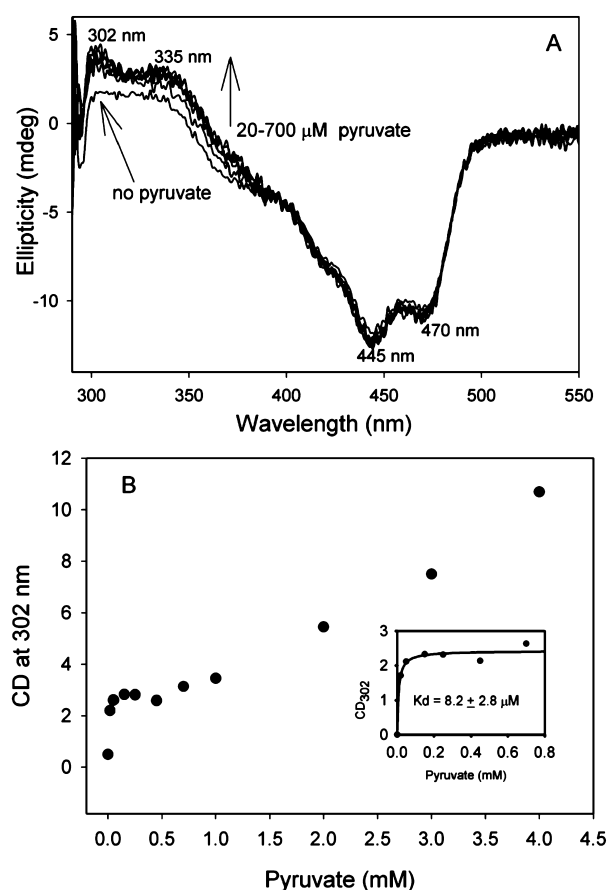


Figure 8. CD titration of I393A GCL by pyruvate at 6 °C. (A) CD spectra of I393A GCL (2.0 mg/mL, active center concentration of $30.8 \text{ } \mu\text{M}$) in $0.1 \text{ M KH}_2\text{PO}_4$ (pH 7.6) containing 0.5 mM ThDP , 2.5 mM MgCl_2 , 1.0 mM DTT , and $10 \text{ } \mu\text{M FAD}$ in the absence and presence of 0.020 – 0.70 mM pyruvate. Each spectrum was recorded after preincubation of I393A GCL with pyruvate for 20 min at 6 °C. (B) Dependence of the ellipticity at 302 nm on pyruvate concentration. The inset shows the plot of the CD_{302} data points vs pyruvate concentration at concentrations of $<0.80 \text{ mM}$. Data were fit to a Hill equation (eq 3).

concentrations of $<500 \text{ } \mu\text{M}$ with an $S_{0.5,\text{pyruvate}}$ of $1.72 \text{ } \mu\text{M}$ (not shown).

The production of (S)-acetolactate requires decarboxylation of pyruvate in the active centers of I393A GCL and V51D/I393A GCL, resulting in the formation of the enamine intermediate, and then subsequent ligation to a second pyruvate (Scheme 1B, bottom). The intermediacy of the enamine was confirmed by the reaction of I393A GCL with pyruvate in the presence of DCPIP under conditions similar to those in the CD experiment. The plot of the initial velocity (slope per minute) of DCPIP reduction versus pyruvate concentration was again biphasic, displaying saturation at low pyruvate concentrations ($K_{d,\text{pyruvate}} = 39 \text{ } \mu\text{M}$) (not shown).

Next, we demonstrated that glyoxylate could also be an acceptor of the enamine intermediate derived from pyruvate on I393A GCL. The positive CD band formed at 302–304 nm on I393A GCL upon addition of stoichiometric amounts of pyruvate was replaced by a negative CD band in the presence of glyoxylate indicating formation of acetylglucolic acid derived from pyruvate as the acetyl donor and glyoxylate as the acceptor (Scheme 1B, top) (Figure 9A). However, the acetylglucolate was not optically stable and could no longer

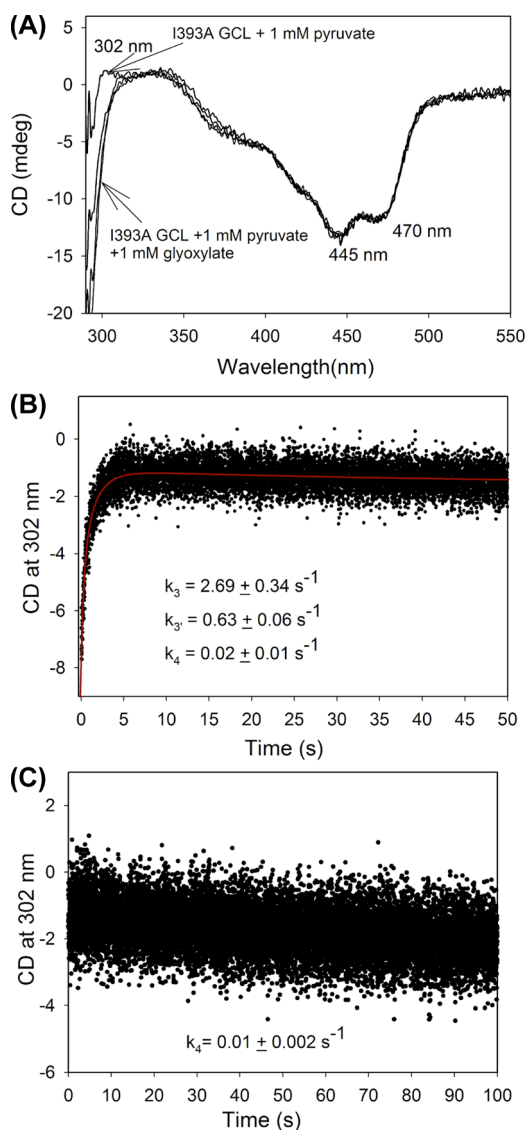


Figure 9. Formation of an acetylglucate product by I393A GCL as detected by CD. (A) CD spectra of I393A GCL recorded in the presence of 1 mM pyruvate and upon addition of 1 mM glyoxylate at 6 °C. The I393A GCL was diluted to a concentration of active centers of 30.8 μM in 0.1 M KH_2PO_4 (pH 7.6) containing 0.5 mM ThDP, 2.5 mM MgCl_2 , 1.0 mM DTT, 10 μM FAD, and 1% glycerol. (B) Time-dependent formation of an acetylglucate–ThDP complex and product release. The I393A GCL (4.69 mg/mL, active center concentration of 72.4 μM) in buffer as in panel A was preincubated with 1 mM pyruvate in one syringe and then mixed at 6 °C with 1.0 mM glyoxylate in the second syringe. Data were fit to a triple exponential (eq 6). (C) Time-dependent release of an acetylglucate product. Conditions were the same as in panel B, but 4 mM glyoxylate was present in the second syringe.

be detected by CD after the protein had been removed from the reaction mixture. This is readily explained as the chiral carbon can undergo racemization, being located between two carbonyl functions, a ketone and a carboxylic acid. The formation of acetylglucate as the only carboligase product was proven by the absence of CD bands corresponding to (S)-acetolactate (positive band at 300 nm) or (R)-TSA (negative band at 290 nm). Both products are optically stable and should be detected if they are formed after the protein had been removed. When I393A GCL incubated with pyruvate in one

syringe was mixed with glyoxylate in the second syringe in a stopped-flow CD experiment, positive ellipticity developed at 302 nm within 5 s with a k_3 of 2.69 s^{-1} and a k_3' of 0.63 s^{-1} , followed by a decrease in ellipticity with a k_4 of 0.02 s^{-1} suggesting formation of an acetylglucate–ThDP complex on the enzyme (it being responsible for the CD band at 302 nm) and its release (Figure 9B and Scheme 1B, top). When the concentration of glyoxylate was increased to 4 mM, only the release of acetylglucate could be detected ($k_4 = 0.01 \text{ s}^{-1}$) (Figure 9C and Scheme 1B, top). These experiments suggest that the acetylglucate–ThDP complex can be detected by stopped-flow CD at 302 nm and its release is the rate-limiting step. In a stopped-flow CD experiment with V51D/I393A GCL, the enzyme in one syringe was mixed with pyruvate in the second syringe but in the absence of glyoxylate (Figure S3 of the Supporting Information). Again, the positive ellipticity at 302 nm was developed within 5 s with a k_3 of 2.22 s^{-1} and a k_3' of 0.50 s^{-1} , which can be assigned to the formation of the acetolactate–ThDP complex. The origin of the CD band at 302 nm will be discussed below.

The I393A Substitution Allows GCL To Stabilize the 1',4'-Iminopyrimidyl Tautomer of Stable Predecarboxylation ThDP-Bound Intermediates from Pyruvate Analogues. Upon addition of AcPhi to I393A GCL (Figure 10) and V51D/I393A GCL (not shown), the positive CD band again developed at 302 nm and reached a plateau, indicating formation of the 1',4'-iminophosphinolactyl–ThDP intermediate and providing half-saturation values ($S_{0.5, \text{AcPhi}}$) of 0.32 mM (I393A GCL) and 0.37 mM (V51D/I393A GCL) (Table 3).

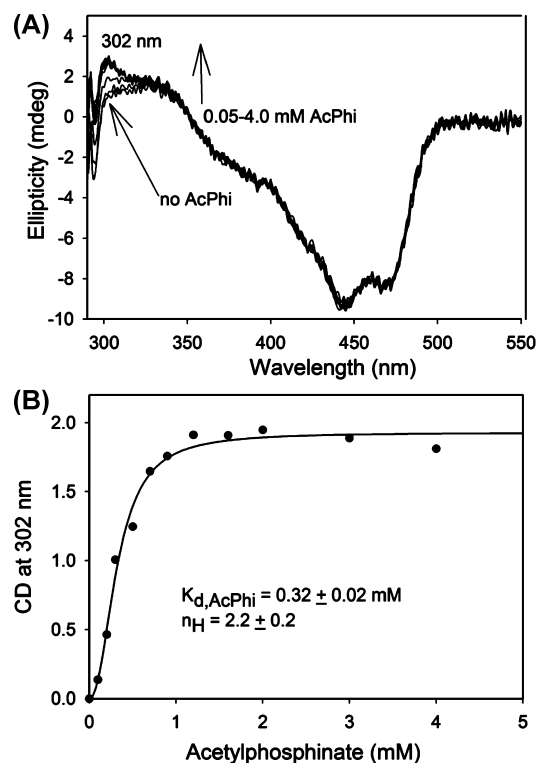


Figure 10. CD spectra of I393A GCL titrated with AcPhi. (A) The I393A GCL (2.0 mg/mL, active center concentration of 30.8 μM) in 0.10 M KH_2PO_4 (pH 7.6) containing 5 mM MgCl_2 and 0.50 mM ThDP was titrated with AcPhi (0.050–4.0 mM) at 25 °C. (B) Dependence of the CD at 302 nm on the concentration of AcPhi. Data were fit to a Hill equation (eq 3).

The I393A GCL displayed positive cooperativity on AcPhi binding ($n_H = 2.23$). In contrast, for I393V GCL and for GCL, there was no significant change in the CD spectra upon addition of AcPhi, most likely because there is little or no binding of AcPhi to the active centers (data not shown).

Similar experiments conducted with I393A and MAP displayed the band at 302 nm, which we ascribe to the 1',4'-iminophosphonolactylThDP (data not shown), and allowed calculation of a $K_{d,MAP}$ of 1.4 mM (Table 3). MAP thus also binds to I393A GCL, although it is a weaker substrate analogue than AcPhi, as was found with other ThDP enzymes.²⁴ For V51D/I393A GCL, the $K_{d,MAP}$ was 0.11 mM.

CD Evidence of the Formation of the 1',4'-IminophosphinopropionylThDP from PrPhi. In addition to AcPhi, the I393A GCL and V51D/I393A GCL could also bind PrPhi, $[CH_3CH_2C(=O)P(H)(=O)O^-]$, an analogue of 2-oxobutanoate, and form the 1',4'-iminophosphinopropionylThDP, providing $K_{d,PrPhi}$ values of 0.46 mM (I393A GCL) and 0.28 mM (V51D/I393A GCL), similar to the value obtained with AcPhi (Table 3). The I393A GCL displayed positive cooperativity on PrPhi binding, similar to that observed with AcPhi. Apparently, the V51D substitution does indeed affect the cooperativity of binding of a pyruvate analogue to the I393A variant (Table 3) for reasons unclear at this time.

A major conclusion from the CD studies cited above is that the predecarboxylation intermediate analogues derived from AcPhi, PrPhi, and MAP can be stabilized on I393A and V51D/I393A variants in their 1',4'-iminopyrimidine tautomeric forms. It is significant that the V51D/I393A variant behaves like the I393A variant in this regard. The observation that the presence or absence of an aspartate at position 51 has little effect on the production of acetolactate by V51D/I393A GCL in comparison with a singly substituted I393A GCL implies that its effect is specifically on the formation of TSA.

DISCUSSION

Following the initial report on the structure of GCL,⁵ we explore two important issues about the enzyme remaining to be elucidated: (1) control of substrate specificity and (2) role of the residue opposite the N1' atom of ThDP, typically occupied by the highly conserved glutamate.

Control of Substrate Specificity at Position 393.

Experiments exploiting different techniques show that the putative higher reactivity of the aldehyde group of glyoxylate is not the important determinant of the substrate specificity of GCL, but, rather, the structure of the active site pocket. In particular, residue Ile393 restricts the ability of GCL to form covalent complexes of ThDP with donor substrates larger than glyoxylate.

Remarkably, once the size of the residue at position 393 is reduced to alanine, pyruvate is accepted as an alternate substrate, and evidence of ThDP-bound intermediates derived from pyruvate could be detected. The CD band at 302 nm observed on I393A at low pyruvate concentrations (Figure 8A) indicates that a covalent intermediate (or intermediates) is formed on GCL at low pyruvate concentrations. We believe this intermediate is one with tetrahedral substitution at C2 α but is not the enamine. Because (R)-acetolactate is produced at higher pyruvate concentrations, the enamine must be present, albeit at a concentration too low to detect. A possible source of the band at 302 nm is the HEThDP, the conjugate acid of and at protolytic equilibrium with the enamine, or the bound carboligase product acetolactylThDP (Scheme 1A,B). An

increase in the pyruvate concentration to >1 mM shifts this equilibrium to acetolactate release. Both the HEThDP and acetolactylThDP would be expected to exist in their 1',4'-iminopyrimidyl tautomeric form on the bases of much precedent for several ThDP enzymes,¹² and either species could be responsible for the CD band at 302 nm.

The V51D/I393A GCL behaves like the I393A GCL according to a kinetic study of acetolactate formation, and from CD studies of the stabilization of the predecarboxylation intermediate analogues derived from AcPhi, MAP, and PrPhi. The predecarboxylation intermediate analogues derived from AcPhi, PrPhi, and MAP could be stabilized on I393A GCL and V51D/I393A GCL in their 1',4'-iminopyrimidine tautomeric forms.

The observation that the presence or absence of an aspartate at position 51 has little effect on the V51D/I393A GCL implies that it is specifically effective on TSA formation from the natural substrate glyoxylate.

With these observations on the I393-substituted GCLs, we suggest that variants engineered at this position would be expected to convert this enzyme to a useful tool in chiral synthesis. For example, both pyruvate and glyoxalate are excellent acceptors for the enamine derived from pyruvate for acetolactate formation ($k_3 = 2.2 \text{ s}^{-1}$) and for acetylglyoxalate formation ($k_3 = 2.7 \text{ s}^{-1}$) (Scheme 1B).

As an aside, we also emphasize that CD has clearly been shown to provide an excellent direct kinetic assay for product formation, much simpler than any coupled assay, or the classical Westerfeld method for acetoin determination.

Role of the Conserved Glutamate. GCL has several properties, which set it apart from the other members of the pyruvate decarboxylase-pyruvate oxidase subfamily of ThDP-dependent enzymes.^{28–31} The conserved glutamate residue hydrogen bonded to N1' of ThDP, found in all the other ThDP-dependent enzymes, is replaced with a valine residue, and the sole substrate of GCL is the 2-aldoacid glyoxylate rather than a 2-ketoacid (Scheme 1A). In addition, not only is the conserved glutamate missing, there are no potential acid–base side chains in the proximity of the ThDP near the C2 thiazolium atom. The recently accepted paradigm regarding the mechanism of ThDP-dependent enzymes^{12,24} suggests that the conserved Glu residue is essential to the tautomerization of ThDP, and that the 1',4'-iminopyrimidine, but not the 4'-aminopyrimidine, tautomer is sufficiently basic to deprotonate the thiazolium C2–H group and thus lead to the active carbanion/ylide form of the coenzyme. The studies reported here provide convincing evidence that with GCL, (1) only the unusual 1',4'-iminopyrimidine tautomer, rather than the canonical AP form, is detected and (2) the IP–APH⁺ proteolytic equilibrium functions in GCL, allowing the IP form to play its central role in the absence and presence of a group that is hydrogen bonded to N1'.

The key ThDP-bound intermediates derived from glyoxylate were identified on the reaction path of V51D GCL. A comparison of the rate constants for 1',4'-iminoglycolyl-ThDP formation, its decarboxylation to the enamine, TSA–ThDP complex formation, and finally release of TSA product from GCL indicates that under the experimental conditions, TSA product release is the rate-limiting step, in agreement with the NMR approach.⁵ In the NMR approach,⁵ covalent ThDP-bound intermediates are released from the enzymes, while in this study, we observe enzyme-bound intermediates directly; both methods lead to the same conclusions.

The 1',4'-iminoglycolyl-ThDP was detected on GCL when the enzyme was titrated by equivalent amounts of glyoxylate. The rate constant of 1',4'-iminoglycolyl-ThDP formation was at least 5 times slower than that for V51D GCL.

A major surprise of our results *in toto* is that all the steps with the exception of product release appear to have rates comparable to or even better than (when adjusted to the pH optimum of the variant) those with Asp in place of Val at position 51 (Table 2).

There is also strong evidence that adding the acceptor substrate significantly increases the rate of decarboxylation, as seen in the glyoxylate concentration dependence of the kinetics of product formation. This may be yet another example of active site communication where glyoxylate in the active site of one subunit increases the rate of decarboxylation in the active site of a second subunit in the "functional dimer" typical of ThDP decarboxylases. Similar observations had already been reported for benzoylformate decarboxylase³² and yeast pyruvate decarboxylase,³³ suggesting that the behavior reflects an alternating active center mechanism in the functional dimer.

The presence of a water molecule in the proximity of the N1' atom might provide an explanation for the ability of GCL to catalyze the tautomeric equilibration required for the reaction. It should be noted that the structures of ThDP-dependent enzymes in the PDB show water molecules in the proximity of the N1' atom.⁵ Given the high concentration of water in all of these crystals, the presence of such water in GCL would not be surprising. The transient charge transferred to the 4'-aminopyrimidine moiety in the course of ThDP activation (Scheme 1A) and other steps in the mechanistic cycle of GCL can presumably be shared with a water molecule H-bonded to N1' and with other groups in its hydrogen bonding network. Significant residual activity is seen in a variant of the homologous enzyme AHAS II when the conserved glutamate (Glu47) is replaced with alanine.³⁴ When the conserved glutamates on the E1 component of the *E. coli* pyruvate dehydrogenase complex¹¹ and the yeast pyruvate decarboxylase³⁵ are replaced with alanine, there is similar residual activity detected. It is reasonable to assume that a water molecule can fit into the gap created by such substitutions and that this water might assist the tautomerization. It is relevant to mention that the pK_a in models for the 4'-aminopyrimidinium ring of ThDP is ~ 4.85 , so that only a modest pK_a perturbation would be required in the active centers of ThDP enzymes to match this.²² It was pointed out that the ThDP binding site in GCL appears in fact to have a larger proportion of aliphatic, nonpolarizable residues in the region of the thiazolium and 4'-aminopyrimidine rings versus what is observed for other ThDP-dependent enzymes, which should favor the stability of the formally charge-neutral ylide on the thiazolium ring (in Scheme 1A).⁵ We suggest that the major need is some manner of protonation of N1' so that the APH⁺ form can be generated, the source of the IP tautomer. The fact that the APH⁺ form was indeed observed by solid-state NMR experiments on each of the three enzymes examined (yeast pyruvate decarboxylase and the E1 components of both the pyruvate and 2-oxoglutarate dehydrogenase complexes from *E. coli*) supports this hypothesis.²⁶

That said, the V51D substitution once more supports the suggestion that the pK_a for the (IP+AP)–APH⁺ equilibrium coincides with the pH optimum of activity, ensuring the presence of all three forms for catalysis. This is the sixth ThDP enzyme to date supporting this notion. Having no other acid–

base candidates near the thiazolium ring allows us to conclude that (a) ThDP itself in its various forms can indeed catalyze all of the reactions of the GCL pathway and (b) the amino acid opposite the N1' atom of ThDP (typically the conserved glutamate) is responsible for both the pK_a of the APH⁺ form and the pH dependence of the activity profiles.

Perhaps the most interesting puzzle remaining concerning GCL is the selective advantage of the valine residue (Val51) in place of the conserved glutamate. It was previously shown that V51D GCL and V51E GCL are less active than GCL in the synthesis of TSA from glyoxylate, despite having higher rates of activation (deprotonation at C2) of the coenzyme.⁵ With the results presented here, it would appear that it is in the product release rate, rather than the rates of any intermediate steps, where the valine has a dramatic effect. The structural basis of these findings remains to be elucidated.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1–S3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Funding

This work was supported by U.S.-Israel Binational Science Foundation Grant 2007-129 (to B.S., D.C., and F.J.) and National Institutes of Health Grant GM050380 to F.J.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

GCL, wild-type glyoxylate carboligase; I393A GCL, V51D GCL, and V51D/I393A GCL, GCL variants at the indicated positions; TSAR, tartronate semialdehyde reductase; AHAS, acetohydroxyacid synthase; ThDP, thiamin diphosphate; TSA, tartronate semialdehyde; AL, acetolactate; MAP, methyl acetylphosphonate; AcPhi, acetylphosphinate; PrPhi, propionylphosphinate; LThDP, C2 α -lactylthiamin diphosphate; PLThDP, phosphonolactylthiamin diphosphate (the MAP–ThDP adduct); DCPIP, 2,6-dichlorophenolindophenol; PEG, polyethylene glycol; AP, 4'-aminopyrimidine tautomeric form of ThDP; IP, 1',4'-iminopyrimidine tautomeric form of ThDP; APH⁺, N1'-protonated 4'-aminopyrimidinium form of ThDP; CD, circular dichroism.

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