Dialkylglycine decarboxylase (DGD)\(^1\) is an intriguing PLP-dependent enzyme because it catalyzes both transamination and decarboxylation reactions in its normal catalytic cycle (Scheme 1). The enzyme was isolated from a soil bacterium (\(^I\)) and transfers the fixed nitrogen from small 2,2-dialkylglycines, which are common in peptide antibiotics \((2)\), onto pyruvate to give L-alanine. The decarboxylation half-reaction is highly specific for oxidative decarboxylation. Thus, the issue of reaction specificity has two main facets with this enzyme: control of oxidative decarboxylation, and control of oxidative vs nonoxidative decarboxylation. The latter is addressed in this work.

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\(^a\) To whom correspondence should be addressed. E-mail: toney@chem.ucdavis.edu. Phone: (530) 754-5282. Fax: (530) 752-8995.

\(^b\)Current address: Monsanto Corporation, St. Louis, MO.

\(^1\)Abbreviations: DGD, dialkylglycine decarboxylase; PLP, pyridoxal 5'-phosphate (PLP)-dependent enzyme dialkylglycine decarboxylase (DGD) were studied individually by multiwavelength stopped-flow spectroscopy. Biphasic behavior was found for the reactions of DGD-PLP, consistent with two coexisting conformations observed in steady-state kinetics [Zhou, X., and Toney, M. D. (1998) \textit{Biochemistry} 37, 5761–5769]. The half-reaction kinetic parameters depend on alkali metal ion size in a manner similar to that observed for steady-state kinetic parameters. The fast phase maximal rate constant for the 2-aminobutyrate (AIB) decarboxylation half-reaction with the potassium form of DGD-PLP is 25 s\(^{-1}\), while that for the transamination half-reaction between DGD-PMP and pyruvate is 75 s\(^{-1}\). The maximal rate constant for the transamination half-reaction of the potassium form of DGD-PLP with L-alanine is 24 s\(^{-1}\). The spectral data indicate that external aldimine formation with either AIB or L-alanine and DGD-PLP is a rapid equilibrium process, as is ketimine formation from DGD-PMP and pyruvate. Absorption ascribable to the quinonoid intermediate is not observed in the AIB decarboxylation half-reaction, but is observed in the dead-time of the stopped-flow in the L-alanine transamination half-reaction. The [1-\(^{13}\)C]AIB kinetic isotope effect (KIE) on \(k_{\text{cat}}\) for the steady-state reaction is 1.043 ± 0.003, while a value of 1.042 ± 0.009 was measured for the AIB half-reaction. The secondary KIE measured for the AIB decarboxylation half-reaction with [C4-\(^{3}\)H]-PLP is 0.92 ± 0.02. The primary [2-\(^{3}\)H]-L-alanine KIE on the transamination half-reaction is unity. Small but significant solvent KIEs are observed on \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_M\) for both substrates, and the proton inventories are linear in each case. NMR measurements of C2-H washout vs product formation give ratios of 105 and 14 with L-alanine and isopropylamine as substrates, respectively. These results support a rate-limiting, concerted Cα-decarboxylation/C4'-protonation mechanism for the AIB decarboxylation reaction, and rapid equilibrium quinonoid formation followed by rate-limiting protonation to the ketimine intermediate for the L-alanine transamination half-reaction. Energy profiles for the two half-reactions are constructed.

Previous work has shown that DGD activity is dependent on alkali metal ions; potassium activates it while sodium inhibits it (3). The crystal structures of four different alkali metal liganded DGD forms have been reported (4–6). In solution, two coexisting, slowly interconverting conformations of homogeneously K\(^{+}\)-liganded DGD were demonstrated (3). These likely correspond to the K\(^{+}\) and Na\(^{+}\) forms observed in the crystal structures. The roles of several catalytically important pKa\(_S\), including one responsible for the interconversion of the two coexisting conformations, have been identified in an extensive pH study (7). Additionally, steady-state and pre-steady-state kinetic analyses of the DGD reactions with alternate substrates have shown that stereo-electronic effects play a role in determining decarboxylation vs transamination reactivity (8, 9).

The first step of all PLP-dependent enzymes is formation of an external aldimine intermediate via a transimination reaction, displacing the active site lysine from its interaction with PLP (Scheme 1). In a classical stepwise decarboxylation mechanism, loss of the AIB Cα-carboxylate group as CO\(_2\) from the external aldimine intermediate leads to the quinonoid intermediate, which, on protonation at C4', gives the ketimine intermediate. These two steps could be concerted, as has been proposed for mechanistically different reactions.
catalyzed by the PLP-dependent enzymes aspartate amionotransferase (10, 11) and tryptophan indole-lyase (12). Regardless of whether decarboxylation is stepwise or concerted, the ketimine formed is hydrolyzed to produce DGD-PMP and acetone. This half-reaction is termed “oxidative decarboxylation” or “decarboxylation-dependent transamination” because the amino acid substrate is oxidized to a ketone instead of yielding an amine as in the reactions catalyzed by classical decarboxylases. The second half-reaction is a transamination between DGD-PMP and pyruvate. Proton abstraction from C4′ of the pyruvate ketimine gives a quinonoid intermediate, which leads to the L-alanine external aldimine via protonation on Cα. The final step of the catalytic cycle is transimination to release L-alanine as the amino acid product.

Here, a series of stopped-flow, kinetic isotope effect, and NMR experiments are described. These studies show that the decarboxylation half-reaction is largely rate-determining for the catalytic cycle and that formation of aldimines and ketimines with all substrates is rapid. For the AIB decarboxylation half-reaction, the lack of a detectable quinonoid intermediate, a large [13C]KIE on decarboxylation, a significant solvent KIE originating from a single proton, a large inverse secondary [2H]KIE on decarboxylation with [2H]PLP, and a greater than unity washout to product formation ratio with isopropylamine all combine to support a concerted decarboxylation/proton-transfer mechanism. For the L-alanine/pyruvate transamination half-reaction, the observation of a quinonoid intermediate in the dead-time of the stopped-flow, the lack of a primary KIE with [2H]alanine, and the large washout to product formation ratio combine to support a mechanism in which, surprisingly, protonation of the quinonoid intermediate is rate determining.

EXPERIMENTAL PROCEDURES

Materials. AIB, pyruvic acid, L-alanine, β-hydroxypyruvate, PLP, NADPH, 2°ADH were purchased from Sigma. Succinic acid, potassium hydroxide, bicine, sodium borodeuteride (99% D), manganese dioxide, and phenetidine hydrochloride were from Aldrich. [2-2H]-L-Alanine (99% D) and deuterium oxide (99.9% D) were from Cambridge Isotope Laboratories. PMP was synthesized as described (3).

Synthesis of [4′-2H]Pyridoxal 5′-Phosphate. Pyridoxal 5’-phosphate (1 g, 4 mmol) was reduced by one-half equivalent of sodium borodeuteride at neutral pH in 25 mL of water. The pH was lowered to 3 and the reaction mixture was rotary evaporated three times from methanol to remove borate. The pH of the reaction mixture was brought to 1 with concentrated HCl (7) and excess aqueous sodium bicarbonate was added. The mixture was heated under nitrogen at room temperature. Manganese dioxide was removed by filtration through celite, and the pH of the filtrate was raised to 7 with KOH. The yield of crude [2-2H]PLP was ~50% as determined spectrophotometrically. The phenetidine Schiff base of PLP was isolated (13). It was hydrolyzed by dissolution in 5 mL of concentrated HCl and purified on Bio-Rex 70 (Bio-Rad) with 1 M HCl as the eluting solvent. The overall yield was ~20%. The purified [4′-2H]pyridoxal-5′-phosphate showed the expected 1H NMR spectrum (14) with ~5% aldehyde remaining.

Synthesis of [1-13C]AIB. The Strecker synthesis with labeled cyanide was employed. To a sealed tube containing 1.7 g of NH₄Cl, 2.2 mL of concentrated NH₄OH, and 2.0 g of K¹³CN in 7.6 mL of water, 2.4 mL of acetone in 9.6 mL of ethanol was added dropwise while stirring. The tube was sealed and heated at 70 °C overnight. The resulting solution was cooled to 0 °C, poured into 34 mL of ice-cold concentrated HCl, and saturated with dry HCl gas. After standing in ice for 5 h, the mixture was refluxed for 3 h. The solvent was evaporated and then coevaporated with a mixture of ethanol-toluene. The residue was suspended in 100 mL of anhydrous ethanol and refluxed for 1 h. The resulting ethanol-insoluble solid was filtered and refluxed with another 100 mL of anhydrous ethanol for an additional hour. After filtration, the two filtrates were combined, concentrated, and neutralized with 10 mL of concentrated
NH₂OH, and evaporated to dryness. The crude product was dissolved in about 1 mL of deionized water and loaded onto a 2 × 30 cm Amberlite IR-120 ion-exchange column. The column was washed with deionized water, and the product eluted with 2 M NH₄OH. The desired [1-¹³C]AIB product was further purified by recrystallization from water-ethanol with a final yield of ~50% based on the starting K¹³CN. ¹H NMR (300 MHz, D₂O) δ 1.60 (d, J = 3.8 Hz); ¹³C NMR (75 MHz, D₂O) δ 23.9, 58.6 (d, J = 54 Hz), 178.3.

1-¹³C]AIB Steady-State Kinetic Isotope Effect. The steady-state KIE on k₄ was measured using the continuous-flow apparatus described previously (15). The conditions were 40 mM bicine-KOH, pH 8.0, 50 mM dipotassium succinate, 5 mM disodium succinate, 25 mM [1-¹³C]AIB or unlabeled AIB, 3 units/mL 2°ADH, 300 μM NADPH, 20 μM PLP, and 25 °C.

Stopped-Flow Kinetic Studies of the Decarboxylation Half-Reaction. An Applied Photophysics SX.17MV stopped-flow spectrophotometer was used. Both DGD-PLP and AIB were in 30 mM bicine-KOH, pH 8.2, 100 mM dipotassium succinate. DGD-PLP (final concentration, 13 μM) was rapidly mixed with an equal volume of AIB of various concentrations ranging from 0.05 to 40 mM. In some experiments, single wavelength kinetic traces were collected with a photomultiplier and monochromator. Generally, absorbance scans from 300 to 550 nm were collected on a logarithmic time scale with a UV-enhanced diode-array detector. The spectra were analyzed globally using SPECFIT (Spectrum Software Associates).

DGD-Rb⁺ was prepared by dialysis of DGD-K⁺ against 30 mM bicine-RbOH, pH 8.2, 100 mM dirubidium succinate. The reactions of the PLP form of DGD-Rb⁺ with AIB were conducted as described above for DGD-K⁺, with Rb⁺ replacing K⁺.

The half-reaction kinetic isotope effects measured with [1-¹³C]AIB and [C₄⁻²H]PLP were measured under identical reaction conditions. A Bio-Logic SFM-400 stopped-flow mixer and a J&M Tidas diode-array detector were employed. Spectra (256) were collected over 4 s and analyzed with SPECFIT to obtain rate constants and spectra for intermediates. The [1-¹³C]AIB KIE was measured by directly comparing the rate constants for AIB and [1-¹³C]AIB. The reaction conditions were 40 mM bicine-KOH, pH 8.2, 50 mM dipotassium succinate, 5 mM disodium succinate, 100 μM PLP. The final concentrations of DGD-PLP and AIB were ~10 μM and 20 mM, respectively.

For the [C₄⁻²H]PLP KIEs, apoDGD was prepared by overnight dialysis at 4 °C against two changes of 40 mM bicine-KOH, 50 mM dipotassium succinate, and 5 mM disodium succinate, pH 8.2. ApoDGD was divided into two parts and 100 μM PLP or [C₄⁻²H]PLP added and allowed to bind for 1 h on ice. Reactions were initiated by mixing equal volumes of DGD-PLP and AIB, each in 40 mM bicine-KOH, 50 mM dipotassium succinate, and 5 mM disodium succinate, pH 8.2. The final concentrations of DGD-PLP and AIB were 24 μM and 20 mM, respectively.

Stopped-Flow Kinetic Studies of the DGD-PMP Transamination Half-Reaction with Pyruvate. DGD-PMP prepared in two ways was compared because of the relatively fast dissociation of PMP from DGD (half-life = ~15 min at pH 8 and 25 °C). In the first method, DGD-PMP was prepared in situ by reaction of limiting AIB with excess DGD-PLP and immediately mixed with various concentrations of pyruvate in the stopped-flow. Multiple single wavelength absorbance traces from 300 to 550 nm (logarithmic time scale; 10 nm interval) were collected using the SX.17MV stopped-flow since the half-life of the fast phase is short compared to the sampling time of the diode-array detector. In the second method, apoDGD was prepared as described above and incubated with a saturating concentration of PMP (6 mM) and 100 mM KCl. This DGD-PMP was mixed with pyruvate, and multiple single wavelength traces from only 370 to 550 nm were taken, due to the high absorbance of free PMP. Traces were fitted both globally and individually using Pro-Kineticist software supplied by Applied Photophysics. The reaction conditions were identical to those for the DGD-PLP reactions.

Stopped-Flow Kinetic Studies of the DGD-PLP Transamination Half-Reaction with L-Alanine. Transamination reactions of DGD-PLP with both L-alanine and [2-²H]L-alanine were followed as described above for AIB using the SX.17MV stopped-flow. The concentrations of L-alanine and [2-²H]L-alanine were accurately measured using L-alanine dehydrogenase in 50 mM borate-KOH, pH 10.0, 5 mM NAD⁺, 4 units/mL AlaDH, and 100 mM KCl. For the solvent isotope effects on these reactions, buffer and reagents were prepared directly in D₂O. The small amount of protium introduced was ignored.

The PLP form of DGD-Rb⁺ was reacted with L-alanine as described above for AIB. Additionally, reactions were performed after incubation for 30 min with 15 mM aminocyclopropane carboxylate, a competitive inhibitor of DGD. The reactions were performed at a single L-alanine concentration of 40 mM, with a final concentration of 1.5 mM aminocyclopropane carboxylate.

Steady-State Kinetics. Solvent isotope effects were measured by coupling the acetone produced from the AIB/pyruvate reaction to 2°ADH and monitoring the decrease in NADPH absorbance at 340 nm using a Kontron Uvikon 9420 spectrophotometer. The reaction conditions were 30 mM bicine-KOH, pH 8.2, 50 mM dipotassium succinate, 0.5 unit/mL 2°ADH, 0.2 mM NADPH. Two stocks of reagents were prepared, one in H₂O, and one in D₂O. These were mixed to give the appropriate atom fraction D₂O in proton inventory experiments. For SIEs on k₄, saturation curves were measured using a substrate mixture of AIB and pyruvate in which the ratio of their concentrations was equal to the ratio of their Kₘ values. For SIEs on k₄/Kₘ, pyruvate was fixed at 1 mM, and AIB saturation curves were measured in various atom fractions of D₂O. For SIEs on k₄/Kₘ, the AIB concentration was fixed at 2 mM, and saturation curves for pyruvate were measured.

The heat of activation for k₄ and k₄/Kₘ was measured in 0.1 M potassium phosphate buffer, pH 7.8, using the 2°ADH coupled assay in the temperature range of 10–40 °C.

NMR Determination of Washout vs Product Formation Ratios. For the transamination reactions of L-alanine and isopropylamine, the peaks for the C2 hydrogens of the substrates and for the methyl groups of the ketone products were followed by NMR at 25 °C. The integrated intensities (normalized by the number of hydrogens contributing to each
peak) vs time were plotted to obtain the relative rates of C2–H washout vs product formation.

A 300 MHz Bruker spectrometer was employed for the characterization of alanine transamination products. Reactions contained 100 mM potassium phosphate, pH 7.8, 50 mM L-alanine, 5 mM \( \beta \)-hydroxybutyrate, 100 mM PLP, 0.4 \( \mu \)M DGD, and 0.2 mM TSP in D\(_2\)O. The reaction was initiated with enzyme and spectra were collected at \( \sim 2 \) min intervals over 1 h. Due to the very low transamination reactivity of isopropylamine, this reaction was measured using a 600 MHz Bruker DRX spectrometer. Mild solvent presaturation was used. One spectrum consisted of 128 scans with a relaxation delay of 2.5 s. Reactions contained 100 mM potassium phosphate, pH 7.8, 25 mM isopropylamine, 10 mM \( \beta \)-hydroxybutyrate, 200 \( \mu \)M PLP, 4 \( \mu \)M DGD, and 0.2 mM TSP in D\(_2\)O. Reactions were initiated with enzyme and spectra were collected at 9 min intervals over 4 h.

**RESULTS**

**Heats of Activation.** The heats of activation for \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_{AIB} \) were found to be 9.8 \( \pm \) 0.7 and 12.7 \( \pm \) 0.9 kcal/mol, respectively, using AIB and pyruvate as substrates.

**DGD-PLP Decarboxylation Half-Reaction with AIB.** Absorbance data at the spectral maxima (410 and 330 nm) clearly show the biphasic behavior of the potassium form of DGD-PLP (Figure 1). Multiwavelength data show a decrease in the absorbance at 410 nm, concomitant with an increase in absorbance at 330 nm and a shift in the \( \lambda_{\text{max}} \) of the remaining aldehydic absorbance to 395 nm. Global analysis of the multiwavelength data using a two-step, serial mechanism (eq 1) in SPECFIT gives spectra for three species (A, B, and C; Figure 2A)

\[
A \xrightarrow{k_1} B \xrightarrow{k_1} C
\]

An isosbestic point at 352 nm is observed with no absorbance at \( \sim 500 \) nm, characteristic of a quinonoid intermediate (16). The observed rate constants from the global fits increase hyperbolically with AIB concentration, with apparent \( y \)-intercepts of zero. An F-test showed that the fit to a rectangular hyperbola with an offset is not significantly better than that without an offset. The kinetic parameters obtained are given in Table 1. The maximal fast phase rate constant is 24.5 \( \pm \) 0.3 s\(^{-1} \) with \( K_{\text{app}} \) = 2.0 \( \pm \) 0.1 mM, while the maximal slow phase rate constant is 0.73 \( \pm \) 0.02 s\(^{-1} \) with \( K_{\text{app}} \) = 0.47 \( \pm \) 0.06 mM.

Figure 2b shows the spectra of species A, obtained from the global analyses of the multiwavelength data, as a function of AIB concentration. The absorbance at 410 and 330 nm on species A increases hyperbolically with AIB concentration (inset Figure 2b); fitting to a rectangular hyperbola with an offset gives \( K_{\text{app}} \) = 2.2 \( \pm \) 0.5 and 2 \( \pm \) 1 mM for the 410 and 330 nm data, respectively. These are identical, within error, to that obtained from the fast phase rate constants (Table 1).

**DGD-PLP Transamination Half-Reaction with L-Alanine.** The transamination half-reaction with L-alanine is also a biphasic process and shows absorbance changes at 410 and 330 nm similar to those observed in the AIB decarboxylation half-reaction. In contrast to the AIB reaction, clear absorbance at 495 nm is observed in the first spectrum collected.
by the diode-array detector. Figure 3 presents results obtained 
with L-alanine. The maximal rate constants for the alanine 
transamination half-reaction are 23.6 ± 0.7 and 1.25 ± 0.05 
s⁻¹, while \( K_{app} \) values are 6.6 ± 0.7 and 2.6 ± 0.4 mM for 
the fast and slow forms, respectively. An F-test showed that 
the fit to a rectangular hyperbola with an offset is not 
significantly better than that without an offset.

Global analysis of the multiwavelength data provides the 
spectra for the three species in the biphase process. The 
spectra of species A have clear absorbance at 495 nm, which 
is characteristic of a quinonoid intermediate \((16)\). Also, the 
spectra of species A show a hyperbolic dependence on 
alanine concentration, similar to that observed with AIB. The 
330 and 410 nm data, in the spectra of the first species both 
give \( K_{app} = 7 \pm 2 \) mM. The 495 nm data, on the other hand, 
give \( K_{app} = 40 \pm 19 \) mM.

**DGD-PMP Transamination Half-Reaction with Pyruvate.** 
DGD-PMP prepared in situ with AIB and from apoDGD 
behaved similarly. Since this half-reaction is relatively fast, 
kinetic traces at 10 nm intervals were individually collected 
using a photomultiplier, and multiwavelength spectra were 
reconstructed from these. The 330 nm absorbance decreased 
and that at 410 nm increased in an apparent single-

The absorbance at 495 nm, on the other hand, increased 
rapidly to a maximum at ~6 ms and then decayed, as seen 
in Figure 5. The fitted lines shown are simple two exponential 
fits. The rate constants obtained from these are plotted against 
pyruvate concentration in the Figure 5, inset. The slow phase 
shows pyruvate dependence with a maximal rate constant 
of 75 ± 2 s⁻¹ and \( K_{app} = 1.1 ± 0.1 \) mM (Figure 
4A, inset, and Table 1). The concentration dependence of the 320 nm absorbance of species (Figure 4B) gives \( K_{app} = 
0.8 \pm 0.4 \) mM.

\[
A + B \rightleftharpoons C \rightleftharpoons D \rightleftharpoons E \tag{2}
\]

of the slow rate constant but a fast phase rate constant that 
is highly correlated with the value of the extinction coeffi-
cient assumed for the quinonoid intermediate. Previous 
work suggests a value of \(~35\, 000\, M^{-1}\, cm^{-1} \) \((16)\), which 
yields values of \( >500\, \text{s}^{-1} \) for the fast phase rate constant. 

**Kinetic Isotope Effects.** The proton inventories on \( k_{cat} \) 
measured here and previously \((15)\) are both linear. Similar 
KIEs of 1.54 ± 0.03 and 1.61 ± 0.01 were obtained from 
these separate experiments (Table 2). The proton inventories 
on \( k_{cat}/K_{AB} \) and \( k_{cat}/K_{PYR} \) are similarly linear. These are shown 
in Figure 6. The KIEs obtained for these parameters are 1.47 
± 0.02 and 1.85 ± 0.02 (Table 2).

The \([1-\text{13C}]\text{AIB} \) steady-state KIE on \( k_{cat} \) was measured to 
be 1.043 ± 0.003 using the continuous-flow method. The 
same KIE was measured for the AIB decarboxylation half-

The \([2-\text{2H}]\)-L-alanine KIE on the fast phase is unity within error in both 
\( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \), while the solvent KIE is ~1.5 with either 
elabeled or unlabeled L-alanine. The \([2-\text{2H}]\)-L-alanine KIE on 
the slow phase is significant, as is the \( \text{D}_2\text{O} \) effect. The data 
suggest an apparent decrease in each KIE when combined 
with the other isotopically labeled species, although the errors 
preclude definitive conclusions.

**NMR Determination of Washout vs Product Formation 
Ratios.** The reactions of DGD-PLP with both L-alanine 
and isopropylamine were followed by 1H NMR (Figure 7). The

<table>
<thead>
<tr>
<th>Table 1: Half-Reaction Kinetic Parameters*</th>
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<tr>
<td><strong>fast phase</strong></td>
</tr>
<tr>
<td>( k_{max}^{fast} ) (s⁻¹)</td>
</tr>
<tr>
<td>DGD-PLP + AIB</td>
</tr>
<tr>
<td>DGD-PLP + AIB + ACPC</td>
</tr>
<tr>
<td>DGD-PLP + L-alanine</td>
</tr>
<tr>
<td>DGD-PMP + pyruvate</td>
</tr>
<tr>
<td>DGD-PLP + AIB</td>
</tr>
<tr>
<td>DGD-PLP + L-alanine</td>
</tr>
<tr>
<td>DGD-PLP + AIB</td>
</tr>
</tbody>
</table>

* Standard errors are given in parentheses. Reaction conditions: 30 mM bicine-alkali metal hydroxide, pH 8.2, 100 mM alkali metal salt of 
succinate, ~12 µM DGD subunits, 50 µM PLP and varying concentrations of substrate. DGD-PMP was prepared by either reaction with 
substrate-specific concentrations of AIB or reconstitution of apoenzyme with excess PMP. PMP was present at a final concentration of 2 mM in 
the latter reactions. The reaction with ACPC preincubation (15 mM) was performed at a single, saturating concentration (100 mM) of AIB, as was 
the reaction of the sodium form of DGD. The amplitudes were calculated from absorbance values at 430 and 330 nm.

<table>
<thead>
<tr>
<th>Table 2: Steady-State Kinetic Isotope Effects*</th>
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<tbody>
<tr>
<td><strong>k_{cat}</strong></td>
</tr>
<tr>
<td>D-O</td>
</tr>
<tr>
<td>[1-\text{13C}]AIB</td>
</tr>
</tbody>
</table>

* Standard errors are given in parentheses. Reaction conditions: 30 mM bicine-alkali metal hydroxide, pH 8.2, 50 mM disodium succinate, 5 mM 
disodium succinate, 50 µM PLP. ° Taken from ref 15, in which this value was measured in a proton inventory experiment.
Table 3: Half-Reaction Kinetic Isotope Effects

<table>
<thead>
<tr>
<th>Isotopically labeled reactant</th>
<th>KIE on $k_{fast}$</th>
<th>KIE on $k_{slow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-13C]-AIB$^b$</td>
<td>1.042 (0.009)</td>
<td></td>
</tr>
<tr>
<td>[4-2H]-PLP$^b$</td>
<td>0.92 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Standard errors are given in parentheses. Values are averages of at least three measurements. $^b$ Reaction conditions: 30 mM bicine-KOH, pH 8.2, 13 μM DGD, 20 mM AIB, or 60 mM L-alanine, 50 mM dipotassium succinate, 5 mM disodium succinate, 50 μM PLP. The concentrations of AIB were determined by copper complexation. The concentrations of alanine were determined using alanine dehydrogenase.

The isomerization of the fast to slow enzyme form was demonstrated through the presence of fast and slow reacting forms in each case. The half-reaction kinetic parameters vary substantially between the potassium and rubidium forms, with the slow form retaining its higher apparent affinity for substrates compared to the fast form.

The data in Table 1 and Figure 1 corroborate, through analysis of the individual half-reactions, that the potassium and rubidium forms of DGD-PLP exist as a mixture of slow and fast reacting enzyme forms. As predicted from the transition from fast to slow enzyme in initial rate measurements, the slow form has a lower $K_{AIB}$.

Analysis of the half-reactions of both the potassium and rubidium forms of DGD-PLP shows no clear indication of a fast-reacting enzyme form, although a few percent of fast-reacting enzyme might not be detectable in the stopped-flow traces. Thus, the structural, steady-state, and half-reaction kinetic data form a self-consistent model in which the larger alkali metal ions activate the enzyme by driving the conformational equilibrium largely but not fully toward the fast reacting conformation, whereas the smaller Na$^+$ and Li$^+$ ions are inhibitors and share a protein structure different from that of the larger ions.

The ratio of washout to product formation was found to be 105 ± 30 for L-alanine and 14 ± 1 for isopropylamine.

**DISCUSSION**

Coexisting Enzyme Forms. Previous work (3) demonstrated that the potassium form of DGD-PLP exists in solution as a mixture of two kinetically distinguishable forms. The slower reacting form has a lower $K_{AIB}$ and a substrate concentration dependent transition of the faster to the slower form is observed in initial rate measurements over ~15 min. DGD has two alkali metal ion binding sites per monomer, one of which is near the active site and will accept a variety of alkali metal ions (4–6). The multiple enzyme forms in solution are consistent with the structural transition observed as a function of alkali metal ion size; the larger Rb$^+$ and K$^+$ ions are activators and share the same protein structure, whereas the smaller Na$^+$ and Li$^+$ ions are inhibitors and share a protein structure different from that of the larger ions.

The data in Table 1 and Figure 1 corroborate, through analysis of the individual half-reactions, that the potassium and rubidium forms of DGD-PLP exist as a mixture of slow and fast reacting enzyme forms. As predicted from the transition from fast to slow enzyme in initial rate measurements, the slow form has a lower $K_{AIB}$. The experiment in which DGD-PLP was preincubated with 1-aminocyclopropane carboxylate, a competitive inhibitor of DGD-PLP, confirms the steady-state observation that preincubation with this inhibitor draws the equilibrium between the two forms toward the tighter binding, slower reacting form. Here, the amplitude of the fast phase of the AIB half-reaction is reduced from 69 to 17% by preincubation with inhibitor.
Unlike DGD-PLP, the apparent absence of a slow reacting enzyme form in the DGD-PMP reaction with pyruvate suggests that this enzyme form exists as a single conformer.

**Rapid Equilibrium Aldimine and Ketimine Formation.**

Previous experiments, in which the half-reaction of the potassium form of DGD-PLP with L-alanine was fully coupled kinetically to the lactate dehydrogenase catalyzed reduction of pyruvate with NADH, demonstrated that ketimine hydrolysis and dissociation of pyruvate from DGD-PMP is rapid. The pH dependence of the steady-state kinetic parameters and inhibition constants demonstrated that AIB binding to the potassium form of DGD-PLP is also a rapid equilibrium process. These previous results are corroborated by the experiments reported here.

Global analysis of the multiwavelength data sets for the half-reactions provides both rate constants and true spectra of the kinetically significant species. The spectrum of the first species is the spectrum of the reactant/product mixture after ∼1.5 ms of reaction (the dead-time of the stopped-flow). The previous results cited above indicate that this species should, in all cases here, be the spectrum of the enzyme rapidly equilibrated with substrate.

The $K_{app}$ values obtained from the concentration dependence of the spectra of the first species agree with those obtained from the concentration dependence of the observed rate constants. The respective values obtained from rate constants and spectra, respectively, are AIB, 2.0 vs 2.2 and 2 (410 and 330 nm, respectively); L-alanine, 6.6 vs 7; and pyruvate, 1.1 vs 0.8. This provides strong independent evidence for these substrates that binding and aldimine or ketimine formation is at equilibrium within the 1.5 ms dead-time of the stopped-flow. Given this, lower limits for these bimolecular rate constants of $3 \times 10^5$ M$^{-1}$ s$^{-1}$ can be estimated. This is well above the steady-state $k_{cat}/K_{AIB}$ value of $7000$ M$^{-1}$ s$^{-1}$ and the $k_{cat}/K_{pyr}$ value of $1 \times 10^5$ M$^{-1}$ s$^{-1}$.

**Transamination Quinonoid Intermediate.**

Table 1 and Figures 2–5 show that the quinonoid intermediate is consistently observed in the L-alanine/pyruvate transamination half-reaction for the various metal ion forms, but not in the AIB decarboxylation half-reaction. Quinonoid absorbance at 495 nm in the L-alanine transamination half-reaction is at initial equilibrium within the 1.5 ms dead-time of the stopped-flow. Thus, within 1.5 ms, L-alanine reacts with DGD-PLP to equilibrate through the reaction sequence of Michaelis complex formation, transimination to the external aldimine intermediate, and deprotonation to the quinonoid intermediate (Scheme 1). This suggests that the rate-limiting step in the L-alanine transamination half-reaction is quinonoid protonation at C4′ to give the ketimine intermediate.

The KIEs measured for the L-alanine half-reaction support the conclusion that quinonoid protonation at C4′ is the rate-limiting step in transamination. The primary KIE has a value of ∼1 in both H$_2$O and D$_2$O, while the solvent KIEs are ∼1.5. This indicates that the Cα proton is not in flight in the rate-limiting step, but that a solvent derived proton is. It is tempting to assign this solvent proton to one on Lys272, the general acid/base catalyst in transamination, but others are possible. For example, proton transfers to or from either the pyridine or the aldimine nitrogens are candidates.
The conclusion that protonation of the quinonoid intermediate on C4′ limits the L-alanine transamination half-reaction predicts, assuming the quinonoid intermediate can exchange the substrate derived proton with solvent, that the rate of exchange of the Cα hydrogen of L-alanine with solvent vs pyruvate formation should be fast. This number was measured in the NMR experiments to be 105 (30. The relatively large error notwithstanding, this shows definitively that quinonoid protonation is the rate-limiting step in the L-alanine/pyruvate transamination half-reaction, and defines the relative heights of the energy barriers facing the quinonoid intermediate. Thus, the protonation at C4′ to go forward to the ketimine intermediate is 2.8 kcal/mol higher in energy than is the reverse reaction to the external aldimine intermediate.

This analysis implies that reprotonation of the quinonoid intermediate at Cα should occur with a rate constant ~100-fold greater than the $k_{\text{cat}}$ value observed in the L-alanine transamination half-reaction, or ~2400 s$^{-1}$. The reverse reaction of DGD-PMP with pyruvate should thus show quinonoid absorbance rise and fall with rate constants of that for ketimine deprotonation at C4′ and ~2400 s$^{-1}$. The values of the slow rate constant obtained from simple two-exponential fits to the 495 nm absorbance increase hyperbolically with pyruvate concentration to a maximal value of 75 s$^{-1}$ (Figure 5). The concentration dependence and the agreement between the $K_{\text{app}}$ values obtained from rate constants vs spectra, discussed above, demonstrates this step to be coupled to pyruvate association and is thus assigned to deprotonation of the ketimine at C4′. The fast phase rate constant, which would thus correspond to quinonoid reprotonation at Cα to give the L-alanine external aldimine intermediate, has a concentration independent value of ~2400 s$^{-1}$. This is substantially different from the expected value of ~2400 s$^{-1}$.

The discrepancy between the rate constants apparently lies in the uncertainties in the enzyme concentration used in the experiment and the extinction coefficient of the quinonoid intermediate, as well as in the error on the ratio measured by NMR. The data in Figure 5 were also fitted using the mechanism of eq 2 and KINSIM/FITSIM with limited success for global analysis of all five data sets. What is clear from this exercise is that the fast-phase rate constant is highly correlated with the values of enzyme concentration and extinction coefficient. Thus, fast-phase values of >500 s$^{-1}$ were obtained in fits where the extinction coefficient was assumed to be >20,000 M$^{-1}$ cm$^{-1}$.

Concerted Decarboxylation/Proton Transfer in the AIB Half-Reaction. DGD shows extreme specificity in its catalysis of AIB decarboxylation; an undetectable amount of isopropylamine (less than 1 in 10⁵ turnovers) is formed via
replacement of the carboxylate group on Cα with a proton, while all detectable product is acetate, which arises from protonation at C4’. This specificity could be determined by enzyme–substrate interactions after formation of the quinonoid intermediate, or, as proposed below, by a concerted transition state in which the C4’-H and the Cα—CO₂⁻ bonds are simultaneously being made and broken (Figure 8).

The pH studies (7) as well as the present data clearly demonstrate that AIB external aldimine formation is rapid and at equilibrium with respect to the rate-limiting step. The large (1.042 ± 0.009) [1-13C]AIB KIE on the decarboxylation half-reaction indicates that this rate-limiting step involves substantial cleavage of the Cα—CO₂⁻ bond, and is thus the decarboxylation step itself.

Absorbance for the quinonoid intermediate at 495 nm is not observed in the AIB decarboxylation half-reaction for any of the alkali metal ion forms of DGD-PLP. This could be due to either rapid reprotonation of the quinonoid, or to a concerted mechanism in which it is never formed. KINSIM simulations using the mechanism of eq 2, 25 mM AIB, 12 μM enzyme, 40 000 M⁻¹ cm⁻¹ for the quinonoid extinction coefficient, and 25 s⁻¹ for the decarboxylation rate constant show that the rate constant for quinonoid protonation at C4’ would have to be > 10 000 s⁻¹ for no quinonoid absorbance to be detected in the stopped-flow traces. Although not impossible, this value is very large compared to that for the same chemical step in the L-alanine transamination half-reaction (25 s⁻¹).

The [4’-2H]PLP KIE and the solvent KIE on k_cat/K_AIB also support a concerted decarboxylation/proton-transfer transition state. The inverse secondary KIE of 8% when [4’-2H]PLP replaces PLP in the AIB decarboxylation half-reaction requires that C4’ undergoes substantial rehybridization from an sp² to an sp³-like geometry in the transition state. No change in geometry at C4’, and thus no KIE, is expected in the decarboxylation step if the quinonoid intermediate, which is sp³ hybridized at C4’, were formed. The solvent KIE of 1.5 on k_cat/K_AIB with a single proton in flight in the transition state (i.e., linear proton inventory) is consistent with a proton being transferred from Ne of Lys272 to C4’ in a concerted transition state, but this KIE is equivocal since other proton transfers (e.g., to or from the pyridine nitrogen) could account for it.

The final evidence presented here for a concerted decarboxylation/proton-transfer transition state is the value of 14 ± 1 for the relative rates of C2—H solvent exchange of isopropylamine vs product formation (acetone via transamination). The deprotonation of the external aldimine at C2 of isopropylamine yields a quinonoid intermediate with a chemical structure identical to that potentially obtained from AIB, assuming rapid CO₂ dissociation. If the mechanism for specificity of protonation at C4’ of PLP vs C2 of isopropylamine (or AIB) originates in enzyme—quinonoid interactions, then the identical quinonoid intermediates formed from isopropylamine or AIB should show identical protonation specificities. The washout-to-turnover ratio for isopropylamine is expected to be unity in this case, based on the observation that less than one in 10⁵ turnovers of AIB occurs with protonation at Cα and the assumption that steps after ketimine formation are fast. This latter assumption is reasonable given the rapidity of pyruvate ketimine hydrolysis (3). The measured value of 14 ± 1 thus clearly indicates that enzyme—quinonoid interactions are not the determinant of C4’ vs C2 protonation specificity.

Combined, the [13C]AIB KIE, the lack of detectable quinonoid absorbance, the secondary [2H]PLP and solvent KIEs, and the large washout to product formation ratio strongly support a concerted transition state in which the Cα—CO₂⁻ bond is being broken and the C4’—H bond is being made simultaneously. This transition state is likely not to be synchronous (i.e., equal bond breaking/bond making), rather Cα—CO₂⁻ bond scission likely leads the process giving it substantial carbanionic character which could be stabilized as in other PLP reactions by the conjugated π system. Definitive proof of a concerted transition state awaits the measurement of multiple KIEs, both [13C]AIB and [2H]-PLP and [13C]AIB and D₂O. [The observation of substantial amounts of nonoxidative decarboxylation with several alternative substrates (8) suggests that these suboptimal reactions do not follow the same concerted mechanism.]

**Half-Reaction Free-Energy Profiles.** The half-reaction kinetic parameters reported here should account well for the steady-state kinetic measurements, if the above interpretations of the kinetic and spectral data are correct. The simplified reaction sequence of Scheme 2 can be employed, given the above discussion. The values of the steady-state kinetic parameters in terms of the rate and equilibrium constants in Scheme 2 are as follows.

\[
k_{\text{cat}} = \frac{k_5 k_5}{k_2 + k_5}
\]

(4)

\[
K_{\text{AIB}} = K_{\text{Pyr}} \frac{k_5}{k_2 + k_5}
\]

(5)

\[
K_{\text{Pyr}} = K_{\text{Pyr}} \frac{k_2}{k_2 + k_5}
\]

(6)

The calculated k_cat is 18.5 s⁻¹. Taking account that only 69% of the total enzyme is in the fast form (3), this value is reduced to 13.9 s⁻¹, in good agreement with the measured
value of $13.1 \pm 0.1$ s$^{-1}$. The calculated value of $K_{\text{AIB}}$ is 1.7 mM while that for $K_{\text{pyr}}$ is 0.17 mM, in good agreement with the measured values of 1.6 ± 0.2 mM and 0.15 ± 0.03 mM, respectively (7).

In terms of Scheme 2, the observed $[^{13}\text{C}]$KIE can be expressed as follows (17).

$$13k_{\text{cat}} = \frac{12k_2/k'_2 + R_f/E_i}{1 + R_f/E_f}$$

(7)

Here $E_i = 1$ and $R_f = k_f/k_c$. Assuming the intrinsic KIE to be 1.06 (18) and that the measured half-reaction KIE of 1.04 ± 0.01 is in agreement with this, the observed $[^{13}\text{C}]$KIE is calculated to be 1.045. This agrees with the measured value of 1.043 ± 0.003.

The half-reaction kinetic parameters thus validated, one can employ them in the construction of half-reaction free energy profiles. Figure 9 presents these. The profile for the AIB decarboxylation half-reaction (Figure 9A) shows the direct conversion of the external aldimine to the ketimine intermediate via a concerted transition state. This is the only kinetically significant transition state, the formation of the AIB external aldimine and hydrolysis of the acetone ketimine being very rapid in comparison. The relative energies of the Michaelis complexes were set by the values of $K_{\text{AIB}}$ and $K_{\text{pyr}}$ (10 mM; ref 7). The former reflects contributions from both the Michaelis complex and external aldimine and the latter only the Michaelis complex (or rather the dead-end pyruvate complex, which is assumed to be indicative of the Michaelis complex). Decarboxylation is assumed to be irreversible, thus the relative energies of the external aldimine and ketimine, are undetermined. The lower energy of DGD-PMP and free acetone compared to the ketimine is based on the lack of significant inhibition of the AIB/pyruvate reaction at relatively low acetone concentrations (<200 mM).

The L-alanine half-reaction is somewhat more complicated (Figure 9B). The relative energies of the free enzyme, Michaelis complex, and external aldimine were set based on the values of $K_{\text{Ala}}$ and $K_{\text{pyr}}$. The data in the inset to Figure 3B, showing that the quinonoid absorbance of species A rises to 0.003 with saturating L-alanine and 13 mM DGD-PLP, provides the energetic difference between the external aldimine and quinonoid intermediates based on a quinonoid extinction coefficient of 35 000 M$^{-1}$ cm$^{-1}$. Protonation of the quinonoid intermediate is the rate determining step in the L-alanine to pyruvate direction and this barrier height is calculated from the $k_{\text{max}}$ value for the L-alanine half-reaction. The value of ~100 for the washout vs product formation ratio determines the relative barrier heights for quinonoid protonation at C$\alpha$ vs C$4'$ to give the external aldimine or ketimine, respectively. The barrier height for ketimine deprotonation at C$4'$, and thus the energetic difference between the quinonoid and ketimine, is obtained from the $k_{\text{max}}$ value for the DGD-PMP reaction with pyruvate. Finally, the position of DGD-PMP and free pyruvate is determined either by the $K_{\text{pyr}}$ value or the half-reaction equilibrium constant calculated from $k_{\text{max}}/K$ for the forward and reverse half-reactions.

Evolutionarily, DGD is an aminotransferase that has gained the ability to catalyze decarboxylation (1). These half-reaction energy profiles paint a picture of an aminotransferase that has compromised its transamination prowess for the sake of adapting to the needs of the rate-limiting C$\alpha$−CO$_2$ cleavage step. The rate constant for C$\alpha$ deprotonation of the L-alanine external aldimine is calculated from the free energy profile to be ~2000 s$^{-1}$. This is faster than even aspartate aminotransferase (19), a highly developed enzyme catalyzing only transamination. A major component of the adaptive restrictions placed on the enzyme by the decarboxylation half-reaction may well be the requirement for a concerted transition state to ensure specific protonation at C$4'$, with ensuing transfer of the substrate amino group to the coenzyme.

**REFERENCES**


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