Kinetic Isotope Effects for Dialkylglycine Decarboxylase via a High-Precision Continuous-Flow Method

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Dialkylglycine decarboxylase (DGD) is an unusual pyridoxal phosphate dependent enzyme that catalyzes the oxidative decarboxylation of 2,2-dialkylglycines coupled to the transamination of pyruvate to L-alanine. With 2-aminoisobutyrate (AIB), the decarboxylation half-reaction fully, or nearly so, limits the overall catalytic cycle.[1] Heavy atom and hydrogen kinetic isotope effects (KIE's) on k_{cat} are used here to probe the extent of rate-limitation by carbon−carbon bond-breaking and proton-transfer steps.

A continuous-flow apparatus comprised of syringe pumps and HPLC detectors, described below, was used to measure the direct KIE's. The stability of the flow system and detectors and the efficiency of the mixer make it possible to measure heavy atom KIE's of a few percent. Uniformly ^{13}C-enriched AIB (Cambridge Isotope Laboratories) was employed here. The ^{12}C- and ^{13}C-labeled substrates were placed in separate pumps at identical concentrations (∼5K_M). During the course of the experiment, the pumps were alternated, switching between ^{12}C- and ^{13}C-labeled AIB. Data from such an experiment are shown in Figure 1. From this and similar data, a value of $^{13}k_{cat}$ = 1.066 ± 0.006 is calculated.

A proton inventory experiment[2] was also performed with DGD. AIB was present at an identical, saturating (∼10K_M) concentration in both syringes. One syringe contained D_2O in place of H_2O. A linear gradient was formed between H_2O and D_2O, giving a continuous variation in the fraction of D_2O present. The data (Figure 2) yield a solvent KIE on k_{cat} of 1.61 ± 0.01.

Intrinsic ^{13}C KIE's for decarboxylation are approximately 6% on the carboxylate carbon.[3] Uniformly labeled AIB will yield a convolution of KIE's from the carboxylate and Cα carbons. This might range up to 12%. The observed KIE of 6.6% suggests that the C−C bond-breaking step in the decarboxylation half-reaction largely limits the rate of the overall catalytic cycle of DGD. This step, or another partially rate-limiting one, also involves a proton transfer as indicated by the solvent KIE of 1.6. The linearity of the proton inventory (Figure 2) shows that a single proton is in flight in the transition state. It remains to be determined whether these two KIE's occur in the same step of the reaction. It is tempting to speculate that they do, and that protonation of the coenzyme C4′ by Lys272, or possibly proton transfer between the coenzyme pyridine nitrogen and Asp243, is responsible for the solvent KIE. The former possibility would explain the strict specificity of the enzyme for oxidative decarboxylation.

Scheme 1 outlines the apparatus employed. In experiments where a variation in the concentration of a given species (e.g. substrate, D_2O) is desired, two syringe pumps are complementarily programmed, giving a linear gradient with a constant flow rate. In a saturation curve, for example, a gradient between (1) buffer and (2) buffer + substrate + dye (included as an internal reference) is formed. Enzyme is flowed at a constant, low rate. The effluents from all three syringes converge at and are mixed in a low-volume, dynamic mixer. This initiates the enzyme-catalyzed reaction and generates a reaction flow that constitutes a continuous substrate concentration gradient. The reaction flow passes through a delay loop. The reaction time for a given volume element is determined by the volume of the delay loop and the flow rate. The extent of reaction is measured by an HPLC absorbance detector. A second detector monitors the absorbance of the dye, giving an experimental determination of the position of the gradient. The outputs from these detectors are digitized by HPLC software on a computer.

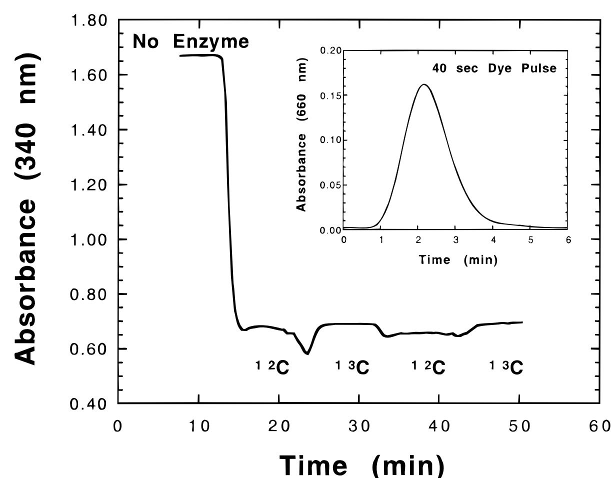

Figure 1. Carbon-13 kinetic isotope effect determination for the decarboxylation of AIB catalyzed by dialkylglycine decarboxylase. Stock solutions of ∼1 M [^{12}C]- and [U-^{13}C]-AIB were prepared in H_2O. AIB concentrations were determined colorimetrically to within 1% by copper complexation. A single solution containing 40 mM BICINE-KOH (pH 8.0), 50 mM dipotassium succinate, 5 mM disodium succinate, 1.5 mM pyruvate, 3 units/mL 2°ADH, and 300 μM NADPH was made. This was divided into two portions. Calibrated [^{12}C]- or [U-^{13}C]-AIB stock solution was added, using an analytical balance, to a portion of the above mixture to give a final AIB concentration of 12 mM. The enzyme solution contained 40 mM BICINE-KOH (pH 8.0), 50 mM dipotassium succinate, 5 mM disodium succinate, 100 μM pyridoxal phosphate, and 1.4 μM DGD. AIB solutions were flowed at 0.15 mL/min, while the enzyme was at 0.05 mL/min. Reaction progress was followed at 340 nm. The measurement consisted of 10 min of [^{12}C]-AIB reagent mixture alone, alternating 10 min periods of either [^{12}C]- or [U-^{13}C]-AIB reagent mixture with enzyme, and 10 min of [U-^{13}C]-AIB reagent mixture alone. (Inset) A 40 s methylene blue dye pulse (0.04 mL/min) was added to a 0.15 mL/min flow of buffer and monitored at 660 nm.

The data from the detector monitoring the extent of the enzymatic reaction represent single time point determinations of initial rates. The application of this technique thus requires that if nonlinear intial rates are observed, the nonlinearity should not depend on the concentration of the varied species (e.g. substrate, D_2O).

The mixer is home-built, and is diagramed in Scheme 1. The central component is a 12 μL mixing chamber with a Teflon-coated magnetic stirrer, which was obtained from Pharmacia Biotech (part No. 18-1801-31). The top of the mixer was constructed from a 1/8 in. thick Lucite disk the diameter of the mixer. Three 1/16 in. holes were drilled from the edge of the disk toward the center. These were opened to the lower face with pinholes. HPLC tubing (1/16 in. o.d., 0.02 in. i.d. PEEK) was affixed into the holes with cyanoacrylate adhesive. The bottom of the mixer was similarly constructed except that a single flow line was introduced. The two plates and their attached flow lines were carefully affixed to the mixer with cyanoacrylate adhesive.

A potential problem with the apparatus is laminar flow, where

(1) (a) Zhou, X.; Kay, S.; Toney, M. D. *Biochemistry* **1998**, *37*, 5761. (b) Sun, S.; Zabinski, R. F.; Toney, M. D. *Biochemistry* **1998**, *37*, 3865.
(2) Schowen, K. B.; Schowen, R. L. *Methods Enzymol.* **1982**, *87*, 551.
(3) O'Leary, M. L. *Acc. Chem. Res.* **1988**, *21*, 450.

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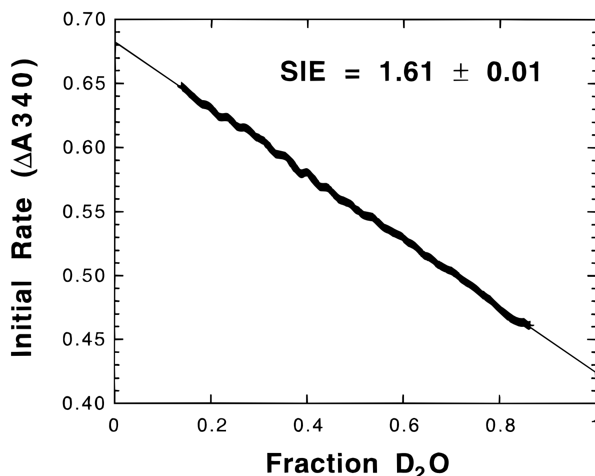
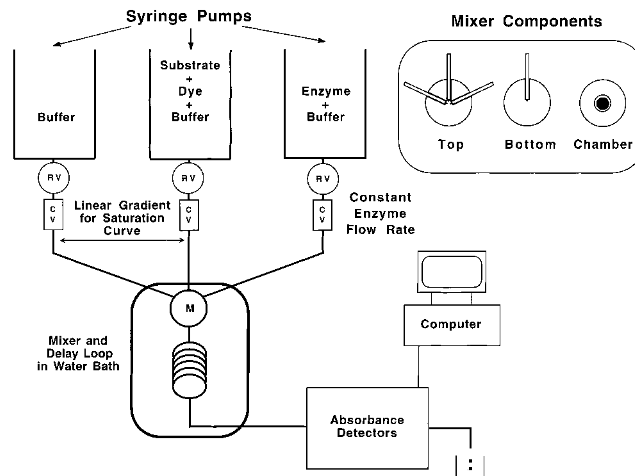


Figure 2. Proton inventory on k_{cat} for dialkylglycine decarboxylase. Identical solutions of 40 mM BICINE-KOH (pH 8.0), 50 mM dipotassium succinate, 5 mM disodium succinate, 20 mM AIB (~10K_M), 5 mM pyruvate, 3 units/mL 2°ADH, and 300 μM NADPH were made in H_2O or D_2O. Methylene blue (~5 μM final concentration) was included as an internal reference in the D_2O solution. The enzyme solution contained 40 mM BICINE-KOH (pH 8.0), 50 mM dipotassium succinate, 5 mM disodium succinate, 100 μM pyridoxal phosphate, and 1.4 μM DGD in 50% D_2O. A linear gradient, with a constant flow rate of 0.15 mL/min, was formed between the H_2O and D_2O solutions by a complementary linear decrease and increase in flow rates. The enzyme solution was flowed at a rate of 0.05 mL/min for a total flow rate of 0.2 mL/min. The measurement consisted of 10 min of H_2O solution alone, 10 min of H_2O solution with enzyme, 20 min of gradient between the H_2O and D_2O solution, 10 min of D_2O solution with enzyme, and 10 min of D_2O solution alone. The ratio of the extinction coefficients of methylene blue at 660 and 340 nm was determined in a separate experiment where a gradient between (1) buffer and (2) buffer + dye was monitored.

the solution near the edges of the tubing flows slower than that in the center. Severe laminar flow is easily detectable by pulsing a dye solution into an optically transparent flow. The inset to Figure 1 presents such an experiment. The 40 s pulse spreads to a half-width of approximately 80 s. The symmetry of the peak and the lack of significant tailing show that laminar flow does not significantly influence the present system. Xue *et al.*[4] previously used a flow system to measure ^{13}C KIE's on formate dehydrogenase. The present system has the advantage over theirs that much smaller volumes are required, making it generally applicable to biochemical problems.

Prudent application of the flow system described here should allow the routine measurement of high-precision enzyme kinetics, including small KIE's.[5] The direct measurement of heavy atom KIE's as described here has the advantage over competitive techniques that effects on k_{cat} can be measured. A disadvantage is the need to obtain highly isotopically enriched substrates. Other applications of this technique are easily imagined. For example, one might convert discontinuous assays into continuous ones by adding, between the reaction loop and detectors, a pump and mixer for the derivatizing reagent. Reduction of the volumes required is forseen.

Scheme 1. Diagram of the Apparatus, Which Is Shown Configured To Perform a Saturation Curve Determination[a]



[a] Three KD Scientific syringe pumps (two Model 200P, one Model 101) are used with Kloehn Series 4000 syringes (5 mL for buffer/substrate, 2.5 mL for enzyme). A linear gradient is formed between (1) buffer and (2) buffer + substrate + dye by programing a complementary decrease and increase in the flow rates over a fixed time such that the sum of the flow rates is constant. Enzyme is flowed at a constant, low flow rate. The three solutions meet and are mixed in a home-built mixer, which is separately diagrammed. The reaction flow spends ~1.5 min in the delay loop before entering the detectors (Spectra-Physics and LDC/Milton Roy). Absorbance data are digitized and stored by Axxiom 727 HPLC datastation hardware and software on an IBM-compatible computer. Syringes are refilled from reservoir syringes attached to three-way valves (RV; Upchurch Scientific). One-way check valves (CV; Upchurch Scientific) are used to prevent back-flow from the mixer into the flow lines.

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(4) Xue, H.; Wu, X. W.; Huskey, W. P. *J. Am. Chem. Soc.* **1996**, *118*, 5804.

(5) The apparatus was originally tested by using a protein tyrosine phosphatase and *p*-nitrophenyl phosphate as substrate. Saturation curves faithfully reproduced both kinetic parameters and a proton inventory, which were reported by: Zhang, Z.-Y.; Malachowski, W. P.; Van Etten, R. L.; Dixon, J. E. *J. Biol. Chem.* **1994**, *269*, 8140.