Fern L-Methionine Decarboxylase: Kinetics and Mechanism of Decarboxylation and Abortive Transamination†

Mahmoud Akhtar, David E. Stevenson, and David Gani*,‡
Department of Chemistry, Institute of Biomolecular Science, The University of Southampton, Southampton SO9 5NH, U.K.
Received October 12, 1989; Revised Manuscript Received March 28, 1990

ABSTRACT: L-Methionine decarboxylase from *Dryopteris filix-mas* catalyzes the decarboxylation of L-methionine and a range of straight- and branched-chain L-amino acids to give the corresponding amine products. The deuterium solvent isotope effects for the decarboxylation of (2*S*)-methionine are $^{D}(V/K) = 6.5$ and $^{D}V = 2.3$, for (2*S*)-valine are $^{D}(V/K) = 1.9$ and $^{D}V = 2.6$, and for (2*S*)-leucine are $^{D}(V/K) = 2.5$ and $^{D}V = 1.0$ at pL 5.5. At pL 6.0 and above, where the value of k_{cat} for all of the substrates is low, the solvent isotope effects on V_{max} for methionine are 1.1–1.2 whereas the effects on V/K remain unchanged, indicating that the solvent-sensitive transition state occurs before the first irreversible step, carbon dioxide desorption. The enzyme also catalyzes an abortive decarboxylation–transamination reaction in which the coenzyme is converted to pyridoxamine phosphate [Stevenson, D. E., Akhtar, M., & Gani, D. (1990a) *Biochemistry* (first paper of three in this issue)]. At very high concentration, the product amine can promote transamination of the coenzyme. However, the reaction occurs infrequently and does not influence the partitioning between decarboxylation and substrate-mediated abortive transamination under steady-state turnover conditions. The partition ratio, normal catalytic versus abortive events, can be determined from the amount of substrate consumed by a known amount of enzyme at infinite time, and the rate of inactivation can be determined by measuring the decrease in enzyme activity with respect to time. For methionine, the values of K_m as determined from double-reciprocal plots of concentration versus inactivation rate are the same as those calculated from initial catalytic (decarboxylation) rate data, indicating that a single common intermediate partitions between product formation and slow transamination. The partition ratio is sensitive to changes in pH and is also dependent upon the structure of the substrate; methionine causes less frequent inactivation than either valine or leucine. The pH dependence of the partition ratio with methionine as substrate is very similar to that for V/K. Both curves show a sharp increase at ~pH 6.25, indicating that a catalytic group on the enzyme simultaneously suppresses the abortive reaction and enhances physiological reaction in its unprotonated state. Experiments conducted in deuterium oxide allowed the solvent isotope effects for the partition ratio and the abortive reaction to be determined. The isotope effect for the partition ratio increased sharply from 0.6 to 1.7 at pL 6.25 while that for the abortive reaction decreased from 1.7 to 0.7, indicating that a postdecarboxylation step on the normal reaction pathway requires a catalytic group on the enzyme to be protonated. This step is probably quinoid protonation at C^{α}. ^{1}H NMR spectroscopic analysis of 3-(methylthio)-1-aminopropane isolated from incubations conducted in 50 molar % deuterium oxide at pL 4.8 and at pL 6.5, where the step for protonation of the quinoid at C^{α} is expected to be kinetically significant in the postdecarboxylation part of the reaction, indicated that the proton donor was monoprotic and, therefore, is probably the imidazolium side chain of a histidine residue. The results of further experiments are described, and a mechanistic scheme in which a His residue operates at C^{α} of the quinoid intermediate in normal decarboxylation and in which a Lys residue operates at C-4′ in abortive reactions is proposed and discussed.

Pyridoxal 5′-phosphate dependent enzymes catalyze many different types of reaction that are of central importance in amino acid metabolism. The major groups of these enzymes are the transaminases and the decarboxylases. While a great deal is known about the mode of action of the transaminase enzymes, and in particular aspartate aminotransferase (Kirsch et al., 1984; Cronin & Kirsch, 1988; Julin et al., 1989; Julin & Kirsch, 1989), details of the mechanism of catalysis by decarboxylases are sparse.

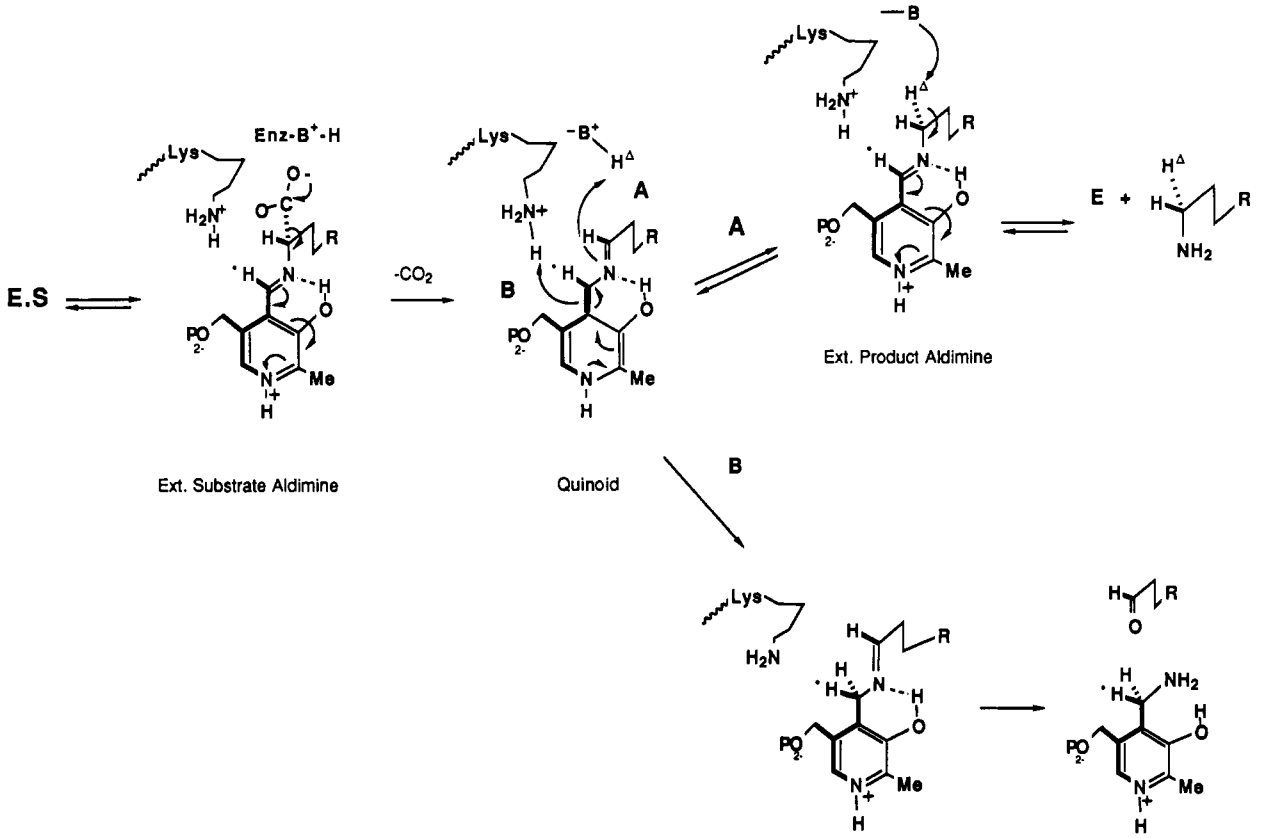
We recently described the purification and properties of L-methionine decarboxylase from the fern *Dryopteris filix-mas*. This enzyme is eminently suited for mechanistic studies and catalyzes the decarboxylation of several nonpolar amino acids. During the decarboxylation process, the quinoid intermediate is occasionally protonated at the C-4′ position of the coenzyme to produce the transamination products, pyridoxamine phosphate and 3-(methylthio)propionaldehyde (Stevenson et al., 1990a). The occurrence of abortive decarboxylation–transamination reactions is not uncommon among PLP[1]-dependent decarboxylases (Sukhareva & Braunstein, 1971; O'Leary & Baughn, 1977; Minelli et al., 1979; Choi & Churchich, 1986; Grant et al., 1987), and indeed, examples of enzymes from evolutionarily diverse sources are known. The possibility that this fact might provide a link with the transaminase group was recently strengthened by the finding that the stereochemical courses of proton transfer to the coenzyme were the same for decarboxylases and transaminases (Stevenson et al., 1990a). On the basis of this ste-

† This work was supported by Science and Engineering Research Council Grants GR/D-21202, GR/E-73512 and GR1/2F-22951 to D.G.
* To whom correspondence should be addressed at the Department of Chemistry, The Purdie Building, The University, St. Andrews, Fife KY16 9ST, Scotland, U.K.
‡ Royal Society University Fellow, 1983–1988.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; NMR, nuclear magnetic resonance; UV, ultraviolet; TLC, thin-layer chromatography; pL, -log [L^{+}].

0006-2960/90/0429-7648$02.50/0

Scheme I: L-Methionine Decarboxylase Reaction Showing the Stereochemical Courses of Protonation at the Quinoid Intermediate at C^{α} and C-4′



reochemical information, arguments were developed to suggest that the ammonium group of an active-site lysine residue protonates the quinoid intermediate at C-4′ in abortive decarboxylation–transamination reactions catalyzed by fern methionine decarboxylase; see Scheme I.

Although in all respects the reaction is analogous to that catalyzed by bona fide transaminase enzymes, for the decarboxylases, protonation at C-4′ is a side reaction. This step may be mediated by the lysine residue, but it is far from clear whether the lysine acts as a proton donor for the quinoid intermediate at C^{α} during the normal course of decarboxylation. Indeed, there is some evidence to suggest that the imidazolium side chain of a His residue fulfills this role, although much of this is rather weak [Yamada and O'Leary (1977) as cited in O'Leary (1977)].

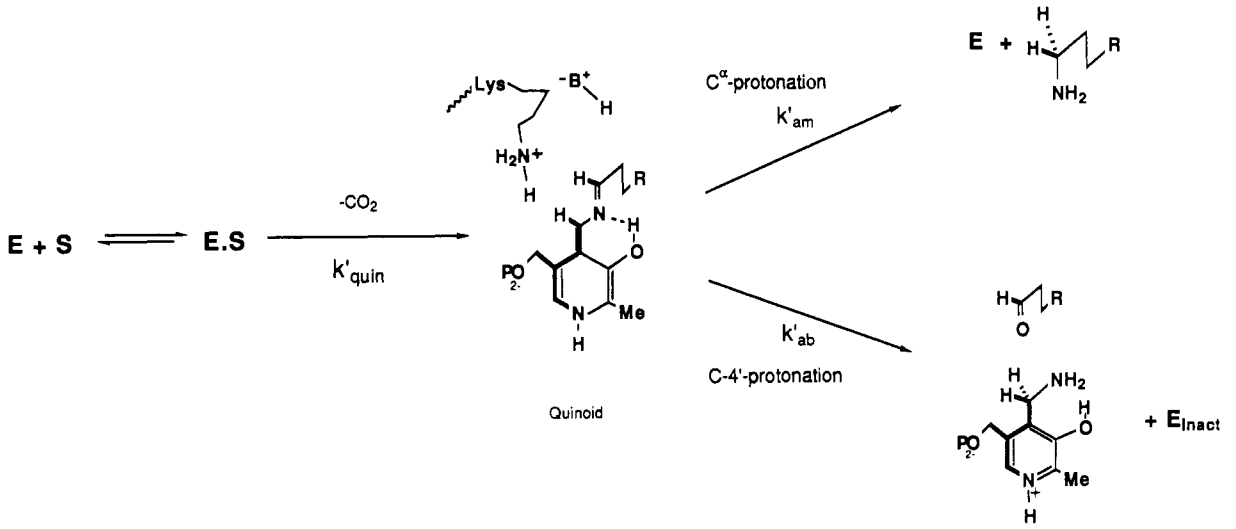
In principle, if certain kinetic features of the decarboxylation and abortive transamination reactions are met (see Theory), it should be possible to titrate the partition ratio if the proton-donating groups for the quinoid intermediate at C^{α} and C-4′ possess different pK_a values. However, the kinetics of PLP-dependent decarboxylase action are extremely complicated and as yet are poorly understood. For the best studied system, *Escherichia coli* glutamate decarboxylase, only the first part of the physiological reaction, up to carbon dioxide desorption, has been examined (O'Leary et al., 1981; Abell & O'Leary, 1988a). The enzyme shows rather broad titration curves for V and V/K (O'Leary et al., 1970; Fonda, 1972), and further studies are hampered because the active hexameric form of the protein dissociates into dimers above pH 6.0 (Tikhonenko et al., 1968; Sukhareva, 1986).

Before embarking on any theoretical predictions of the pH dependence of the partition ratio for fern methionine decarboxylase, it was necessary to establish whether the decarboxylation product, the amine, is able to promote coenzyme transamination through reverse steps. Aside from complicating the kinetic analysis by decreasing the apparent partition ratio, amine-promoted transamination might prove to be useful as a mechanistic probe.

Our earlier studies of the decarboxylation of (2*S*)-methionine revealed that the measured partition ratios in three different types of experiment varied significantly (Stevenson et al., 1990a). It was evident that the partition ratio was pH dependent, but this information alone could not account for the differences in the apparent partition ratio between experiments. The major differences in the conditions employed in the three types of experiments were the concentrations of coenzyme used and the extent to which the product amine was allowed to accumulate. The most consistent argument that we could put forward to account for these observations was that the decarboxylase possessed a product amine transaminase activity. While the enzyme used in the experiments was not homogeneous, and thus could have been contaminated with an as yet undiscovered ω-transaminase activity, the results of other experiments, notably the lack of solvent hydrogen wash-in into the product pool, suggested that this was not the case. We were, therefore, compelled to accept that the product amine could promote transamination of the coenzyme in a reaction catalyzed by the decarboxylase in a manner similar to but less pronounced than that reported for porcine brain glutamate decarboxylase (Porter et al., 1985). Indeed, the result obtained with the porcine brain enzyme was particularly interesting because the product amine mediated transamination was so facile. Other decarboxylases, namely *E. coli* glutamate decarboxylase [Yamada & O'Leary, 1977; also see Mandeles et al. (1954)] and fern methionine decarboxylase (Stevenson et al., 1990a) apparently showed no such activity.

In order to rationalize the dichotomy for the fern enzyme, we suggested that a solvent-shielded monoprotic conjugate acid might be formed at the active site of the decarboxylase on the removal of a hydrogen from the amine through reverse steps.

Scheme II: Minimal Kinetic Mode for Quinoid Protonation at C^{α} and C-4′[a]



[a] k'_{quin}, k'_{am}, and k'_{ab} represent the composite rate constants for multistep processes leading to actual decarboxylation (quinoid formation), amine formation, and abortive transamination, respectively; see Theory.

It might then be possible to return only the same hydrogen to the quinoid intermediate at C^{α} as the one that was originally removed; see Scheme I. The reaction would be invisible to solvent hydrogen wash-in experiments but should be detectable by other methods. For example, the use of C-1 tritiated amines in product-promoted transamination would potentially allow hydrogen washout to be measured, while the use of a ^{35}S-labeled 3-(methylthio)-substituted amine would allow the radiolabeled aldehyde transamination product to be detected. The loss of enzyme activity due to the transamination of the coenzyme by the product amine could also be measured.

These experiments are described here (under Materials and Methods) as a necessary prelude to the construction of a kinetic model for PLP-dependent decarboxylase action. The results indicate that the amine is able to promote coenzyme transamination but only at very high concentration. Thus, for the purpose of investigating the pH dependence of the partition ratio, product-promoted transamination, at least for the amine product from methionine, can be ignored.

Theory

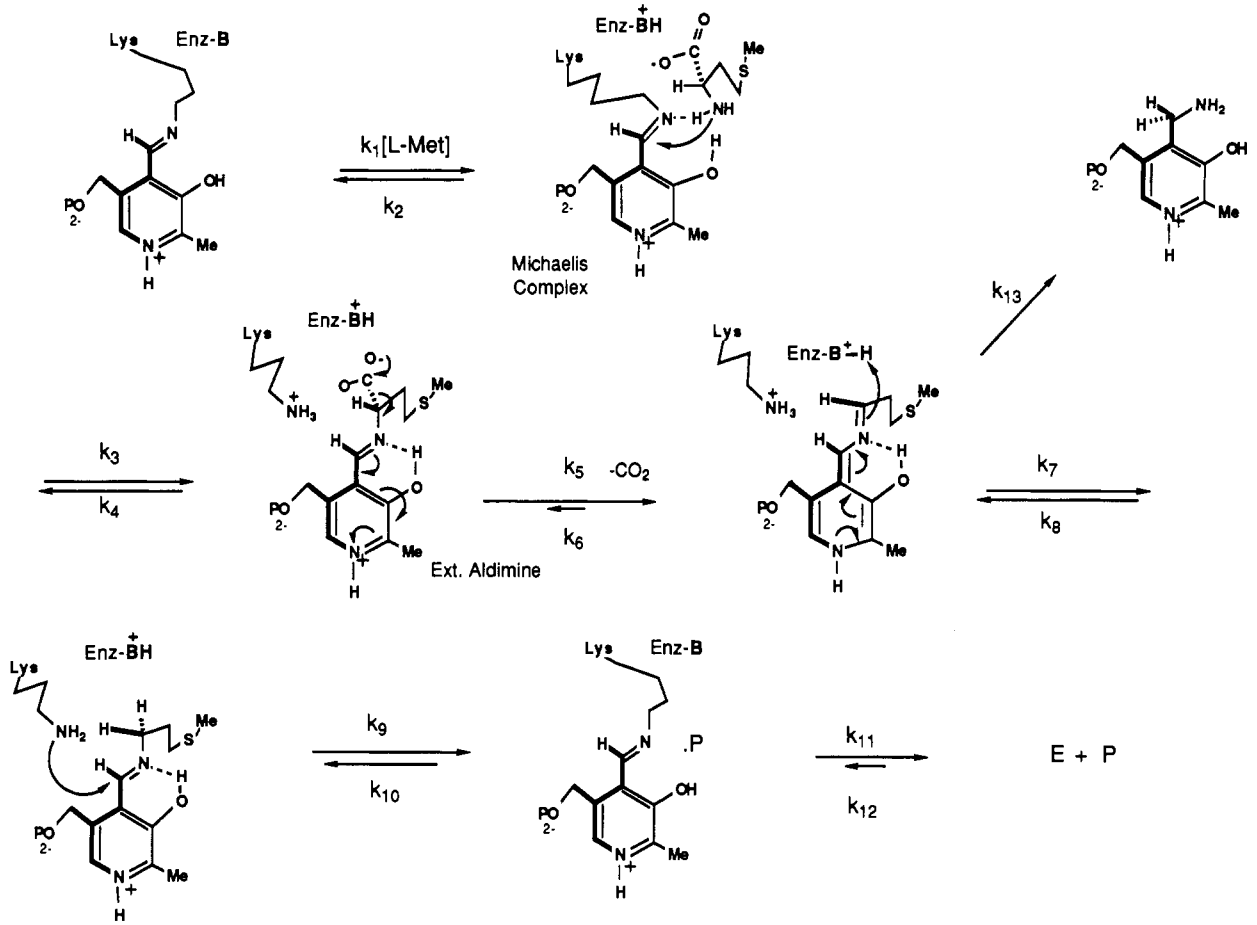
Intuitively, the construction of the kinetic model for the partition ratio for the fern methionine decarboxylation reaction must consist of at least three parts: (1) formation of the quinoid intermediate from the external aldimine (this is an irreversible process since CO_2 is lost), (2) protonation at C^{α} to give, eventually, the product amine, and (3) protonation at C-4′ to give PMP; see Scheme II. If the reactions of the quinoid intermediate to give the product amine and PMP are effectively irreversible, which is certainly true at early times, then this simple model predicts that the partition ratio is k'_{am}/k'_{ab}, where k'_{am} and k'_{ab} are composite rate constants. The *ratio* will not be affected by independent changes in the value of k'_{quin} even if the observed rates of PMP and amine formation change, as they will do so equally; see Scheme II. Furthermore, the values of K_m determined from the substrate concentration dependencies of v_{am} and v_{ab} will be identical. [These predictions have been verified by computer simulation with the simple model of Scheme II and the ACUCHEM computer program (Braun et al., 1986). The partition ratio was unaffected by changes in substrate concentration for a range of assumed values of each constant.]

With respect to k'_{ab}, it is reasonable to expect that PMP formation is irreversible, since aldehydes do not restore activity to inactivated PMP/apoenzyme (Stevenson et al., 1990a). It is also reasonable to expect that the slowest step en route to free PMP from the quinoid intermediate is C-4′ protonation for many reasons. First, it is the only chemical step in a side reaction that must occur at the active site of the enzyme. Second, the products, PMP and aldehyde (or the aldimine), are not physiological substrates or products for the enzyme; therefore, their release rates should not be slow compared to the release of the product amine. Third, the overall rate of transamination is very slow: $\sim 10^{-2}$–10^{-3} s^{-1}, i.e., $\sim 10^4$ times slower than k_{cat} (Stevenson et al., 1990a). Hence, the transition state for proton transfer should dominate the transamination part of the reaction coordinate profile, and the abortive reaction should show a primary deuterium solvent isotope effect.

The situation is very much more complex for k'_{am}, which must contain contributions for the rate of protonation at C^{α}, the two parts of the product amine transaldimination process, and product debinding. Aside from product debinding (at early times when there is no product), all of these processes are also reversible, and two, protonation at C^{α} and transaldimination, should show primary deuterium solvent isotope effects that are detectable on the overall decarboxylation rate if the transition states of these components of k'_{am} dominate the reaction coordinate profile for k'_{am} and if k'_{am} is smaller than k'_{quin}. Even if k'_{am} is larger than k'_{quin}, the partition ratio should show an isotope effect under certain pH conditions unless the proton donor for the quinoid intermediate at C^{α} and C-4′ is the same group.

Finally, with regard to the simple kinetic scheme, k'_{quin} should also contain kinetic terms for the reversible isomerization of the Michaelis complex to the external aldimine via transaldimination, in addition to the term for C–C bond cleavage. The transaldimination step should show a detectable primary deuterium isotope effect on V/K if the substrate is not sticky and if the reverse commitments due to C–C bond cleavage and CO_2 dissociation steps are not large, regardless of the magnitude of other rates in the reaction. V/K isotope effects can reflect the full intrinsic value even if a very slow step occurs after the step of interest, provided that the slow step occurs after the first irreversible step, in this case CO_2 dissociation (Klinman, 1978; O'Leary, 1978). Small external commitments are also important in facilitating the interpretation of observed molecular pK_a values; see Discussion. The

Scheme III: Possible Mechanistic Scheme for the Decarboxylation and Transamination Reactions[a]



[a] Transaldimination reactions, which are in fact multistep processes, are represented by single steps.

solvent isotope effect on V will be suppressed toward unity unless a proton transfer step somewhere in the reaction coordinate profile from substrate through to product is at least partially rate limiting. Thus, a more realistic minimal kinetic scheme can be derived which takes account of all 13 of the steps that can be anticipated to influence the rate of decarboxylation, the rate of abortive transamination, and the ratio of these rates. The results of the experiments described below are interpreted and discussed by using Scheme III.

Materials and Methods

Fronds of *D. filix-mas* were collected from local areas during May to October and were stored, after removing midveins, at −70 °C prior to use. Amino acid substrates, PLP, PMP, buffers, salts, and deuterium oxide were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Amberlite IR45(OH) and Dowex 1X8(OH) ion-exchange resins were obtained from British Drug Houses (Poole, Dorset, U.K.), and [1-^{14}C]-L-amino acid substrates, [^{35}S]methionine, and tritiated water were obtained from Amersham International (Amersham, Bucks, U.K.). Water-miscible scintillant (ES-199) was obtained from Canberra Packard (Pangbourne, Berks, U.K.). All other chemicals were of analytical grade or were recrystallized or redistilled before use. ^{1}H NMR spectra were recorded by using a Brucker AM360 instrument.

Enzyme. L-Methionine decarboxylase was isolated and purified as described previously (Stevenson et al., 1990a).

Activity Assays. L-Methionine decarboxylase activity was determined by using the radioactivity assay (Stevenson et al., 1990a).

3-(Methylthio)-1-aminopropane Hydrochloride. (2*S*)-Methionine (100 mg, 0.67 mmol) was incubated with fern acetone powder (1 g) in 200 mM acetate buffer (15 mL) containing PLP (5 mg, 0.02 mM) at pH 4.8 and 25 °C for 24 h with gentle stirring. The particulate matter was removed by filtration on a pad of prewashed Celite, and the filtrate was adjusted to pH 11 by the addition of 2 M sodium hydroxide. The aqueous phase was extracted with dichloromethane (3 × 25 mL), and the organic phase was extracted with 500 mM hydrochloric acid (2 × 15 mL). The acid solution was reduced in volume in vacuo to give 3-(methylthio)-1-aminopropane hydrochloride. The crude product was purified on Dowex 1X8(OH) and was then converted to the hydrochloride by the addition of 6 M HCl, in 65% overall yield. The compound showed the expected spectral and analytical properties.

[^{35}S]-3-(Methylthio)-1-aminopropane Hydrochloride. The labeled amine was freshly prepared from (2*S*)-[^{35}S]methionine by the method described previously (Stevenson et al., 1990a). The compound was recrystallized to constant activity and was homogeneous both chemically and radiochemically, as judged by TLC on cellulose eluted with 2-propanol/aqueous NH_3 (sp gr 0.88)/H_2O (26:6:5).

(1R)-[1-^{3}H]-3-(Methylthio)-1-aminopropane Hydrochloride. (1*R*)-[1-^{3}H]-3-(methylthio)-1-aminopropane was prepared from (2*S*)-methionine (50 mg, 0.34 mmol) as described above, with tritium oxide as the solvent. The amine was isolated and purified in the usual manner and was recrystallized to constant radioactivity (36.9 × 10^6 dpm mmol^{-1}) from 2-propanol/ethyl acetate (1:1).

(1S)-[1-^{3}H]-3-(Methylthio)-1-aminopropane Hydrochloride. (2*RS*)-[2-^{3}H]Methionine (250 μCi, 6 Ci mmol^{-1}) was decarboxylated in water as described above. The tritiated

Table I: Incubation of ^{35}S-Labeled 3-(Methylthio)-1-aminopropane with Enzyme[a]

plate region (R_f)	amount (dpm)	species
0.0–0.1	1180	amine
0.1–0.2	37	
0.2–0.3	39	
0.3–0.4	30	DNP reagent (unreacted)
0.4–0.5	59	
0.5–0.6	1123	DNP aldehyde
0.6–0.7	29	
0.7–0.8	47	

[a] See Materials and Methods for details.

amine was isolated in the usual manner and was recrystallized to constant specific radioactivity (20.1×10^6 dpm mmol^{-1}) from 2-propanol/ethyl acetate (1:1).

Transamination of the Product Amine. (*A*) [^{35}S]-3-(Methylthio)-1-aminopropane hydrochloride (3.2 mg, 18×10^6 dpm) was incubated with the enzyme (1 unit, 7 units mg^{-1}) in 0.2 M succinate buffer at pH 5.0 and 37 °C for 24 h. Excess 2,4-dinitrophenylhydrazine reagent was added, and the solution was allowed to stand for 1 h and was then extracted with ethyl acetate (3×5 mL). The extract was concentrated in vacuo to 0.2 mL, and carrier hydrazone (10 mg) was added. The solution was analyzed by TLC on fluorescent silica plates, with diethyl ether as eluent. The developed chromatogram was visualized under a UV lamp, and 10 horizontal sections of the TLC plate were removed. The radioactivity content of each section was determined by liquid scintillation counting, as shown in Table I.

(*B*) Enzyme (0.25 nmol, 7 units mg^{-1}, freed of unbound coenzyme) was preincubated with isobutylamine, 3-(methylthio)-1-aminopropane, or on its own in 200 mM succinate buffer at pH 6.0 under various conditions prior to assay. The assay procedure was designed to determine the activity in the absence and in the presence of PLP for each preincubation under essentially steady-state conditions, which simplify data analyses. The assay was based on the radioactivity method previously described (Stevenson et al., 1990a) except the reactions were followed for several hours (against controls) so that the extent of methionine turnover at the end point, when the enzyme was completely inactive due to abortive decarboxylation–transamination, could be accurately measured or estimated. The data from the experiments containing added PLP gave essentially straight line plots for turnover versus time. Experiments that did not contain PLP gave curved time courses (Figure 1; supplementary material, Figures 1 and 2) and were analyzed as described below; see Data Processing.

(*C*) *Transamination and Hydrogen Exchange.* (1*R*)-[1-^3H]-3-(methylthio)-1-aminopropane (20×10^6 dpm) was incubated with the enzyme (2 units) and PLP (2 mg, 0.01 mM) in 200 mM succinate buffer (2 mL) at pH 6.0 at 37 °C in a total volume of 3 mL. A control experiment contained tritiated amine and coenzyme but no enzyme. Aliquots (0.5 mL) of the solutions were removed after 36, 72, and 108 h. Each aliquot was adjusted to pH 2 with 6 M hydrochloric acid and was lyophilized. The tritium content of the solvent and the amine was determined by scintillation counting. The experiment was repeated with (1*S*)-[1-^3H]-3-(methylthio)-1-aminopropane.

Solvent Isotope Effect. Several similar incubations contained (2*S*)-[^{14}C]methionine (5–40 mM), enzyme (0.01 nmol, 7 units mg^{-1}), and PLP (0.02 mM) in 100 mM succinate and 100 mM phosphate buffer at pL 4.5, 5.5, and 6.5 in either protium or deuterium oxide, in a total volume of 200 μL at 37 °C. Reactions were initiated by the addition of enzyme and were followed by the radiochemical assay technique (Stevenson et al., 1990a). The determinations were repeated, the kinetic data were analyzed by the method of Eisenthal and Cornish-Bowden (1974), and the solvent isotope effects on V and V/K were calculated. The experiments were repeated at pL 5.5 with (2*S*)-valine and (2*S*)-leucine as substrates.

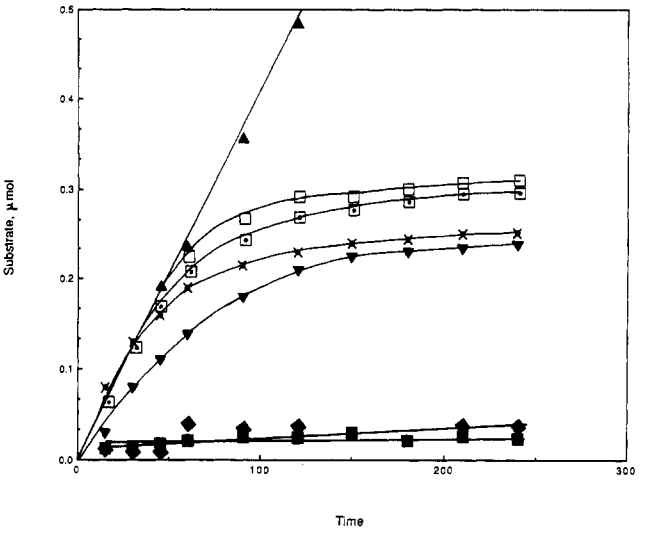


FIGURE 1: Time course for incubations containing substrate (methionine) and potential substrates for transamination. The concentration of the substrate was 20 mM. The preincubation of enzyme with potential substrate for transamination was conducted for 240 min. Reaction incubations contained no additive (□); 1 mM PLP (▲); 25 mM isobutylamine (⊡); 50 mM isobutylamine (◆); 100 mM isobutylamine (■); 25 mM isobutylamine, preincubated (▼); and enzyme preincubated with buffer (×).

Concentration and Substrate Structure Dependence of the Partition Ratio. Several, typically 15–21, identical incubations containing (2*S*)-[1-^{14}C]methionine (5 mM, 50 000 dpm) and enzyme (0.01 nmol, 7 units mg^{-1}, freed of unbound coenzyme) were set up in 100 mM succinate and 100 mM phosphate buffer at pH 5.5 in a total volume of 200 μL. One-third of the incubations contained PLP (1 mM) and were straight-line activity versus time controls. The remaining two-thirds contained no added coenzyme. After various time intervals, the reactions were quenched with sulfuric acid and scintillation counted in the usual way (Stevenson et al., 1990a). The entire experiment was repeated with 10, 20, and 50 mM methionine at pH 5.5 and with 2, 5, 10, and 20 mM methionine at pH 6.5. The rates of decarboxylation were determined from the straight-line controls, the rates of abortive transamination were determined from ln (enzyme activity) versus time plots, and the partition ratio was determined from the amount of substrate consumed at infinite time. Double-reciprocal plots, rate versus substrate concentration, were used to determine the apparent K_m for the substrate for decarboxylation and for abortive transamination.

The entire analysis was repeated for valine at pH 5.0 (at 10, 25, 50, and 75 mM), 5.5 (at 5, 10, and 20 mM), and 6.5 (at 2, 5, 10, and 20 mM) and for leucine at pH 5.5 (at 5, 10, and 20 mM) and 6.5 (at 5, 10, and 20 mM).

pH Dependence of the Partition Ratio. Several, typically 15–21, identical incubations containing (2*S*)-[1-^{14}C]methionine (20 mM, 50 000 dpm) and enzyme (0.01 nmol, 7 units mg^{-1}, freed of unbound coenzyme) were set up in 150 mM succinate and 150 mM phosphate buffer in a total volume of 300 μL. One-third of the incubations contained PLP (1 mM) and were straight-line activity versus time controls. The remaining two-thirds contained no added coenzyme. After various time intervals, the reactions were quenched with sulfuric acid and

scintillation counted in the usual way (Stevenson et al., 1990a). Each set of determinations was conducted at pH 4.5, 5.0, 5.5, 6.0, 6.75, and 7.5. Data were analyzed as described above for both straight-line and curved plots, and the decarboxylation and abortive transamination rates and their ratios were determined. The entire experiment was repeated with (2*S*)-[1-^{14}C]leucine and (2*S*)-[1-^{14}C]valine for the same pH range.

Deuterium Solvent Isotope Effects for Abortive Transamination. Incubations of the type described above were repeated in both protium and deuterium oxide in parallel experiments at pL 5.0, 5.5, 5.75, 6.0, 6.25, 6.5, and 7.0 for methionine and 5.0, 5.5, and 6.0 for valine and leucine, at 20 mM substrate. The solvent isotope effects on the rates of decarboxylation, the rates of abortive transamination, and the partition ratio were determined as described above.

Decarboxylation in 50% Deuterium Oxide. (2*S*)-Methionine (50 mg, 0.34 mmol) was incubated with enzyme (2 units, 7 units mg^{-1}) and PLP (1 mM) in succinate or phosphate buffer (200 mM) containing exactly 50 molar % deuterium oxide (allowing for exchangeable hydrogens) at pL 4.8 or 6.5 and 37 °C in a total volume of 10 mL. The partially deuteriated amine was isolated as the hydrochloride, as described above, and the deuterium content was assessed by integration of the C-1 proton signals in the 360-MHz ^{1}H NMR spectrum, with the remaining signals serving as an internal reference.

Results

Transamination of the Product Amine. The results of the incubation of the ^{35}S-labeled amine are shown in Table I. The total radioactivity associated with the hydrazone band (1123 dpm) is 20-fold higher than that for the background of the plate or for the control, but it represents the conversion of only 0.1% of the amine to aldehyde. Thus, the enzyme preparation appears to be able to catalyze the product-promoted transamination of the coenzyme but very slowly. It should be noted that the K_i for the amine, determined as a competitive inhibitor, is extremely high, >250 mM, whereas the concentration in the experiment was necessarily low, 5 mM; hence, the enzyme was far from saturated. The chiral C-1 tritiated 3-(methylthio)-1-aminopropanes released tritium to the solvent at the same rate as control experiments that contained PLP but no enzyme.

The results of the experiments in which the enzyme was incubated and/or preincubated with potential substrates for transamination are shown in Figure 1 and in Figures 1 and 2 in the supplementary material. At low concentration, neither isobutylamine nor 3-(methylthio)-1-aminopropane caused a reduction in the amount of substrate consumed at infinite time relative to controls. However, at higher concentrations, preincubation of the enzyme with isobutylamine caused complete inactivation. Dilution of the inactivated enzyme solution followed by the addition of PLP regenerated activity, and during the steady-state turnover of methionine in the presence of PLP, the enzyme was protected against inactivation. These results indicate that at high concentrations isobutylamine undergoes enzyme-mediated transamination.

Substrate Structure versus Partition Ratio. The apparent K_m values for methionine as derived from the abortive transamination rates are identical with the values derived from initial velocity measurements of decarboxylation at pH 5.5 and 6.5. The values of K_m obtained for valine from abortive rate data at pH 5.5, 6.0, and 6.5 are consistently lower than the values determined from initial velocity data. This result complicates the analysis of the mechanism and suggests that more than one intermediate may be able to partition away from the main reaction coordinate to give transamination products or that recombination of the product amine, isobutylamine, with the enzyme to give the quinoid intermediate occurs when valine is the substrate.

Table II: Partition Ratios (×10^5) for (2*S*)-Methionine, (2*S*)-Valine, and (2*S*)-Leucine over the pH Range 4.0–7.5[a]

pH	methionine	valine	leucine
4.0	1.22	>1.5	0.35
4.5	2.13	>8.4	0.41
5.0	2.01	1.04	0.47
5.5	1.71	0.96	0.41
6.0	1.45	0.89	0.41
6.75	>4.50	0.81	0.38
7.5	>5.00	0.66	0.42

[a] Several, typically 15–21, identical incubations containing (2*S*)-[1-^{14}C]methionine (20 mM, 50 000 dpm) and enzyme (0.01 nmol, 7 units mg^{-1}) free of unbound coenzyme were set up in 150 mM succinate and 150 mM phosphate buffer in a total volume of 300 μL. One-third of the incubations contained PLP (1 mM) and were straight-line activity versus time controls. The remaining two-thirds contained no added coenzyme. After various time intervals the reactions were quenched with sulfuric acid and scintillation counted in the usual way (Stevenson et al., 1990a). The values of the partition ratio presented here are derived from the amount of substrate consumed by the enzyme at t_∞. These values agreed well with the values of the partition ratio calculated from the rates of the catalytic and abortive reactions; see Table III.

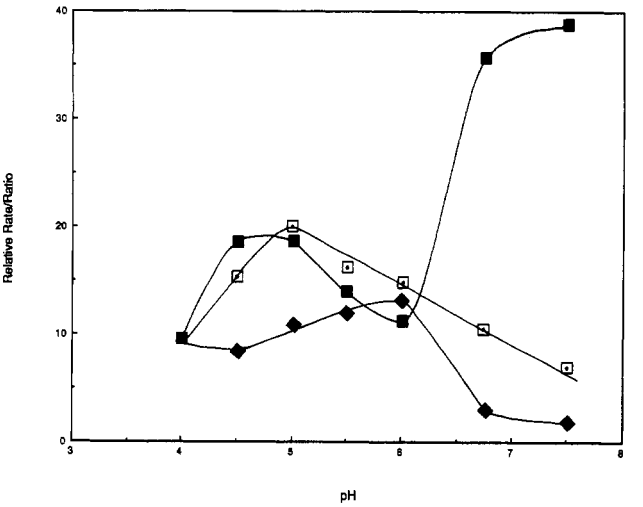


FIGURE 2: pH dependence of the normal reaction (⊡), the abortive reaction (◆), and the partition ratio, v_{am}/v_{ab} (■), with methionine as the substrate.

The partition ratio between normal decarboxylation and abortive transamination is generally lower for (2*S*)-methionine than for (2*S*)-leucine or (2*S*)-valine, which show similar partition ratios. Table II gives the calculated partition ratios for each substrate over the pH range 4.0–7.5, and Figure 2 depicts the pH dependence of the normal reaction, the abortive reaction, and the partition ratio (v_{am}/v_{ab}) for methionine. Figure 3 compares the pH dependence of v_{am}/v_{ab} with the dependence of V/K for methionine.

Solvent Isotope Effects. The decarboxylase showed deuterium solvent isotope effects on V/K and V of 3.4 and 1.46 at pL 4.5, 6.5 and 2.3 at pL 5.5, and 5.48 and 1.0 at pL 6.5 with (2*S*)-methionine as substrate. (2*S*)-Valine gave V/K and V isotope effects of 1.9 and 2.6 at pL 5.5 and 6.76 and 1.15 at pL 6.0, and (2*S*)-leucine showed effects of 2.5 and 1.0 at pL 5.5. Errors are within ±10–15% of the stated values.

The observed rates of decarboxylation and abortive transamination, and the partition ratios, at pL 5.0, 5.5, 5.75, 6.0, 6.25, 6.5, and 7.0 with methionine (20 mM) as substrate in both protium and deuterium oxide are tabulated in Table III.

Table III: Rates of Decarboxylation and Abortive Transamination and Partition Ratios with (2*S*)-Methionine, (2*S*)-Valine, and (2*S*)-Leucine as Substrates in both Protium and Deuterium Oxide[a]

	methionine			valine			leucine		
pL, solvent	V_{am}[b]	V_{ab}[c]	V_{am}/V_{ab}[d]	V_{am}[b]	V_{ab}[c]	V_{am}/V_{ab}[d]	V_{am}[b]	V_{ab}[c]	V_{am}/V_{ab}[d]
5.00, H	21.2	10.0	2.12	1.61	4.60	0.35	7.1	13.45	0.53
5.00, D	8.5	5.67	1.49	0.82	5.81	0.14	2.3	7.56	0.30
5.50, H	20.7	12.16	1.70	4.29	8.06	0.53	10.2	5.28	1.93
5.50, D	13.2	7.90	1.76	1.50	4.98	0.30	9.6	5.43	1.77
5.75, H	19.1	13.07	1.46						
5.75, D	15.8	7.79	2.03						
6.00, H	18.4	13.76	1.34	6.22	6.19	1.00	7.9	8.55	0.92
6.00, D	18.1	7.94	2.28	3.11	3.76	0.83	6.0	13.87	0.43
6.25, H	16.4	4.83	3.40						
6.25, D	13.2	5.24	2.52						
6.50, H	12.7	3.50	3.63	4.93	0.98	0.99	6.0	18.43	0.33
6.50, D	11.1	4.90	2.27						
7.00, H	9.4	2.01	4.68						
7.00, D	7.5	2.89	2.60						

[a] Incubations of the type described for Table I were repeated in both protium and deuterium oxide in parallel experiments at pL 5.0, 5.5, 5.75, 6.0, 6.25, 6.5, and 7.0 with methionine as the substrate and at selected pH values for valine and leucine. [b] 10^{-9} mol min^{-1}. [c] 10^{-14} mol min^{-1}. [d] 10^{5}.

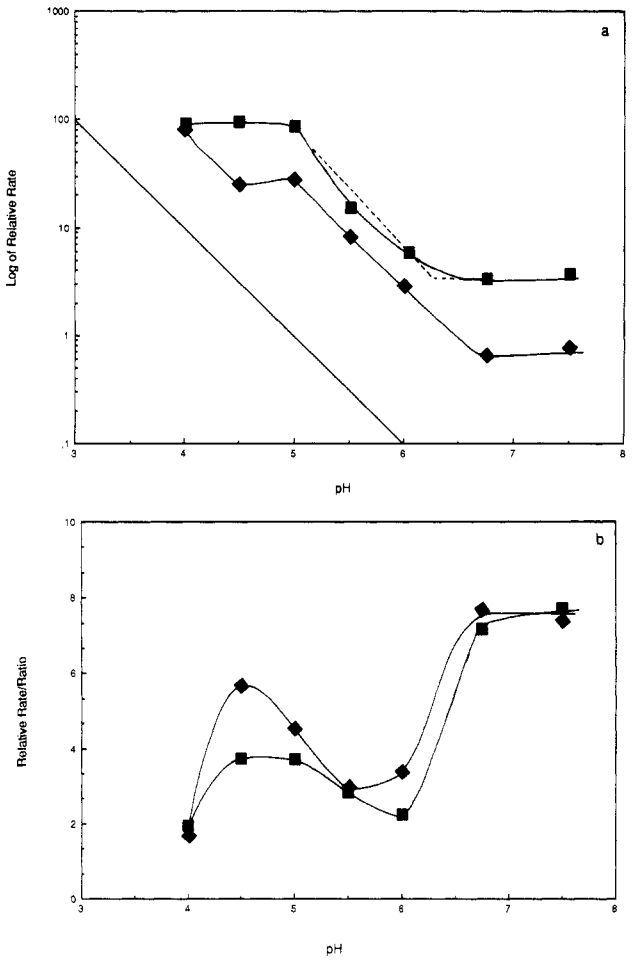


FIGURE 3: Comparison of pH dependencies of (a) log V_{app} (■) and log K_{app} (◆) [unit slope (—)] and (b) v_{am}/v_{ab} (■) and V/K (◆) with methionine as the substrate.

The corresponding data for valine and leucine at pL 5.0, 5.5, and 6.0 are also presented. The pH dependence of the deuterium solvent isotope effects for the observed rates of decarboxylation and transamination with methionine as substrate, as calculated from Table III, is depicted in Figure 4. The pK_a values derived from these results are discussed in the context of the kinetic scheme (Scheme III) proposed in Theory.

Product Amine Solvent Hydrogen Incorporation via Decarboxylation. Examination of the ^1H NMR spectrum of 3-(methylthio)-1-aminopropane isolated from incubations

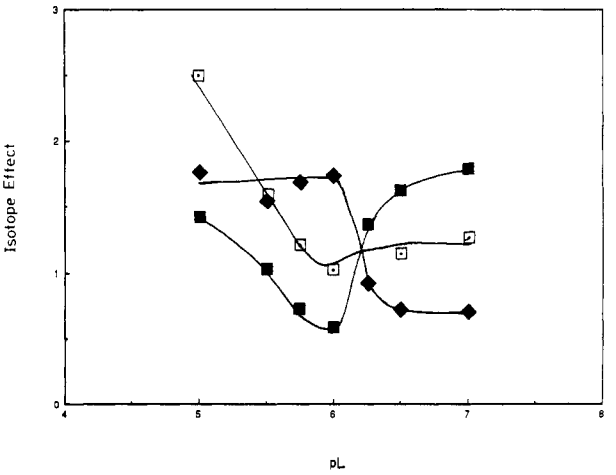


FIGURE 4: pH dependence of the deuterium solvent isotope effects for the observed rates of decarboxylation (⊡) and transamination (◆) and their ratio, v_{am}/v_{ab} (■) with methionine as the substrate.

conducted in 50 molar % deuterium oxide at pL 4.8 and 6.5 showed that 53% ± 5% of one of the C-1 methylene positions [the *pro-R* hydrogen; Stevenson et al., 1990a (first paper of three in this issue)] was labeled with deuterium. These results, in the light of the kinetic analysis of the reaction commitments discussed below, indicate that a monoprotic acid possessing a fractionation factor close to unity protonates the quinoid intermediate at C^{α}.

Data Processing. Incubations containing added coenzyme, typically 6–10 for each determination, were sequentially terminated by the addition of sulfuric acid. After all of the $^{14}CO_2$ had evolved, scintillant was added to the actual incubation vessel and the residual radioactivity was determined. Typically 80 000 dpm were present at the start of a kinetic run, and counting errors were less than 1%. The data were converted to moles and plotted against time. The data gave very shallow curves, and tangents to these were used to determine the initial velocities. These initial velocities served as controls for the determination of partition ratios and abortive transamination rates. Note that the product amine from methionine is not a reversible inhibitor for the decarboxylase and the curve obtained up to 50% substrate conversion resulted from substrate depletion only. Long incubations of up to 5 h duration, conducted in the presence of PLP, did not show a significant reduction in reaction rate that might have arisen through protein denaturation. The incubations containing gel-chromatographed holoenzyme, typically 9–14 of each, were treated

exactly as described above. The total incubation time was 5 or 6 h. The counting data were converted to moles and a time course was plotted. In most cases the amount of substrate consumed during the complete inactivation of the enzyme could be determined directly from the time course by drawing a horizontal tangent to the curve. In other cases, the amount consumed at infinite time was estimated and then later fitted as described below. The experiment was designed so that the amount of substrate consumed at infinite time, s_∞, was small compared to the total substrate, s_0. This ensured that curvature due to substrate depletion was minimal and that the loss of enzyme activity ($e_0 - e_i$), measured as the progression of substrate consumption ($s_\infty - s_t$), was first order with respect to enzyme. However, the experimental error is large where s_∞/s_0 is small, so that the reactions were allowed to progress to s_∞/s_0 = 6–20% [see Figure 3 in the supplementary material, for example]. The experimental data was then fitted to the equation:

$$\ln (s_\infty - s_t) = \ln (s_\infty) - {}^*k_{ab}t \quad (1)$$

which is derived from the rate equation for inactive enzyme (e_i) formation, $de_i/dt = {}^*k_{ab}(e_0 - e_i)$. Integration with respect to time and application of the limits yields $\ln [(e_0 - e_i)/e_0] = -{}^*k_{ab}t$, and so $(e_0 - e_i)/e_0 = \exp(-{}^*k_{ab}t)$.

The concentration of inactive enzyme cannot be measured directly. However, since the *enzyme activity* can be measured as the rate of substrate consumption ($v_{am} = e_0{}^*k_{dec}$), under conditions where the decarboxylation reaction is zeroth order with respect to substrate and first order with respect to enzyme, the rate of loss of enzyme activity will be reflected by the rate of change in the substrate decarboxylation rate. Thus, the substrate decarboxylation rate becomes $-ds/dt = (e_0 - e_i){}^*k_{dec} = e_0{}^*k_{dec} \exp(-{}^*k_{ab}t)$. Separation of the variables, integration, and then application of the limits gives

$$s_0 - s = s_t = -(e_0{}^*k_{dec}/{}^*k_{ab})[\exp(-{}^*k_{ab}t) - 1] \quad (2)$$

and, therefore, at very large times, $s_\infty = e_0{}^*k_{dec}/{}^*k_{ab}$. Substituting with s_∞ in eq 2 and rearranging gives $s_\infty - s_t = s_\infty \exp(-{}^*k_{ab}t)$, the exponential form of eq 1. ${}^*k_{ab}$ is the apparent first-order rate constant for the abortive reaction, and its reciprocal is a linear function of $1/[s_0]$ according to

$${}^*k_{ab} = k_{ab}[s_0]/(K_m' + [s_0]) \quad (3)$$

s_0, s_t, and s_∞ are the amounts of substrate present at $t = 0$ and the amount of substrate *consumed* at time t and at infinite time, measured in moles. The square-bracketed substrate terms are concentrations, and k_{ab} is the first-order rate constant for the abortive reaction. Plots of $\ln (s_\infty - s_t)$ versus t gave straight lines of slope ${}^*k_{ab}$ and intersected the y-axis ($t = 0$) at $\ln s_\infty$. No data quoted here gave regression factors of less than 0.985.

The value of V_{ab}, the rate of the abortive reaction at saturating substrate, was determined from double-reciprocal plots of the apparent inactivation rates v_{ab} versus [s] at pH 5.5 and 6.5. The value of K_m' for methionine, also determined from these plots (see Results) was identical with the value of K_m determined from initial velocity (v_{am}) versus [s] double-reciprocal plots at pH 5.5 and 6.5 in accordance with the predictions of the simple model; see Theory. Thus, for methionine:

$$v_{am} = e_0k_{am}[s]/(K_m + [s]) \quad (4)$$

$$v_{ab} = e_0k_{ab}[s]/(K_m' + [s]) \quad (5)$$

and, therefore, where $K' = K_m$:

$$v_{am}/v_{ab} = k_{am}/k_{ab} = R \quad (6)$$

where R is the partition ratio. The important result of this analysis is that the effect of substrate depletion on the observed rate v_{ab} is exactly matched by the decrease in the rate v_{am} due to substrate depletion, and the partition ratio, R, is concentration independent.

The fact that the curves behave essentially as pure first-order exponential decays of enzyme activity, even though a significant proportion of the starting amount of substrate (s_0) is consumed during the course of the reaction, can also be understood in terms of eq 5 by considering its time dependence. Since the abortive reaction obeys the Michaelis–Menten equation and since decarboxylation reaction products do not inhibit the enzyme, the effect of substrate depletion on the rate of the abortive reaction at any given time can be calculated; see eq 7. For methionine under the worst conditions, at low

$$v_{ab(t)} = e_0k_{ab}[s_0 - s_t]/(K_m' + [s_0 - s_t]) \quad (7)$$

pH (see Figure 3 for pH dependence of k_m) where k'_m = 20 mM, s_∞/s_0 = 0.1 and $[s_0]$ = 20 mM; the term $[s_0 - s_t]/(K_m' + [s_0 - s_t])$ varies from $20/(20 + 20) = 0.5$ at the start of the reaction to $18/38 = 0.474$ at t_∞; the error in assuming first-order behavior over several half-lives is less than 5%. Typically in the experiments described here this error is ~2%. Valine and leucine also gave curves that fit eq 1. Analysis in terms of eq 7 indicates that the error in assuming first-order behavior is low, largely because the frequency of the abortive event for these substrates is higher than for methionine and less substrate is consumed over the course of the reaction. Valine, and under some conditions leucine, does not give the same apparent K_m values when determined by each of the two methods, vide supra, and indeed K_m is larger than K'_m. Thus, the normal reaction and the abortive reaction are affected differently by substrate depletion and the partition ratio, R, becomes a variable *possibly* defined by

$$R_t = (v_{am}/v_{ab})_t = \frac{e_0k_{am}[s_0 - s_t]/(K_m + [s_0 - s_t])}{e_0k_{ab}[s_0 - s_t]/(K_m' + [s_0 - s_t])} = \frac{(k_{am}/k_{ab})(K_m' + [s_0 - s_t])}{K_m + [s_0 - s_t]} \quad (8)$$

Clearly, if K_m and K_m' are nonequivalent, the partition ratio will only be effectively constant over the course of the experiment if s_0 is very large compared to both K_m values and if s_t is very small. Fortunately, for both valine and leucine the rate constant for inactivation is ~10-fold higher than for methionine, so that s_∞ occurs at earlier s_t. However, the values of K_m are higher than those for K_m' and so the decarboxylation reaction is slowed more by substrate depletion than the abortive reaction. A further complication in the analyses of the kinetic data for these substrates is that the origins of the differences in the values of K_m are not known. The complex dependence of the values of K_m and K_m' on pH and on the solvent, together with the concentration-dependent nature of the partition ratio, limits the usefulness of the data obtained for valine and leucine. The discussion is, therefore, mainly concerned with the properties of the methionine system.

Discussion

In the preceding paper we reported that L-methionine decarboxylase from *D. filix-mas* catalyzed an occasional abortive decarboxylation–transamination reaction during sustained methionine turnover, which gave PMP and 3-(methylthio)-propionaldehyde as products (Stevenson et al., 1990a). It was noted that the frequency of the abortive event was sensitive to pH and to the level of product amine that was allowed to accumulate during the decarboxylation reaction. It was also noted that the amine was not an inhibitor for the decarbox-

ylation reaction and that on no occasion could enzyme-catalyzed tritium exchange from the solvent into the amine pool be demonstrated. We reasoned that the transamination of the coenzyme by the product amine through reverse steps might account for the enhanced frequency of abortive events in experiments in which the amine was allowed to accumulate (Porter et al., 1985). Furthermore, we suggested that this observation was not inconsistent with our own inability to demonstrate solvent hydrogen incorporation into the product pool since the conjugate acid formed after the abstraction of a proton from C^{α} of the external product aldimine intermediate might be both monoprotic and also shielded from the solvent (Stevenson et al., 1990a).

In this paper it is clearly demonstrated, by using [^{35}S]-3-(methylthio)-1-aminopropane, that the enzyme can labilize a proton from C^{α} of the external product aldimine to give the expected transamination products, albeit slowly. The low frequency of the product-promoted abortive transamination at low concentrations of the amine is not surprising given the high K_i values for the amines (Stevenson et al., 1990a), the fast rate of conversion of the external product aldimine through to product (see discussion below), and the high intrinsic value of the partition ratio. We are confident that this activity is not associated with a contaminating transaminase activity, since no solvent hydrogen incorporation into the amine pool was catalyzed by the purified enzyme or by acetone powders under very favorable conditions (Stevenson et al., 1990a) and neither of the two C-1 labeled enantiomers of [1-^{3}H]-3-(methylthio)-1-aminopropane showed any tritium washout to the solvent relative to controls. Transaminases are known to catalyze extremely rapid solvent hydrogen exchange with hydrogens in the labile positions of their substrates (Cooper, 1976; Gehring, 1984). Furthermore, the finding that high concentrations of product amines can cause complete inactivation of the decarboxylase holoenzyme cannot be accounted for by the presence of a contaminating transaminase or oxidase and indicates that the decarboxylase itself catalyzes the transamination of the coenzyme.

Given that L-methionine decarboxylase is able to remove a proton from the external product aldimine and having established that the occurrence of the transamination reaction is negligible in the absence of very high product amine concentrations, it was possible to examine the mechanism of the reactions catalyzed by the decarboxylase in detail. (Note that the actual deprotonation must occur more frequently than the actual transamination by a factor equal to the partition ratio.) Specifically, we wanted to verify that the conjugate acid was monoprotic (Stevenson et al., 1990a) and to determine its identity, if possible, from its molecular pK_a value.

Incubation of the enzyme with (2*S*)-methionine in 50 molar % deuterium oxide at pL 4.8 and 6.5 gave 3-(methylthio)-1-aminopropane that contained 53% deuterium in the *pro*-1*R* position. Since it is unlikely that a polyprotic acid such as the ϵ-ammonium group of a lysine residue would transfer protium and deuterium to the quinoid intermediate with equal facility, the result supports the notion that a monoprotic acid such as the imidazolium side chain of a histidine residue protonates the quinoid (Yamada & O'Leary, 1977). However, if the product off rate or some other step subsequent to protonation was rate limiting for the overall reaction, the quinoid protonation step might possess a high reverse commitment and, therefore, could be brought into equilibrium (Klinman, 1978). The equilibrium isotope effect for the protonation would be close to unity unless a thiol group acted as the proton donor (Schowen, 1978; Cleland, 1987). Hence, an estimate of the reverse commitment for the step for the protonation at C^{α} of the quinoid intermediate was required.

Three steps follow quinoid protonation: transaldimination, in two parts, and product amine release, as illustrated in Scheme III. Product release is the last step before the enzyme is recycled, and this step should not show a primary deuterium isotope effect since its transition state does not involve X–H bond cleavage. If the product release step was cleanly rate limiting, the solvent isotope effect on V_{max} for any step prior to product release would be suppressed toward unity. (Discussion concerning the magnitude of the isotope effect upon V/K is deferred since, for irreversible reactions, V/K isotope effects are not influenced by events that occur after the first irreversible step.) Thus, a simple test for a single question, avoiding the consideration of which transition state gives rise to the effect, would be to measure the magnitude of the solvent isotope effect for V_{max}. Accordingly, the deuterium solvent isotope effect was determined at a range of substrate concentrations at pL 5.0, 5.5, and 6.0; see Results. With methionine as substrate the values of ^{D}V were 1.46, 2.3, and 1.1, respectively, indicating that at low pH the product off rate is not *the* rate-determining step. However, in the absence of a knowledge of the quinoid intermediate C^{α} protonation rate and the subsequent transaldimination rate, it is not possible to complete the determination of the magnitude of the reverse commitment to step 7. To obtain information on these postdecarboxylation steps it is necessary to examine the partition ratio, but first we should establish why the deuterium solvent isotope effect on V_{max} is low and falls to 1.1 at pL 6.0 when the values for V/K are large.

High forward commitments suppress V/K isotope effects toward unity, and high reverse commitments suppress (or enhance) observed V/K isotope effects toward their equilibrium value (Cleland, 1987). Therefore, isotope effects on steps that occur after the first irreversible step, for example, steps 7 and 9, must possess very high forward commitments and should not influence V/K. The large size of $^{D}(V/K)$ for methionine at pL 5.0–6.0 coupled with the low ^{D}V values strongly suggests that the deuterium-sensitive step occurs prior to decarboxylation and has low forward and low reverse commitments but is not rate limiting for the overall decarboxylation reaction. The only steps that are expected to show deuterium isotope effects prior to decarboxylation are the two parts of the transaldimination reaction, step 3 in Scheme III.

We have already eliminated the possibility that product release is cleanly rate limiting, and indeed, on the basis of the high K_i values for the product and in view of the low forward commitment for the substrate (the reverse equivalent to the actual reverse commitment for the product), we would expect that product release is not rate limiting at all. Thus, another step must be responsible for the suppressed value of ^{D}V. This other step cannot be either protonation at C^{α} of the quinoid intermediate or external product aldimine transaldimination, since these steps, if partially rate limiting for the overall reaction, would be expected to enhance the observed value of ^{D}V. The only reasonable explanation is that the C–C bond cleavage step is rate limiting and that this step becomes completely rate determining above pH 5.5. Note that in chemical models C–C bond cleavage is much slower than transaldimination [see O'Leary (1988) for a recent review]. Examination of the pH dependence of V_{max} for methionine (Figure 3), and indeed, of leucine and valine also (Stevenson et al., 1990a), shows that the rate decreases rapidly by a factor of more than 30 above pH 5.1. Thus, it appears that the decarboxylation process requires a form of the enzyme–sub-

strate complex to be protonated, that the pK_a of the molecular acid is ~5.1, and that this step is rate limiting. The protonated pyridinium ring of the external substrate aldimine would be expected to titrate at ~pH 5.1 if it is not closely associated with the β-carboxylate group of an aspartate residue, as it is in aspartate aminotransferase (Kirsch et al., 1984) and (probably) in *Streptomyces* methionine decarboxylase (Stevenson et al., 1990b).

The large value of $^D(V/K)$ and the small value for ^DV, 5.5 and 1.1, respectively, at pL 6.0, put very tight constraints on the values for the rate constants for the isotopically sensitive step and the rate-limiting step that follows it. For the relevant part of Scheme III analyzed as outlined above, the equations relating the observed solvent isotope effects to the intrinsic value are

$$^D(V/K) = [^Dk_3 + k_3/k_2 + (k_4/k_5)^DK_{eq}]/(1 + k_3/k_2 + k_4/k_5)$$

and

$$^DV = [^Dk_3 + k_3/k_5 + (k_3/k_7)(1 + k_8/k_9) + (k_3/k_9)(1 + k_{10}/k_{11}) + (k_3/k_{11}) + (k_4/k_5)K_{eq}]/[1 + k_3/k_5 + (k_3/k_7)(1 + k_8/k_9) + (k_3/k_9)(1 + k_{10}/k_{11}) + (k_3/k_{11}) + k_4/k_5]$$

where the transaldimination processes are treated as a single isotopically sensitive step. The value of $^Dk_3$ should be in the range 6.5–7.0, and the forward commitment, k_3/k_2, must be near zero. If we assume that the fractionation factors for the species involved in the isotopically sensitive step are close to unity, which is reasonable if a thiol is not involved, $^DK_{eq}$ will be 1.0 and the reverse commitment, k_4/k_5, can be no larger than 0.2 in order to satisfy the observed value of $^D(V/K)$ of 5.5 at pL 6.0. O'Leary and co-workers obtained a value of 0.5 for k_4/k_5 for *E. coli* glutamate decarboxylase (O'Leary et al., 1981) and a value of 1.0 for *Morganella morganelli* histidine decarboxylase (Abell & O'Leary, 1988b), although they did not take account of any steps subsequent to carbon dioxide desorption in their kinetic analysis.

For ^DV, we already know that k_4/k_5 is low. Since k_7 and k_9 represent steps after the irreversible desorption of carbon dioxide that would show isotope effects if they were slow, both must be quite large, much larger than k_5. If this analysis is incorrect, it will come to light in the analysis of the isotope effects on the partition ratio; see below. The rate constant for the release of the amine product, k_{11}, could suppress the value of ^DV if it were small. However, the value of ^DV decreases from 2.3 to 1.1 from pL 5.5 to 6.0, where the rate of reaction decreases ~4-fold. At pL 5.0, ^DV is 1.5 and it seems unlikely that the product would be sticky at a pL value where V_{max} is about 10% of its optimum value and where a significant isotope effect is detected (see above). Hence, the term k_3/k_{11} is quite small and the most important term in the equation for ^DV is k_3/k_5. In order to satisfy the observed value of 1.1 for ^DV at pL 6.5, the minimum value of k_3/k_5 must be close to 20. Clearly, as the pL is lowered, the ratio decreases in value as the C–C bond-breaking process becomes less rate limiting and the observed value of ^DV increases. The analysis of the reaction coordinate profile up to the first irreversible step is now refined well enough to allow the partition mechanism to be tackled.

It is important in the interpretation of pH versus kinetic parameter curves that the above analysis allows the stickiness of the substrate to be calculated from the commitments. In general, sticky substrates and products cause the apparent pK_a value for a molecular acid to be displaced outwards (away from neutrality) from its real value by log $(1 + S'_r)$ (Cleland, 1986). If the substrates and products are not sticky, then we should expect to observe the true pK_a values of the molecular acids, which may be, after very careful consideration of the effects of the pH changes on kinetic parameters, assigned to specific species. Clearly, the pK_a values can still be displaced, but the effect will be due to the local protein environment [see Knowles (1976) for a critical review].

Cleland has defined the stickiness of a substrate according to the equation:

$$S'_r = (c_{f\text{-}ex} + c_{r\text{-}ex})/(1 + c_{f\text{-}in} + c_{r\text{-}in})$$

where $c_{f\text{-}ex}$, $c_{r\text{-}ex}$, $c_{f\text{-}in}$, and $c_{r\text{-}in}$ refer to the external and internal parts of the forward and reverse reaction commitments, respectively (Cleland, 1986). For a decarboxylase, where decarboxylation is irreversible, the factor $k_{carboxylation}/k_{CO_2,off}$ is zero (in Scheme III these steps are not shown separately but are incorporated in steps 5 and 6). Therefore, the reaction has no $c_{r\text{-}ex}$ and the only term in the numerator is $c_{f\text{-}ex}$. The external part of the forward commitment is the ratio k_3/k_2. At pL 6.0 the deuterium solvent isotope effect for V/K is near intrinsic and thus k_3/k_2 must be very small, probably less than 0.1. The values in the denominator, for example, $c_{f\text{-}in}$, k_5/k_4, may be quite large; note from above that the value of k_4/k_5 is ~0.2. Hence, the value of S'_r is small (its maximum value is ~0.1) and we can expect to observe the true pK_a values for molecular acids.

Before examining the partition mechanism in detail, it was important to check that the partition ratio was not concentration dependent. Any such dependence would severely complicate the analysis of the mechanism and would negate the implicit assumption in the simple model (see Theory) that only a single intermediate could leak away from the main reaction coordinate to give transamination products or that the protonation steps were irreversible. Fortunately, at pH 5.5 and 6.5, methionine gave values for K_m when calculated from the abortive transamination rate (determined from log plots) identical with those that were obtained from initial rate data for the decarboxylation reaction. Valine and leucine gave different values for the two types of K_m determination and therefore cannot be analyzed on the basis of the kinetic model set out in Scheme III. Nevertheless, some of the results obtained with valine and leucine are useful in analysing the mechanism for methionine. Since methionine showed the same saturation kinetics for both rates, all subsequent reactions could be conducted at a single concentration and the decarboxylation rate, the abortive transamination rate, and the partition ratio could be determined from the same series of miniincubations performed at the same time, hence minimizing experimental errors.

The pH dependence of the partition ratio is depicted in Figure 3. The similarity of the profile to that for V/K is striking. The fact that there is no gross change in the partition ratio just above pH 5.1, where the rate of decarboxylation reduces drastically (~30-fold), indicates that the slow step occurs before the partition can occur and strengthens the validity of our previous arguments. The small decrease with increasing pH that occurs here in both profiles is probably due to the effect of deprotonating the pyridinium ring on the rate of transaldimination of the holoenzyme Michaelis complex, in the case for V/K, and the rate of transaldimination of the external product aldimine, in the case of the partition ratio. Both processes are expected to be slightly slower when the electrophilicity of the aldimine carbon is reduced by deprotonation at N-1.

In both profiles the largest change occurs at ~pH 6.25. For the V/K profile this corresponds to an increase in V/K with

increasing pH, while for the partition ratio, the frequency of the abortive reaction decreases relative to the decarboxylation reaction with increasing pH. Recalling that the ϵ-ammonium group of a Lys residue is expected to protonate the quinoid intermediate at C-4′ to give transamination products, it seems strange that the rate of protonation at C-4′ should titrate at 3 pK_a units below the expected value. Under Theory we stated that the abortive reaction was so slow that it was reasonable to expect that the only transition state that needed to be considered was that for the actual protonation at C-4′ of the quinoid. If this reasoning is correct, then either the enzyme possesses a very low pK_a Lys residue or the titration is due to an increase in rate along the latter part of the reaction pathway for the normal reaction. On the basis of the similar (indeed, within experimental error, identical) values for the titration in the V/K profile, it is tempting to suggest that the latter part of the physiological reaction speeds up at ~pH 6.25 and hence causes a decrease in the partition toward transamination. Extreme caution must be exercised if we are to pursue this argument, since the two profiles are complementary in the information they provide. Recall that V/K is not affected by events that occur after the first irreversible step and that the partition ratio only gives information on the relative ease of the routes through the decarboxylation and transamination pathways after the first irreversible step. Thus, the same *actual* event cannot give rise to the two titrations, but similar events might. Considering that the decarboxylase reaction is symmetrical in appearance, at least with regard to transaldimination, vide supra, it is possible that the titrations are due to an increase in the rate of both transaldimination reactions. Nevertheless, it is difficult to imagine why this should occur at pH 6.2 in the partition ratio profile, where we would expect the most likely candidate for protonation at C^α of the quinoid, a His residue, to lose its proton and therefore slow down the reaction, unless the His residue also serves as a base in the transaldimination reaction.

The pH dependence of the decarboxylation and abortive transamination reactions were studied in parallel incubations in protium and deuterium oxide so that the deuterium solvent isotope effect could be determined on each rate and on their ratio; see Results. Before analyzing the results, it is useful to try to predict how the partition ratio will be affected by the deuteriated solvent. First, from the discussion before, it is evident that the sizable solvent isotope effect on the transaldimination of the external substrate aldimine, previously detected as an effect on V/K, should not affect the partition ratio. Likewise, any isotope effect on C–C bond cleavage, if one exists, will not affect the partition ratio. However, en route to the decarboxylation products, both the quinoid intermediate C^α protonation step and the two parts of the transaldimination step have deuterium-sensitive transition states and should reveal isotope effects on the partition ratio even if they were not detectable under steady-state conditions where C–C bond cleavage is rate-limiting. The transamination pathway is expected to show a deuterium solvent isotope effect also. We have argued on the basis of the slow rate of the reaction (see Theory) that the most important step on this pathway is quinoid intermediate C-4′ protonation. If we are correct, this step should show a large, near intrinsic isotope effect. Since it is expected that the ϵ-ammonium group of the Lys residue, with a pK_a value of ~9.5, provides the proton and that the pK_a of the relevant conjugate carbon acid is ~14–18, we can expect to observe a primary deuterium isotope effect of ~4–5 (Bordwell & Bolye, 1975), assuming deuterium fractionation factors of unity for both species. Thus, the deuterium solvent isotope effect on the partition ratio will reflect a balance between the isotope effect on the latter part of the decarboxylation process and the transamination process. When product debinding is the slowest step in these pathways, the partition ratio in deuterium oxide will decrease the frequency of the abortive events. When either protonation at C^α or transaldimination of the external product amine are the slowest steps, then the frequency of abortive events will directly reflect the size of the relevant isotope effects on both pathways and should be either unchanged or decreased relative to the situation in protium oxide.

Figure 4 shows the pL dependence of the isotope effects on the overall decarboxylation, the abortive reaction, and their ratio. Analysis of the partition ratio is most reliable, since it is not affected by the change in the value of K_m over the pL range of study and can be compared directly with the profile for the partition ratio in water, shown in Figure 3b. However, it is useful to look at all three isotope effects together. At pL 5.0 the isotope effect for the overall decarboxylation begins to falls from a value of ~2.5 to a value of ~1.1 at pL 6.0. This is in excellent agreement with the values obtained for V_{max}, vide supra. At pL 6.25 the isotope effect for the abortive rate decreases from ~1.7 to ~0.7 with increasing pH, indicating that the pathway leading to the formation of the product amine is expressing a larger isotope effect above pL 6.0 than the transamination pathway by a factor of ~1.43. Below pL 6.25 the transamination pathway is expressing an isotope effect ~1.5 times that for the latter part of the physiological reaction pathway.

The partition ratio is not sensitive to isotope effects that occur prior to the formation of the quinoid intermediate, and its profile titrates at ~pL 5.5 and 6.25. The change at pL 5.5, which corresponds with a decrease in the observed isotope effect, indicates that a step on the normal reaction pathway which is not isotopically sensitive, or which is at least less sensitive than the step for abortive transamination, has increased importance. The effect results in protection against abortive transamination in deuterium oxide compared to protium oxide and may be derived from the slowing of the nucleophilic attack on the external product aldimine by the Lys side chain due to the deprotonation of the pyridinium ring. Note that from pL 5.0–6.0 the isotope effect for abortive transamination does not change, whereas, as noted previously, the isotope effect for decarboxylation decreases by a factor of ~2.2, clearly confirming that for the overall reaction the rate-limiting processes occur before carbon dioxide desorption.

The titration that occurs at pL 6.25 in the profile for the isotope effect for the partition ratio corresponds to an increase in the kinetic importance of a deuterium-sensitive step in the conversion of the quinoid intermediate to the product amine relative to the conversion of the quinoid to PMP.

The value of the titration is highly significant and is in keeping with the idea that the imidazolium side chain of a histidine residue serves to protonate the quinoid intermediate at C^α. Thus at pL 6.2 and above, the protonation at C^α of the quinoid intermediate is kinetically important in the postdecarboxylation portion of the reaction coordinate profile and is not followed by slow steps that could bring it into equilibrium. Therefore, for the reactions conducted in 50 molar % deuterium oxide at pL 6.5 (see Results), the extent of deuterium incorporation into the product amine should reflect partitioning between deuteration at C^α and protonation at C^α. The deuterium content of the solvent will be matched by the partitioning only if the conjugate acid is monoprotic. A polyprotic acid would supply a proton to the quinoid more

readily than a deuteron, by a factor equivalent to the size of the isotope effect, so that the deuterium incorporation into the amine would be much less than that in the bulk solvent. The isolated amine contained 53% deuterium in the *pro*-1*R* position, confirming that a monoprotic conjugate acid operates at C^{α} of the quinoid intermediate.

Given that the imidazolium side chain of an active-site His residue with a pK_a value of ~6.25 serves to protonate the quinoid intermediate at C^{α} on the 4′-*si* face of the coenzyme, the same face on which the Lys residue is disposed, the two conjugate acids must be close in space and might be expected to interact. The finding that, in protium oxide, both V/K and the decarboxylation:transamination partition ratio increase at ~pH 6.25 suggests that the His residue might serve an additional function. Since the ϵ-ammonium group of the Lys residue must act as a nucleophile in the first step of product transaldimination, the step that immediately follows quinoid C^{α} protonation, and must first lose a proton, it seems quite reasonable that the nascent neutral imidazole ring should act as the base in the transaldimination reaction. This would account for the increase in the partition ratio at pH ~6.25. The magnitude of a postirreversible step cannot affect V/K, and it must be assumed, therefore, that the titration in the V/K profile is due to a similar process operating on the transaldimination step in the formation of the external substrate aldimine.

Overview of the Mechanism. The results of experiments with fern L-methionine decarboxylase described in this and the previous paper indicate the following points: (1) The PLP-dependent fern L-methionine decarboxylase catalyzes the decarboxylation of its substrates with retention of configuration at C^{α}. (2) The proton donor for the quinoid intermediate at C^{α} is probably the imdiazolium side chain of a His residue. (3) The abortive transamination of the coenzyme is slightly reversible and occurs on the 4′-*si* face, similar to the reaction catalyzed by the transaminases. (4) The proton donor at C-4′ is probably the ϵ-ammonium group of the active-site Lys residue that forms part of the internal aldimine. (5) The two bases/conjugate acids are on the same face of the coenzyme and interact. (6) The His residue serves as a base in both of the transaldimination reactions. (7) At pH 6.0, C–C bond cleavage is the slowest step and the deprotonation of the pyridinium N-atom in the external substrate aldimine determines the magnitude of V_{max}. (8) The substrates and products are not sticky, so that the measured pK_a values should reflect the true molecular acid dissociation constants. The titration of V_{max} gives a value of 5.1, which indicates that the pyridinium proton is not associated with the β-carboxyl group of an aspartate residue as it is in aspartate aminotransferase.

Efforts toward establishing whether these features are general for the decarboxylases, with the goal of comparing the two major PLP-dependent groups, decarboxylases and transaminases, are underway in our laboratory.

Acknowledgments

We thank the SERC for financial support and the Royal Society for a Royal Society University Fellowship to D.G. We are grateful to Prof. Dominic Tildesley for useful discussion.

Supplementary Material Available

Figures 1 and 2, showing the time course for incubations containing substrate (methionine) and potential substrates for transamination, and Figure 3, showing typical progress curves of the abortive reaction for methionine, valine, and leucine in the presence and absence of added PLP (3 pages). Ordering information is given on any current masthead page.

References

Abell, L. M., & O'Leary, M. H. (1988a) *Biochemistry 27*, 3325–3330.
Abell, L. M., & O'Leary, M. H. (1988b) *Biochemistry 27*, 5927–5933.
Bordwell, F. G., & Bolye, W. J. (1975) *J. Am. Chem. Soc. 97*, 3447–3452.
Braun, W., Herron, J. T., & Kahaner, D. (1986) *ACUCHEM, A computer program for modelling complex reaction systems*, National Bureau of Standards, Gaithersburg, MD.
Choi, S. Y., & Churchich, J. E. (1986) *Eur. J. Biochem. 160*, 515–520.
Cleland, W. W. (1986) in *Investigation of Rates* and *Mechanisms of Reactions* (Bernasconi, C. F., Ed.) Part 1, p 791, Wiley-Interscience, New York.
Cleland, W. W. (1987) *Bioorg. Chem. 15*, 283–302.
Cooper, A. J. L. (1976) *J. Biol. Chem. 251*, 1088–1096.
Cronin, C. N., & Kirsch, J. F. (1988) *Biochemistry 27*, 4572–4579.
Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.
Fonda, M. L. (1972) *Biochemistry 11*, 1304–1309.
Gehring, H. (1984) *Biochemistry 23*, 6335–6340.
Grant, P. L., Basford, M., & John, R. A. (1987) *Biochem. J. 241*, 699–704.
Julin, D. A., & Kirsch, J. F. (1989) *Biochemistry 28*, 3825–3833.
Julin, D. A., Heinrich, W., Toney, M. D., & Kirsch, J. F. (1989) *Biochemistry 28*, 3815–3821.
Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. (1984) *J. Mol. Biol. 174*, 497–525.
Klinman, J. P. (1978) in *Transition States in Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 165, Plenum Press, New York.
Knowles, J. R. (1976) *CRC Crit. Rev. Biochem. 4*, 165–173.
Mandeles, S., Koppelman, R., & Hanke, M. E. (1954) *J. Biol. Chem. 209*, 327–332.
Minelli, A., Charteris, A. T., Voltattorni, C. B., & John, R. A. (1979) *Biochem. J. 183*, 361–368.
O'Leary, M. H. (1977) in *Bioorganic Chemistry: Enzyme Action* (van Tamelen, E. E., Ed.) Vol. 1, p 259, Academic Press, New York.
O'Leary, M. H. (1978) in *Transition States in Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 285, Plenum Press, New York.
O'Leary, M. H. (1988) *Acc. Chem. Res. 21*, 450–455.
O'Leary, M. H., & Baughn, R. L. (1977) *J. Biol. Chem. 252*, 7168–7173.
O'Leary, M. H., Richards, D. T., & Hendrickson, D. W. (1970) *J. Am. Chem. Soc. 92*, 4435–4440.
O'Leary, M. H., Yamada, H., & Yapp, C. J. (1981) *Biochemistry 20*, 1476–1481.
Porter, T. G., Spink, D. C., Martin, S. B., & Martin, D. L. (1985) *Biochem. J. 231*, 705–712.
Schowen, K. B. J. (1978) in *Transition States in Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 225, Plenum Press, New York.
Stevenson, D. E., Akhtar, M., & Gani, D. (1990a) *Biochemistry* (first paper of three in this issue).
Stevenson, D. E., Akhtar, M., & Gani, D. (1990b) *Biochemistry* (third paper of three in this issue).
Sukhareva, B. S. (1986) in *Vitamin B_6 Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects* (Dolphin, D.,

Poulson, R., & Avramovic, O., Eds.) Part B, p 325, Wiley-Interscience, New York.
Sukhareva, B. S., & Braunstein, A. E. (1971) *Mol. Biol. (Engl. Transl.)* *5*, 302–317.
Sukhareva, B. S., Dunathan, H. C., & Braunstein, A. E. (1971) *FEBS Lett.* *15*, 241–244.
Tikhonenko, A. S., Sukhareva, B. S., & Braunstein, A. E. (1968) *Biochim. Biophys. Acta* *167*, 476–479.
Yamada, H., & O'Leary, M. H. (1977) *J. Am. Chem. Soc.* *99*, 1660–1661.

Streptomyces L-Methionine Decarboxylase: Purification and Properties of the Enzyme and Stereochemical Course of Substrate Decarboxylation†

David E. Stevenson, Mahmoud Akhtar, and David Gani*,‡
Department of Chemistry, Institute of Biomolecular Science, The University of Southampton, Southampton SO9 5NH, U.K.
Received October 12, 1989; Revised Manuscript Received March 28, 1990

ABSTRACT: L-Methionine decarboxylase from *Streptomyces* species ATCC 21020 has been purified to near homogeneity, characterized, and compared to the enzyme from the fern *Dryopteris filix-mas* [Stevenson, D. E., Akhtar, M., & Gani, D. (1990) *Biochemistry* (first paper of three in this issue)]. The enzyme catalyzes the decarboxylation of a range of alkylamino acid substrates, but the substrate specificity is different from that for the fern enzyme. In accord with the properties of the fern enzyme, the *Streptomyces* enzyme is also a homodimer of M_r 100 000 ± 5000 and requires PLP for activity. At low pH, where the value of V_{max} for both enzymes is maximal and essentially pH independent, k_{cat} for the *Streptomyces* enzyme with (2*S*)-methionine as substrate is slightly higher (60 s^{-1}) than the value for the eukaryotic protein (50 s^{-1}). The pH optimum for V/K is much higher than that for the fern enzyme although many features of the pH dependence are similar, including the shape of the curve for the pH dependence of K_m. When the decarboxylations of (2*S*)-methionine, (2*S*)-norleucine, and (2*R*)-*S*-ethyl-L-cysteine were conducted on a preparative scale in protium and deuterium oxide, unlabeled and deuteriated amines were formed. ^{1}H NMR spectroscopic analysis of the stereochemistry at C-1 of the camphanamide derivatives of the products [Stevenson, D. E., Akhtar, M., & Gani, D. (1990) *Biochemistry* (first paper of three in this issue)] indicated that each conversion was stereospecific and occurred with retention of configuration at C-2 of the substrates. The *Streptomyces* enzyme does not catalyze abortive transamination reactions and is unable to catalyze the incorporation of tritium from the solvent into the product amine or into (2*R*)-methionine. A monoprotic acid appears to serve as the proton donor in the conversion of the quinoid intermediate to the external product aldimine during decarboxylation. The differences and similarities of the two enzymes are discussed in mechanistic terms.

Dryopteris filix-mas L-methionine decarboxylase catalyzes the decarboxylation of L-methionine and a range of alternative alkylamino acids to give amines and carbon dioxide. The enzyme also catalyzes an occassional abortive transamination event in which the coenzyme is converted to PMP[1] and the amine, prior to release as product, is converted to an aldehyde (Stevenson et al., 1990). Analysis of the mechanism and the stereochemical courses of reactions catalyzed by the decarboxylase (Stevenson et al., 1990) indicated that the enzyme shared many common features with transaminase enzymes. For example, for fern methionine decarboxylase, it was apparent that the coenzyme was protonated at C-4′ on the 4′-*si* face quinoid intermediate derived from the physiological substrate during the abortive transamination reaction and that a polyprotic base, probably the ϵ-ammonium group of the active-site aldimine-forming lysine residue, served as the proton donor. Furthermore, from the retentive mode of decarboxylation and the high chiral integrity of the C-2 deuteriated products derived from the decarboxylation of a range of substrates, it appeared that decarboxylation (carbanion/quinoid intermediate generation) occurred on the same (4′-*si*) face of the coenzyme. This chemistry exactly parallels the well-established mode of catalysis by transaminases (Kirsch et al., 1984).

In order to extend our understanding of the PLP-dependent enzymes as a group and, in particular, to identify common mechanistic and structural features, we wished to compare the chemistry of the fern enzyme to a similar broad substrate specificity enzyme from an evolutionarily distant species. Hagino and Nakayama (1968) described an L-methionine decarboxylase activity in *Streptomyces* strains grown in the presence of L-methionine. Several years later the enzyme was purified 582-fold, in 5% yield, and characterized (Misono et al., 1980). The reported properties of the enzyme indicated that the system was similar to the fern enzyme in many re-

† This work was supported by Science and Engineering Research Council Grants GR/D-21202 and GR/E-73512 to D.G. and a studentship to M.A.
* To whom correspondence should be addressed at the Department of Chemistry, The Purdie Building, The University, St. Andrews, Fife KY16 9ST, Scotland, U.K.
‡ Royal Society University Fellow, 1983–1988.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; NMR, nuclear magnetic resonance; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DEAE, (diethylamino)ethyl; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; AAT, aspartate aminotransferase; pL, -log [L^+] (Schowen, 1978).

0006-2960/90/0429-7660$02.50/0