Stereochemical Imperative in Enzymic Decarboxylations. Stereochemical Course of the Decarboxylation Catalyzed by Acetoacetate Decarboxylase

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Abstract: The stereochemical course of the decarboxylation of acetoacetate catalyzed by the enzyme acetoacetate decarboxylase (AAD) has been studied by using samples of optically active 2-tritioacetoacetate, prepared by enzymatic oxidation of samples of enantiomeric pairs of diastereomeric 2-tritio-3-hydroxybutyrates. A correlation is proposed connecting the stereochemical course of enzymatic decarboxylation (retention or inversion) with the structure of the substrate. Acetoacetate decarboxylase was found to catalyze decarboxylation with net racemization.

The stereochemical course of an enzymic reaction is determined by the geometrical arrangement of substrates and amino acid residues in the active site. This arrangement presumably arises from evolutionary pressures selecting for enzymes that best contribute to the survival of their host organism.[1-3] Thus, stereochemical choices made by enzymes are likely to reflect underlying principles of protein structure and catalysis and their role in adaptation and evolution. An important theme in recent work of ours and others is to explore how the stereochemical details of enzymic reactions might reflect underlying catalytic and structural principles that explain in evolutionary terms how stereochemical choices made by enzymes came to be.[2,3]

A common stereochemical course is often shared by different enzymes catalyzing similar reactions. For example, all aliphatic hydroxylases proceed with retention of configuration; all enzymes using pyridoxal phosphate catalyze the transfer of the *pro-R* hydrogen of pyridoxamine.[2] In these uniformities, several authors have seen an underlying catalytic principle. For example, Hanson and Rose[2] recently suggested that there may be an optimal stereochemical course for particular classes of reactions and that stereochemical similarities in enzymes catalyzing reactions in each class reflect convergent evolution to conform to this "mechanistic imperative".

An alternative to this teleological explanation for stereochemical uniformities within a mechanistic class is a historical explanation, also noted by Hanson and Rose.[2] If all modern enzymes catalyzing a certain type of reaction evolved from a common ancestral enzyme displaying a particular stereochemical preference, a common preference in all modern enzymes might reflect the conservation of the original preference during the evolutionary process.

This pair of explanations is merely a molecular example of a problem frequently encountered in biology: are the structural features common in different species best understood as arising by convergent evolution direct toward a purpose or by divergent evolution from a common precursor with conservation of details?

This question has special importance in those classes of enzymes where stereochemical heterogeneity, not uniformity, is the rule. Hanson and Rose[2] note two such classes: dehydrogenases dependent on nicotinamide cofactors, where some enzymes transfer the *pro-R* hydrogen of NADH while others transfer the *pro-S* hydrogen, and β-keto acid decarboxylases, where some enzymes produce inversion of configuration at the decarboxylating center, while others produce retention. In classes of enzymes not displaying a uniform stereochemical choice, it is difficult to ascribe either a mechanistic or evolutionary rationale to the choices observed. The temptation therefore exists to describe stereochemical choices made by these enzymes as "random". However, this "random" appearance may only be an indication of how little we know about the factors governing the stereoselectivity of these enzymes.

Table I. Substrates, Stereoselectivities, and Requirements for Metal Ions in Decarboxylases

enzyme	EC no.	α-keto acid substrate	stereoselectivity	metal ion?	ref
isocitrate dehydrogenase ($NADP^+$)	1.1.1.42	yes	retention	yes	22
isocitrate dehydrogenase (NAD^+)	1.1.1.41	yes	retention	yes	23
malic enzyme	1.1.1.40	yes	retention	yes	24
phosphogluconate dehydrogenase	1.1.1.44	no	inversion	no	25
UDP glucuronate decarboxylase	4.1.1.35	no	inversion	no	26
acetolactate decarboxylase	4.1.1.5	no	inversion	no	27

We recently suggested that the stereochemical choices made by dehydrogenases can be explained by assuming four mechanistic hypotheses[3] and that the stereochemical heterogeneity reflects enzymes adjusting the redox potential of a bound cofactor to achieve a desired free energy profile. The second class of enzymes, the β-decarboxylases, is the subject of this report.

The stereochemical preferences of six enzymes catalyzing the decarboxylation of β-keto acids are known (Table I). Three produce retention of configuration; three produce inversion of configuration. These results were interpreted by Hanson and Rose to mean that β-decarboxylases choose their stereochemical course "randomly".[2]

However, from studies of reactions in solution that model enzymic decarboxylation, at least two mechanisms for catalyzing β-decarboxylation are known. First, metal ions chelated to an α-keto acid unit may act as an "electron sink", facilitating decarboxylation.[4] The decarboxylation of oxaloacetate catalyzed by divalent manganese apparently proceeds via such a mechanism.[4] Alternatively, decarboxylation can be catalyzed by amines, via an intermediate Schiff's base.[5] Presumably, the protonated Schiff's base acts as an "electron sink".

Both forms of catalysis are apparently exploited by enzymes, depending on the structure of the substrate. If the substrate possesses an α-keto acid moiety capable of coordinating a metal ion, a metal ion is apparently required for enzyme-catalyzed decarboxylation[6] (Table I). Whenever the substrate does not bear

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(2) (a) Rose, I. A.; Hanson, K. R. *Crit. Rev. Biochem.* **1973**, *1*, 33–60; (b) *Acc. Chem. Res.* **1975**, *8*, 1–10.

(3) (a) Benner, S. A. *Experientia* **1982**, *38*, 633–637. (b) Nambiar, K. P.; Stauffer, D. M.; Kolodziej, P. A.; Benner, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 5886–5890.

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an α-keto acid unit (and presumably cannot coordinate a metal ion in a fashion productive to catalysis), a metal ion is apparently not required.

This observation permits a correlation to be drawn from the data in Table I. *Whenever a metal ion is required for catalysis, the decarboxylation proceeds with retention* of configuration, while *whenever a metal ion is not required, decarboxylation proceeds with inversion* of configuration. *The two stereochemical modes observed in β-decarboxylases appear to reflect the existence of two mechanisms for enzyme-catalyzed decarboxylation.* As a working hypothesis, this correlation predicts the stereochemical preference of any β-decarboxylase whose substrate is known.

The enzyme acetoacetate decarboxylase (AAD) appears to be an opportune system for testing this hypothesis. No metal is required for enzymic catalysis; rather, a Schiff's base is known to be an intermediate in the enzymatic conversion of acetoacetate to acetone and carbon dioxide by AAD.[7] Therefore, our hypothesis predicts that AAD catalyzes decarboxylation with inversion of configuration. However, studies on the decarboxylation of the substrate analogue cyclohexane-2-carboxylate found that retention was the predominant, but not the exclusive, stereochemical pathway.[8] Since the stereochemical course of an enzymatic reaction of an unnatural substrate need not be the same as for the natural substrate, and because the stereochemistry was not cleanly retention, we decided to determine the stereochemical course of the reaction catalyzed by AAD on its natural substrate, acetoacetate.

Determining the stereochemical course of the decarboxylation of acetoacetate catalyzed by AAD is complicated by two factors. First, because the decarboxylation converts a methylene into a methyl group, three isotopes of hydrogen must be used to make the methyl group isotopically chiral. Furthermore, as acetoacetate enolizes at 22 °C at pH 6.4 with a half-life of approximately 1.5 min, the doubly labeled isotopically chiral starting materials (e.g., (*R*)- and (*S*)-2-deuterio-2-tritioacetoacetate) are extremely difficult to obtain.

We report here the solution to these problems and the surprising conclusion that AAD catalyzes the decarboxylation with 50 ± 5% retention and 50 ± 5% inversion.

Experimental Section

Preparation of Labeled 3-Hydroxybutyrates. *E* and *Z* enol acetates **1** and **2** were prepared by the reaction of acetyl chloride with ethyl acetoacetate in pyridine.[9] To a solution of ethyl acetoacetate (1.32 g, 0.010 mol) in 3 mL of dry pyridine under an argon atmosphere at 0 °C was added acetyl chloride (1.17 g, 0.015 mol) dropwise with stirring. The reaction mixture was allowed to warm to room temperature and stand for 12 h. The solid product was then extracted 3 times with 15 mL of anhydrous ether, and the combined extracts were washed with water and then with saturated aqueous $CuSO_4$ to remove pyridine. The solution was dried over anhydrous $MgSO_4$, and the ether was removed by evaporation to yield an orange-red liquid containing a 70:30 mixture of the *E* and *Z* enol acetates. These were separated by preparative thin-layer chromatography on silica gel (methylene chloride eluant). The higher R_f band corresponded to the *E* isomer, and the lower R_f band corresponded to the *Z* isomer as identified by proton NMR.[9]

Using the custom labeling service of New England Nuclear, the *cis*- and *trans*-3-acetoxycrotonates were hydrogenated separately with isotopically pure T_2 gas in dry tetrahydrofuran over a 5% rhodium/carbon catalyst (Englehard) (Figure 1). One of us has previously shown this hydrogenation yields stereospecifically cis addition of hydrogen to the double bond.[10] The product ethyl 3-acetoxybutyrates were analyzed by tritium NMR (New England Nuclear) to confirm their structures.

The separate samples of tritiated ethyl 3-acetoxybutyrates were hydrolyzed in methanolic NaOH (10 mg NaOH in 5 mL of methanol) and purified after acidification to pH 3 by ion-exchange chromatography on a Bio-Rad AG 1 X8, eluting with 1 N HCl_2H. Lyophilization left the 2,3-ditritio-3-hydroxybutyric acids, which were converted to sodium salts by titration with NaOH. Because the hydrogenation proceeds with cis stereochemistry, (*E*)-3-acetoxycorotonate yields a racemic mixture of 2(*S*)-tritio-3(*R*)-tritio-3-hydroxybutyrate (**3**) and 2(*R*)-tritio-3(*S*)-tritio-3-hydroxybutyrate, (**4**). Hydrogenation of the *Z* isomer yields a racemic mixture of 2(*R*)-tritio-3(*R*)-tritio-3-hydroxybutyrate (**5**) and 2(*S*)-tritio-3(*S*)-tritio-3-hydroxybutyrate (**6**) (Figure 1).

Isolation of Acetone from the Coupled Enzymatic Reaction and Its Conversion to Acetate. 3-Hydroxybutyrate dehydrogenase (3-HBDH) is known to catalyze the oxidation of only 3(*R*)-hydroxybutyrate to acetoacetate.[11] Therefore, as the cis tritiation introduced label at the 2-position of hydroxybutyrate with known stereochemistry relative to the chemical stereochemistry at the 3-position, enzymatic oxidation of a mixture of 2-tritiated hydroxybutyrates would yield (*S*)-2-tritioacetoacetate (**7**), if the hydroxybutyrate originated from the (*E*)-corotonates, and (*R*)-2-tritioacetoacetate (**8**), if the hydroxybutyrates originated with the (*Z*)-crotonate (Figure 1). These labeled acetoacetates were prepared in situ in the presence of large excess of AAD, so that they were rapidly converted to acetone and carbon dioxide.

The potassium phosphate buffers, labeled 3-hydroxybutyrate, and the NAD^+ were dissolved separately in D_2O, the pH was adjusted to 6.5, and the solutions were twice lyophilized to replace exchangable protons with deuterium. The enzymes were centrifuged from suspensions in ammonium sulfate, resuspended in previously equilibrated ammonium sulfate/D_2O solutions, and recentrifuged.

To a mixture of NAD^+ (25 mg) in 3.0 mL of potassium phosphate buffer in D_2O (pH readjusted to 6.5) where added 20 mg of AAD (20 000 units) and 1.0 mg of 3-HBDH (14 units). The reaction mixture was contained in a two-neck round-bottom flask equipped with a stir bar, a condenser coodled with ice water, and a serum cap over one of the two necks. From the top of the condenser, a short length of rubber hose led to a solution of bromine (1.0 g) and sodium hydroxide (3.0 g/10 mL). The reaction was initiated by injection via syringe of labeled hydroxybutyrate (0.8 mg, 300 mCi) below the surface of the liquid. The mixture was incubated at room temperature for 5 min, at which time carrier acetone (10 μL) was added by syringe through the serum cap, and nitrogen was bubbled through the solution and into the alkaline bromine solution for 5 min.

After a half hour, the bromoform reaction was quenched with excess sodium metabisulfite and the mixture acidified to pH 1.5 with 85% phosphoric acid. Acetic acid was distilled from the mixture under reduced pressure to provide acetic acid. After three such distillations, the aqueous acetic acid, contaminated with HBr and sulfurous acid, which inevitably codistilled, was titrated to pH 7 with sodium hydroxide and lyophilized. Radioactive yields were 3–5% based on starting 3-hydroxybutyrate.

The acetate was converted to the *p*-phenylphenacyl ester by reaction in refluxing ethanol with *p*-phenylphenacyl bromide (2-fold molar excess, 2 h). The derivative was twice purified by chromatography on 0.25-mm silica gel plates (CH_2Cl_2 as eluant) and extracted with anhydrous ether. The homogeneous radioactive *p*-phenylphenacyl ester was then hydrolyzed in methanolic KOH, the reaction mixture acidified with phosphoric acid, and the acetic acid distilled as before. The product acetic acid was neutralized with NaOH and lyophilized to yield sodium acetate, which was stored at −20 °C.

Exchange Reactions. Rates of exchange of acetoacetate were followed by NMR in D_2O, in phosphate buffers (50–200 mM) at room temperature.

Exchange of acetone (with NaOD in D_2O) and hexadeuterioacetone (with NaOH in H_2O) under conditions of the bromoform reaction were followed both by mass spectroscopy on acetone and also by mass spectroscopy on isolated acetate samples. The acetates were isolated by acidification of the reaction mixture with phosphoric acid, low-temperature bulb-to-bulb distillation, titration to form sodium acetate, lyophilization, and conversion to crystalline *p*-phenylphenacyl acetate derivatives as described above.

In further control experiments, acetone was generated in D_2O from unlabeled 3-hydroxybutyrates under the same experimental conditions used for the tritiated samples and described above. Acetone was transfered in a stream of nitrogen without carrier directly into a solution containing Br_2 and NaOD. The acetic acid formed was purified as described above and analyzed by mass spectroscopy.

Mass Spectroscopy. Specimens (1–5 mg) of labeled sodium acetate prepared as described above were acidified with 50 μL of 85% phosphoric acid. The samples were then evacuated briefly to remove SO_2 arising from the bisulfite used to quench the bromoform reaction. The mixture was then introduced into a mass spectrometer (MS-9) through an evaporative inlet. Relative intensities of unlabeled, monodeuterated, and

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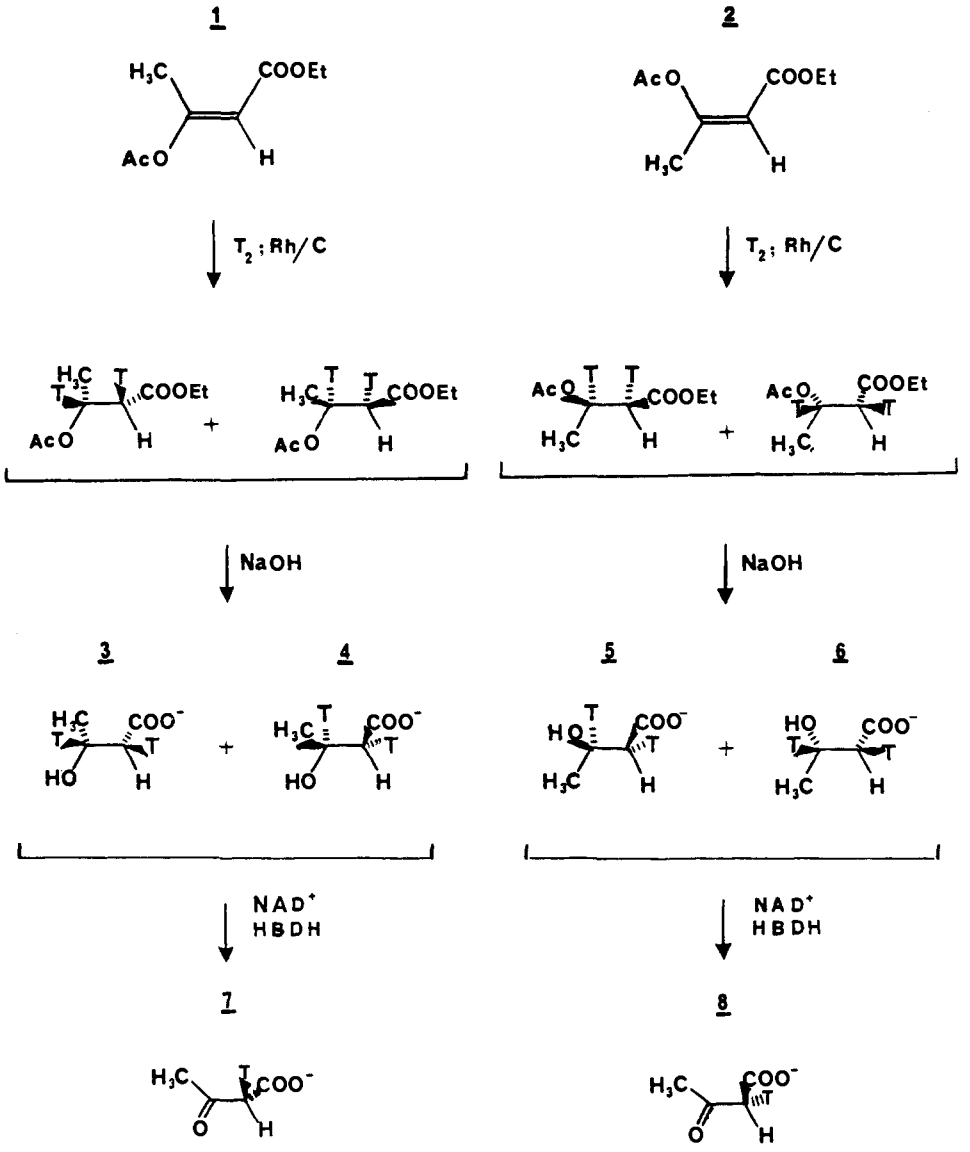

Figure 1. Scheme for enzymatic oxidation of 3-hydroxybutrate, prepared by catalytic tritiation of *E* and *Z* enol acetates.

polydeuterated acetates were corrected for background and natural isotopic substitution, using data from control experiments with unlabeled acetates.

Analysis of Chiral Acetates. The absolute configuration of acetates prepared as described above labeled with hydrogen, deuterium, and tritium was determined by the method of Cornforth et al. and Luthy et al.[12] Malate synthetase was purified from yeast and heated at 50 °C for 1 h (50 mM $MgCl_2$) to remove fumarase in a modification suggested by Eggerer. All batches of malate synthetase were assayed for contaminating fumarase and were found to produce satisfactory chiral methyl analyses on authentic samples of chiral methyl acetate. Fumarase was obtained from Sigma.

Results

Acetone labeled with tritium was generated by the coupled enzymatic oxidation and decarboxylation starting with labeled hydroxybutyrates and converted to labeled acetic acid by a bromoform reaction, as described in the Experimental Section.

Radioactivity appearing in the acetate corresponded to a 3–5% enzymatic conversion of substrate, consistent with the known amounts of enzymatic activities present in the reaction mixture.

Results of analyses of chiral methyl acetate derived from isotopically chiral 2,3-ditritio-3-hydroxybutyrates are shown in Table II. All samples of acetate produced by decarboxylation of acetoacetate were essentially racemic. The upper limit for the enantiomeric excess present in the mixtures was 5%. These data suggest prima facie that acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetate with no predominating preference for either inversion of retention of configuration.

Table II. Analyses of Isotopically Chiral Acetates[a]

source of acetate	% tritium retained	expts
E enol acetate	52	3
Z enol acetate	48	1

[a] Analysis by method of Cornforth, Arigoni, and co-workers.[12] Radioactive malates were purified by ion exchange chromatography (Dowex 1, eluted with dilute formic acid) and assayed to determine specific activity by using malate dehydrogenase and citrate synthase in a coupled assay. This procedure was found to correctly assign the stereochemistry of authentic samples of isotopically chiral methyl acetate, using the identical enzymes, substrates, and purification systems.[28]

Control Experiments. No evidence was found to suggest that the hydrogens of substrate, precursor, or products underwent any exchange with hydrogens in the medium, under the conditions of the reaction. Consistent with the data of Pederson,[5] the rate of enolization of acetoacetate was found to depend on buffer concentration, with a half-life at 22 °C 1.4 min in 100 mM potassium phosphate buffers at pH 6.4. Using known kinetic constants for AAD and 3-HBDH, the steady-state concentration of acetoacetate is approximately 4.5×10^{-6} M and is decarboxylated with a rate of approximately 9×10^{-6} M/s under conditions of its in situ

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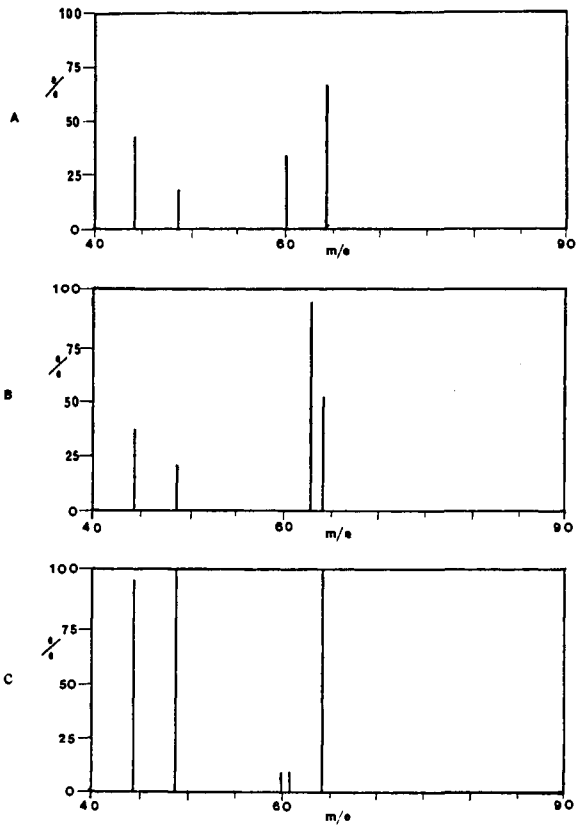

Figure 2. Mass spectra of samples of acetic acid (A) from bromoform oxidation of acetone in D_2O, (B) from bromoform oxidation of hexadeuteroacetone in H_2O, and (C) from the sequence of reactions starting from unlabeled hydroxybutrates executed at each state in D_2O. These experiments reveal no exchange of protons with solvent in the transformations. Background spectra displayed no ions with either mass 60 or 61.

generation: 3-hydroxybutyrate, 400 mCi, 1.0–1.5 mg; AAD, 10–15 mg/mL, 20000–30000 IU per mL). This rate is thus faster than the rate of enolization of acetoacetate (4×10^{-8} M/s) under these conditions by a factor of 200, and no appreciable exchange of the 2-protons with solvent was expected.

No exchange of the α-protons of either acetone or hexadeuterioacetone with solvent was observed under the conditions of the bromoform reaction, either with acetone, Br_2, and NaOD in one experiment or hexadeuterioacetone, Br_2, and NaOH in the complementary experiment. By mass spectroscopy, the bromoform reaction of acetone in D_2O yielded greater than 95% unlabeled acetic acid, while the identical reaction of hexadeuterioacetone yielded greater than 95% trideuterioacetate (Figure 2). This result is consistent with the mechanism of the bromoform reaction, in which enol is formed in a rate-limiting step and rapidly trapped by even modest concentrations of bromine.[13]

In the final control experiments, acetate was generated from acetoxybutyrates in a sequence where deuterium was the only exchangeable species in the solvent for *all of the transformations*. The mass spectrum of the acetates produced in this process showed only acetate and monodeuterioacetate, in an approximate ratio of 1:1 (Figure 2C). No di- or trideuterioacetate was observed. These data are entirely consistent with the data of Hammons et al.,[14] who showed that the AAD-catalyzed exchange of the α-protons of hexadeuterioacetone proceeded one proton at a time.

Discussion

It is commonly accepted that "all enzyme-catalyzed reactions are stereospecific".[2a] Acetoacetate decarboxylase appears to catalyze a reaction with no apparent stereospecificity. This result is surprising, and careful attention to the controls is deserved.

The precursors of the substrates used in this work are indisputably chiral. Tritium NMR of specimens of the *O*-acetyl ethyl esters of **3–6** actually used in these experiments showed greater than 95% isotopic labeling of a single diastereotopic 2-hydrogen of ethyl 3-acetoxybutyrate. The assignment of absolute configuration was made by using two independent tests; the details of these tests are discussed elsewhere.[10]

Furthermore, control experiments rule out the possibility that isotopic chirality was lost at steps intermediate in the conversion of chiral 3-hydroxybutyrate to acetate. No exchange occurred under the conditions of the bromoform reaction or in subsequent manipulations of the acetates. Acetoacetate itself is not expected to racemize at a rate sufficient under these conditions to lead to racemization. Most important, the product acetic acids produced in media containing deuterium as the only exchangeable species of hydrogen contain only a *single deuterium*. This fact requires that if racemization did occur at any step other than the decarboxylation, *it must have occurred without exchange*. Thus, racemization at a step either before or after decarboxylation cannot explain the racemic products.

These controls appear to require that an isotopically chiral acetoacetate be converted to an achiral acetone with single deuterium incorporation at the decarboxylation step. While the simplest model to account for this result involves a nonstereoselective protonation of the enamine of acetone believed to be an intermediate in the reaction,[7] other mechanisms not involving such a stereorandom step should be considered.

For example, it is conceivable that AAD might produce the net effect of racemization if it catalyzes a rapid, reversible proton transfer between the enzyme and the intermediate enamine. In its simplest form, this possibility is excluded by the mass spectral data showing incorporation of a single deuterium atom in D_2O, as such exchange should produce substantial fractions of di- and trideuterioacetone, which were not observed.

Also possible is that the protons being rapidly transferred are sequestered during this process from exchange with the protons in bulk solvent. However, even if the protons are "sticky," this explanation is still unsatisfactory. Racemization without major conformational exchange is expected to require either a di- or triprotic acid in the active site. (In the absence of major conformational change, a monoprotic acid would presumably return the abstracted hydrogen to the same face of the enamine, a process that would not produce racemization.) In either case, such a process should produce substantial fractions of dideutero and trideuteroacetone, given reasonable assumptions for the magnitude of equilibrium isotope effects. These are not observed.

An alternative mechanism involves the release of the enol into solution from the enzyme, which is then ketonized by a nonselective proton transfer from the solvent to give racemic acetone with incorporation of a single deuterium. While this possibility does not require the existence of a nonstereoselective enzyme-catalyzed step, the principle of microscopic reversibility, together with the fact that AAD catalyzes the exchange of the α-protons of acetone,[15] and the presumption that the exchange reaction is the microscopic reverse of the last steps of the decarboxylation reaction argue against this possibility. If AAD releases the enol form of acetone, by microscopic reversibility, it must also bind enol. As enolization is the slow step in the solution-catalyzed exchange process, a protein that binds only the enol form of acetone cannot catalyze exchange.

A further mechanistic possibility is that decarboxylation occurs via an intermediate carbinolamine to produce directly the enol form of acetone. While a similar mechanism has been previously suggested for base-catalyzed enolizations,[16] it is not obviously consistent with either labeling studies using oxygen-18,[17] or the

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(17) Hamilton, G. A.; Westheimer, F. H. *J. Am. Chem. Soc.* **1959**, *81*, 6332–6333.

hydrogen exchange reactions of acetone catalyzed by AAD.[15]

Finally, it may be that AAD catalyzes the racemization of isotopically chiral acetoacetate by a process irrelevant to decarboxylation. However, such racemization would presumably proceed via the enol form of acetoacetate, incorporate exchangeable deuteriums from solvent, and lead to the formation of multiply deuterated acetone as a major product. Again, this product is not observed.

We therefore must conclude that decarboxylation occurs with racemization due to stereorandom protonation of the intermediate enamine, that the racemization of enzyme-bound acetoacetate without concomitant exchange, processes requiring major conformational flexibility in the active site, or that there are two different active sites in the enzyme, each catalyzing half of the decarboxylation with complete but opposite stereospecificities. In the absence of contradicting evidence, we prefer the first explanation as the simplest.

If this conclusion is correct, AAD is unusual as an enzyme catalyzing a microscopic step with nearly complete absence of stereoselectivity; we exclude in this connection those enzymes whose physiological function is the racemization of optically active substances.[18] It is certainly one of the first examples of an acknowledged absence of stereoselectivity in a stereochemically "cryptic" microscopic reaction step.[19] As such, it appears that racemization must enter the lexicon of stereochemical alternatives accessible to enzymes catalyzing β-decarboxylations.

The stereorandom protonation of the enamine formed in the active site of AAD may mean that the enzyme has two proton sources of roughly equal acidity accessible to the enamine or that the enzyme does not catalyze this protonation at all. Interestingly, the protonation of the enamine may not need catalysis. The second-order rate constant for protonation in solution of a simple enamine of acetone and an aliphatic amine apparently has not been measured. However, it is clearly large and, at pH 6, the protonation by solvent may be kinetically competent in comparison with the first-order turnover number (k_{cat}) of 1400 s^{-1} for the enzymic reaction.[20]

Possibly relevant to this discussion is an interesting recent result of Chang et al.,[21] who found that aspartate β-decarboxylase appeared to produce somewhat racemic chiral methylalanine from isotopically chiral aspartate labeled at the 3-position. These workers attributed this loss of optical activity to enzyme-catalyzed exchange of product that presumably had left the active site and then returned. Although they did demonstrate the existence of enzyme-catalyzed exchange of the methyl protons of alanine, they did not report whether alanine produced by β-decarboxylation of aspartate in D_2O contained a single deuterium. Therefore, we are unable at this time to determine whether substantial loss of optical activity occurred in the decarboxylation step itself. However, it is interesting that the mechanism by which aspartate β-decarboxylase is presumed to act is precisely analogous to that used by AAD, even though the "electron sink" is formally the Schiff's base of an α-keto acid.

Thus, the correlation suggested in the introduction is, as yet, unconfirmed. Importantly, the mechanistic details of the decarboxylases producing inversion have yet to be explored with the same degree of rigor as those of AAD. For the three enzymes listed in Table I as producing inversion, it has never been conclusively demonstrated that decarboxylation is mediated via a Schiff's base or whether a third mechanism is available to these enzymes for catalyzing decarboxylation. It is clear that much further work, both mechanistic and stereochemical, will be needed before the β-decarboxylase puzzle can be definitively solved.

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Registry No. 1, 27750-19-2; **2**, 26805-39-0; **3**, 91112-33-3; **4**, 91112-34-4; **5**, 91112-35-5; **6**, 91112-36-6; **7**, 91112-37-7; **8**, 91112-38-8; AAD, 9025-03-0; ethyl acetoacetate, 141-97-9.

(18) (a) Adams, E. *Adv. Enzymol.* **1976**, *44*, 69–138.

(19) In fact, there are many cases in which only partial stereoselectivity is reported for enzyme-catalyzed reactions. Most experimentalists nevertheless conclude in these cases that the enzyme is completely stereospecific and the predominant stereochemical outcome is that of the enzyme and assume that any stereochemical infidelity observed is the result of experimental artifact. In most cases, this approach is probably reasonable.

(20) The tautomerization of the imine formed with acetone and *n*-butylamine at pH 4 is reportedly "faster than the halogenation of the enamine". While the concentrations of halogen were not specified in this report, the pseudo-first-order rate constant for halogenation at normal concentrations of halogen is quite large, making the solvent-catalyzed rate of enolization of the imine still larger. Thus, if the imine–enamine equilibrium favors imine, the rate of the reverse tautomerization (enamine to imine) in this system must be still larger: Bender, M. L.; Williams, A. *J. Am. Chem. Soc.* **1966**, *88*, 2502–2508.

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