pyranoside, which does not bind to the receptor, had no effect on antibody binding as measured by gold localization at the cell surface (Figure 4f). These results demonstrate that antibodies can be targeted to the *E. coli* specifically through their mannose receptors.

In summary, we have exploited a carbohydrate receptor to target antibodies to the surface of a pathogenic strain of *E. coli* that would not otherwise recognize the organism. Carbohydrate–protein conjugates similar to BCM–avidin may be used as a general strategy to prime pathogenic organisms for killing by host defense mechanisms. The use of these conjugates to mediate cell killing by complement proteins and macrophage cells is currently being investigated.

Experimental Section

Agglutination Assays. *E. coli* K1 *pilA+::tetR* was grown for 24 h at 37 °C on solid LB media supplemented with tetracycline and suspended with a cotton swab in 4 mL of Dulbecco's PBS to a final dilution of 2×10^8 cells/mL. Yeast (*Saccharomyces cerevisiae*, wild type) was grown for 36 h on solid YPD media at 30 °C and suspended with a cotton swab in 4 mL of Dulbecco's PBS to a final dilution of 1×10^8 cells/mL. Protein concentrations were determined by BCA Protein Assay (Pierce). Agglutination assays were performed on a 20-well ceramic ring plate. Typically, 90 μL of a solution of the test compound was combined with 30 μL of the bacterial suspension. After 30 s, 30 μL of the yeast suspension was added to give a final volume of 150 μL and the wells were allowed to develop for 3 min with agitation. A 5-μL aliquot was removed from each well and spread onto a standard microscope slide. The slides were quickly heat fixed and mounted with 10 μL of glycerol. The slides were examined under phase contrast at 500× magnification using a Zeiss Axioskop microscope. Agglutination was observed as clusters of cells. Total inhibition of agglutination was determined by the observation of single cells only. Determination of the concentrations necessary for total inhibition of agglutination was accurate within 20%.

Transmission Electron Microscopy. *E. coli* K1 *pilA+::tetR* was cultivated on solid LB media containing tetracycline at 37 °C for 18–20 h and suspended in PBS using a sterile swab to a final dilution of 10^9 cells/mL immediately prior to use. TEM samples were prepared in the following manner. Copper electron microscopy specimen grids (200 mesh) were coated with polyvinyl Formvar, carbon stabilized, and ionized by argon plasma discharge. A drop of the bacterial suspension was placed onto the grids, and after 2 min the drop was removed via pipet and the excess fluid blotted with filter paper. The grids were washed three times with PBS before negative staining with a 1% solution of phosphotungstic acid (pH 6.9). All specimens were examined with a Zeiss EM-109 electron microscope. The BCM–avidin conjugate was used as a 1.5×10^{-6} M solution in PBS. Monoclonal anti-avidin IgG (Sigma) (1.2×10^{-5} M in PBS) was preincubated with the BCM–avidin conjugate overnight at 0 °C before use. Protein A–15 nm colloidal gold conjugate (GpA, E-Y Laboratories) was diluted 1:10 in PBS containing 1% BSA.

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α- and β-Deuterium Kinetic Isotope Effects on the Inactivation of the General Acyl-Coenzyme A Dehydrogenase from Pig Kidney by (2-Methylenecyclopropane)acetyl-CoA

John E. Baldwin* and Wayne C. Widdison

Contribution from the Department of Chemistry, Syracuse University, Syracuse, New York 13244-4100. *Received September 4, 1991*

Abstract: Seven deuterium-labeled and unlabeled forms of (2-methylenecyclopropane)acetyl-CoA (MCPA-CoA) have been employed in kinetic studies to assess α- and β-deuterium isotope effects on the inactivation of the general acyl-CoA dehydrogenase from pig kidney. The racemic forms of α-d_2-MCPA-CoA and β-*d*-MCPA-CoA do not show any significant kinetic isotope effects. When inactivations of the enzyme caused by (*R*)-MCPA-CoA and by (*R*)-β-*d*-MCPA-CoA or those involving (*S*)-MCPA-CoA and (*S*)-β-*d*-MCPA-CoA are compared, only slight secondary deuterium isotope effects on the time versus extent of inactivation profiles are detected. These observations mitigate against one proposed mechanism for the inactivation which postulates dehydrogenation of the thioester substrate to form (2-methylenecyclopropylidene)acetyl-CoA, a reactive electrophilic Michael acceptor.

Introduction

Over the past decade there has been a growing interest in the chemistry of acyl-CoA dehydrogenases and in the biochemistry and molecular biology associated with various acyl-CoA dehydrogenase deficiencies, for the clinical significance of dehydrogenase impairments have become more strikingly evident.[1-3] An early harbinger of these interrelated chemical and clinical concerns may now be recognized in epidemeological, clinical, and chemical work related to the Jamaican vomiting sickness extending back more than 100 years.[4-7] The unripe fruit of the ackee tree,

(1) Tanaka, K.; Rosenberg, L. E. In *Metabolic Basis of Inherited Disease*, 5th ed.; Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., Brown, M. S., Eds.; McGraw-Hill: New York, 1983; pp 440–473.
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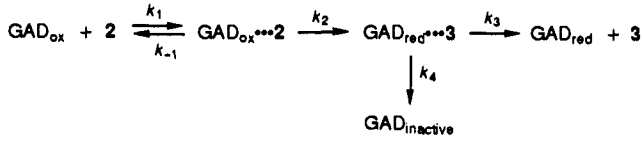
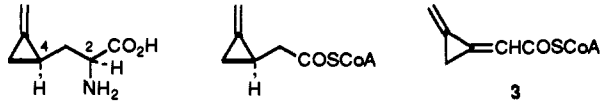
Scheme I



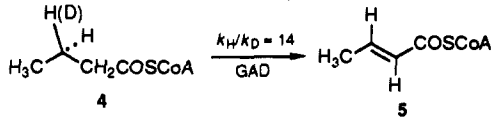
Blighia sapida, contains relatively high concentrations of hypoglycin (**1**), which manifests toxicity through its metabolite



MCPA-CoA (**2**). Inactivation of acyl-coenzyme A dehydrogenases by MCPA-CoA causes the biochemical derangements associated with the Jamaican vomiting sickness. Investigations of the in vitro "suicide" (or "Trojan horse", or "mechanism-based") inactivation[8] of acyl-CoA dehydrogenases by MCPA-CoA have shown that most or all of the inactivation involves covalent modifications of the flavin adenine dinucleotide (FAD) cofactor of the dehydrogenase,[9,10] but the mechanistic and structural aspects of the inactivation process still seem imperfectly understood.[11]

Perhaps the first mechanistic proposal for the inactivation was offered in 1978 by Abeles,[13] who suggested that the normal oxidative reaction mediated by the enzyme could convert MCPA-CoA to (2-methylenecyclopropylidene)acetyl-CoA (**3**), a reactive electrophilic Michael acceptor, which should be very susceptible to nucleophilic attack and could therefore inactivate the dehydrogenase.

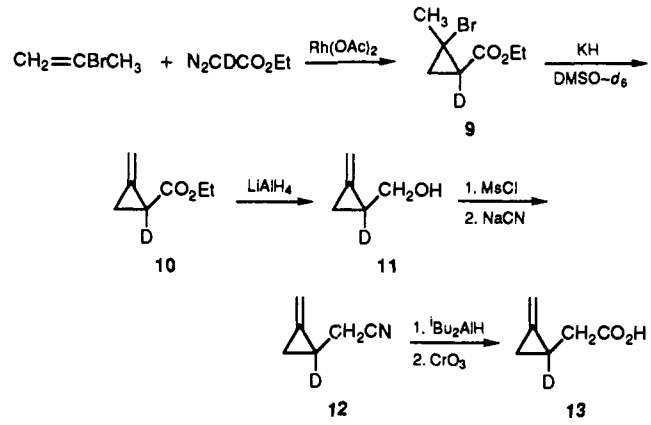
Subsequent mechanistic work on the normal reactions of GAD from pig kidney with deuterium-labeled versions of butyryl-CoA has shown that a β-hydrogen is lost in the rate-determining step; substitution of deuterium for the *pro-R* β-hydrogen lost in the oxidative process is associated with a substantial primary deuterium kinetic isotope; values of k_H/k_D as large as 14 have been reported.[14,15]



Reports on α-deuterium kinetic isotope effects for such oxidations have been mixed: There may be an isotope effect of about 2 for cases where the elimination of hydrogen from Cα and Cβ seems concerted,[14,15] or there may be no significant effect, when *pro-R* Cα deprotonation/reprotonation appears to precede the rate-determining cleavage of *pro-R* Cβ-H.[16]

The minimal kinetic scheme for "suicide" substrate inactivations suggested by Walsh and co-workers[17] adapted to the Abeles mechanistic proposal for inactivation by MCPA-CoA[13] is outlined in Scheme I. The rate constant for the slow step, k_2, according to this model, would be much smaller than the rate constants k_1, k_{-1}, k_3, and k_4.

Scheme II



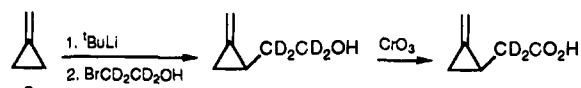
The scheme does not include the normally rapid reoxidation of the reduced enzyme, nor does it distinguish among possible forms of the substrate in the complex immediately preceding the rate-determining step: it could be an enol or an enolate.

We have tested this model by looking for a substantial primary deuterium kinetic isotope effect on the inactivation process associated with loss of a β-hydrogen from MCPA-CoA in the rate-determining step; it was not seen, and accordingly the mechanism shown in Scheme I seems an inadequate rationale for the inactivation.

Results

In earlier work we developed synthetic routes to MCPA-CoA[18] and to carbon-13- and carbon-14-labeled analogues of MCPA-CoA,[19] (2-methylenecyclopropane)acetic acid was resolved,[20] and absolute stereochemistry for the antipodes was established through an X-ray crystallographic study.[20] The derived (*R*)-MCPA-CoA and (*S*)-MCPA-CoA esters both proved to be inactivators of the enzyme GAD (general acyl-CoA dehydrogenase from pig kidney; EC 1.3.99.3; also named medium-chain acyl-CoA dehydrogenase (MCADH)), with (*R*)-MCPA-CoA being the faster and more efficient inactivator.[20] These precedents guided and facilitated the present work.

Syntheses. Reaction of methylenecyclopropane[21] (**6**) with *tert*-butyllithium in THF/TMEDA gave (methylenecyclopropyl)lithium,[22] which was reacted with 2-bromoethanol-d_4; the product 2-(2-methylenecyclopropane)ethanol-d_4 (**7**) was converted through Jones oxidation to α-d_2-MCPA (**8**). The proton NMR absorption seen for the hydrogens α to the carbon in unlabeled MCPA at δ 2.40 ppm was not apparent in the product, and thus deuterium incorporation at this position was estimated to be essentially quantitative.



Condensation of 2-bromopropene with ethyl α-deuteriodiazoacetate[23,24] (Scheme II) gave a mixture of diastereomers of ethyl

(6) Sherratt, H. S. A. *Trends Pharmacol. Sci.* **1986**, *7*, 189–191.
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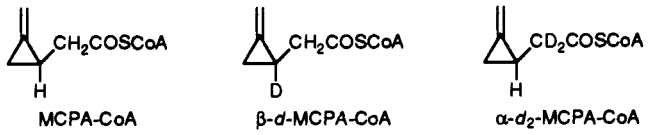
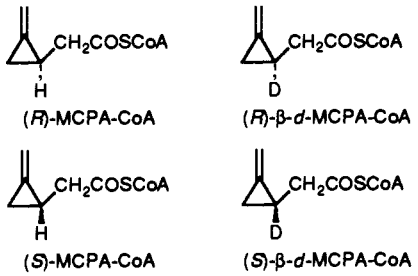
(18) Baldwin, J. E.; Parker, D. W. *J. Org. Chem.* **1987**, *52*, 1475–1477.
(19) Baldwin, J. E.; Widdison, W. C. *J. Labelled Compounds Radiopharm.* **1990**, *28*, 175–179.
(20) Baldwin, J. E.; Ostrander, R. L.; Simon, C. D.; Widdison, W. C. *J. Am. Chem. Soc.* **1990**, *112*, 2021–2022.
(21) Salaun, J. R.; Champion, J.; Conia, J. M. *Org. Synth.* **1977**, *57*, 36–40.
(22) Leandri, G.; Monti, H.; Bertrand, M. *Bull. Soc. Chim. Fr.* **1974**, 3015–3020.
(23) Carbon, J. A.; Martin, W. B.; Sweet, L. R. *J. Am. Chem. Soc.* **1958**, *80*, 1002.

Chart I

Coenzyme A Esters from Racemic Acids



Coenzyme A Esters from Enantiomerically Pure Acids



1-deuterio-2-bromo-2-methylcyclopropanecarboxylate (**9**) in good yield and isotopic incorporation, but there was substantial loss of deuterium label when this intermediate was reacted with potassium hydride in DMSO. The initial elimination presumably gave ethyl 2-methylcyclopropenecarboxylate which then isomerized under the basic reaction conditions to the more stable isomer having an exocyclic double bond. When the elimination was conducted in DMSO-d_6, the (2-methylenecyclopropane-*1-d*)-carboxylate **10** formed was found by NMR spectroscopy to be 99% deuterium labeled at C1 of the cyclopropane. This ester was reduced with $LiAlH_4$ to afford (2-methylenecyclopropane-*1-d*)-methanol (**11**), which was converted in turn to the corresponding mesylate,[25] nitrile (**12**),[26] aldehyde,[27] and carboxylic acid, *β-d*-MCPA (**13**).

Samples of unlabeled MCPA and of the racemic deuterium-labeled acid **13** were resolved by way of the amides derived from (*R*)-(−)-2-phenylglycinol;[20,28,29] these diastereomeric amides may be readily separated by HPLC on a preparative scale, and the absolute stereochemistries of these amides and of the acids they give upon hydrolyses are known.[20]

All seven of the MCPA forms used in this study were converted to the corresponding coenzyme A esters (Chart I) by a standard method[30] and were purified thoroughly prior to the kinetic work through reversed-phase HPLC.

The concentration of a solution of unlabeled racemic MCPA-CoA was determined using a spectrophotometric assay developed by Stadtman.[31] Various dilutions of this sample were then analyzed by HPLC; an excellent linear correlation between integrated HPLC detector absorbance response at 254 nm and concentration was thus established. The concentrations of solutions of the other six versions of MCPA were then established by HPLC analyses using a phosphate buffer as the mobile phase and reference to the concentration versus detector response correlation. This technique was advantageous because of its high precision and convenience.[32] Concentrations were determined in duplicate and were in all cases reproducible to within 5%.

Enzyme Isolation and Purification. The enzyme used in this work was isolated from fresh pig kidneys.[33] Procedures described

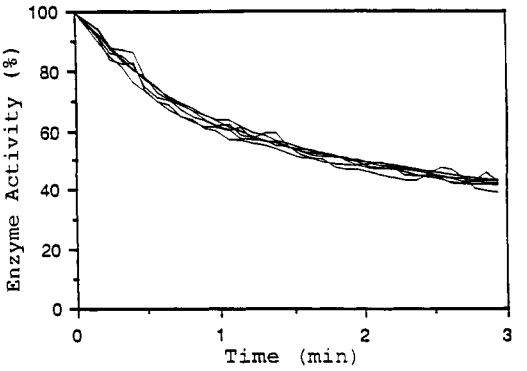


Figure 1. Duplicate inactivations of 4.03 μM GAD by 20.1 μM racemic MCPA-CoA, α-d_2-MCPA-CoA, and β-*d*-MCPA-CoA.

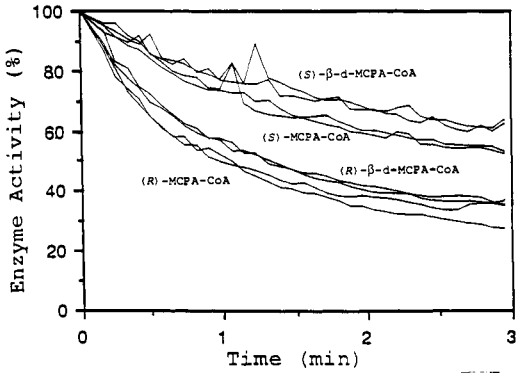


Figure 2. Duplicate inactivations of 4.03 μM GAD by 20.1 μM (*R*)-MCPA-CoA, (*R*)-β-*d*-MCPA-CoA, (*S*)-MCPA-CoA, and (*S*)-β-*d*-MCPA-CoA.

by Thorpe and co-workers[34,35] for the isolation and purification of GAD from this source involving DE-52, hydroxylapetite, Affi-gel blue, and Sephacryl chromatography were generally followed; the enzyme secured was subjected to protein and SDS-PAGE analyses, which showed only one major band with a molecular weight of approximately 45 000 (GAD subunit weight, about 42 000) and several weak bands of higher molecular weight. Freshly purified enzyme had an absorbance ratio at 272, 373, and 446 nm of 5.5:0.67:1.0, which compares closely with that recorded in the literature: 5.7:0.65:1.0.[35] The enzyme used in this work had been stored in a freezer for 9 months; it showed some loss of activity and altered absorbance ratios. The concentrations of active FAD in enzyme solutions were determined through enzyme activity assays.[34,35]

Kinetics. Portions of 5 equiv of each of seven versions of MCPA-CoA (Chart I) were combined with GAD, and the inactivation reactions were monitored by following changes in absorbance at 446 nm, a strong chromophore in the FAD cofactor known to be bleached as the inactivation takes place.[9,10]

Percent inactivation values estimated spectrophotometrically at 446 nm are equal to, or are only very slightly less than, extents of inactivation determined through enzyme assays. The slight inequality may be due to a minor component of inactivation through covalent modification of an amino acid of the enzyme resulting in inactivation, but this point is still unresolved.[10,11,36] All, or almost all, of the inactivation involves reaction between an activated compound derived from MCPA-CoA and the FAD cofactor of the enzyme.

Inactivation reactions between the racemic substrates MCPA-CoA, β-*d*-MCPA-CoA, and α-d_2-MCPA-CoA (each 20.1 μM)

(24) Hubert, A. J.; Noels, A. F.; Anciaux, A. J.; Teyssie, P. *Synthesis* **1976**, 600–602.
(25) Crossland, R. K.; Servis, K. L. *J. Org. Chem.* **1970**, *35*, 3195–3196.
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(36) Zeller, H. D.; Ghisla, S. In *Flavins and Flavoproteins 1987*; Edmondson, D. E., McCormick, D. B., Eds.; Walter de Gruyter: New York, 1987; pp 161–164.

and GAD (4.03 μM) were followed spectrophotometrically, and the enzyme activity versus time profiles seen were identical within experimental uncertainty. No significant kinetic isotope effects were apparent (Figure 1).

Enzyme activity was based on the active FAD present, compared with active FAD at the beginning of the inactivation. Percent activity was calculated as $100\,(1 - \Delta A_{446}/A^*_{446}(t_0))$, where $A^*_{446}(t_0)$ represents the absorbance at that wavelength at the start of the inactivation due to enzymatically active FAD cofactor; $A^*_{446}(t_0)$ was calculated by multiplying the active FAD concentration in a stock solution determined by enzyme activity assays[34,35] times the molar extinction coefficient for enzyme-bound active FAD (0.0154 cm^{-1} μM^{-1})[34,35] times a dilution factor to account for diminution of the 446-nm absorbance simply caused by dilution of the enzyme solution by the solution containing substrate.

Further work with the coenzyme A esters of the separate enantiomers of MCPA and of β-*d*-MCPA, substrate pairs thought to offer a more sensitive test for possible kinetic isotope effects, did serve to uncover some modest differences in inactivation versus time profiles. Inactivations of 4.03 μM GAD by 5 equiv of (*R*)-MCPA-CoA, (*R*)-β-*d*-MCPA-CoA, (*S*)-MCPA-CoA, and (*S*)-β-*d*-MCPA-CoA were run in duplicate; the inactivation versus time profiles observed are shown in Figure 2. These plots show the raw data, without editing or smoothing of any kind; the few unsightly spikes in activity percent values are annoying, but they do not in any way obfuscate the essential qualitative or quantitative results. The stereospecific reactivity advantage of (*R*)-MCPA-CoA over (*S*)-MCPA-CoA, reported earlier[20] and subsequently confirmed in another laboratory,[37] is evident, as are slight differences in profiles associated with deuterium labeling at cyclopropane C1, the carbon β to the thioester function.

Analysis and Conclusions

The absence of a significant α effect (Figure 1) in the racemic substrates suggests a rapid preequilibrium between MCPA-CoA and the enol form of the thioester. This view is consistent with recent tritium exchange studies demonstrating that both (*R*)-MCPA-CoA and (*S*)-MCPA-CoA exchange α-*pro*-*R* labels rapidly and to essentially the same extent under comparable conditions.[38]

To progress from a visually apparent perception of slight isotope effects (Figure 2) to assessments of magnitudes and possible mechanistic significance, the analytical expression appropriate to suicide inactivation[17] by the Abeles mechanism[13] (Scheme I), the Tatsunami equation,[39,40] was employed. This expression models the dependence between time (t) and fractional enzyme activity remaining ($x = (1 - e_i/e_0)$) as a function of the initial substrate concentration (s_0), the initial enzyme to substrate concentration ratio ($\mu = e_0/s_0$), and three parameters related to the rate constants of Scheme I: r, B, and C.

$$t = rB[\ln\,[1 - (1 + r)\mu(1 - x)]/x]/Cs_0[1 - (1 + r)\mu] - r(\ln x)/C \quad (1)$$

$$r = k_3/k_4 \quad (2)$$

$$B = (k_{-1} + k_2)(k_3 + k_4)/k_1(k_2 + k_3 + k_4) \quad (3)$$

$$C = k_2k_3/(k_2 + k_3 + k_4) \quad (4)$$

Equation 1 will give theoretical inactivation profiles based on the minimal kinetic scheme for suicide inactivation and the necessary concentrations and parameters, or the parameters r, B, and C may be estimated when experimental inactivation extent versus time data are available. For inactivations caused by (*R*)-MCPA-CoA, the predominant stereoisomeric form of MCPA-CoA derived from natural hypoglycin,[18] and by (*R*)-β-*d*-MCPA-CoA substrates, such modeling leads to satisfactory matches between observed and calculated profiles when the parameters r, B, and C are 5.6, 36, and 0.26 for the unlabeled substrate and 6.2, 36, and 0.23 for (*R*)-β-*d*-MCPA-CoA (Figure 3). Very similar small changes in r and C will model the corresponding changes in inactivation profiles observed for the unlabeled and β-*d*-labeled (*S*)-MCPA-CoA esters.

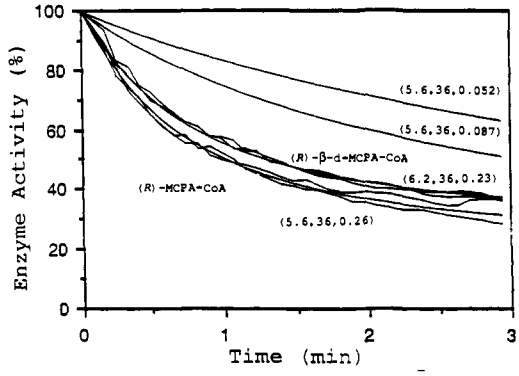

Figure 3. Duplicate inactivations of GAD by (*R*)-MCPA-CoA and (*R*)-β-*d*-MCPA-CoA from Figure 2 and four theoretical inactivation versus time functions based on the Tatsunami equation[39] and the given r, B, and C parameters.

Model calculations with larger assumed changes in parameter C (C = 0.087, 0.052) appropriate to primary isotope effects on k_2 of 3 and of 5 are included in Figure 3; they show that such isotope effects would have produced significant, readily perceived alterations in the inactivation versus time profiles. Such profound changes in the kinetics of inactivation are not seen.

The significance of these variations in parameters becomes clear when one considers how they relate to the rate constants of Scheme I. According to the mechanistic model summarized in the scheme, k_2, the rate constant governing cleavage of the β C–H or C–D bond, is much smaller than the other rate constants; thus, an isotope effect on k_2 could well have no impact on parameter B, for it appears in eq 3 only in additive combinations with other, much larger rate constants. But the parameter C is directly proportional to k_2; with $k_2 + k_3 + k_4$ approximated by $k_3 + k_4$, eq 5 results.

$$C = k_2[r/(r + 1)] \quad (5)$$

Changes in this parameter associated with an added deuterium label would be dominated by changes in k_2 values: If the β C–H or C–D was lost as the reactive intermediate formed with rate constant k_2, r should not change and the ratio of C parameters $C(H)/C(D) = k_2(H)/k_2(D)$; if the β C–H or C–D bond was still present in the reactive intermediate, there might be small secondary deuterium isotope effects on the partitioning ratio $r = k_3/k_4$, and $C(H)/C(D) \approx k_2(H)/k_2(D)$. Small changes in r have little impact on C.

Thus, the 11% change in parameter C and the 12% change in partitioning ratio r noted for the inactivation of (*R*)-β-*d*-MCPA-CoA relative to that of (*R*)-MCPA-CoA imply that the β-hydrogen is *not* lost in the rate-determining step having rate constant k_2 and that the β-hydrogen is still present in the [reduced GAD···reactive intermediate] product which partitions between k_3 and k_4. Hence, Scheme I as formulated, with (2-methylenecyclopropylidene)acetyl-CoA (**3**) as the oxidation product and reactive intermediate, cannot be correct. One must resort to other hypothetical mechanisms for the inactivation of GAD by MCPA-CoA.

The alternative mechanism most frequently mentioned in recent years[10,11,41] postulates a unimolecular isomerization of an enolate

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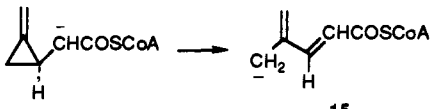
anion derived from MCPA-CoA (**14** → **15**) through cleavage of



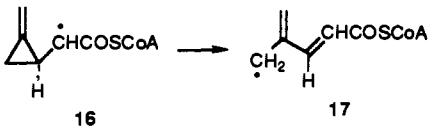
14 **15**

the three-membered ring; the cross-conjugated anion **15** then presumably attacks FAD nucleophilically to give covalently modified products and to inactivate the enzyme. But this formulation seems less and less likely. It does not readily account for the absence of coenzyme A 4-methylpenta-2,4-dienethioate in inactivation reaction product mixtures;[42] nor is it compatible with other evidence showing that FAD is reduced as the reactive intermediate is formed.

Under conditions giving complete inactivation of the enzyme with MCPA-CoA under aerobic conditions, incubation of GAD with MCPA-CoA under anaerobic conditions inactivates only about half of the enzyme, the other half being reduced.[10] The implication is that the FAD cofactor is reduced as the reactive intermediate is generated. An artificial GAD made from apo-GAD and 5-deaza-5-carba-FAD does not react with MCPA-CoA.[43] Yet, according to the anionic rearrangement followed by nucleophilic attack hypothesis, an altered cofactor should make no difference until after **15** is formed.

These experimental facts[10,43] suggest that an oxidation of MCPA-CoA and a simultaneous reduction of FAD give the reactive intermediate which then partitions between turnover product and an inactivation reaction with the reduced form of FAD; they serve to weaken the case for the **14** → **15** → inactive enzyme possibility.

A variant of the anionic mechanism involving unimolecular cleavage of a radical (**16** → **17** → inactive enzyme) has recently been suggested[37] and deserves further consideration. It is not



16 **17**

in conflict with the present kinetic isotope effect studies. Perhaps its greatest weakness is a total lack of precedent supporting the involvement of substrate-derived radical species in any chemistry catalyzed by GAD.[15] Reduction of GAD by acyl-CoA substrates generates a reduced GAD···enoyl-CoA charge-transfer complex having absorption spectral characteristics quite unlike the neutral or anionic semiquinones derived from the FAD cofactor in GAD. Both ESR and other evidence suggest that this complex may react with electron transferring flavoprotein (ETF) to form a semiquinone radical.[44-47]

Further work and new hypotheses regarding the inactivation of GAD by MCPA-CoA seem warranted. One needs to consider especially possible oxidative transformations of MCPA-CoA generating reactive intermediates which retain a β C–H bond.

Experimental Section

Reactions were performed under a nitrogen atmosphere with magnetic stirring. Tetrahydrofuran (THF) was distilled from sodium–benzophenone; diethyl ether was distilled from sodium–benzophenone, stored over sodium, and decanted immediately before use. Chemicals were purchased from Aldrich Chemical Co., Sigma Chemical Co., or Fluka Chemie AG. Proton and carbon-13 spectra were recorded on a GE QE 300 spectrometer. Chemical shifts (δ) for $CDCl_3$ solutions are expressed relative to $CDCl_3$ (δ(^{1}H) 7.26). High-performance liquid chromatographic purifications and analyses were performed on a Hewlett-Packard (HP) Series 1090 or a Rainin HPX/Gilson Model 112 based system. Analytical gas chromatographic (GC) analyses were done with an HP 5780 instrument, using cross-linked 5% phenyl methyl silicone and methyl silicone 0.2-mm-i.d. 25-m fused silica capillary columns connected to a single-injection port through a 0.4-mm two-hole Supeltex M-2A ferrule (Supelco, Inc.: 2-2467) and to two flame ionization detectors leading to HP 3390A and 3392A series reporting integrators. Preparative gas chromatographic separations were performed with a Varian A-90-P3 instrument. Thin-layer chromatography (TLC) plates (20 × 20 cm silica gel 60 F254, 0.25-mm thickness for analytical plates, 1.0-mm thickness for preparative plates) were purchased from EM Science Industries.

2-(2-Methylenecyclopropane)ethanol-1,1,2,2-d_4 (7). A 50-mL flask containing 15 mL of THF was cooled to −78 °C. Methylenecyclopropane[21] (0.5 mL, d, 0.9, 10 mmol) was added to the flask using a dry ice cooled syringe. *tert*-Butyllithium (5 mL, 1.7 M in pentane) and 1 mL of TMEDA were injected into the flask. The mixture was stirred at −78 °C for 30 min and then at 0 °C for 40 min. The solution was cooled to −78 °C, and a solution of 2-bromoethanol-d_4 (0.25 g, 1.9 mmol, 99+% deuterium incorporation, Cambridge) was injected into the flask. The mixture was stirred at −78 °C for 30 min and then warmed slowly to room temperature. The mixture was quenched with 1 mL of H_2O and extracted twice with 15-mL portions of ether. The organic layer was dried over $MgSO_4$ and concentrated to about 5 mL by distillation though a 15-cm Vigreux column. The major product had identical analytical GC retention times as authentic unlabeled alcohol.

2-(2-Methylenecyclopropane)acetic-α,α-d_2 acid (8) was prepared from the 5-mL solution of crude 2-(2-methylenecyclopropane)ethanol *1,1,2,2-d_4* in ether by Jones oxidation.[18] ^{1}H NMR: δ 0.87–0.94 (m, 1 H), 1.35–1.44 (m, 1 H), 1.68–1.78 (m, 1 H), 5.42 (d, J = 1.1 Hz, 1 H), 5.52 (t, J = 1.3 Hz, 1 H).

Ethyl α-Deuteriodiazoacetate. Ethyl diazoacetate (30 mL, 285 mmol) was combined with 50 mL of CH_2Cl_2 and 50 mL of D_2O in a 250-mL flask. Sodium deuteroxide (10%, 5 drops) was then added followed by 400 mg of the phase-transfer catalyst hexadecyltrimethylammonium bromide. The mixture was vigorously stirred at room temperature for 48 h. The organic layer was separated, and the aqueous layer was washed with two 25-mL portions of CH_2Cl_2. The organic layers were combined and dried over $MgSO_4$ and then carefully concentrated by rotary evaporation at 10–15 °C. This procedure was repeated once more using fresh D_2O; comparisons of ^{1}H NMR integrations of the residual 4.73 ppm signal versus the methylene quartet at 4.22 ppm indicated 98+% deuterium incorporation.

Ethyl (2-Bromo-2-methylcyclopropane-*1-d*)carboxylates (9). Rhodium(II) acetate dimer (35 mg) was added to 2-bromopropene (17 g, 140 mmol). Ethyl α-deuteriodiazoacetate (10 mL) was added to the alkene at room temperature over 3 h using a syringe pump. The mixture was stirred for an additional 1 h and then filtered through Celite to remove the catalyst. The filtrate was concentrated by short-path distillation. The pot residue was purified by Kugelrohr distillation (60 °C, 1 Torr) to give 20 g (68% yield) of ester products as a 1.3:1 mixture of diastereoisomers. Each diastereomer was purified by GC on a 2.5 m × 6.3 mm 14% Apiezon L on 60/80-mesh Chromosorb G column. Data for first eluted isomer follow. ^{1}H NMR: δ 1.28 (t, J = 7.0 Hz, 3 H), 1.42 (d, J = 6.4 Hz, 1 H), 1.58 (d, J = 6.4 Hz, 1 H), 1.83 (s, 3 H), 4.25 (q, J = 7.0 Hz, 2 H). Data for second isomer follow. ^{1}NMR: δ 1.22 (d, J = 6.4 Hz, 1 H), 1.29 (t, J = 7.4 Hz, 3 H), 1.74 (d, J = 6.4 Hz, 1 H), 1.83 (s, 3 H), 4.21 (q, J = 7.4 Hz, 2 H).

Ethyl (2-Methylenecyclopropane-*1-d*)carboxylate (10). A mixture of diastereomers of ethyl (2-bromo-2-methylcyclopropane-*1-d*)carboxylate (10 g, 48 mmol) was dissolved in 25 mL of dimethyl-d_6 sulfoxide. Potassium hydride (6 g, 35 wt % dispersion in mineral oil, 50 mmol) was then added to the ester in four portions. Ether (30 mL) was then added to the mixture followed by 10 mL of D_2O. The organic layer was removed, and the aqueous layer was extracted twice with 15-mL portions of ether. The organic layers were combined and washed with 5 mL of D_2O. The organic layer was then dried over $MgSO_4$ and concentrated by distillation through a 15-cm Vigreux column. This procedure was repeated to give 4 g of product (65% yield) with 99% deuterium incorporation as judged by comparing the integrations of the 1.79 ppm doublet of triplets to the residual 2.25 ppm signal. ^{1}H NMR: δ 1.25 (t, J = 7.2 Hz, 3 H), 1.61 (d, J = 8.8 Hz, 1 H), 1.79 (tt, J = 9.0 Hz, J = 2.4 Hz), 2.25 (m, 0.03 H), 4.13 (q, J = 7.0 Hz, 2 H), 5.50–5.51 (m, 2 H).

(2-Methylenecyclopropane-1-*d*)methanol (11). A solution of ethyl (2-methylenecyclopropane-*1-d*)carboxylate (3 g, 23 mmol) in 10 mL of ether was added dropwise to a slurry of lithium aluminum hydride (1.4 g, 36 mmol) in 15 mL of ether at 0 °C. Addition was complete after 20

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min, and the mixture was stirred for an additional 1 h at room temperature; then, it was subjected to a standard workup. The ethereal alcohol solution was concentrated by distillation through a 15-cm Vigreux column to give 1.8 g of crude product (90% yield). A sample of the crude alcohol was purified by preparative GC on the Apiezon L column. ^{1}H NMR: δ 0.95 (d, J = 8.8 Hz, 1 H), 1.31 (d, J = 8.4 Hz, 1 H), 1.65 (br s, 1 H), 3.49 (d, J = 11.2 Hz, 1 H), 3.60 (d, J = 11.2 Hz, 1 H), 5.42 (s, 1 H), 5.47 (t, J = 2.1 Hz, 1 H).

(2-Methylenecyclopropyl-*1-d*)methyl Mesylate. Methanesulfonyl chloride (3 g, 30 mmol) was added dropwise to a solution of (2-methylenecyclopropane-*1-d*)methanol (**11**; 1.8 g, 21 mmol) in 15 mL of CH_2Cl_2 and triethylamine (3 g, 30 mmol) at 0 °C.[25] The addition was complete after 5 min, and the reaction mixture was stirred for an additional 20 min. The mixture was then extracted successively with 10 mL of ice water, 10 mL of ice-cold 10% HCl, and 10 mL of ice-cold saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$ and concentrated by rotary evaporation (bath temperature 10–20 °C) to give 3 g (80% yield) of the crude product.

(2-Methylenecyclopropane-*1-d*)ethanenitrile (12). A solution of (2-methylenecyclopropyl-*1-d*)methyl mesylate (1 g, 5.6 mmol) in 2 mL of DMSO was added dropwise to a slurry of NaCN (0.3 g, 6.1 mmol) in 15 mL of DMSO at room temperature.[26] The addition was complete after 5 min, and the mixture was stirred for an additional 30 min. Ether (50 mL) was added, and the mixture was extracted successively with 10 mL of H_2O followed by 10 mL of 2 N HCl. The organic layer was dried over $MgSO_4$ and concentrated by distillation through a 15-cm Vigreux column to give 1.2 mL of a solution which GC analysis showed was approximately 40% the nitrile product. A sample was purified by preparative GC on the Apiezon L column. ^{1}H NMR: δ 1.02 (d, J = 10 Hz, 1 H), 1.45 (d, J = 10 Hz, 1 H), 2.45 (s, 2 H), 5.51 (s, 1 H), 5.60 (t, J = 2.5 Hz, 1 H).

(2-Methylenecyclopropane-*1-d*)acetaldehyde. The crude ethereal solution of (2-methylenecyclopropane-*1-d*)ethanenitrile (1.1 mL) was dissolved in 5 mL of THF at 0 °C. Diisobutylaluminum hydride (0.6 M in hexane, 16 mL, 9.6 mmol) was then added to the solution over 5 min, and the mixture was stirred at 0 °C for an additional 30 min.[27] The reaction was quenched by the addition of 2 mL of saturated NH_4Cl followed by 2 mL of 10% H_2SO_4. The mixture was stirred at room temperature for 2 min and was then extracted with three 25-mL portions of ether. The organic layers were combined and dried over $MgSO_4$, filtered, and concentrated to approximately 3 mL by distillation through a 15-cm Vigreux column. Analysis by GC/MS verified that the concentrate contained the aldehyde: m/e 97 (M^+, 1.8%), 96 (M − 1, 18%), 82 (M^+ − 15, 16%), 69 (M − 28, 31%), 68 (M^+ − 29, 100%), 54 (M^+ − 43, 36%), 42 (M^+ − 54, 66%), 40 (M^+ − 57, 68%), 39 (M^+ − 58, 77%).

(2-Methylenecyclopropane-*1-d*)acetic Acid (13). The ethereal solution of crude (2-methylenecyclopropane-*1-d*)acetaldehyde prepared above was dissolved in 15 mL of acetone and cooled in an ice water bath. Jones reagent (1 mL, 2 mol equiv) was added dropwise to the mixture. The mixture was stirred for 2 h, then 15 mL of ether was added, and solvent was decanted from the green residue. The decanted solution was concentrated to approximately 2 mL by distillation using a 15-cm Vigreux column. An aqueous chromium ion containing phase formed a lower layer, and the top organic layer was separated, dried over $MgSO_4$, and filtered. Preparative GC isolation of pure deuterium-labeled acid from this filtrate (about 1.5 mL) on a 10% FFAP on 60/80-mesh Chromosorb G column gave 150 mg of product. ^{1}H NMR: δ 0.90 (d, J = 9.3 Hz, 1 H), 1.39 (d, J = 9.2 Hz, 1 H), 2.40 (s, 2 H), 5.43 (s, 1 H), 5.52 (t, J = 2.4 Hz, 1 H).

***N*-(2-Hydroxy-1(*R*)-phenylethyl)-(*R*)-(2-methylenecyclopropane-*1-d*)acetamide and *N*-(2-Hydroxy-1(*R*)-phenylethyl)-(*S*)-(2-methylenecyclopropane-*1-d*)acetamide.** A solution of the GC-purified (2-methylenecyclopropane-*1-d*)acetic acid (**13**; 100 mg, 0.89 mmol) in 2 mL of THF was cooled to −20 °C in a dry ice/carbon tetrachloride bath. *N*-Methylmorpholine (120 mL, 1.1 mmol) was added to the solution, and after 15 min isobutyl chloroformate (120 mL, 1.2 mmol) was added. The solution was stirred for 15 min and then (*R*)-(−)-2-phenylglycinol (190 mg, 1.4 mmol) was added. The mixture was stirred for 30 min at −20 °C and then for 1 h at room temperature. The solvent was removed by rotary evaporation, and the residue was dissolved in 5 mL of CH_2Cl_2. The solution was washed successively with 0.5 mL of saturated $NaHCO_3$, 0.5 mL of 1 N HCl, and finally 0.5 mL of H_2O. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give 160 mg of crude product (71% yield). The diastereomeric amides were separated by HPLC on the Rainin system using a 20 × 200 mm Machery-Nagel Nucleosil 50-5 column and 6.5:3.5 EtOAc/isooctane. A final HPLC analysis of each of the separated amides on a 4 × 200 mm analytical Nucleosil column showed the compounds to be diastereomerically pure. The separation yielded 50 mg (62% recovery, 44% yield overall) of the first amide, the *R,R* isomer.[20] ^{1}H NMR: δ 0.85 (d, J = 9.0 Hz, 1 H), 1.35 (d, J = 9.0 Hz, 1 H), 2.13 (d, J = 15.4 Hz, 1 H), 2.33 (d, J = 15.4 Hz, 1 H), 2.53 (t, J = 5.1 Hz, 1 H), 3.85 (br s, 2 H), 5.04 (m, 1 H), 5.39 (s, 1 H), 5.43 (t, J = 2.4 Hz, 1 H), 6.34 (br s, 1 H), 7.20–7.34 (m, 5 H). A 43-mg yield (54% recovery, 38% yield overall) of the second, *R,S* amide, was also achieved. ^{1}H NMR: δ 0.85 (d, J = 9.0 Hz, 1 H), 1.35 (d, J = 9.0 Hz, 1 H), 2.17 (d, J = 15.6 Hz, 1 H), 2.39 (d, J = 15.6 Hz, 1 H), 2.48 (t, J = 5.9, 1 H), 3.80–3.90 (m, 2 H), 5.04 (q, J = 5.0 Hz, 1 H), 5.38 (s, 1 H), 5.41 (t, J = 2.0 Hz, 1 H), 6.35 (br s, 1 H), 7.19–7.34 (m, 5 H).

***N*-(2-Hydroxy-1(*R*)-phenylethyl)-(*R*)-(2-methylenecyclopropane)-acetamide and *N*-(2-Hydroxy-1(*R*)-phenylethyl)-(*S*)-(2-methylenecyclopropane)acetamide.** The (*R*)-(−)-phenylglycinol amide derivatives of unlabeled MCPA were prepared and separated on the same scale as described for the amides derived from **13**. The separation supplied 45 mg (40% yield) of the d_0 *R,R* amide. ^{1}H NMR: δ 0.82–0.89 (m, 1 H), 1.32–1.38 (m, 1 H), 1.65–1.68 (m, 1 H), 2.13 (dd, J = 15.4 Hz, J = 7.3 Hz, 1 H), 2.33 (dd, J = 15.4 Hz, J = 7.3 Hz, 1 H), 3.55 (br s, 1 H), 3.78 (br s, 2 H), 4.95–5.03 (m, 1 H), 5.42 (s, 1 H), 5.47 (s, 1 H), 6.80 (d, J = 7.0 Hz, 1 H), 7.2–7.4 (m, 5 H). It also supplied 38 mg (34% yield) of the d_0 *R,S* amide. ^{1}H NMR: δ 0.82–0.89 (m, 1 H), 1.32–1.39 (m, 1 H), 1.64–1.69 (m, 1 H), 2.17 (dd, J = 15.6 Hz, J = 7.7 Hz, 1 H), 2.39 (dd, J = 15.6 Hz, J = 6.8 Hz, 1 H), 2.49 (t, J = 6.0 Hz, 1 H), 3.80–3.90 (m, 2 H), 5.04 (q, J = 5.0 Hz, 1 H), 5.39 (d, J = 1.6 Hz, 1 H), 5.42 (q, J = 2.0 Hz, 1 H), 6.35 (br s, 1 H), 7.19–7.34 (m, 5 H).

(*R*)-(2-Methylenecyclopropane-*1-d*)acetic Acid ((*R*)-13). A solution of *N*-(2-hydroxy-1(*R*)-phenylethyl)-(*R*)-(2-methylenecyclopropane-*1-d*)acetamide (50 mg, 0.22 mmol) in 0.5 mL of dioxane was added to a glass storage ampule followed by 0.5 mL of 3 N H_2SO_4. The ampule was cooled in a dry ice/acetone bath and sealed with a torch. The sample was allowed to come to room temperature and then was heated in an oil bath at 140 °C for 1 h. The sample was cooled to room temperature, and the ampule was broken at the neck. The sample was extracted with four 0.5-mL portions of ether. The organic layers were combined, dried over $MgSO_4$, and filtered; the product was purified by preparative GC on a 10% FFAP on 60/80-mesh Chromosorb G column to give 15 mg of product (50% yield). The *R* d_1 acid had analytical GC retention times identical with those of the racemic d_1 acid **13**.

(*S*)-(2-Methylenecyclopropane-*1-d*)acetic acid ((*S*)-13) was prepared by hydrolysis of *N*-(2-hydroxy-1(*R*)-phenylethyl)-(*S*)-(2-methylenecyclopropane-*1-d*)acetamide (43 mg, 0.19 mmol) with 3 N H_2SO_4 as described for the hydrolysis of the (*R*)-(−)-2-phenylglycinol amide of (*R*)-(2-methylenecyclopropane-*1-d*)acetic acid. Purification by preparative GC gave 14 mg (66% yield) of product. The analytical GC retention times were identical to those characteristic of racemic acid **13**.

(*R*)-(2-Methylenecyclopropane)acetic acid ((*R*)-2) was prepared by hydrolysis of *N*-(2-hydroxy-1(*R*)-phenylethyl)-(*R*)-(2-methylenecyclopropane)acetamide (45 mg, 0.20 mmol) with 3 N H_2SO_4 as described for the hydrolysis of the (*R*)-(−)-2-phenylglycinol amide of (*R*)-(2-methylenecyclopropane-*1-d*)acetic acid. Purification by preparative GC gave 12 mg (54% yield) of product. The analytical GC retention times were identical to those found for racemic MCPA (**2**).

(*S*)-(2-Methylenecyclopropane)acetic acid ((*S*)-2) was prepared by hydrolysis of *N*-(2-hydroxy-1(*R*)-phenylethyl)-(*S*)-(2-methylenecyclopropane)acetamide (38 mg, 0.17 mmol) with 3 N H_2SO_4. Purification by preparative GC gave 10 mg (52% yield) of product. The analytical GC retention times were identical to those found for racemic MCPA (**2**).

***N*-[(*R*)-(2-Methylenecyclopropyl-*1-d*)acetoxy]succinimide.**[30] A solution of *N*-hydroxysuccinimide (30 mg, 0.26 mmol) in 3 mL of ethyl acetate was added to a solution of (*R*)-(2-methylenecyclopropane-*1-d*)-acetic acid (15 mg, 0.13 mmol) in 1 mL of ethyl acetate. A solution of dicyclohexylcarbodiimide (36 mg, 0.17 mmol) in 2 mL of ethyl acetate was then added, and the resulting solution was swirled a little without magnetic stirring. The reaction mixture was then left undisturbed at room temperature for 24 h. The dicyclohexylurea that precipitated during this time was removed by filtration, and the filtrate was concentrated to approximately 0.5 mL by rotary evaporation at room temperature. Some *N*-hydroxysuccinimide precipitated during the concentration and was removed by filtration through a glass wool plug. The crude dilute filtrate was then purified by HPLC on the Rainin system using a 4.6 × 250 mm Dynamax 60-Å 8-nm silica column and 65:35 ethyl acetate/isooctane as the mobile phase, a flow rate 1.5 mL/min, and a 50-μL sample loop. The product-containing fractions were combined, and solvent was removed by rotary evaporation to give 20 mg of product (71% yield). ^{1}H NMR: δ 0.98 (dt, J = 9.4 Hz, J = 2.1 Hz, 1 H), 1.45 (d, J = 9.4 Hz, 1 H), 2.56 (d, J = 16.5 Hz, 1 H), 2.72 (d, J = 16.5 Hz, 1 H), 2.84 (s, 4 H), 5.47 (s, 1 H), 5.60 (t, J = 2.4 Hz, 1 H).

***N*-[(*S*)-(2-Methylenecyclopropyl-*1-d*)acetoxy]succinimide** was prepared from (*S*)-(2-methylenecyclopropane-*1-d*)acetic acid (14 mg, 0.13 mmol), *N*-hydroxysuccinimide (30 mg, 0.26 mmol), and dicyclohexylcarbodiimide (36 mg, 0.17 mmol). Purification by HPLC gave 21 mg

(80% yield) of product. ^{1}H NMR: δ 0.98 (dt, J = 9.4 Hz, J = 2.1 Hz, 1 H), 1.45 (d, J = 9.4 Hz, 1 H), 2.56 (d, J = 16.5 Hz, 1 H), 2.72 (d, J = 16.5 Hz, 1 H), 2.84 (s, 4 H), 5.47 (s, 1 H), 5.60 (t, J = 2.4 Hz, 1 H).

***N*-[(±)-(2-Methylenecyclopropyl-*1-d*)acetoxy]succinimide** was prepared from (±)-(2-methylenecyclopropane-*1-d*)acetic acid (**13**; 15 mg, 0.13 mmol), *N*-hydroxysuccinimide (30 mg, 0.26 mmol), and dicyclohexylcarbodiimide (36 mg, 0.17 mmol). Purification by HPLC gave 20 mg (71% yield) of product. ^{1}H NMR: δ 0.98 (d, J = 9.4 Hz, 1 H), 1.45 (d, J = 9.4 Hz, 1 H), 2.56 (d, J = 16.5 Hz, 1 H), 2.72 (d, J = 16.5 Hz, 1 H), 2.84 (s, 4 H), 5.47 (s, 1 H), 5.60 (t, J = 2.4 Hz, 1 H).

***N*-[(±)-(2-Methylenecyclopropyl)acetoxy]succinimide** was prepared from (±)-(2-methylenecyclopropane)acetic acid (**2**; 30 mg, 0.26 mmol), *N*-hydroxysuccinimide (58 mg, 0.53 mmol), and dicyclohexylcarbodiimide (77 mg, 36 mmol). Purification by HPLC gave 40 mg (70% yield) of product. ^{1}H NMR: δ 0.85–1.21 (m, 1 H), 1.45 (tt, J = 9.2 Hz, J = 2.1 Hz, 1 H), 1.73–1.79 (m, 1 H), 2.56 (dd, J = 16.5 Hz, J = 7.5 Hz, 1 H), 2.72 (dd, J = 16.5 Hz, J = 7.5 Hz, 1 H), 2.84 (s, 4 H), 5.47 (d, J = 1.5 Hz, 1 H), 5.61 (t, J = 2.2 Hz, 1 H). ^{13}C NMR: 9.66 (s), 10.30 (s), 25.4 (s), 34.3 (s), 104.4 (s), 132.6 (s), 167.6 (s), 169.1 (s).

***N*-[(*R*)-(2-Methylenecyclopropyl)acetoxy]succinimide** was prepared from (*R*)-(2-methylenecyclopropane)acetic acid (12 mg, 0.11 mmol), *N*-hydroxysuccinimide (30 mg, 0.26 mmol), and dicyclohexylcarbodiimide (36 mg, 0.17 mmol). Purification by HPLC gave 17 mg (74% yield) of product. ^{1}H NMR: δ 0.85–1.21 (m, 1 H), 1.46 (tt, J = 9.2 Hz, J = 2.1 Hz, 1 H), 1.73–1.79 (m, 1 H), 2.56 (dd, J = 16.5 Hz, J = 7.5 Hz, 1 H), 2.72 (dd, J = 16.5 Hz, J = 7.5 Hz, 1 H), 2.84 (s, 4 H), 5.47 (d, J = 1.5 Hz, 1 H), 5.61 (t, J = 2.1 Hz, 1 H).

***N*-[(*S*)-(2-Methylenecyclopropyl)acetoxy]succinimide** was prepared from (*S*)-(2-methylenecyclopropane)acetic acid (10 mg, 0.09 mmol), *N*-hydroxysuccinimide (30 mg, 0.26 mmol), and dicyclohexylcarbodiimide (36 mg, 0.17 mmol). Purification by HPLC gave 16 mg (85% yield) of product. ^{1}H NMR: δ 0.85–1.21 (m, 1 H), 1.46 (tt, J = 9.2 Hz, J = 2.1 Hz), 1.73–1.79 (m, 1 H), 2.56 (dd, J = 16.5 Hz, J = 7.5 Hz, 1 H), 2.72 (dd, J = 16.5 Hz, J = 7.5 Hz, 1 H), 2.84 (s, 4 H), 5.47 (d, J = 1.5 Hz, 1 H), 5.61 (t, J = 2.1 Hz, 1 H).

***N*-[(±)-(2-Methylenecyclopropyl)acetoxy-α,α-d_2]succinimide** was prepared from (2-methylenecyclopropane)acetic-α,α-d_2 acid (**8**; 17 mg, 0.15 mmol), *N*-hydroxysuccinimide (34 mg, 30 mmol), and dicyclohexylcarbodiimide (50 mg, 0.23 mmol) to give 24 mg (76% yield) of product. ^{1}H NMR: δ 0.93–1.0 (m, 1 H), 1.43 (t, J = 9.1 Hz, 1 H), 1.72–1.76 (m, 1 H), 2.80 (s, 4 H), 5.44 (d, J = 1.5 Hz, 1 H), 5.57 (d, J = 2.0 Hz).

(*R*)-(2-Methylenecyclopropyl-*1-d*)acetyl-CoA ((*R*)-β-*d*-MCPA-CoA). Nitrogen gas was bubbled through a solution of $NaHCO_3$ (50 mg) in 3 mL of H_2O for 10 min to remove dissolved oxygen. The lithium salt of coenzyme A (68 mg, 0.090 mmol; Sigma) was then added to the solution. The solution tested positive for a free thiol group according to the nitroprusside test.[48] A solution of *N*-[(*R*)-(2-methylenecyclopropyl-*1-d*)acetoxy]succinimide (20 mg, 0.088 mmol) in 1 mL of THF was then added over approximately 20 s while the nitrogen bubbling was maintained. The mixture was allowed to stand for 2 h as nitrogen bubbling providing agitation. The solution no longer tested positive for a free thiol group, and it was brought to pH 5 with 5% perchloric acid. The solution was concentrated to approximately 1 mL by rotary evaporation (bath temperature 30 °C). The crude product was purified by HPLC using a Waters 3.9 mm × 30 cm C_{18} μ-Bondapak column, C_{18} μ-Bondapak guard column insert, and a 50-μL injection loop. The mobile phase consisted of methanol and a 20 mM HCO_2NH_4 pH 6.0 buffer. A linear gradient of 20–35% methanol was applied over 30 min; the mobile phase was then maintained at 35% methanol for an additional 30 min. A flow rate of 0.5 mL/min was used with spectrophotometric monitoring at 254 nm. The thiol ester product from each 50-μL injection was collected in approximately 1.7 mL of eluting buffer solution, and most of the methanol was removed from this solution by rotary evaporation for about 15 min before an additional fraction was collected. After product in buffer was collected from three injections, the solution of thioester was concentrated to approximately 1 mL by rotary evaporation (bath temperature 25 °C). Analysis of the resulting solution by HPLC with detection at 254 nm showed the desired product accounting for 98% of the total area.

(48) Stadtman, E. R. *Methods Enzymol.* **1957**, *3*, 931–941.

(*S*)-(Methylenecyclopropyl-*1-d*)acetyl-CoA ((*S*)-β-*d*-MCPA-CoA) was prepared from *N*-[(*R*)-(2-methylenecyclopropyl-*1-d*)acetoxy]-succinimide (21 mg, 0.092 mmol), LiSCoA (50 mg, 0.07 mmol), and 50 mg of $NaHCO_3$ and was purified by HPLC.

(±)-(2-Methylenecyclopropyl-*1-d*)acetyl-CoA (β-*d*-MCPA-CoA) was prepared from *N*-[(2-methylenecyclopropyl-*1-d*)acetoxy]succinimide (20 mg, 0.088 mmol), LiSCoA (52 mg, 0.07 mmol), and $NaHCO_3$ and was purified by HPLC. Similarly prepared and purified from the requisite precursors were **(±)-(2-methylenecyclopropyl)acetyl-CoA (MCPA-CoA)**, **(±)-(2-methylenecyclopropyl)acetyl-α,α-d_2-CoA (α-d_2-MCPA-CoA)**, **(*R*)-(2-methylenecyclopropyl)acetyl-CoA ((*R*)-MCPA-CoA)**, and **(*S*)-(2-methylenecyclopropyl)acetyl-CoA ((*S*)-MCPA-CoA)**.

Enzyme Stock Solution. A sample of GAD which had been isolated from pig kidneys and purified following the literature[34,35] and which had been stored in a −20 °C freezer for 9 months was allowed to thaw at room temperature. Analysis by ultraviolet/visible spectroscopy showed peaks at 272, 373, and 446 nm in a ratio of 11:0.9:1.0, and the concentration of active FAD was found to be 12 μM. A stock solution was prepared from this batch of enzyme by diluting it with 50 mM KH_2PO_4/100 mM KCl, pH 7.6, buffer and filtering the diluted enzyme through a glass-wool plug to remove particulate matter and denatured protein. The activity of the stock solution of GAD was taken as 4.60 ± .05 μM, the average of three measured values,[34,35] 4.63, 4.55, and 4.61 μM. The absorbance at 446 nm due to active FAD in the stock solution was calculated to be 0.0708 based on the molar extinction coefficient for enzyme-bound FAD (0.0154 cm^{-1} μM^{-1}).[34,35] The total absorbance at 446 nm for this enzyme solution was 0.1397.

Enzyme Inactivation Kinetics. The HP diode array spectrometer was programmed to acquire spectra at wavelengths of 446, 320, and 360 nm and store the data on 3.5-in. disks in a HP 9122 dual-disk drive. Kinetic experiments were run with a repetition time of 5 s. The spectrometer cell was maintained at a constant temperature of 25 °C using a Lauda-Thermostat K 2 constant-temperature bath which pumped 25 °C water through the spectrometer cell holder. One cuvette was used for a given set of kinetic experiments, and the cell was washed with distilled water between runs. An Eppendorf 10–100-μL adjustable-volume pipet was used to measure and transfer buffer volumes of 100 μL or less; to transfer larger volumes, an Eppendorf 100–1000-μL adjustable-volume pipet was used. The spectrometer absorbances were referenced (zeroed) on a 7:1 solution of 50 mM KH_2PO_4/100 mM KCl, pH 7.6, and 20 mM HCO_2NH_4, pH 5.5 (blank solution); pH analysis showed the blank solution to be pH 7.6. Concentration of a 400-μL stock solution of (*R*)-MCPA-CoA was determined by HPLC analysis to be 240 ± 5 μM.

A 161 μM sample of (*R*)-MCPA-CoA was prepared by adding an 80.5-μL aliquot of the stock substrate to 39.5 μL of 20 mM HCO_2NH_4 pH 5.6 buffer to give a total volume of 120 μL. The enzyme (4.60 μM, 700 μL) was added to a cuvette and allowed to stand in the cell holder for 8 min, after which 100 μL of the 161 μM substrate was added to the enzyme. The resulting time-zero enzyme concentration in the reaction mixture was 4.03 μM in active FAD, and the resulting substrate concentration was 20.1 μM: $A^*_{446}(t_0)$ = 0.0620. The reaction solution was mixed rapidly by repeatedly drawing and dispensing the mixture, by use of the Eppendorf pipet, for approximately 3 s, and then the cover of the diode array spectrometer was closed; the entire operation took approximately 4 s. The reaction was monitored for 20 min, and an enzymatic activity assay was taken of the mixture 24 min after initiating the reaction: 0.89 μM of active FAD remained (78% inactivation). This procedure was repeated for a duplicate kinetic experiment; an enzyme activity assay 23 min after initiating the reaction showed that 1.11 μM of active FAD remained (72% inactivation). The time versus extent inactivation profiles for these two kinetic runs over the first 3 min are displayed in Figure 2.

The other 12 kinetic runs for the other coenzyme A esters of Chart I were conducted in the same fashion, using the same enzyme, freshly analyzed each day by enzyme activity assays to determine active FAD concentrations and by ultraviolet/visible spectroscopy to determine total A_{446} and $A^*_{446}(t_0)$ values; the inactivation profiles are shown in Figures 1 and 2. For six of the eight inactivations presented in Figure 2, enzyme activities after 23 or 24 min were determined both by spectrophotometric means and through activity assays; the percent activity values agreed to ±4%.

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