data would imply that either AAD and HBDH have not evolved from a common precursor or, if they did, the original enantioselectivity was lost in the course of that evolution. In any case, we believe that this is the first case where the conformational enantioselectivity has been examined for two different enzymes catalyzing different reactions of the same substrate.

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Supplementary Material Available: Kinetic data for 2-oxocyclohexanecarboxylate as an enzyme substrate (4 pages). Ordering information is given on any current masthead page.

Stereospecificity and Stereochemical Infidelity of Acetoacetate Decarboxylase (AAD)

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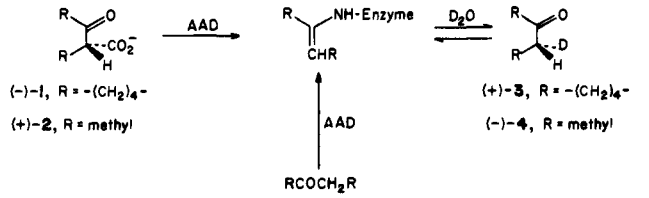
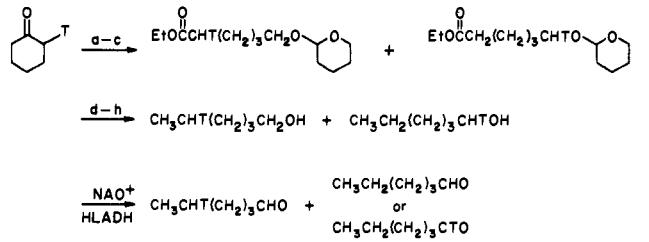
The stereospecificity observed in enzymic catalysis provides both essential clues for understanding the mechanisms of enzymic reactions[1] and opportunities for synthesizing optically active molecules that would otherwise not be easily accessible.[2] Our studies of the stereoselectivity of acetoacetate decarboxylase (AAD, EC 4.1.1.4) from *Clostridium acetobutylicum*[3] have been particularly fruitful in these respects, and we report here the following findings: (1) Decarboxylation of the two β-keto carboxylates **1** and **2** proceeds with net retention of stereochemistry; (2) AAD catalyzes the exchange of *pro-R* α-hydrogens of a variety of ketones, making it a versatile reagent for the synthesis of optically active α-deuterio ketones;[4] (3) the AAD-catalyzed exchange reaction proceeds with a small but detectable level of stereochemical infidelity, which results from competing reaction pathways at the active site.

Westheimer and co-workers[5] have shown that AAD forms a protein-bound enamine as an intermediate in the enzymic decarboxylation of β-keto carboxylates, as shown in Scheme I. When incubated with racemates of 2-oxocyclohexanecarboxylate (**1**) or 2-methyl-3-oxobutyrate (**2**), AAD catalyzes the selective decarboxylation of (−)-**1**, leaving behind (+)-**1**, and (+)-**2**, leaving behind (−)-**2**.[6] We have assigned the stereochemistries of the reactive enantiomers by determining the absolute configurations of the unreacted antipodes. Unreactive (+)-**1** has the *S* configuration;[7] likewise, we have found that unreactive (−)-**2** also has the *S* configuration by reducing it with sodium borohydride to *erythro*- and *threo*-2-methyl-3-hydroxybutyrates,[6] which have known configurations at the 2 position.[8]

Scheme I

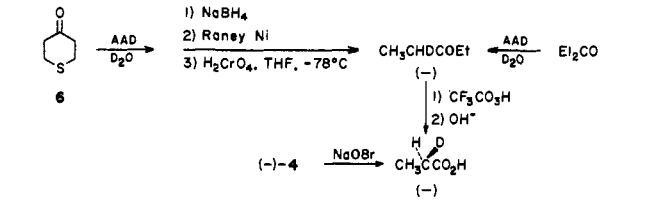


Scheme II[a]

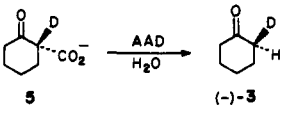


[a] (a) CF_3CO_3H, CH_2Cl_2, 0 °C; (b) EtOH, H_2SO_4, reflux; (c) dihydropyran, *p*-TsOH, benzene; (d) $LiAlH_4$, ether, reflux; (e) *p*-TsCl, pyridine, 0 °C; (g) $LiAlH_4$, ether, reflux; (h) MeOH, *p*-TsOH, reflux.

Scheme III



When AAD catalyzes the decarboxylation of racemic **5** (the 1-deuterio analogue of **1**) in H_2O, (−)-**3**, $[\alpha]^{25}_D$ −0.6° (*c* 14, ether), is recovered, which is 40% d_1.[9] Similarly, when racemic



(1)

2,4,4,4-tetradeuterio-**2** is decarboxylated in H_2O, dextrorotatory deuterated 2-butanone is recovered, while decarboxylation of racemic **2** in D_2O yields levorotatory deuterated 2-butanone. From the absolute configurations that we assign below to these chiral, deuterated ketones, we conclude that the decarboxylations of both (−)-**1** and (+)-**2** by AAD proceed with net retention of configurations, with protonation of the intermediate enamine in either case occurring on the same face from which CO_2 departed.[10]

AAD also catalyzes the exchange of deuterium or tritium into the α positions of cyclohexanone or 2-butanone to produce (+)-**3** or (−)-**4** or their tritio analogues. Three specimens of [2-^{3}H]-cyclohexanone were prepared: the first by enzymic exchange from tritiated water into cyclohexanone, the second by hydrolysis of commercial *N*-morpholino-1-cyclohexene to yield a stereorandomly labeled sample, and the third by AAD-catalyzed exchange of tritium out of the stereorandomly labeled material. These labeled

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(1) Popjak, G. *Enzymes, 3rd Ed.* **1970**, *2*, 116–214.

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(4) AAD-catalyzed exchange of one of the methylene hydrogens of 2-butanone has been reported: Hammons, G.; Westheimer, F. H.; Nakaoka, K.; Kluger, R. *J. Am. Chem. Soc.* **1975**, *97*, 1568–1572, 4152.

(5) (a) Westheimer, F. H. *Proc. Robert A. Welch Found. Conf. Chem. Res.* **1971**, *15*, 7–50. (b) Guthrie, J. P. *J. Am. Chem. Soc.* **1972**, *94*, 7020–7024; 7024–7029. (c) Guthrie, J. P.; Jordan, F. *Ibid.* **1972**, *94*, 9132–9136; 9136–9141.

(6) A 0.75 M solution of racemic **2** was incubated in 50 mM phosphate buffer (pH 5.95) at 22 °C with 7200 IU of AAD; after 30 min the solution showed a rotation $[\alpha]_D$ −2.0°, excess sodium borohydride was added, and the reaction mixture was incubated, then acidified at 0 °C to pH 1 with HCl, filtered, lyophilized, and extracted with ether to yield *erythro*- and *threo*-2-methyl-3-hydroxybutyric acids.

(7) Benner, S. A.; Morton, T. H. *J. Am. Chem. Soc.* **1981**, *103*, preceding paper in this issue.

(8) Tai, A.; Imaida, M. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 1114–1117.

(9) All isotopic purities reported herein are based on relative molecular ion intensities (corrected for ^{13}C natural abundance) in 70-eV mass spectra. Incomplete labeling of **3** from reaction 1 is a consequence of exchange of the label in **5** with solvent.

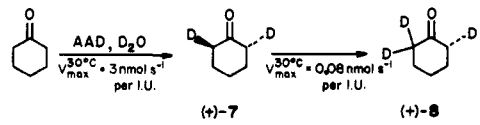
(10) The stereochemical preferences of a number of similar β-decarboxylases have been reviewed: Rose, I. A. *Crit. Rev. Biochem.* **1972**, *1*, 33–58.

0002-7863/81/1503-993$01.25/0

cyclohexanones were then converted to labeled 1-hexanols (Scheme II), diluted with 1-[1-^{14}C]hexanol as internal standard, and oxidized enzymically with NAD^+ and horse liver alcohol dehydrogenase (HLADH).[11] The $^{14}C/^3H$ ratios for the *N*-phenylcarbamate of 1-hexanol and the semicarbazone of hexanal produced by HLADH-catalyzed oxidation were compared for each sample. Upon enzymic oxidation, the stereorandomly labeled sample lost 23% of its tritium label, the sample from "exchanged in" [2-^{3}H]cyclohexanone lost 39% of its label, and the sample from "exchanged out" lost none of its label. Assuming that the Baeyer-Villiger oxidation in Scheme II proceeds with retention of configuration at the migrating center,[12] HLADH catalyzes removal of the *pro-R* hydrogen from hexanol,[2] and approximately half the original label in [2-^{3}H]cyclohexanone ultimately appears in an unexchangeable methylene group,[13] we conclude that AAD catalyzes exchange of *pro-R* hydrogens at the α positions of cyclohexanone.[14]

We have confirmed this assignment by preparing optically active α-deuterated ketones via AAD-catalyzed exchange of cyclopentanone, 4-isopropylcyclohexanone, and 4-thiacyclohexanone (**6**) with D_2O. Absolute configurations for α-deuterated cyclopentanone[15] and 4-isopropylcyclohexanone[16] have been reported and are consistent with our assignment. Both enantiomers of α-deuterated **6** were prepared and absolute configurations assigned by conversion to the known optically active [2-^{2}H]propionic acid[17] via α-deuterated 3-pentanone,[18] as shown in Scheme III. The assignment of absolute stereochemistry to **3** agrees with the octant rule[19] and independent work from Djerassi's laboratory.[20] The *R* configuration was assigned to (-)-**4** by its conversion to (-)-[2-^{2}H]propionic acid,[21] shown in Scheme III. This assignment was confirmed by preparation of (+)-2-[3-^{2}H]butanone by chromic acid oxidation of an authentic sample of 2-[3-^{2}H]butanol known to have 3*S* configuration.[22]

We have studied the kinetics of AAD-catalyzed exchange of the α-hydrogens of cyclohexanone and 2-butanone by polarimetry and mass spectrometric analysis of the extent of deuteration. Surprisingly, we observe that AAD catalyzes a slow but detectable exchange of the *pro-S* hydrogens at a rate 1-3% that of the exchange of *pro-R* hydrogens.[23] Exchange reaction 2 gives excellent yields of (+)-**7**,[24] but at longer times (+)-**8** can be seen both by mass spectrometry and ^{2}H NMR.[25] The *pro-S* and *pro-R*



(+)-**7** (+)-**8** (2)

exchange are both inhibited completely by 5 μM acetopyruvate, a known inhibitor of the decarboxylation reaction.[5b] Both exchanges are slowed by a factor of 5 in 10 μM 5-nitrosalicylaldehyde, a competitive inhibitor of AAD.[5a] Furthermore, perchlorate,[26] in concentrations from 1 to 10 μM, inhibits in parallel both the *pro-R* and *pro-S* exchanges. These experiments strongly suggest that *pro-S* exchange occurs at the active site and proceeds via the same intermediate responsible for stereospecific *pro-R* exchange.

The broad substrate specificity of AAD makes this enzyme valuable in the synthesis of a wide variety of compounds that are optically active by virtue of asymmetric isotopic substitution. The small amount of nonspecific exchange is especially interesting, since it appears to be an exception to the general rule that enzyme stereoselectivity is absolute once a substrate is bound.[10,27] For AAD, unlike many enzymes, stereochemical infidelity is not easily understood in terms of alternative modes of binding.[2] If both the predominant *pro-R* exchange and the minor *pro-S* exchange occur by the attack of a proton on the same enamine intermediate, we are forced to conclude that two stereochemically distinct reactions can occur at the same active site on the same bound intermediate to yield the same chemical result. We know of no other case where this has been shown for an enzyme.

Acknowledgment. We thank Professor Frank H. Westheimer, in whose laboratories this work was performed, and Dr. Walter Blättler for helpful discussions. The support of the NSF (to J.D.R. through a predoctoral fellowship), the Xerox Corporation (to S.A.B. through a postdoctoral fellowship), the NIH (through Grants NS-14773 to T.H.M. and GM-04-71223 to F.H.W.), and the Rockefeller Foundation (to T.H.M. as an IPH Visiting Scholar) are gratefully acknowledged.

(11) Enzymic oxidations were carried out under nitrogen in 0.1 M ammonium carbonated buffer (pH 10.1) containing 0.2 M acetaldehyde, 0.8 mM NAD^+, and 0.18 mg mL^{-1} HLADH. Experimental details are reported in: Polavarapu, P. L.; Nafie, L. A.; Benner, S. A.; Morton, T. H. *J. Am. Chem. Soc.*, submitted for publication.

(12) Mislow, K.; Brenner, J. *J. Am. Chem. Soc.* **1953**, *75*, 2318-2322.

(13) The presence of tritium in both the 1 and 5 positions of the radiolabeled hexanols was confirmed by chemical oxidation to hexanoic acid, by which ~40% of the ^{3}H label was lost.

(14) Degradation of (-)-**7**, $[\alpha]^{28}_D$ -3.9° (neat), which was prepared by AAD-catalyzed exchange of cyclohexanone-*2,2,6,6-d*$_4$ with H_2O, to *n*-pentyl camphanate, followed by NMR analysis (Gerlach, H.; Zagalak, B. *J. Chem. Soc., Chem. Commun.* **1973**, 274-275) confirms our assignment. We are grateful to Processor John M. Schwab (*J. Am. Chem. Soc.*, submitted for publication) for communicating these results to us prior to publication.

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(18) α-Deuterated **6**, $[\alpha]^{37}_D$ +6.4° (*c* 2, D_2O) was converted to α-deuterated 3-pentanone, $[\alpha]^{25}_D$ -1.6° (*c* 4, ether). Levorotatory α-deuterated 3-pentanone was also prepared by AAD-catalyzed exchange of 3-pentanone with D_2O.

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(20) Sundararaman, P.; Barth, G.; Djerassi, C. *J. Org. Chem.* **1980**, *45*, 5231-5236. We are grateful to Professor Djerassi for communicating these results to us prior to publication.

(21) Retey, J.; Umani-Ronchi, A.; Arigoni, D. *Experientia* **1966**, *22*, 72-73.

(22) We are grateful to Professor Duilio Arigoni for supplying a sample of (2*R*,3*S*)-2-[3-^{2}H]butanol.

(23) After reaching a peak value, the optical rotation of a reaction mixture slowly declines and ultimately vanishes. The *pro-S* exchange has also been monitored by loss of radioactivity from (2*S*)-[2-^{3}H]cyclohexanone. Both the *pro-R* and *pro-S* exchange rates are independent of pH in the range pH 5.2-6.8. Both rates are also independent of phosphate buffer concentration from 15 to 50 mM at pH 5.95.

(24) Exchange of 1.5 g of cyclohexanone in 30 mL of buffered D_2O containing 1 mg (800 IU) of AAD at 38 °C reached peak optical activity after 1 h, and ethereal extraction followed by distillation afforded 1.1 g of (+)-**7**, $[\alpha]^{25}_D$ +3.7° (neat), which showed a d_1:d_2:d_3 ratio of approximately 2:9:1 by mass spectrometry.

(25) The proton-decoupled 41.44-MHz deuterium NMR spectrum of (+)-**7** in $CHCl_3$ shows a single peak at δ 1.62, while a mixture of (+)-**7** and (+)-**8** shows two singlets at δ 1.60 (CD_2) and 1.62 (CHD). We are indebted to Mr. Peter Demou of Yale University for recording these spectra at the Southern New England High Field NMR Facility, supported by the Biotechnology Resources Program of the NIH (RR-798).

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Increase of ^{13}C NMR Relaxation Times in Proteins due to Picosecond Motional Averaging†

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The nature of atomic motions in the interior of proteins is currently a topic of great interest.[1-3] It has been shown by

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