Mechanism and Stereochemistry of α,β-Dihydroxyacid Dehydratase

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Received June 20, 1990

Abstract: A mechanistic model of spinach α,β-dihydroxyacid dehydratase (EC 4.2.1.9) based on the processing of a number of synthetic substrate analogues has been developed. These analogues include dihydroxy acids with β-substituents of various electronic and chemical properties and high-energy intermediate (carbocation) analogues. Their properties suggest the enzyme uses a polar but concerted elimination mechanism which should proceed through an anti transition state. Study of the stereochemistry of the enzyme has shown insufficient discrimination between isomeric substrates or enol analogues to permit the proof of an anti mechanism.

Introduction

The biosynthetic pathway for branched-chain amino acids (Figure 1) in higher plants has become an important topic of study due to the recent discoveries that several classes of herbicidal compounds interfere with valine, leucine, and isoleucine production.[2-9] Much of the focus has been on acetolactate synthase (EC 4.1.3.18, ALS or AHAS), the first enzyme of the pathway and the target of the herbicides, but the appearance of pest species possessing enzymes resistant to sulfonylureas after less than a decade of their use[10] has brought attention to the basic enzymology of the whole pathway. The readily available bacterial AHAS isozymes have been studied with a number of the active herbicides. The plant enzyme has been purified to homogeneity, and the existence of isozymes from this source has also recently been demonstrated.[11] The cloning and sequencing of the *Nicotiana* and *Arabidopsis* AHAS genes represent a major advance,[12] and the recent expression of the *Arabidopsis* AHAS in *Escherichia coli*[13] provided quantities of enzyme for study.

The next enzyme in the pathway, acetohydroxyacid reductoisomerase (EC 1.1.1.86, AHAIR), has not been purified from plants; consequently, a recent mechanistic study by Chunduru et al.[14] used the *E. coli* enzyme. The complete sequence of the *E. coli ilv* operon, whose transcription is regulated by attenuation,[15] had been recently reported,[16] and it provided the coding sequence used to construct an AHAIR overproducer. An inhibitor of both the bacterial and plant reductoisomerase, herbicide Hoe 704, has recently been discovered.[17]

The third enzyme of the pathway, α,β-dihydroxyacid dehydratase (EC 4.2.1.9, DHAD), has been purified to homogeneity from spinach by two groups.[18,19] The final enzyme of the pathway, branched-chain amino acid transaminase or transaminase B (EC 2.6.1.42, BCAATA), has been sequenced from the DNA in *E. coli* and from the protein in *Salmonella*,[20] but nothing is known about the plant enzyme.

Dihydroxyacid dehydratase activity has been found in bacteria,[21] algae and higher plants,[22] and yeast.[23] Auxotrophic mutants lacking DHAD have been characterized in bacteria,[24] fungi,[25] and higher plants. *Datura innoxia* and *Nicotiana plumbaginifolia* cell lines that are DHAD deficient[26] have been isolated. The *E. coli ilv*D gene that encodes DHAD has been cloned by complementation and sequenced as part of the *ilv* operon as mentioned above. This gene encodes a 66-kDa polypeptide, and its gene product has a molecular mass of 65-66 kDa on the basis of SDS-PAGE.[27] The genes encoding DHAD in *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* have been cloned by complementation but have not yet been sequenced for comparison to the bacterial sequence.[28]

The *Neurospora crassa* enzyme as obtained in homogeneous form is slightly larger than 66 kDa and appears to be multimeric.[29] Flint and Emptage[19] reported the spinach enzyme's monomer molecular mass as 63.5 kDa, and gel filtration and nondenaturing gel electrophoresis indicated it exists as a dimer. We previously

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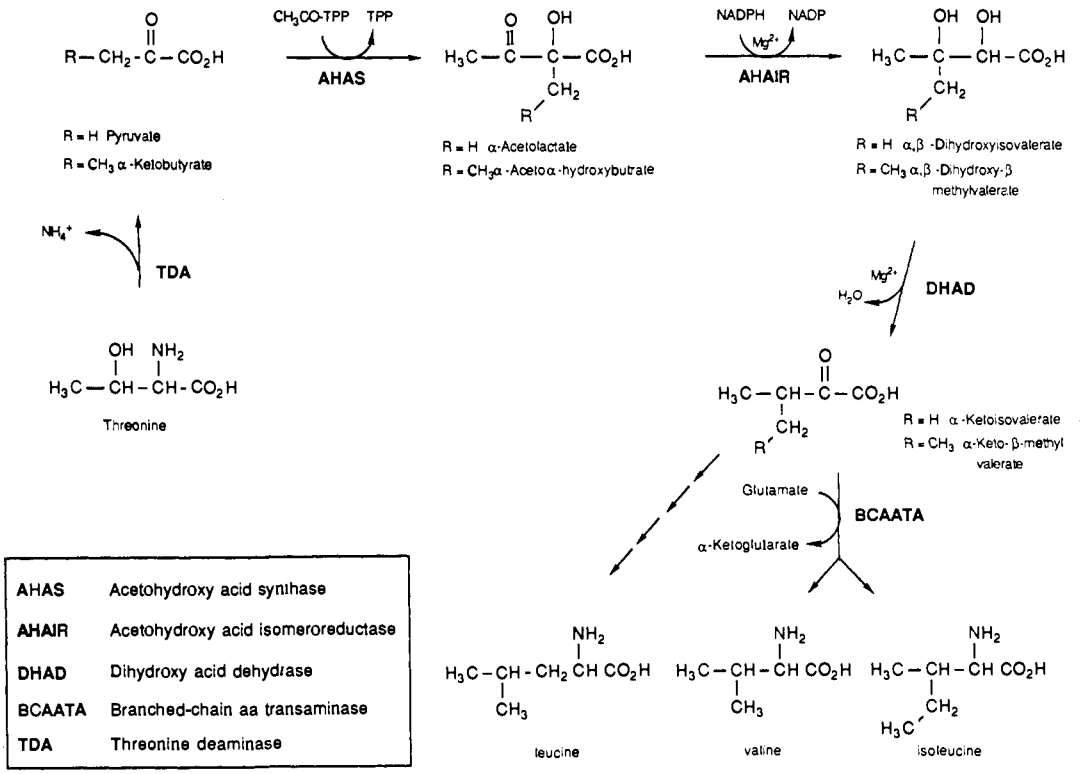

Figure 1. Biosynthetic pathway for branched-chain amino acids in bacteria and higher plants.

postulated that the enzyme is disulfide-linked on the basis of its hydrodynamic properties under reducing conditions.[18] Purification of DHAD from prokaryotic sources has not been possible due to its instability. Other plant sources have included green gram[30] and wheat seedlings,[31] from which DHAD has been obtained only in partially purified form of unknown M_r.

The DHADs examined thus far share many common features. A divalent metal ion is required for activity; thus, Mg^{2+} is generally included in purification and assay solutions. Heavy metals (copper, silver, mercury) are commonly inhibitors, as are fluoride, sulfhydryl reagents, and EDTA. The optimal pH is usually around 8. Superoxide inhibits the enzyme in plants[18] and bacteria,[32] presumably by reducing the iron–sulfur cluster discovered associated with DHAD by Flint and Emptage. All known DHADs can process both dihydroxyisovalerate (valine precursor) and β-methyl dihydroxyvalerate (isoleucine precursor).[29] Several lines of evidence suggest a single enzyme is at work. The two activities have similar chromatographic properties, temperature sensitivity, and pH optima, and DHAD-deficient mutants accumulate both substrates. Generally, the K_m is 2–3 times higher for dihydroxyisovalerate.

Mechanistic studies of DHAD from any source are meager, and no true kinetics have been reported. On the basis of the change in the UV and EPR spectra of the iron–sulfur cluster of the spinach enzyme upon substrate binding, Flint and Emptage[19] postulated that iron acts as a Lewis acid to facilitate the departure of the β-hydroxyl group. This idea has analogies in mechanistic postulates for aconitase. It has been demonstrated in both the *Salmonella*[33] and spinach enzymes[18] that the α-proton is lost to the medium during dehydration. This result is best explained by a pathway involving elimination to form an enol which is enzymatically protonated. Two studies of inhibitors of DHAD have been made. Our previous work focused on the purified spinach enzyme,[18] while crude yeast and *E. coli* enzymes were studied by Westkaemper and Zenk.[34]

The work described in this paper was undertaken with two purposes. The first, to address the sequence of bond-making and -breaking steps in the elimination reaction, has impact on the expected stereochemical course of the elimination. The kinetic dependence on stereochemistry was the second issue to address, in particular the distinctions of molecules differing only in the presence and placement of a methyl group.

Materials and Methods

General. 2-(Dimethylphosphinyl)-2-hydroxyacetate (Hoe 704) was a gift from Dr. Arno Schulz of Hoechst AG. Dimethylammoniomethanesulfonate (**5**) was obtained by the method of King and Skonieczny.[35] ^{19}F NMR data were recorded in parts per million upfield from $CFCl_3$ by using CF_3CO_2H ($\delta = +76.55$) as an internal, secondary standard.

Assays. DHAD activity was measured as previously described.[18] Specific activity (units) is given in micromoles of 2-oxo-3-methylbutanoate formed per hour per milligram of protein at an α,β-dihydroxyisovalerate concentration of 10 mM. All substrates and chiral inhibitors were studied as racemates. Ten substrate concentrations around K_m were used for the determination of K_m and k_{cat}. Twenty data points at five different inhibitor concentrations were used for the determination of K_i/K_m. Since our original publication, we have determined that β-mercaptoethanol is an inhibitor of DHAD, so purification and assay buffers now omit it.

Ethyl (*Z*)-3-Methyl-2-pentenoate. Ethyl 2-pentynoate (2.8 g, 22.2 mmol) in 35 mL of dry THF was added dropwise over 15 min to a −78 °C solution of lithium dimethyl cuprate (prepared at 0 °C from 4.46 g of CuI, 35 mL of THF, and 32 mL of 1.4 M MeLi in ether). The reaction was stirred an additional 10 min and quenched at −78 °C with 5 mL of methanol. The cooling bath was removed, and 50 mL of 15% ammonium chloride and 50 mL of ether were added. The mixture was filtered through Celite and washed with ether. The ether layer was

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washed with saturated ammonium chloride and with brine and then dried over Na_2SO_4. Rotary evaporation followed by flash chromatography on silica gel provided 2.81 g (84%) of a colorless oil. The structure was confirmed by comparison with literature data.[36]

(*Z*)-3-Methyl-2-pentenoic Acid. The ethyl ester was hydrolyzed with barium hydroxide by the method of Crout and Whitehouse.[37]

Sodium (2*RS*,3*RS*)-2,3-Dihydroxy-3-methylpentanoate. Oxidation of (*Z*)-3-methyl-2-pentenoic acid with H_2WO_4/H_2O_2 was performed by the method of Crout and Whitehouse[37] to provide **9**.

Ethyl (2*RS*,3*SR*)-2,3-Dihydroxy-3-methylpentenoate. Ethyl (*Z*)-3-methyl-2-pentenoate (680 mg, 4.78 mmol) was dissolved in 14 mL of acetone/water (2:1). A small crystal of OsO_4 (~5 mg) was added. $Ba(ClO_3)_2 \cdot H_2O$ (775 mg, 2.39 mmol) was added and the solution stirred 18 h at room temperature. The solution was diluted with water and extracted with ether (6×). The combined ether layers were dried over Na_2SO_4. The solvent was evaporated, and flash chromatography on silica gel provided 380 mg (45%) of a colorless oil: IR (thin film) 3430, 2960, 1725, 1375, 1270, 1100 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.36–4.24 (m, 2 H), 4.03 (d, J = 6.7, 1 H), 3.09 (d, J = 6.7, 1 H), 1.67–1.58 (m, 1 H), 1.48–1.39 (m, 1 H), 1.34 (t, J = 7.2, 3 H), 1.22 (s, 3 H), 0.97 (t, J = 7.5, 3 H). HRMS calcd for $C_6H_{11}O_4$ [M - Et]: 147.0657. Found: 147.0665. HRMS calcd for $C_4H_8O_3$: 104.0473. Found: 104.0485.

Sodium (2*RS*,3*SR*)-2,3-Dihydroxy-3-methylpentanoate. The above ethyl ester (800 mg, 4.54 mmol) was dissolved in 3 mL of methanol. Sodium hydroxide (5 mL of 1 N) was added, and the system was stirred for 1 h at room temperature. The solution was washed with dichloromethane (2×) and then passed down a Dowex 50X8-200 column (H+ form). The pH of the effluent was adjusted to 7 with 1 N NaOH, and the solvent was evaporated to give 733 mg (95%) of **8** as a white powder. The structure was confirmed by comparison with literature data.[38]

Stereochemical Preferences. Racemic α,β-dihydroxyisovalerate (20 mg, 120 μmol) was incubated at 35 °C with 45 units of DHAD in 2.5 mL of Tris buffer. Aliquots (5 μL) were removed periodically and assayed for keto acid production. After 3.5 h, 48% conversion had occurred. No further change had occurred after an additional 3.5 h. The solution was evaporated to dryness, the residue was suspended in 0.1 N HCl (2 mL), and the optical rotation was measured as $[\alpha]_D$ +11.8° (*c* 0.5%, 0.1 N HCl) (lit.[39-42] $[\alpha]_D$ +13.5° for the 2*S* isomer).

Stereochemical Course. (2*RS*,3*RS*)-Dihydroxy-3-methylvalerate (21.3 mg, 125 μmol) was incubated at 35 °C with 45 units of DHAD for 5 h, whereupon 48% of the substrate had been consumed. A solution of 2,4-dinitrophenylhydrazine (23 mg, 81 μmol) in 3.5 mL of 2 N HCl was added. After standing for 1 h at room temperature, the precipitate was collected by filtration. Its specific rotation [$[\alpha]_D$ +16.5° (*c* 0.88%, EtOH)] indicates high optical purity of the 3*S* isomer ($[\alpha]_D$ +16.5°).[43] The filtrate was evaporated, and the residue was resuspended in 2 mL 0.1 N HCl. The residual substrate had $[\alpha]_D$ +20.2° (*c* 0.5%, 0.1 N HCl), compared to a literature value for the 2*S*,3*S* isomer of +23.2°.[42] When the same experiment was performed with the 2*RS*,3*SR* isomer, the dinitrophenylhydrazone of the product had $[\alpha]_D$ -15.1° (*c* 0.89%, EtOH) compared to the $[\alpha]_D$ -16.7° reported by Meister[43] for the 3*R* isomer.

2-Fluoro-3-methyl-2-pentenoic Acid. To a solution of 4.8 g (41.2 mmol) of chlorotrifluoroethylene in 50 mL of ether cooled to -60 °C was added 28 mL (36.4 mmol) of a 1.3 M solution of *s*-BuLi in cyclohexane. The resultant green-brown solution was stirred for 15 min and then treated with 3.90 mL of 2-butanone. The reaction mixture was stirred at -60 °C for 20 min, warmed to room temperature, and partitioned between ether and 1 M HCl. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to afford 6.5 g of a brown oil. This residue was added to a mixture of 30 mL of concentrated H_2SO_4 and 1.5 mL of water cooled to -20 °C. THF (4 mL) was added to make the solution homogeneous. The reaction mixture was allowed to warm to room temperature over 20 min and was treated with 20 mL of water. After 1 h of stirring, the solution was partitioned between ether and water. The organic layer was washed with brine, dried with $MgSO_4$, and evaporated to afford 2.6 g of a brown oil. Chromatography on silica gel (74% hexanes/25% ethyl acetate/1% acetic acid) afforded 719 mg (15%) of the diastereomeric mixture of acids: IR (thin film) 2980, 1701, 1655, 1437, 1300, 1159 cm^{-1}. 7: ^1H NMR ($CDCl_3$) δ 1.09 (t, J = 7.6, 3 H), 2.13 (d, J = 3.1, 3 H), 2.30 (dq, J = 3.4, 7.6, 2 H): ^1H NMR (D_2O, Na^+ salt) δ 1.00 (t, J = 7.6, 3 H), 1.96 (d, J = 3.0, 3 H), 2.16 (dq, J = 3.4, 7.6, 2 H); ^{19}F NMR (D_2O, Na^+ salt) δ 121.36. **6**: ^1H NMR ($CDCl_3$) δ 1.09 (t, J = 7.5, 3 H), 1.91 (d, J = 4.3, 3 H), 2.56 (dq, J = 1.2, 7.5, 2 H); ^1H NMR (D_2O, Na^+ salt) δ 0.99 (dt, J = 1.0, 7.5, 3 H), 1.74 (d, J = 4.2, 3 H), 2.44 (dq, J = 1.4, 7.5, 2 H); ^{19}F NMR (D_2O, Na^+ salt) δ 119.22. Comparison with literature data[44] permits the assignment of stereochemistry. HRMS Calcd for $C_6H_9FO_2$: 132.0587. Found: 132.0597.

Benzyl 2-Fluoro-3-methyl-2-pentenoate. A solution of 719 mg (5.44 mmol) of 2-fluoro-3-methyl-2-pentenoic acid in 60 mL of methylene chloride was cooled to 0 °C and treated with 1.2 mL (11.6 mmol) of benzyl alcohol, 57 mg (0.47 mmol) of dimethylaminopyridine, and 1.30 g (6.29 mmol) of DCC. The solution was stirred for 30 min and warmed to room temperature for 2 h. Petroleum ether (150 mL) was added, and the reaction mixture was filtered and concentrated to give a brown oil. Silica gel chromatography (2% ether/hexanes) afforded 1.12 g (93%) of the title compound as a colorless oil. HPLC (Zorbax Sil preparative column, 1% ether/5% chloroform/94% hexanes) gave pure samples of each isomer. *E* (first to elute): IR (thin film) 2980, 1729, 1667, 1300, 1232, 1106, 790, 770 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.07 (dt, J = 0.8, 7.5, 3 H), 1.86 (d, J = 4.3, 3 H), 2.54 (dq, J = 1.5, 7.5, 2 H), 5.25 (s, 2 H), 7.30–7.42 (m, 5 H); ^{19}F NMR ($CDCl_3$) δ 126.98. HRMS Calcd for $C_{13}H_{15}O_2F$: 222.1057. Found: 222.1061. *Z* (second to elute): IR (thin film) 2980, 1722, 1667, 1315, 1275, 1240, 1106, 790, 770 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.06 (t, J = 7.6, 3 H), 2.10 (d, J = 3.2, 3 H), 2.25 (dq, J = 3.5, 7.6, 2 H), 5.25 (s, 2 H), 7.30–7.42 (m, 5 H); ^{19}F NMR ($CDCl_3$) δ 128.67. HRMS Calcd for $C_{13}H_{15}O_2F$: 222.1057. Found: 222.1061.

Sodium (*E*)-2-Fluoro-3-methyl-2-pentenoate. A solution of 61 mg (0.274 mmol) of the benzyl ester in 2 mL of THF was treated with 0.30 mL of 1 N NaOH solution, 1 mL of water, and 0.1 mL of methanol (until solution was homogeneous). After 15 min, the solvent was removed. The residue was taken up in 4 mL of water, and the pH was adjusted to 7.0 with 1 M HCl. The solvent was again removed, and the solid was triturated with warm absolute ethanol to afford after evaporation 35.7 mg (85%) of **12** as a hygroscopic white solid.

Sodium (*Z*)-2-Fluoro-3-methyl-2-pentenoate. The saponification was carried out as described above with 172 mg (0.774 mmol) of the benzyl ester and 1 mL (1.0 mmol) of 1 N NaOH in 3 mL of THF, 2 mL of water, and 1 mL of methanol to afford after trituration 113 mg (95% yield) of **13** as a hydroscopic white solid.

Benzyl 2-(Benzyloxy)-3-cyclopropyl-3-hydroxybutanoate. To 3.74 mL of a 1.0 M solution of lithium bis(trimethylsilyl)amide in THF cooled to -78 °C under nitrogen was added dropwise by syringe over 5 min a solution of 871 mg (3.4 mmol) of benzyl (benzyloxy)acetate in 1 mL of THF. The solution was stirred for 90 min at -78 °C, and then a solution of 315 mg (3.74 mmol) of cyclopropyl methyl ketone in 1 mL of THF was added dropwise over 5 min by syringe. The reaction was stirred for an additional 30 min at -78 °C, at which point the cooling bath was removed, and the reaction was quenched with 4 mL of 15% aqueous ammonium chloride. The mixture was extracted with ether. The ether layer was washed with water and then with brine and dried over $MgSO_4$. The solvent was removed by rotary evaporation. Flash chromatography on silica gel, eluting with hexanes/ethyl acetate (9:1), provided 585 mg (51%) of a colorless oil, shown by NMR to be a 1:1 mixture of diastereomers: IR (thin film) 3520, 3030, 3000, 1745, 1460, 1125, 765 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.38–7.3 (m, 10 H + 10 H), 5.27–5.19 (m, 2 H + 2 H), 4.74 (d, J = 11.5, 1 H) + 4.73 (d, J = 11.6, 1 H), 4.44 (d, J = 11.5, 1 H) + 4.34 (d, J = 11.6, 1 H), 3.94 (s, 1 H) + 3.89 (s, 1 H), 1.22 (s, 3 H) + 1.17 (s, 3 H), 1.05–0.91 (m, 1 H + 1 H), 0.39–0.18 (m, 4 H + 4 H). HRMS Calcd for $C_{16}H_{16}O_3$ (McLafferty fragment): 256.1099. Found: 256.1089.

Sodium 3-Cyclopropyl-2,3-dihydroxybutanoate. The benzyl ester above (580 mg, 1.7 mmol) was placed in a 25-mL flask with 200 mg of 10% palladium on carbon. Twice the system was evacuated and then flushed with hydrogen. Absolute ethanol (6 mL) and one drop of concentrated HCl were added, and the system was stirred for 48 h under 1 atm of hydrogen. The catalyst was removed by filtration through Celite. Flash chromatography on silica gel, eluting with methanol/dichloromethane (1:9), provided a colorless oil, shown by NMR to be a 1:1 mixture of diastereomers: IR (thin film) 3390, 2980, 1730, 1390, 1230, 1105 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.12 (s, 1 H) + 4.10 (s, 1 H), 1.34 (s, 3 H) + 1.24 (s, 3 H), 1.19–1.10 (m, 1 H + 1 H), 0.56–0.36 (m, 4 H +

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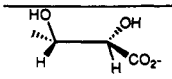
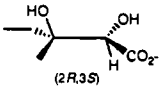
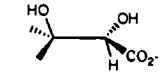
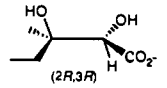
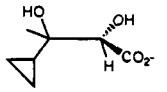
Table I. Michaelis Constants, k_{cat}, and k_{cat}/K_m for substrates of DHAD[a]

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
	0.55 ± 0.04	4.6	8.3
 (2R,3S)	0.71 ± 0.04	12.3	17.3
	0.97 ± 0.03	11.5	11.9
 (2R,3R)	0.33 ± 0.02	8.3	25.2
	1.78 ± 0.08	5.7	3.2

[a] Our earlier report of the Michaelis constant for dihydroxyisovalerate was substantially higher. All of these kinetic constants were determined with the same high-quality enzyme preparation in the absence of the inhibitor mercaptoethanol and are quite reliable.

4 H). HRMS Calcd for $C_6H_9O_4$ ($M^+ - CH_3$): 145.0501. Found: 145.0505. Neutralization with 1 N NaOH, followed by solvent evaporation, provided 249 mg (80%) of **7** as a white powder.

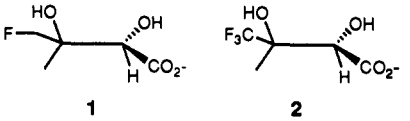
3-Cyclopropyl-2-oxobutanoic Acid Dinitrophenylhydrazone. 3-Cyclopropyl-2,3-dihydroxybutanoate (20 mg, 110 μmol) was dissolved in 2.5 mL of Tris buffer and incubated overnight with 35 units of DHAD at 35 °C. A solution of 2,4-dinitrophenylhydrazine (14 mg, 71 μmol) in 3.1 mL of 2 N HCl was added. After 1 h, the precipitate was collected by filtration and washed with a little cold 2 N HCl to provide 14 mg (79%) of hydrazone: mp 149–151 °C dec; IR (KBr) 3000, 1610, 1505, 1410, 1340, 1270, 1220, 1150, 1125, 1050, 930, 855, 730 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.17 (d, J = 2.5, 3 H), 8.40 (dd, J = 9.5, 2.5, 1 H), 8.14 (d, J = 9.5, 1 H), 2.34 (m, 1 H), 1.38 (d, J = 7.0, 3 H), 1.07 (m, 1 H), 0.61 (m, 1 H), 0.52 (m, 1 H), 0.31 (m, 1 H), 0.24 (m, 1H). MS (LSIMS: 321 [M−H]. HRMS Calcd for $C_{12}H_{14}N_4O_4$ [M − CO_2H]: 278.1015. Found: 278.1007.

3-(Trifluoromethyl)-2-oxobutanoic acid. 3-(Trifluoromethyl)-2,3-dihydroxybutyrate (10 mM)[18] was incubated with 8.8 units of enzyme for 20 h and assayed as before. The OD_{538} indicated that 40 nmol of keto acid had been formed. For comparison, this quantity of DHAD will produce 8800 nmol/h from dihydroxyisovalerate.

Results

Mechanism. A number of mechanistic possibilities for DHAD exist within the β-elimination/enol tautomerization pathway established in our previous work. The postulate of Flint and Emptage that the iron–sulfur cluster in DHAD acts as a Lewis acid in facilitating the departure of the β-hydroxyl group suggests that DHAD operates by an E_1 mechanism. We have used three different types of molecules to probe the accumulation of positive charge at the β-carbon in the elimination reaction. The first are dihydroxy acid analogues with β-groups of varying electron-donating ability. The second are positively charged mimics of a putative β-carbocation. The final compound bears a cyclopropyl group which would signal the formation of a β-carbocation by undergoing ring opening and/or inactivating the enzyme.[45]

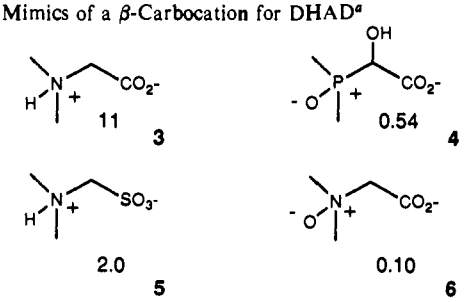
We previously reported that the fluorinated and trifluorinated analogues **1** and **2** are not turned over by DHAD. In the presence



1 **2**

of large quantities of enzyme, however, both are converted into keto acid. For **2**, the rate is $>10^3$ times slower than for dihydroxyisovalerate. When one of the alkyl groups is replaced by hydrogen, as in α,β-dihydroxybutyrate (Table I), there is a similar but much smaller drop in rate.

Chart I. Mimics of a β-Carbocation for DHAD[a]



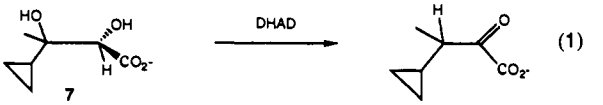
11 **3** 0.54 **4**

2.0 **5** 0.10 **6**

[a] Included are K_i/K_m values, where K_i is for the inhibitor in question and K_m is for the substrate dihydroxyisovalerate.

β-Carbocation mimics are able to bind fairly strongly to DHAD as demonstrated in Chart I. Given with each compound is its K_i/K_m value. The experimental herbicide Hoe 704 (**4**) is included in this set. We now have several compounds that are respectable inhibitors which are based on such mimicry.

A final test of the E_1 mechanism used compound **7**, which was prepared as a racemic diastereomeric mixture by condensation of benzyl (benzyloxy)acetate with cyclopropyl methyl ketone, followed by hydrogenolysis of the benzyl groups. Its kinetic properties with DHAD are shown in Table I; no kinetic evidence for mechanism-based inactivation was obtained. Given the increased bulk of the cyclopropyl group, the respectable turnover was surprising. Cyclopropyl is evidently a much "smaller" group than its acyclic analogues,[46] so there is little cost in binding compared to the ethyl group. The propyl and isopropyl analogues have poor "activity" in bacterial extracts.[38,42] In preparative runs, the cyclopropyl keto acid can be isolated as its DNP derivative in 80% yield based on the 2R isomers (eq 1). Because **7** is a 1:1



7 (1)

mixture and spinach DHAD retains stereochemistry at C-3 (vide infra), it is presumed that the keto acid which is produced is racemic.

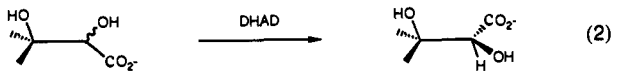
The foregoing data allow the formation of a coherent picture of the mechanism of DHAD in which C–O bond cleavage leads C–H bond cleavage but in which a full carbocation is not generated. This inference then impacts on the expected stereochemistry of the reaction. There are rather consistent trends that enzymes which conduct eliminations through enamine or charged intermediates do so via syn stereochemistry, while those which do not benefit from special charge stabilization in the substrate have anti stereochemistry.[47] The latter generally use concerted mechanisms. The mechanistic results suggest DHAD would fall into the anti stereochemical class.

Stereochemistry. It was first necessary to establish the stereochemical preference at the α-carbon. Three lines of evidence indicate that the 2R configuration is required by the spinach enzyme. We previously reported the inhibition of DHAD by (2R)-pantoic acid.[18] The racemic compound was demonstrated to have a K_i value twice that of the 2R isomer. This is predicted if the 2S acid does not fit the active site. Second, the exhaustive incubation of racemic α,β-dihydroxyisovalerate with spinach DHAD proceeds to 48% conversion with essentially the same rate throughout. The optical rotation of the residual substrate indicates it has the 2S configuration and is of high optical purity (eq 2). Finally, in the study of the overall stereochemical course as de-

(45) Suckling, C. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 537.

(46) Bruch, M.; Jun, M. Y.; Leudtke, A. E.; Schneider, M.; Timberlake, J. W. *J. Org. Chem.* **1986**, *51*, 2969–2973.

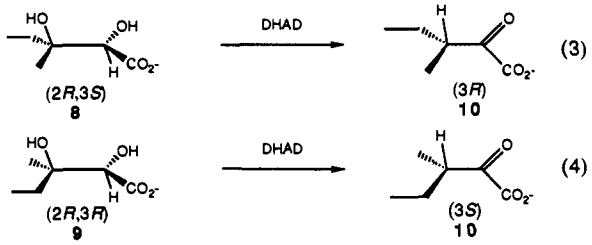
(47) Schwab, J. M.; Klassen, J. B.; Habib, A. *J. Chem. Soc., Chem. Commun.* **1986**, 357–358.



(2)

scribed below, the recovered substrate from the incubation of (2*RS*,3*RS*)-β-methyl dihydroxyvalerate was shown to have the 2*S*,3*S* configuration and to be of high optical purity.

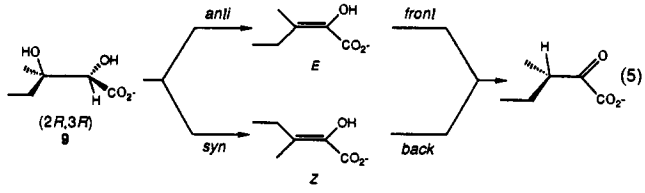
The overall stereochemical outcome was established by incubation of the diastereomerically pure racemates **8** and **9** (shown as the 2*R* enantiomers) with DHAD and determination of the absolute configuration of keto acid **10** (via the optical rotation of the derived dinitrophenylhydrazone). The method used to prepare **8** and **9** was a hybrid based on several reports (see Materials and Methods).[37,48,49] Addition of lithium dimethyl cuprate (20 min, −78 °C, 1:1 THF/ether) to ethyl 2-pentynoate gives ethyl (*Z*)-3-methylpentenoate. Either hydrolysis in refluxing 5% barium hydroxide followed by oxidation with H_2WO_4/H_2O_2 or oxidation with $OsO_4/Ba(ClO_3)_2$ followed by saponification gives **8** and **9**, respectively. As in the bacterial DHAD, the transformation of **8** and **9** occurs with retention of configuration at C-3: proton addition occurs on the same face of the molecule from which hydroxyl departs (eqs 3 and 4). For the 2*R*,3*S* isomer, the 3*R* keto acid is produced, which has the same configuration as L-*allo*-isoleucine.



(3)

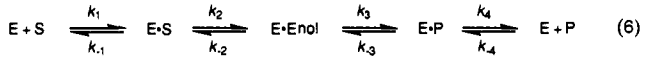
(4)

The high stereochemical preference for processing of **8** and **9** to keto acid indicates that both the elimination and protonation steps are stereospecific. Both reactions must adopt the same syn or anti course, as expected on the basis of an invariant arrangement of catalytic groups in the active site. As eq 5 shows, if anti



(5)

elimination occurs from **9**, the (*E*)-enol will be produced as an intermediate. Its enzyme-catalyzed protonation would then be required to occur from the front face to produce the keto acid observed. Conversely, when **8** is eliminated, it must produce an enol isomeric to the intermediate in the elimination of **9** (*Z*) if the elimination is anti). It was therefore of interest to determine the kinetics of processing of **8** and **9** to quantitate the diastereomeric interactions at each stage (substrate binding, transition state for elimination, enol binding, and transition state for protonation) of catalysis. A minimum of five states describes the catalytic cycle for DHAD (eq 6), and such data provide information on six of



(6)

the eight rate constants. The lack of product inhibition is direct evidence of the magnitude of k_4/k_{-4}.

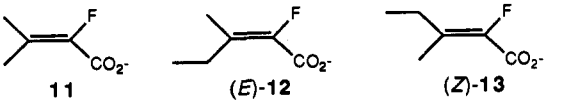
As summarized in Table I, the 2*R*,3*R* isomer **9** is indeed the better substrate, but by only a small margin. The 2*R*,3*S* isomer **8** is *still* a better substrate than dihydroxyisovalerate. This result suggests that the stereochemistry of isoleucine biosynthesis is dictated only by the stereochemistry of the reductoisomerase.

(48) Siddall, J. B.; Biskup, M.; Fried, J. H. *J. Am. Chem. Soc.* **1969**, *91*, 1853–1854.
(49) Neusiadter, V. *Monatsh. Chem.* **1906**, *27*, 879–934.

Independent confirmation of the stereochemistry of the DHAD substrate as 2*R*,3*R* in *Datura* auxotrophs which accumulate it has recently been accomplished.[50] Previous work with *Salmonella* crude extracts showed no discrimination in "activity" between **8** and **9** at 10 mM concentration.[38]

On the basis of these data, only small differences between **8** and **9** in binding energy (~0.5 kcal/mol) and kinetic barriers are inferred. If this is the case, we would expect there to be a small difference in binding energies for the (*E*)- and (*Z*)-enols. Indeed, just the stereochemical data demonstrate that the enzyme can accommodate both. While it is obviously difficult to determine directly their binding energies due to the instability of enols, it may be possible to estimate them by using stable analogues. The properties of such analogues could thereby inform us as to which branch of eq 5 is followed. Furthermore, an enol analogue that is processed would permit determination of the enantiomeric selectivity in the protonation step, a second opportunity to determine the pathway through eq 5. The strategy of using a stable analogue to infer stereochemistry of an unstable intermediate has already seen application in other arenas.[51] We have previously suggested the vinyl fluoride functionality[18] as an analogue for enols in enzymatic transformations.[52] The 2-fluoro acid **11** has superior inhibitory characteristics with DHAD compared to many other 2-substituted dimethylacrylic acid derivatives. Functional groups able to serve as stable enol analogues are uncommon. Some previously used include amides and acrylates.[53]

Vinyl fluorides **11–13** were prepared by conventional methods. Initially, **11** was examined as a substrate for DHAD, but no keto



acid was produced even on incubation with large quantities of enzyme for considerable periods. Furthermore, no statistically significant difference between **12** and **13** in inhibition of DHAD could be discerned.

Discussion

It is difficult to explain the rate data for the substrate analogues we hve prepared if there is not positive charge accumulation at the β-carbon and if the elimination step is not an important contributor to rate limitation. Preliminary primary isotope effect studies support the latter idea ($^DV = 1.95 \pm 0.05$). Along with the inhibitory properties of the high-energy intermediate analogues in Chart I, these data lend support to the Lewis acid mechanism proposed by Flint and Emptage. There was some concern that the heteroatom oxides might inhibit the enzyme by chelating the required magnesium ion, but appropriate control experiments eliminate this possibility. Among the ammonium analogues, the superior inhibition obtained by replacing the carboxylic acid in dimethylglycine with a sulfonic acid parallels trends we have seen previously with α-hydroxy acid analogues.[18] The phosphine oxide

(50) Pirrung, M. C.; Horowitz, D. M.; King, J. F. Unpublished results.
(51) Alberg, D. G.; Bartlett, P. A. *J. Am. Chem. Soc.* **1989**, *111*, 2337–2338. Andrews, P. R.; Cain, E. N.; Rizzardo, E.; Smith, G. D. *Biochemistry* **1977**, *16*, 4848–4852. Boger, J. *Peptides: Structure and Function. Proceedings of the Eighth American Peptide Symposium*; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; p 569. Bott, R.; Subramanian, E.; Davies, D. R. *Biochemistry* **1982**, *21*, 6956–6962. Cha, S.; Agarwal, R. P.; Parks, R. E., Jr. *Biochem. Pharmacol.* **1975**, *24*, 2187–97. Frieden, C.; Kurz, L.; Gilbert, H. *Biochemistry* **1980**, *19*, 5303–5309. Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1979**, *254*, 10839–10845. Logusch, E. W.; Walker, D. M.; McDonald, J. F.; Leo, G. C.; Franz, J. E. *J. Org. Chem.* **1988**, *53*, 4069–4074. Marciniszyn, J., Jr.; Hartsuck, J. A.; Tang, J. *J. Biol. Chem.* **1976**, *251*, 7088–7094. Marshall, G. R. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1976**, *35*, 2494. Pecoraro, V. L.; Rendina, A. R.; Cleland, W. W. *Biochemistry* **1985**, *24*, 1619–1622.
(52) Rose, I. A. *Methods Enzymol.* **1982**, *87*, 84–97.
(53) Bertics, P. J.; Edman, C. F.; Karavolas, H. J. *Endocrinology (Baltimore)* **1984**, *114*, 63–9. Metcalf, B. W.; Holt, D. A.; Levy, M. A.; Erb, J. M.; Heaslip, J. I.; Brandt, M.; Oh, H.-J. *Bioorg. Chem.* **1989**, *17*, 372–376. Northrop, D. B.; Cleland, W. W. *J. Biol. Chem.* **1974**, *249*, 2928–2931. Novoa, W. B.; Schwert, G. W. *J. Biol. Chem.* **1961**, *236*, 2150–2153. Rasmusson, G. H.; Liang, T.; Brooks, J. R. *Gene Regulation by Steroid Hormones 2*; Roy, A., Clark, J. H., Eds.; Springer: New York, 1983; pp 311–334.

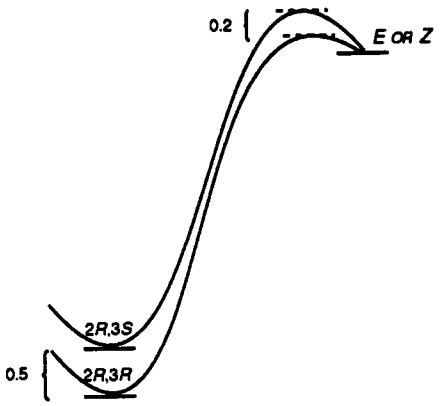
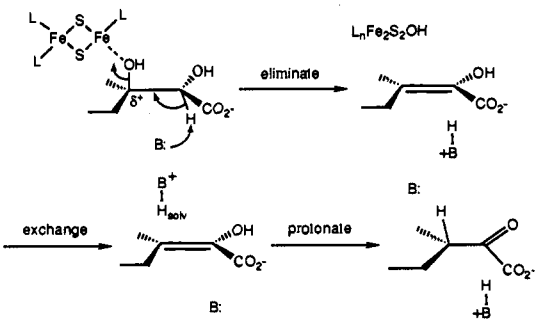


Figure 2. Free energy profile for the processing of diastereomeric substrates by DHAD.

herbicide Hoe 704 appeared to have every characteristic we had found previously in powerful inhibitors. It is clear that α-hydroxy acid functionality is important, and the heteroatom oxide group should also be beneficial. While Hoe 704 is a good inhibitor of DHAD, its in vivo activity must be mostly due to its inhibition of the reductoisomerase. It causes accumulation of acetoin and diminution of dihydroxy acid.[17]

It is disconcerting on first examination that, throughout the mechanism, there is little diastereomeric discrimination. The ability to differentiate molecules bearing groups even closer in size than methyl and hydrogen is a demonstrated property of many enzymes. Often such discrimination is crucial metabolically. Those involved in studying enzyme stereochemistry have come to expect high selectivity, but here that is not the observation. If there has never been pressure to discriminate between these substrates, i.e., by luck, the only substrate DHAD has ever been presented is one which leads to the correct metabolite, then it is merely an accident that the diastereomeric substrate is well accepted. The result has no more *metabolic* significance than if some other man-made chemical were turned over as rapidly as the natural substrate. This observation is also in concert with ideas relating specificity to catalytic efficiency.[54] Here, both are fairly low.

It may be hazardous to extrapolate the stereochemistry for DHAD on the basis of comparison to enzymes of primary metabolism. However, all precedent suggests that DHAD should operate with the anti elimination mechanism shown in eq 7. The



(7)

evidence from substrate analogues and isotope effects that the elimination step is a major contributor to rate limitation permits a free energy profile for the stereoisomeric substrates to be proposed (Figure 2).

Acknowledgment. Financial support from NSF [Graduate Fellowship to D.M.H., support for the purchase of an NMR spectrometer (CHE-8109064), and Presidential Young Investigator Award (CHE 84-51324)] and American Cyanamid is greatly appreciated.

(54) Benner, S. A. *Chem. Rev.* **1989**, *89*, 789–806.