Kinetic Mechanism and Reaction Pathway of *Thermus thermophilus* Isopropylmalate Dehydrogenase

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Received October 8, 1993 (Revised Manuscript Received March 2, 1994•)

Mechanistic studies of isopropylmalate dehydrogenase (IMDH, EC 1.1.1.85), the penultimate enzyme in leucine biosynthesis in bacteria and yeast, have been conducted. It performs two chemical operations, oxidation and decarboxylation, on isopropylmalate to produce α-ketoisocaproate. A recombinant enzyme encoded by the *leuB* gene of the thermophilic bacterium *T. thermophilus* was used for experiments addressing the kinetic mechanism and chemical pathway of IMDH. A new, asymmetric synthesis of the substrate, (2*R*,3*S*)-isopropylmalate, has been developed starting from (2*R*,3*R*)-tartaric acid. On the basis of kinetic inhibition patterns and exchange experiments, it has been shown that the enzyme follows an ordered sequential bi-tri mechanism, with cofactor NAD binding before substrate β-isopropylmalate. The release of products occurs in the order CO_2, α-ketoisocaproate, and NADH. The enzyme catalyzes the exchange of solvent protons into the α-position of the product, implying that an enol/enolate intermediate is formed. The enzyme conducts two discrete steps: dehydrogenation to isopropyloxaloacetate, which was prepared and shown to be a competent substrate, and decarboxylation to give product.

Introduction[1]

The biosynthetic pathway to leucine in bacteria and yeast (Figure 1) departs from that of the two other branched-chain amino acids at α-ketoisovalerate.[2] It is condensed with acetylCoA to produce α-isopropylmalate, which is equilibrated with β-isopropylmalate through the action of isopropylmalate isomerase, a transformation analogous to that conducted by aconitase. The β-isopropylmalate is then oxidatively decarboxylated to produce α-ketoisocaproate, the leucine keto acid, which is subsequently transaminated. Thus, the steps of leucine biosynthesis recall the tricarboxylic acid cycle with α-ketoisovalerate substituted for oxaloacetate. The central boxed compound of the pathway, β-isopropylmalate, is metabolized by the penultimate enzyme of Figure 1, β-isopropylmalate dehydrogenase (IMDH, EC 1.1.1.85).

Isopropylmalate dehydrogenase has recently been the subject of intense study in genetics. It is the product of the *Leu2* gene of *Saccharomyces cerevisiae*, which was among the first eukaryotic genes to be cloned and expressed in *Escherichia coli*.[3] Subsequently, homologous genes have been cloned from many genera of bacteria (*leuB* from *Bacillus*[4] and *Thermus*[5]) and yeast (*Candida*[6]). Sequences are available for bacterial[7,8] yeast,[6,9] and plant[10] IMDH genes. The *Candida* and *Saccharomyces* amino acid sequences are 85% identical, and the sequences of this whole class of proteins show a great deal of homology. They range in length from 345 to 363 residues and have monomer unit M_rs of 38-45 kDa based on the gene sequences. *Leu2* has proven to be a convenient, selectable genetic marker in yeast transformation.[11] Based on this precedent in yeast, IMDH has been applied in host-vector systems for *Acetobacter*[12] and *Candida*.[6]

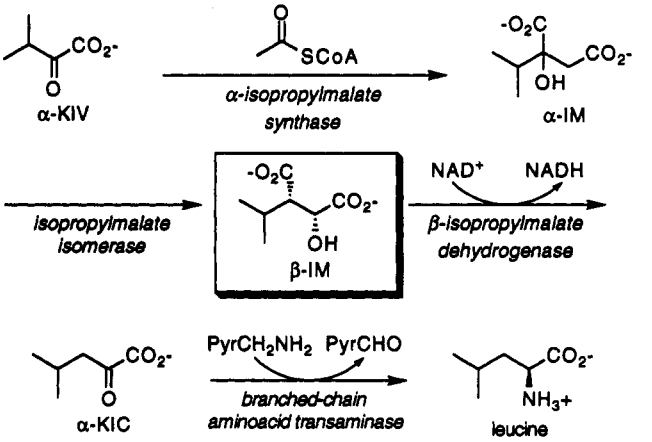

Figure 1. Biosynthetic pathway to leucine in plants, yeast, and bacteria.

• Abstract published in *Advance ACS Abstracts*, April 1, 1994.

(1) Abbreviations: IMDH: isopropylmalate dehydrogenase; ICDH: isocitrate dehydrogenase; α-KIC: α-ketoisocaproate; α-IM: α-isopropylmalate; β-IM: β-isopropylmalate; α-KG: α-ketoglutarate; NAD: nicotine adenine dinucleotide; Ser: serine; Leu: leucine; Asn: asparagine; Thr: threonine; DNB-Cl: 3,5-dinitrobenzoyl chloride; MTPA-Cl: methoxy-(trifluoromethyl)phenylacetyl chloride (Mosher's reagent).

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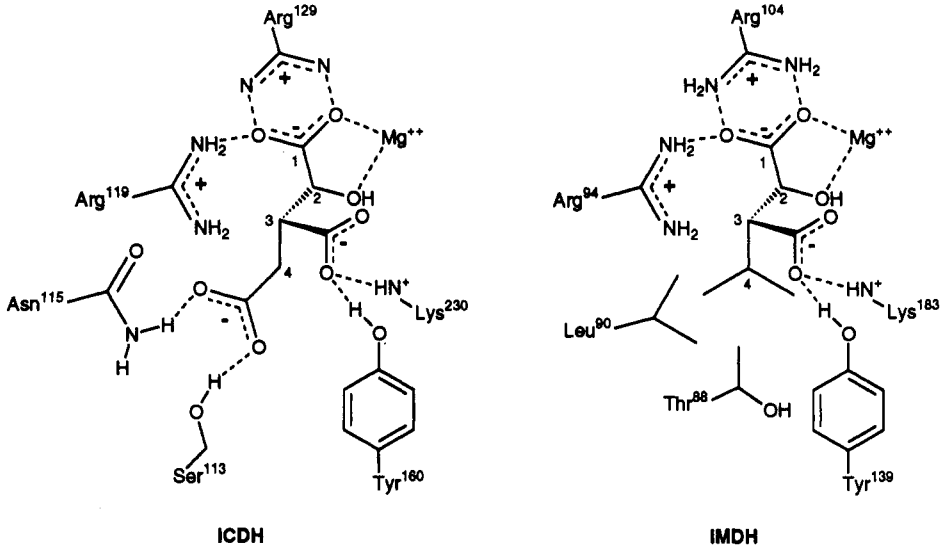


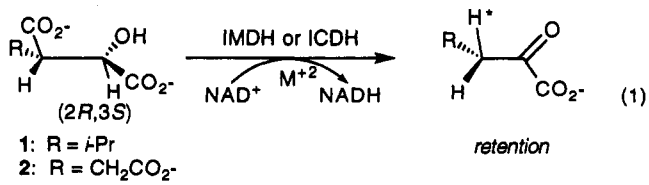
Figure 2. Comparison of the active site residues of isopropylmalate dehydrogenase (IMDH) and isocitrate dehydrogenase (ICDH).

Table 1. Isopropylmalate Dehydrogenase: Sources and Properties

source	M_r	K_m (IM)	K_m (NAD)	optimum pH	metals	ref
Salmonella	35 kDa	19 μM	0.1 mM	9.0	K, Mn	13
C. maltosa	–	0.42 mM	0.34 mM	6.8	K, Mn	12
C. utilis	38.7 kDa (DNA)					6
S. cerevisiae	45 kDa? 39 085 (DNA)	42 μM	0.1 mM	9.5	Mn	28
B. coagulans	44 kDa (DNA)	–	–	9.5	Mn, Mg	4
B. subtilis	44 kDa (DNA)					7
T. thermophilus	40 kDa 36 825 (DNA)	80 μM	0.6 mM	9.0	K, Mg/Mn	24
T. aquaticus	–	–	–	–	K, Mg	40

Table 1 summarizes the current knowledge of the IMDH protein family. The enzme has been directly purified from *Candida maltosa*,[13] *Salmonella*,[14] *Saccharomyces*,[14] *Bacillus coagulans*,[4] *and Thermus thermophilus*.[5] In the last three cases, overproducers have been gained by recombinant DNA techniques. This achievement has been important to obtain quantities of pure enzyme for study, as expression in the wild-type is strongly repressed by leucine. A simple purification procedure is available for the recombinant thermophilic protein wherein host proteins are inactivated by heat treatment.[5] Common characteristics of the proteins are the requirement for a divalent cation, preferably manganese, and a quite basic pH optimum. Generally, assays are conducted at a lower pH to maintain solubility of the metals. A rapid, continuous optical assay based on the reduction of NAD is available.[14] All of the IMDH proteins so far studied are dimers in their active form. The yeast enzyme exhibits a unique cold sensitivity (particularly at lower pH) and a dynamic monomer/dimer equilibrium that causes its activity to be concentration dependent. The 345 amino acid *T. thermophilus* IMDH has been crystallized and the structure solved at 2.2-Å resolution.[15] The structure is unlike other known dehydrogenases, but its secondary, tertiary, and quaternary structure and its cofactor and substrate binding sites resemble *E. coli* isocitrate dehydrogenase (ICDH).[16] The most significant difference in active site residues between ICDH and IMDH is the substitution of Asn115 by Leu90 (Figure 2). It can be presumed that the basis of substrate selectivity for isocitrate in ICDH is hydrogen bonding and for isopropylmalate in IMDH is hydrophobic interaction.

The simple transformations conducted by IMDH and ICDH are shown in eq 1. The substrates (2*R*,3*S*)-isocitrate (**2**)[17] and (2*R*,3*S*)-isopropylmalate (**1**),[18] constituting a single carbon skeleton substituted by a carboxymethyl group or an isopropyl group, are oxidatively decarboxylated to produce an α-keto acid. Overall retention of stereochemistry in the replacement of the carboxylate by solvent



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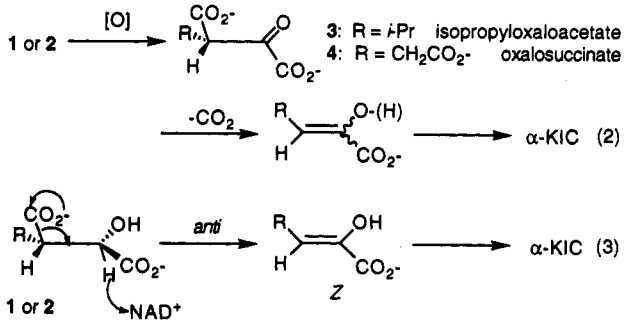
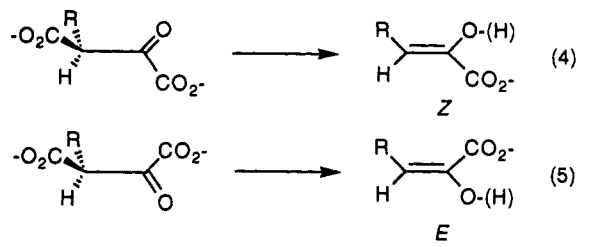
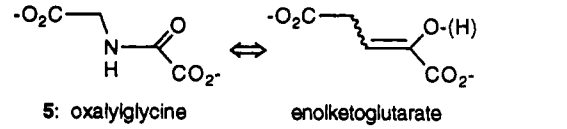
hydrogen has been demonstrated for both ICDH[19] and IMDH.[20] The reaction may occur through two half-reactions: dehydrogenation to give isopropyloxaloacetate (3) or oxalosuccinate (4) (*vide infra*) and decarboxylation of the keto acid (eq 2); or through a concerted process where decarboxylation is concomitant with cofactor reduction (eq 3). An important distinction between these



mechanisms is that the two-step process could produce the enolate or the enol, depending on the protonation state of other active site residues. The concerted mechanism should produce the enol. Stereoelectronic considerations suggest that a concerted reaction should prefer an antiperiplanar orientation of the C–C and the C–H bonds being broken. This constraint enforces the (*Z*) stereochemistry on the enol so produced. The stepwise mechanism requires the decarboxylation to occur with the C–CO_2 bond to be parallel to the carbonyl p-orbitals, an orientation that can be achieved via two conformations (eq 4, 5) that differ by a 180° rotation of the α-keto acid unit. Of course, it is likely that 3 and 4 are unable to rotate freely within their respective enzyme active sites and that therefore only one pathway (*via* the (*E*) or (*Z*) enol/enolate) is followed.



It has not been possible to demonstrate the presence of oxalosuccinate on ICDH by exchange or trapping experiments; either it is nondissociable or it has no existence (i.e., the reaction is concerted). A proposed mimic of the enol-ketoglutarate intermediate in ICDH, oxalylglycine (5),[21] is a rather poor competitive inhibitor with a K_i of 29



μM compared to the K_m of 1.1 μM for substrate. The kinetics of oxalylglycine inhibition earlier supported a random mechanism. More recent studies have shown ICDH follows a compulsory-ordered mechanism with a substrate binding order of cofactor, isocitrate and a product release order of CO_2, ketoglutarate, and reduced cofactor.[22] Only meager studies of IMDH have been reported. It is known that hydrogen transfer to NAD is A-specific[23] and that IMDH does not bind malic acid, thiomalic acid, citraconic acid, ketoglutarate, (2*R*,3*R*)-isopropylmalate, α-KIC, or isocitrate as substrates or inhibitors.[24]

This study was conducted in preparation for efforts to rationally design, synthesize, and test IMDH inhibitors on the basis of the enzyme mechanism. One aspect of the study of IMDH that has not been adequately addressed is the substrate. Synthesis provides a mixture of racemic diastereomers[25] or a racemate,[26] and isolation of the natural isomer *via* fermentation is not very practical.[27] Consequently, several studies have been conducted with mixtures.[28] It has been reported that the L-enantiomer inhibits the *Salmonella* enzyme[25] but not the *T. thermophilus* enzyme.[24] Material of unquestioned stereochemistry (high optical and diastereomeric purity) was important to these kinetic studies, so this report includes a synthesis of the natural (2*R*,3*S*) isomer of isopropylmalate.

Results and Discussion

The substrate was prepared by a stereospecific synthesis from tartaric acid. Following a literature route, holemic (*R*,*R*)-epoxysuccinate is prepared from (*R*,*R*)-diethyltartrate as previously described.[29,30] Attempts to add diisopropylcopperlithium or isopropylcyanocopperlithium (THF, −78 °C) to this epoxide gave none of the desired diethyl isopropylmalate. That the problem was not the quality of the isopropyllithium (synthesized from 2-chloropropane and Li in pentane) was confirmed by its successful reaction with piperonal (80% yield). Attempted additions of higher order cuprates[31] (either isopropyl-2-thienylcyanocopperdilithium or isopropyl-2-thienylcyanocopperlithiummagnesium bromide, THF, −78 °C → rt) met with similar results. Finally, efforts were focused on a copper cyanide-promoted Grignard addition reaction with isopropylmagnesium bromide. A serendipitous discovery was made that a somewhat faster mixing of the reagents and a somewhat higher temperature allow an increase in the yield of the diethyl ester 7 to 60% (eq 6). This result was reproduced several times on as much as 9 g of epoxysuccinate. In order to insure the diastereomeric and optical purity of the substrate, the liquid diethyl isopropylmalate so formed was converted to the 3,5-dinitrobenzoate (DNB-Cl, pyridine, CH_2Cl_2; 92% crude yield). This compound crystallizes spontaneously on isolation and is available in 76% yield following one

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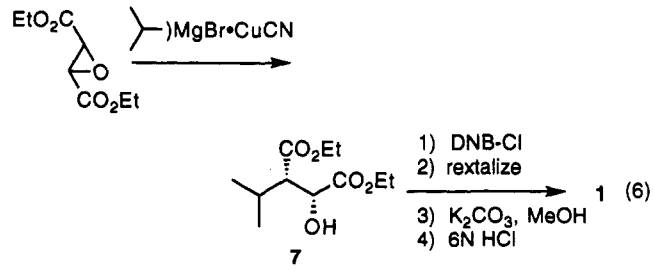
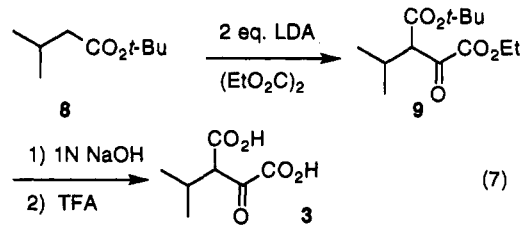


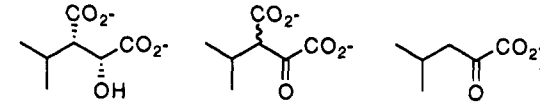
recrystallization from hexane/ethyl acetate. Crude material shows $[\alpha]_D$ −12.5° (c = 1.5, EtOAc), which increases to −13.1° following recrystallization and does not change with further recrystallization. A further indication of the optical purity of this material is that reaction with MTPA-Cl gives a single compound (GC, ^{1}H NMR). Although the dinitrobenzoyl group was untouched by HBr/HOAc/EtOH, it was readily removed with K_2CO_3/EtOH. More than 98% of the ethyl dinitrobenzoate so formed could be removed by filtration following one recrystallization from hexane, producing diethyl isopropylmalate. Cleavage of the remaining ethyl esters is difficult. Basic conditions (10 equiv of LiOH, 60 °C, 18 h) hydrolyze both esters but provide an impure product. The difficulties were overcome by switching to acidic conditions wherein isopropylmalic acid is formed in 82% yield (6 N HCl, 80 °C, 6 h). In summary, (2*R*,3*S*)-3-isopropylmalic acid was prepared in 16% overall yield in seven steps from diethyl (L)-tartrate. The route permitted the production of over 3 g of isopropylmalic acid.

One way to address the question of a stepwise or concerted mechanism for IMDH is detection of the oxidized but non-decarboxylated substrate, isopropyloxaloacetate (**3**). As mentioned above, the putative intermediate in the ICDH transformation, oxalosuccinate **4** (the product of the oxidation half-reaction), has never been detected. The intermediate in the IMDH transformation was readily prepared, despite its envisioned instability toward decarboxylation, as shown in eq 7. The key to



preventing its decomposition is conducting all of the workup at low temperature (4–10 °C). Isopropyloxaloacetate is a substrate for the reverse reaction to produce isopropylmalate, which is followed by NADH oxidation. This material was prepared and used as the racemate, but the kinetic data were not corrected because of the likely epimerization of the α-CH center in **3** due to its high acidity. The kinetic parameters describing the processing of IM in the forward reaction, its inhibition by **3**, and the processing of **3** as well as α-KIC (in the presence of CO_2) in reverse reactions are collected in Chart 1. The kinetic competence of **3** can be evaluated by comparison of its rate with the rate for α-KIC. The similarity of the second-order rate constants (k_{cat}/K_m, 10^4 M^{-1} s^{-1} and 1.58×10^4 M^{-1} s^{-1}, respectively) provides support for the stepwise mechanism. We recognize that intermediates tightly

Chart 1. Kinetic Parameters for Substrates for IMDH



1: K_m = 40 μM, k_{cat} = 2.57 s^{-1}

3: K_i = 0.68 mM; reverse reaction: K_m = 22-27 μM, k_{cat} = 0.27 s^{-1}

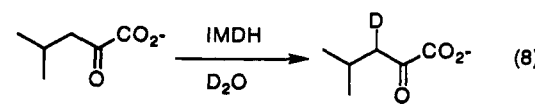
reverse reaction: K_m = 55 μM, k_{cat} = 0.87 s^{-1}

bound in internal states might not show kinetic competence, so this is not necessarily a discriminating criterion.[32]

The kinetic mechanism of a multisubstrate enzyme can be determined by well-established procedures based on determination of the inhibition pattern (competitive, noncompetitive, or mixed) for each substrate against the other(s).[33] The isopropylmalate dehydrogenase used for this purpose was the homogeneous, recombinant *T. thermophilus* protein expressed in *E. coli*. When NAD^+ is the fixed substrate and isopropylmalate the variable substrate, a double reciprocal plot of the initial velocity produces a family of lines intersecting close to (mixed inhibition) or on (competitive inhibition) the $1/v$ axis (Figure 3). When isopropylmalate is the fixed substrate and NAD^+ the variable substrate, a pattern intersecting markedly to the left of the $1/v$ axis (mixed inhibition) is obtained (Figure 4). A steady state model defines substrate binding as ordered bimolecular but does not permit definition of the order based on these data.

Further differentiation among possible ordered mechanisms for substrate binding and product release was made on the basis of inhibition studies. No product inhibition by α-KIC is observed, but NADH is a competitive inhibitor against NAD^+ (Figure 5), showing that they compete for the same form of the enzyme. Substrate inhibition[34] by isopropylmalate is observed at concentrations above ~100 μM (~2 × K_m), whereas no substrate inhibition is observed with [NAD^+] > 30 × K_m, suggesting that NAD^+ binds first and NADH is released last. These data are consistent with catalysis of the oxidative decarboxylation of isopropylmalate by IMDH *via* a sequential ordered bi–tri mechanism. Further definition of the order of product release will be addressed below.

With the earlier demonstration that the reverse of the redox half-reaction could be observed, we examined the decarboxylation step. Since the first step in the reverse reaction would be tautomerization/deprotonation to produce the enol/enolate in either the IMDH–NADH–α-KIC complex or the IMDH–NADH–α-KIC–CO_2 complex, we evaluated exchange of the α-protons of α-KIC in the presence of IMDH. The 3-methylene protons are exchanged for solvent isotope (both with d_2-α-KIC/H_2O and α-KIC/D_2O) by IMDH as evidenced by a change of the integrated signal for these protons compared to the 4-methine proton (eq 8). The α-protons are exchanged to



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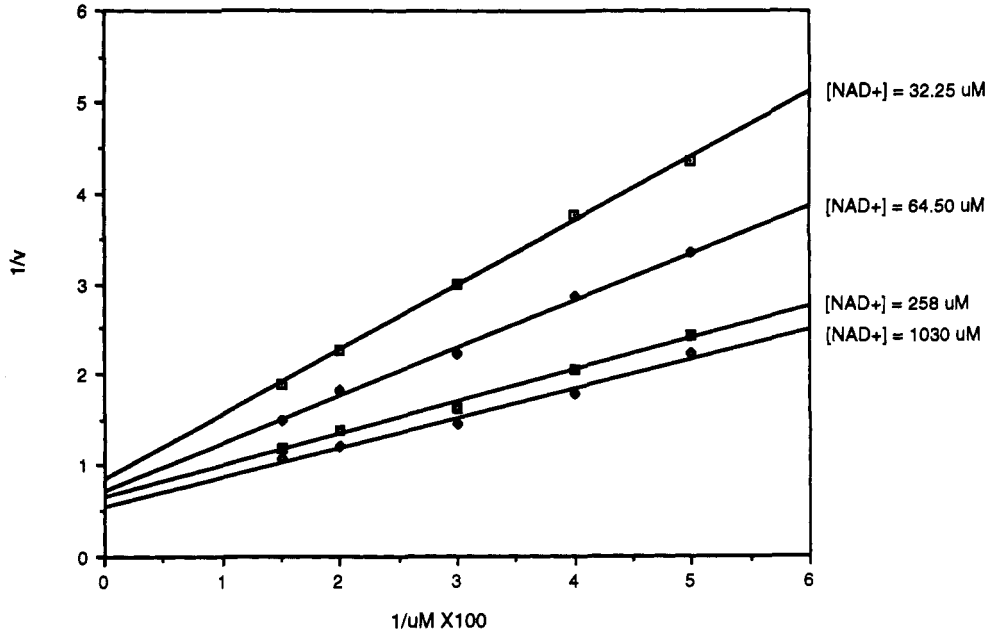


Figure 3. Kinetics of IMDH with isopropylmalate as variable substrate.

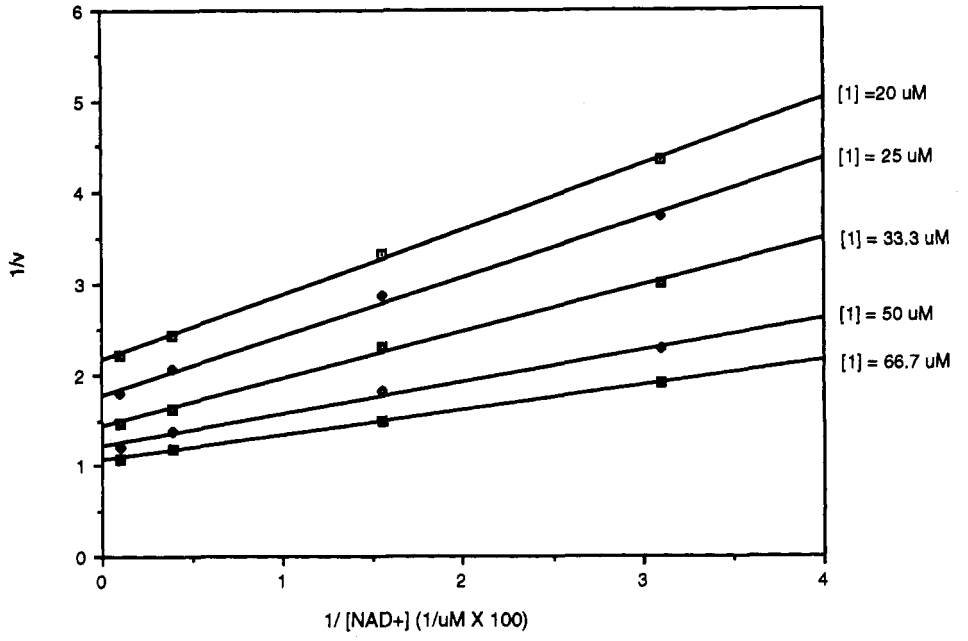
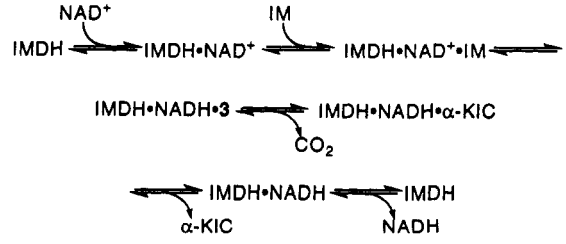


Figure 4. Kinetics of IMDH with NAD^+ as variable substrate.

the extent of 20% over a 5-h incubation. The rate of exchange is 0.052 s^{-1} in the presence *and* the absence of CO_2. Exchange is completely dependent on the presence of reduced cofactor, showing that it occurs in a binary product complex. With the earlier result that NADH binds first in the reverse reaction, CO_2 of necessity binds last. These data are consistent with the forward reaction of IMDH following a bi–tri mechanism that is ordered sequential for both substrate binding (order is NAD^+, isopropylmalate) and product release (order is CO_2, α-KIC, and NADH, eq 9).

In interpreting the results of the foregoing experiments, it is worthwhile to consider kinetic studies of related enzymes. Isocitrate dehydrogenase (ICDH) from *E. coli*, which is structurally homologous to IMDH, had long been considered to have a sequential ordered mechanism, but recent experiments have demonstrated that it is in fact steady-state random.[35] Detailed studies with mutant



(9)

proteins were required for this determination. It seems unlikely that such extensive efforts to test the sequential ordered mechanism of IMDH are necessary, since further data supporting it has come from inhibition studies reported in the accompanying manuscript. A number of anionic compounds that are believed to mimic a putative

(35) Dean, A. M.; Koshland, D. E., Jr. *Biochemistry* **1993**, *32*, 9302–9309.

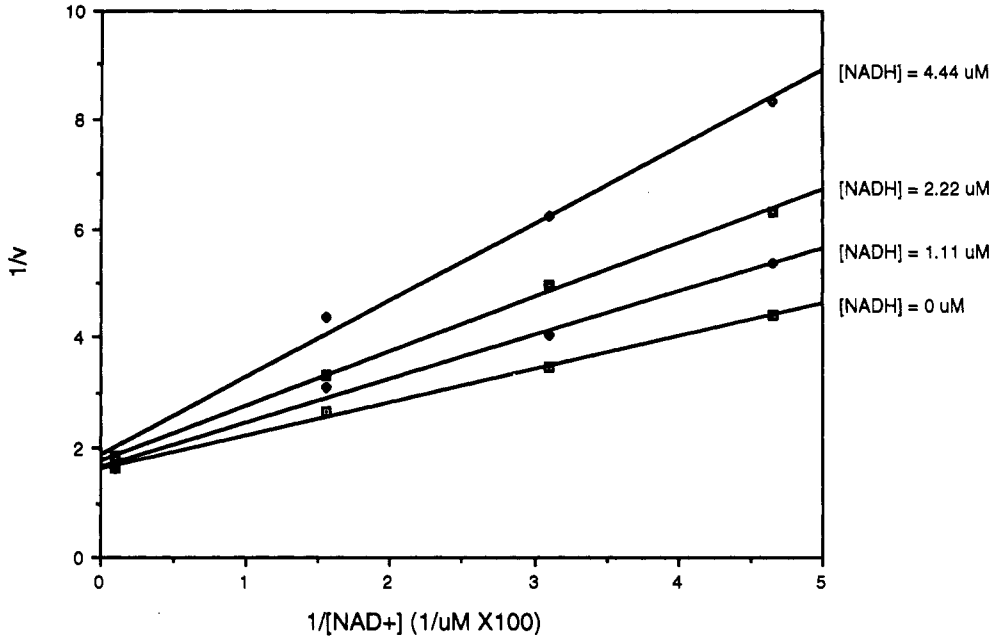


Figure 5. Competitive inhibition of IMDH by NADH.

product enolate show uncompetitive inhibition against IM. An ordered kinetic mechanism is a necessity to observe uncompetitive inhibition.

Experimental Section

General. Isopropylmalate dehydrogenase was a gift from Professor T. Oshima. Unless otherwise noted, other materials were obtained from commercial suppliers and were used without further purification. Dichloromethane, ether, pentane, 2-bromopropane, 2-chloropropane, and triethylamine were distilled from calcium hydride. Ethanol was dried over molecular sieves and distilled. THF was distilled from sodium/benzophenone ketyl. Reactions were conducted in oven-dried glassware under an atmosphere of dry nitrogen or, in the case of reactions using lithium metal, argon. Copper(I) cyanide was dried azeotropically with toluene. Brine refers to a saturated aqueous solution of sodium chloride. Flash chromatography refers to the method of Still[36] and was performed using EM Reagents 0.042–0.063-mm grade silica gel (Kieselgel 60). Infrared spectra were recorded on a Bomem MB-100 instrument. Only the largest or most diagnostic lines are reported. NMR spectra were recorded on Varian XL300 or GE QE300 instruments.

Diethyl (2*S*,3*S*)-2-Acetoxy-3-bromosuccinate. A volume of 300 mL of a 30% solution of HBr in HOAc was added over 30 min to 108 g (0.52 mol) diethyl L-tartrate, with mechanical stirring. The solution was stirred for 15 min and then warmed to room temperature and stirred for 14 h. The solution was poured onto 1.2 L of ice–water, the phases were separated, and the aqueous phase was extracted (3×) with ether. The combined ether layers were washed with brine and dried over $MgSO_4$, and solvents were removed *in vacuo*. Simple distillation (145–150 °C, 0.5 torr) gave 124 g (76%) of a colorless oil: ^{1}H NMR ($CDCl_3$) δ 5.6 (d, 1H), 4.8 (d, 1H), 4.32–4.21 (m, 4H), 2.1 (s, 3H), 1.33–1.28 (m, 6H); IR (neat) 2984, 1752, 1374, 1229, 1027 cm^{-1}.

Diethyl (2*S*,3*S*)-3-Bromomalate.[37] An amount of 119 g (0.38 mol) of diethyl acetoxybromosuccinate was refluxed for 6 h with 1.2 L of dry ethanol and 35 mL of 30% HBr/HOAc. Solvents were then removed *in vacuo*. The residue was taken up in ether and washed with saturated $NaHCO_3$ and with brine and dried over Na_2SO_4, and then solvent was removed by rotary evaporation. Simple distillation (133–137 °C, 0.5 torr) gave 97.4 g (94%) of a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 4.73–4.64 (m, 2H), 4.32–4.23 (m, 4H), 3.41 (d, J = 7.4 Hz, 1H), 1.31 (t, J = 7.1 Hz, 6H); IR (neat) 3465, 2984, 1739, 1372, 1242 cm^{-1}.

Diethyl (2*R*,3*R*)-Epoxysuccinate (6).[38] Diethyl bromomalate (77 g, 0.29 mol) was dissolved in 300 mL of CH_2Cl_2. Triethylamine (77 mL, 0.55 mol) was added in one portion and the reaction warmed noticeably but did not reflux. The reaction was stirred overnight at ambient temperature. The mixture was washed with 1 N HCl and brine and dried over Na_2SO_4, and the solvent was removed by rotary evaporation. Distillation (123–125 °C, 2.7 torr) gave 33.6 g (62%) of a colorless liquid: ^{1}H NMR ($CDCl_3$) δ 4.32–4.22 (m, 4H), 3.67 (s, 2H), 1.32 (t, J = 7.1 Hz, 6H); IR (neat) 2985, 1747, 1455, 1371, 1198, 1096, 1029, 901 cm^{-1}; $[\alpha]_D$ −111.5° (c = 1.44, ether).

Diethyl (2*R*,3*S*)-3-Isopropylmalate (7). Isopropylmagnesium bromide was prepared by dropwise addition of 38 g (0.31 mol) of 2-bromopropane in 120 mL of ether to a suspension of 7.5 g (0.31 mol) of magnesium powder in 40 mL of ether. The reaction was stirred for 1 h at ambient temperature and allowed to settle for 8 h. The supernatant was withdrawn by syringe for use. An aliquot was titrated for ~1.6 M.

CuCN (20 g, 0.22 mol) was suspended in 225 mL of ether at −20 °C with mechanical stirring. Grignard reagent (125 mL) was added quickly in a stream by syringe, and the cuprate was stirred for 15 min. Diethyl epoxysuccinate (9.0 g, 47.8 mmol) in 30 mL of ether was added dropwise and the reaction was stirred 1 h. The reaction mixture was quenched with aqueous NH_4Cl/NH_4OH and filtered through Celite, and the phases were separated. The aqueous phase was extracted twice more with ether. The combined organic layers were washed with brine and dried over Na_2SO_4, and the solvent was removed by rotary evaporation. Flash chromatography on a column of silica gel (7.5 × 15 cm) provided 6.7 g (60%) of the desired DEIPM. See characterization below.

Diethyl (2*R*,3*S*)-2-[(3′,5′-Dinitrobenzoyl)oxy]-3-isopropylsuccinate. To a solution of 9.02 g (38.8 mmol) of DEIPM and 9.5 g (41.2 mmol) of 3,5-dinitrobenzoyl chloride in 60 mL of CH_2Cl_2 at 0 °C were added a few crystals of DMAP and 15 mL of pyridine dropwise. The reaction was slowly warmed to room temperature and stirred overnight. The reaction mixture was partitioned between CH_2Cl_2 and water and the aqueous layer discarded. The organic layer was washed sequentially with 1 N HCl (2×), with saturated $NaHCO_3$, and with brine, dried over $MgSO_4$, and filtered, and the solvent was removed by rotary evaporation to give 14.2 g (86%) of an amber oil, which crystallized on standing. Recrystallized from hexanes/EtOAc (~95:5) at 0

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(37) Mori, K.; Iwasawa, H. *Tetrahedron* **1980**, *36*, 87–90.

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°C. (12.6 g, 76%) as large needles: mp 75.7–77.3 °C; ^{1}H NMR ($CDCl_3$) δ 9.26 (dd, J = 2.1 Hz, 1H), 9.17 (d, J = 2.1 Hz, 2H), 5.59 (d, J = 5.1 Hz, 1H), 4.32–4.21 (m, 4H), 2.97 (dd, J = 7.8 Hz, 1H), 2.20 (J ≈ 7, oct, 1H), 1.33 (t, J = 7.1 Hz, 3H), 1.32 (t , J = 7.1 Hz, 3H), 1.08 (d, J = 6.7 Hz, 3H), 1.04 (d, J = 6.8 Hz, 3H); IR: 3102, 2955, 1743, 1630, 1548, 1462, 1347, 1230, 1031 cm^{-1}; $[\alpha]_D$ −13.1° (c = 1.5, EtOAc); HRMS calcd for ($C_{18}H_{22}N_2O_{10}$ + H) 427.1353, found 427.1360.

Diethyl (2*R*,3*S*)-3-Isopropylmalate (7). The above dinitrobenzoate (12.6 g, 29.5 mmol) was suspended in 250 mL of dry ethanol, and 0.25 g (1.8 mmol) of K_2CO_3 was added, giving a wine red solution. After 1 h, the reaction mixture was cooled to −20 °C and filtered. Solvent was removed by rotary evaporation. Further ethyl dinitrobenzoate was removed by filtration following recrystallization from hexane/EtOAc. The yield was 6.6 g (95%): ^{1}H NMR ($CDCl_3$) d 4.36 (dd, J = 3.2, 9.5 Hz, 1H), 4.23 (dq, J = 2.4, 7.1 Hz, 2H), 4.15 (q, J = 7.1 Hz, 2H), 3.37 (d, J = 9.5 Hz, 1H), 2.53 (dd, J = 3.2, 9.2 Hz, 1H), 2.31–2.18 (m, 1H), 1.30 (t, J = 7.1 Hz, 3H), 1.25 (t, J = 7.1 Hz, 3H), 1.07 (d, J = 6.7 Hz, 3H), 1.00 (d, J = 6.7 Hz, 3H); IR (neat) 3493, 2969, 1733, 1465, 1376, 1200, 1031 cm^{-1}.

(2*R*,3*S*)-Isopropylmalate (1). DEIPM (4.95 g, 21.3 mmol) was stirred with 250 mL of 6 N HCl at 80 °C for 6 h and solvent was removed *in vacuo*. The residue was purified by precipitation from EtOAc/hexane at −20 °C, giving 3.1 g (82%) of a white powder: ^{1}H NMR (D_2O) δ 4.52 (d, J = 4.3 Hz, 1H), 2.60 (dd, J = 4.3, 8.8 Hz, 1H), 2.11–1.99 (m, 1H), 0.98 (d, J = 6.9 Hz, 3H), 0.96 (d, J = 6.8 Hz, 3H); $[\alpha]^{25}_D$ −2.1° (c = 1.4, H_2O); HRMS calcd for ($C_7H_{12}O_5$ + H) 177.0763, found 177.0760.

***tert*-Butyl 3-Methylbutanoate (8).**[39] 3-Methylbutanoic acid (8.58 g, 84 mmol), liquefied isobutylene (12 mL, 150 mmol), and H_2SO_4 (0.7 mL) were mixed in ether (20 mL) and stirred overnight at room temperature. The reaction mixture was poured into an ice–water mixture containing 10 g of NaOH. Ether extraction, drying over $MgSO_4$, and distillation under aspirator pressure gave the desired product boiling between 75–80 °C (aspirator) (8.63 g, 65%): ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.5 Hz, 6H), 1.44 (s, 9H), 2.08 (m, 3H).

Ethyl 2-Oxo-3-(*tert*-butyloxycarbonyl)-4-methylpentanoate (9). To diisopropylamine (3.84 g, 37.98 mmol) in dry THF (20 mL) at 4 °C was slowly added *n*-BuLi (20 mL of 2.1 M solution, 37.98 mmol). After stirring for 15 min, the yellowish solution was cooled to −78 °C with a dry ice–acetone bath and treated with *tert*-butyl 3-methylbutanoate (3 g, 18.99 mmol) in 2 mL of THF. After 10 min, diethyl oxalate (2.77 g, 18.99 mmol) in 3 mL of THF was slowly added to the reaction mixture. The resulting mixture was stirred for 15 min, poured into 1 N HCl solution (50 mL), extracted with ether, and dried over $MgSO_4$. Evaporation of the ether and column chromatography of the residue ($CHCl_3$) gave the desired product (R_f ~ 0.5) (3.87 g, 79%): ^{1}H NMR ($CDCl_3$) δ 1.01 (d, J = 6.8 Hz, 3H), 1.02 (d, J = 6.9 Hz, 3H), 1.36 (t, J = 7.1 Hz, 3H), 1.43 (s, 9H), 2.43 (m, 1H), 3.74 (d, J = 6.4 Hz, 1H), 4.43 (q, J = 7.1 Hz, 2H); IR (neat) 2953, 1733, 1464, 1392, 1369 cm^{-1}; HRMS calcd for ($C_{13}H_{22}O_5$ + H) 259.1545, found 259.1538.

2-Oxo-3-(*tert*-butyloxycarbonyl)-4-methylpentanoic Acid. Ethyl 2-oxo-3-(*tert*-butyloxycarbonyl)-4-methylpentanoate (1 g, 3.88 mmol) was placed in 1 N NaOH (30 mL) and stirred at room temperature until the solution nearly reached homogeneity. The reaction mixture was extracted with ether to remove unreacted mixed ester. The aqueous phase was acidified with cold concd HCl at 4 °C and extracted with ether. The ether was dried over $MgSO_4$ and evaporated to give the title compound (0.81 g, 91%): ^{1}H NMR ($CDCl_3$) δ 1.03 (d, J = 6.9 Hz, 3H), 1.04 (d, J = 6.8 Hz, 3H), 1.44 (s, 9H), 2.45 (m, 1H), 3.87 (d, J = 6.3 Hz, 1H), 8.03 (broad s, 1H); ^{13}C NMR (78 MHz, $CDCl_3$) δ 190, 167, 160, 83, 60, 27.8, 27.6, 21, 20; IR (neat) 3113, 1722, 1465, 1251 cm^{-1}; HRMS calcd for ($C_{11}H_{18}O_5$ + H) 231.1232, found 231.1237.

2-Oxo-3-isopropylsuccinic Acid (3). 2-Oxo-3-(*tert*-butyloxycarbonyl)-4-methylpentanoic acid (0.5 g, 2.17 mmol) was placed in a 1:1 mixture of CH_2Cl_2 and CF_3COOH (10 mL) and stirred at room temperature for 70 min. The solvent was removed *in vacuo* in a 10 °C water bath to produce the oxo acid (0.360 g, 95%: ^{1}H NMR ($CDCl_3$) δ 1.07 (d, J = 6.8 Hz, 3H), 1.08 (d, J = 6.8 Hz, 3H), 2.53 (m, 1H), 4.01 (d, J = 6.5 Hz, 1H), 9.05 (broad s, 2H); ^{13}C NMR (78 MHz, $CDCl_3$) δ 188, 174, 161, 59, 28, 21, 20; IR (neat) 3059, 1701, 1468, 1265 cm^{-1}. This compound was unstable, preventing analysis of its molecular formula.

Enzyme Assays. All kinetic measurements were, unless mentioned, performed at 25 °C in a buffer solution containing 0.5 mM $MgCl_2$, 0.05 M KCl, 0.67 mM NAD^+, and 0.1 M KP_i, pH 7.6. The reaction was followed by measuring the appearance or disappearance of NADH at 340 nm. The reaction was initiated by adding enzyme (0.75 μg) to the reaction mixture with all required components including the substrate (and the inhibitor) present. The initial linear portion of the reaction progress curve (less than 5% substrate conversion) was used for data analysis. Data were graphically analyzed by Lineweaver–Burk double reciprocal plots. The kinetic data we obtained: K_m (IM) = 40 μM at [NAD^+] = 670 μM (Chart 1) and K_m (NAD^+) = 25 μM at [IM] = 67 μM; compare to the following data from Oshima: K_m (IM) = 80 μM at [NAD^+] = 714 μM and K_m (NAD^+) = 630 μM at [IM] = 413 μM. In the product inhibition study with NADH, the concentration of isopropylmalate was 33.33 mM. For the enzymatic reduction of intermediate **3**, more enzyme (7.5 μg) was used, and the concentration of NADH was 0.15 mM.

Isotope Exchange. Exchange-in: α-Ketoisocaproate (α-KIC, 20 mg), IMDH (0.6 mg), and NADH (15 mg) were added to the same buffer (5 mL) as above except D_2O was used. The resulting mixture was stirred for 10 h at room temperature, acidified, and extracted with ether. After evaporation of the ether, the residue was subjected to NMR analysis. The integration of the C2-methylene protons versus the C3-methine proton was used to determine the extent of isotope exchange. A control experiment omitting enzyme showed no exchange. Exchange-out: 3-[$^2H_2$]-α-KIC was prepared by H–D exchange of α-KIC in basic D_2O solution. NMR showed deuterium incorporation >98.5%. This 3-[$^2H_2$]-α-KIC was incubated with enzyme in H_2O buffer under several conditions: with NADH, with NADH and CO_2, and with CO_2. NMR of the isolated product showed a double-triplet at δ = 2.80 ppm due to C3 methylene ($^3J_{H-H}$ = 6.7 Hz, $^2J_{H-D}$ = 2.6 Hz). Control experiments without enzyme or without NADH showed no 2.8 ppm signal.

Acknowledgment. Financial support by NIH GM-43816 is appreciated. We gratefully acknowledge the provision of IMDH by Prof. Oshima.

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