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Stereochemistry of the Reaction of Sheep Liver Threonine Dehydratase. A Nuclear Magnetic Resonance and Optical Rotatory Dispersion Study of Its Reaction Pathway and Products†

Gordon Kapke‡ and Leodis Davis*

ABSTRACT: Products, substrates, and inhibitors of the threonine dehydratase from sheep liver (EC 4.2.1.16) have been investigated by proton nuclear magnetic resonance and optical rotation. The α-ketobutyrates produced from L-threonine and L-allothreonine in ^2H_2O have been shown to incorporate a single deuterium into the β position. The dehydratase forms *R*-α-ketobutyrate-β-*d* from L-threonine and L-allothreonine. The α protons of the substrates, threonine and allothreonine, do not exchange in the presence of the dehydratase. In the presence of dehydratase, the competitive inhibitors L-cysteine and L-alanine undergo α-proton exchange. Highly purified dehydratase has been used to determine kinetic parameters for the substrates L-threonine, L-allothreonine, L-serine, and L-chloroalanine. L-Chloroalanine, in addition to being a substrate, inhibits the dehydratase in a manner kinetically identical with that of L-serine.

Sheep liver threonine dehydratase converts L-threonine to α-ketobutyrate and requires an α-ketobutyryl group as a cofactor rather than pyridoxal phosphate (Kapke and Davis, 1975). With this report and the one by Cohn and Phillips (1974) of a similar microbial serine–threonine dehydratase, further investigations of the mechanism by which keto acids function in dehydration is warranted. Presumably, keto acid cofactors function in catalysis by forming a Schiff base with the amino acid substrate and promoting α-proton labilization. A study of the chirality of the products was made to obtain information about the stereochemistry of the elimination reaction. The labilization of the α proton of substrates and inhibitors has been studied by proton nuclear magnetic resonance. The results presented here add support to the suggested

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TABLE I: Kinetic Parameters for Sheep Liver Threonine Dehydratase.[a]

Substrate	K_m[b]	V_{max}[c]	K_i[b]
L-Threonine	12.5	30.0	
L-Allothreonine	13.5	12.0	
L-Serine	64.1	88.3	
L-β-Chloroalanine	0.4	22.3	
Inhibitor			
L-Cysteine		*d*	3.2
L-Alanine		*d*	13.6
2-Carboxypyrrole		*d*	3.3

[a] All measurements made at pH 7.2 in 0.05 M phosphate buffer. [b] Units, mM; determined in the presence of 0.1 M L-threonine. [c] Units, μmol min^{-1} mg^{-1}. [d] Not detectable; <0.001.

mechanism of dehydration by enzymes in this class (Davis and Metzler, 1972).

Experimental Procedures

Materials. Sheep livers were purchased fresh from Wilson and Co. of Cedar Rapids, Iowa. L-Allothreonine was obtained from Calbiochem. L-Threonine, L-serine, L-methionine, L-cysteine, *S*-adenosyl-L-methionine, L-chloroalanine, L-alanine, and glycine were obtained from Sigma Chemical Co. 2-Carboxypyrrole was obtained from Aldrich.

Methods. Sheep liver serine threonine dehydratase was isolated by the method of Kapke and Davis (1975). The assays have been previously described by Davis (1965). One dehydratase unit is the amount of enzyme that will produce 1 μmol of keto acid from L-threonine per min at 37 °C and pH 7.2. K_m, K_i, and V_{max} were determined with a least-squares computer program.[1] Nuclear magnetic resonance spectra were obtained using a Varian A-60 NMR[2] spectrometer. Optical rotation was determined on a Cary 60-60001 ORD/CD spectropolarimeter.

Results

Using purified dehydratase, K_m and V_{max} were determined for various substrates (Table I). Also included in Table I are the K_i values determined by competitive inhibition experiments with threonine as the substrate for various inhibitory amino acids. L-Methionine and *S*-adenosyl-L-methionine were tested as feedback inhibitors, since the dehydratase removes potential one-carbon units from the transhydroxymethylase pathway. However, they had no effect on the dehydratase

Nonoxidative deamination of L-threonine catalyzed by the dehydratase was monitored by proton nuclear magnetic resonance (Figure 1). The ratio of the peak heights of the α-proton signal and methyl protons signal of L-threonine remained constant, hence, no α-proton exchange had occurred. One deuterium atom was found to be incorporated into the β position of the α-ketobutyrate from the NMR spectrum. However, with long incubations, spontaneous exchange between the

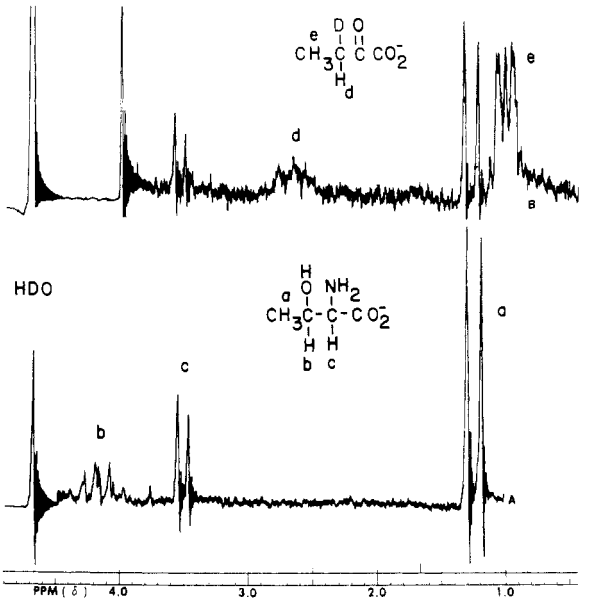


FIGURE 1: L-Threonine. Spectrum A is the proton nuclear magnetic resonance spectrum of L-threonine in ^2H_2O. Spectrum B was taken 4 h after the addition of 33 units of dehydratase activity to a volume of 0.5 ml of 0.1 M L-threonine, pH 7.4. Part of the L-threonine has been converted to α-ketobutyrate with a deuteron at the β position. The β proton is labile and hence some dideuterated product is observed as indicated by the singlet between the doublet marked e.

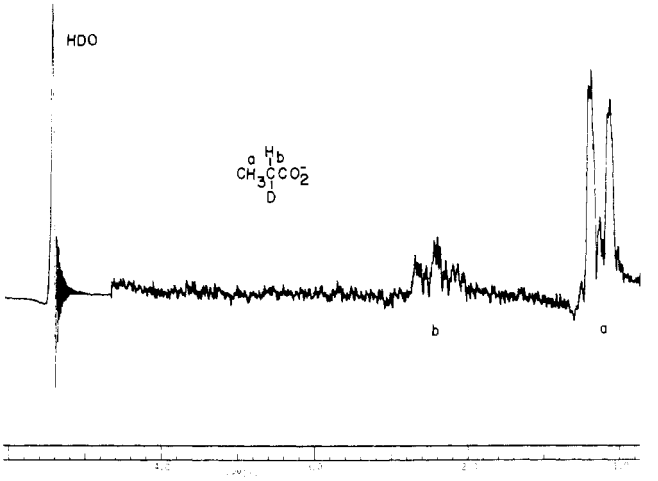


FIGURE 2: Proton nuclear magnetic resonance spectrum of propionic acid formed from decarboxylation of α-ketobutyrate produced by threonine dehydratases in ^2H_2O.

proton at the β position of α-ketobutyrate and solvent was observed (Figure 1b). The methyl group protons signal of α-ketobutyrate is a doublet, indicating that one ^2H is occupying a position on the β carbon. Spontaneous double deuteration of the α-ketobutyrate is observed, as indicated by the singlet between the doublet peaks. This double deuteration could be reduced by using large amounts of enzyme and allowing the reaction to proceed for a short period of time. By oxidatively decarboxylating the α-ketobutyrate containing the deuterium atom in the β position with hydrogen peroxide one eliminates the ability of the hydrogen at the β position to exchange with solvent. The NMR spectrum of propionate produced from α-ketobutyrate is shown in Figure 2.

L-Threonine and L-allothreonine were converted to α-ketobutyrate by the dehydratase in deuterated solvent. The α-ketobutyrate produced in separate reactions using these sub-

[1] The square of the difference between the observed and calculated product concentrations was minimized by means of a computer program (Los Alamos Publication LA-2367 and addenda). The present modification was written in Fortran IV by Dr. G. Gordon, Miami University, and the enzyme kinetic subroutines were written by Dr. K. Sando, University of Iowa.

[2] Abbreviations used are: NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; CD, circular dichroism.

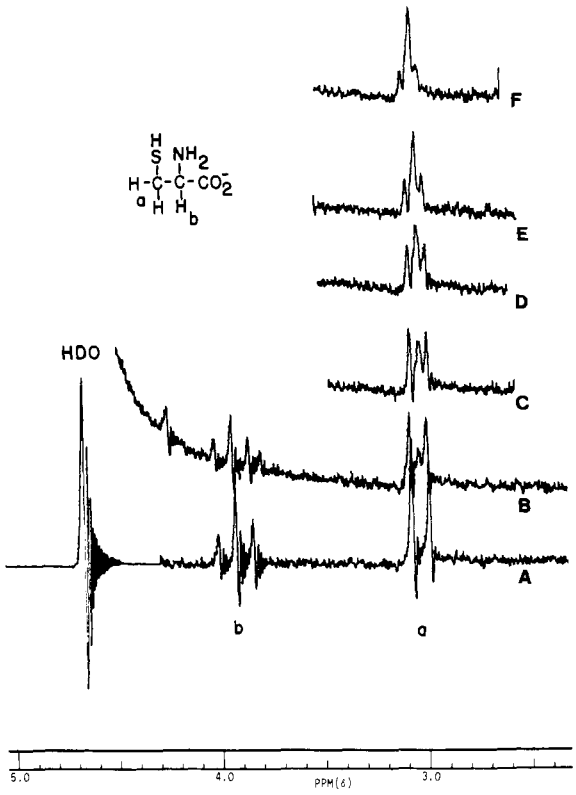


FIGURE 3: L-Cysteine. The proton nuclear magnetic resonance spectrum of 0.2 M L-cysteine was recorded in 0.1 M sodium phosphate buffer, pH 7.2, in D_2O (A). Nine units of dehydratase activity was added and spectra (B–F) were recorded at 15-min intervals.

TABLE II: Specific Rotation of α-Deuteriopropionates from Threonine and Allothreonine.[a]

	[α]		
λ(nm)	Enzyme	Products	2(*R*)-Deuteriopropionate[b]
280	−3.1[c]	−3.0[d]	−3.9
270	−4.0	−4.0	−5.0
260	−4.8	−4.6	−6.1
250	−6.6	−6.5	−8.3
240	−9.8	−9.7	−12.3

[a] Determined on a Cary Model 60 spectropolarimeter 20 mg/ml. [b] Krongelb et al. (1968). [c] Threonine. [d] Allothreonine.

strates was immediately decarboxylated with peroxide to form propionate-α-*d*. Decarboxylation limits the ability of the deuterium to exchange and does not affect any asymmetry that existed in the ketobutyrate-β-*d*. The propionate-α-*d*'s were isolated and purified by the method of Krongelb et al. (1968). The NMR spectra and optical rotatory dispersion spectra of the propionates were determined.

The optical rotations of the propionates were both observed to have the same negative values between 240 and 280 nm (Table II). Since the rotation of 2(*R*)-deuteriopropionate is negative in this spectral region (Krongelb et al., 1968) it was concluded that (*R*)-deuterioketobutyrate was generated by action of the dehydratase on both L-threonine and L-allothreonine. The values in Table II are lower than those reported for pure 2(*R*)-deuteriopropionate due to some dideuterated product, as evidenced in the NMR spectra.

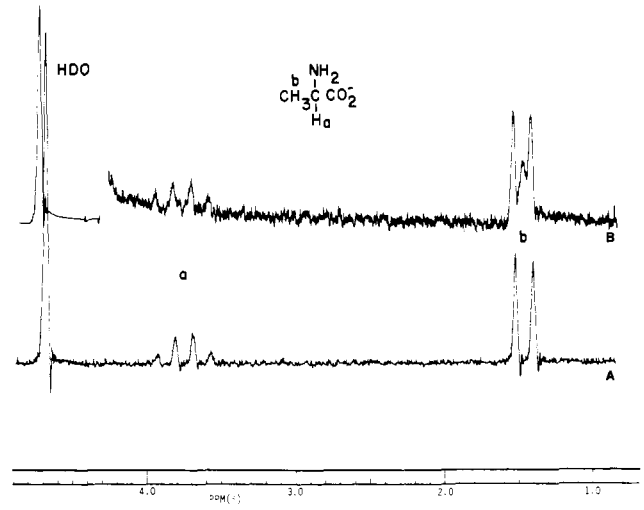


FIGURE 4: L-Alanine. Spectrum A is the proton nuclear magnetic resonance spectrum of 0.2 M L-alanine in D_2O after 20 h. Spectrum B is the proton nuclear magnetic resonance spectrum of L-alanine 20 h after the addition of nine units of dehydratase. Both samples were incubated at 37 °C.

A solution of 0.2 M L-cysteine in ^2H_2O in 0.1 M potassium phosphate buffer, pH 7.2, was monitored using proton nuclear magnetic resonance after the addition of dehydratase. No keto acid was formed; however, α-proton exchange was observed as indicated by the conversion of the β-protons' doublet to a singlet (Figure 3). The α-proton exchange did not affect the optical rotation of the L-cysteine; consequently, the sheep liver dehydratase can be used to prepare α-^{2}H-labeled L-cysteine. L-Alanine was similarly treated and found to undergo a much slower exchange. The nuclear magnetic resonance spectrum of a mixture of dehydratase and L-alanine after 20 h is shown in Figure 4. An attempt was made to exchange the α protons of glycine with the dehydratase. The reaction was monitored by peak height of the glycine's α-protons signal by nuclear magnetic resonance against a control with no dehydratase. After 24 h of incubation at pH 7.2 and 37 °C with 33 dehydratase units in 2 ml of 0.1 M glycine, no enzyme mediated exchange was detected.

Chloroalanine was shown to be a substrate of the dehydratase and to produce the type of inhibition found with serine (Davis and Metzler, 1972). At pH 7.2, an initial rapid rate of keto acid production that decreases to zero was observed. At pH 8.9, the initial rapid rate of keto acid production was followed by a slower rate that remained constant. L-Serine and L-chloroalanine inhibit the dehydratase completely after approximately 10 000 catalytic cycles have occurred at pH 7.2. This inhibition can be reversed by pH adjustment and/or incubation with high concentrations of pyridoxal phosphate as reported by McLemore and Metzler (1968).

Discussion

In this manuscript we have investigated the mechanism of dehydration of substrates by the sheep liver serine dehydratase. The mechanism of the pyridoxal phosphate requiring *E. coli* L-serine dehydratase has previously been studied by Phillips and Wood (1965). Using ^3H_2O and $H_2{}^{18}O$ in exchange studies they found ^3H was incorporated into L-threonine faster than was ^{18}O, which is consistent with the steps in the reaction being reversible and the reaction proceeding by proton abstraction followed by β elimination. In contrast, we could not show incorporation of ^2H into L-threonine nor L-allothreonine by the sheep liver enzyme. In spite of the lack of exchange of the α

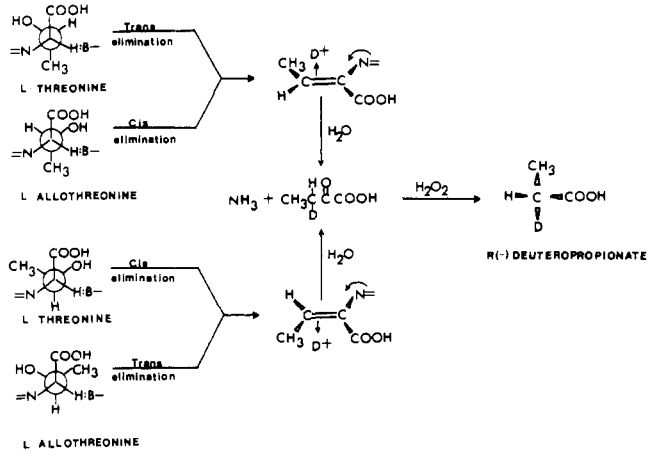


FIGURE 5: Proposed mechanism for the formation of (*R*)-deuteriobutyrate by the sheep liver serine threonine dehydratase.

proton of L-threonine and L-allothreonine with solvent in the presence of the dehydratase, proton removal does occur during the course of the reaction, since the α carbon of threonine is converted from a primary amine to a carbonyl function.

L-Cysteine is not a substrate for the dehydratase (Nishimura and Greenberg, 1961) in spite of the fact that it undergoes rapid α proton exchange (Figure 3). Also, L-alanine undergoes dehydratase-mediated α-proton exchange. The α proton of L-cysteine is more than 80% exchanged after 1 h, while the α proton of L-alanine is exchanged at approximately 5% of the L-cysteine rate. The observation that α-proton removal occurs with these inhibitors, which do not β eliminate, lends support to the idea that α-proton labilization is involved in the reaction mechanism.

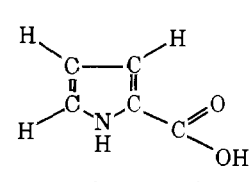
A number of pathways have been proposed for the enzyme-catalyzed elimination reaction of serine and threonine. Shown in Figure 5 is one possible mechanism that is consistent with the data reported in this communication. Previous work has shown that the anion forms of L-threonine and L-allothreonine are bound by the enzyme (Davis and Metzler, 1962). Presumably, a Schiff base is formed between the enzyme's α-ketobutyryl group and the amino acid anion. Because α-hydroxybutyric acid can be isolated after NaB^3H_4 reduction of the dehydratase (Kapke and Davis, 1975), the α-ketobutyryl cofactor is not bound as an aldimine to an ϵ-amino group of lysine as is the case for pyridoxal phosphate-containing dehydratases (Davis and Metzler, 1972).

Results shown in Table II establish that in the conversion of threonine and allothreonine to α-ketobutyrate, catalyzed by the dehydratase, the β proton is added stereospecifically to an enzyme-bound intermediate. Both chemical considerations and experimental evidence would suggest that the intermediate is an enamine (Flavin and Slaughter, 1966). Additional evidence for the existence of α-β-unsaturated intermediates in the conversion of β-substituted amino acids to α-keto acids is the finding by Kapke and Davis (1975) and Miles (1975) that β-γ-unsaturated amino acids are substrates for enzymes catalyzing β eliminations.

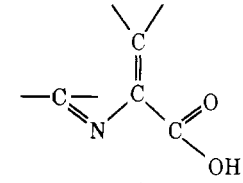
Assuming the stereospecificity of product arises because only one side of the enzyme-bound intermediate is accessible to solvent, the observations in Table II can arise from two possible mechanistic pathways. The elimination of water from threonine and allothreonine can occur in identical manners (both eliminations cis or trans) giving two different enzyme-bound enamines. The two different enzyme-bound enamines would then be deuterated from opposite sides to give identical products. Alternatively, dissimilar eliminations could occur, resulting in identical enzyme-bound intermediates which are deuterated from the same side to give identical products. Consider the stereochemistry of the substrates as shown in Figure 5. Since hydroxyl is eliminated, it probably remains accessible to solvent. Hence, if the spatial relationships of the nitrogen, carboxyl group, and methyl group are maintained by the enzyme, one would expect opposite eliminations of water to take place from threonine and allothreonine, generating a common enzyme-bound enamine. The alternative explanation of similar eliminations producing opposite enzyme-bound enamines and protonation occurring from opposite sides would require much more flexibility at the active site of the dehydratase. Also, one proton or deuteron addition would have to occur from the opposite side of the enamine from which the hydroxyl departed. Hence, this seems to be a less likely explanation of the observed stereochemistry of the products. Similar stereochemistry has been observed with the D-serine dehydratase from *E. coli* by Yang et al. (1975) and with tryptophanase from *E. coli* by Schleicher et al. (1976). The formation of α-ketobutyrate from D-threonine by the *E. coli* D-serine dehydratase, the formation of pyruvate from L-serine and L-tryptophan by the *E. coli* tryptophanase, and the formation of α-ketobutyrate from L-threonine by the sheep liver dehydratase proceed with retention of configuration. However, the formation of α-ketobutyrate from L-allothreonine by the sheep liver dehydratase proceeds with inversion of configuration. Results from dehydratase-catalyzed elimination reactions suggest that *R* products are formed from L amino acids and *S* products are formed from D amino acids. The D-serine dehydratase acting on D-threonine as a substrate in ^2H_2O produces 3(*S*)-deuteriobutyrate, while the sheep liver L-threonine dehydratase acting on L-threonine and L-allothreonine as a substrate in ^2H_2O produces 3(*R*)-deuteriobutyrate.

The final attack of water on an enzyme-bound enamine could occur either at the α carbons of the substrate or the coenzyme. At present, the point of attack is unknown. However, attack at either position would be consistent with stereospecificity of proton attachment at the β position.

2-Carboxypyrrole was tried as an inhibitor on the basis of its similarity to the suggested enzyme-bound enamine. However, it was found to be a relatively poor competitive inhibitor.



2-carboxypyrrole



enamine

Serine also serves as substrate for L-threonine dehydratase from sheep liver (Davis and Metzler, 1962). However, the enzyme is inactivated as a result of action on serine. At pH 7.2, an initial rapid rate of pyruvate production that decreases to zero was observed. At pH 8.9, the initial rapid rate of pyruvate production was followed by a slower rate that remained constant. The fact that L-β-chloroalanine shares the inhibition characteristics of L-serine suggests that an enzyme-generated intermediate is responsible for the inhibition. Both L-serine and L-β-chloroalanine would generate the same intermediate upon α-β-elimination. The observations that threonine dehydratase from *Salmonella typhimurium* is inhibited by L-chloroalanine

(Arfin and Koziell, 1971) and that *N*-ethylmaleimide protects the threonine dehydratase from *C. tetanomorphum* (Phillips, 1968) from serine inhibitions, along with the results reported here, would suggest that alkylation of an enzyme-generated intermediate could be responsible for the L-chloroalanine and L-serine inhibition of many of the threonine dehydratases.

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Studies on the Intramolecular and Intermolecular Kinetic Isotope Effects in Pyruvate Carboxylase Catalysis†

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ABSTRACT: A deuterium kinetic isotope effect of 2.1 was observed when [$^2H_3$]pyruvate was used as the substrate for pyruvate carboxylase. The effect is on V_{max}/K_m alone and disappears at infinite substrate concentration. This is interpreted to mean that the slowest step in the overall catalysis is in the half-reaction involving the carboxylation of enzyme–biotin by ATP and HCO_3^-. A tritium *intra*molecular isotope effect of 4.8 and an *inter*molecular effect of 1.2 were also observed. The former was interpreted as the isotope effect on the "effective k_{cat}", while the latter the one on V_{max}/K_m. With these data, the rate constant for binding of pyruvate was estimated to be 4.5×10^6 M^{-1} min^{-1}, and the deuterium kinetic isotope effect on the catalytic step to be 3.1. Relative values for various rate constants were also obtained. Fluoropyruvate was also shown to be a substrate, reacting six times slower. A deuterium kinetic isotope effect of 1.5 was observed, which remained even at infinite substrate concentration. This is interpreted to mean that the slowest step in the overall catalysis is now the carboxylation of fluoropyruvate.

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-containing enzyme purified from liver mitochondria (Utter and Keech, 1963). It catalyzes the ATP[1]-dependent carboxylation of pyruvate by HCO_3^-, forming oxalacetate, ADP, and inorganic phosphate. The methyl group of pyruvate is converted to the C3 prochiral methylene group of oxalacetate. Our interests in this enzyme stem from two unique features of this class of enzymatic reactions: (i) pyruvate carboxylase is conveniently used in establishing the absolute configurations of chirally labeled [^{1}H,^{2}H,^{3}H]pyruvate samples (Cheung and Walsh, 1976a; Creighton and Rose, 1976). We have been interested in determining the kinetic isotope effect with respect to the *intra*molecular discrimination against deuterium, and thus be able to predict accurately (for chiral pyruvate samples) the theoretical ratio of (3*R*)- to (3*S*)-[3-^{3}H]oxalacetate formed, and after enzymatic reduction 3*R*- and 3*S*-[3-^{3}H]-L-malate in such experiments (Cornforth et al., 1969; Luthy et al., 1969). (ii) This enzymatic reaction, involving reaction at a torsiosymmetric methyl group, offers a unique situation where one can separately determine the kinetic isotope effects on both the catalytic step (an *intra*molecular effect) and the overall reaction (an *inter*molecular effect), thus probing certain kinetic aspects of this enzyme which is not possible experimentally in many other cases.

We report here results on these two issues and relate this information to some of the previous kinetic information on the enzyme (Scrutton and Young, 1972; Utter et al., 1975).

Experimental Section

Materials. Sodium pyruvate, sodium fluoropyruvate, lactic dehydrogenase, and malate dehydrogenase were purchased

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[1] Abbreviations used are: ATP, ADP, adenosine tri- and diphosphate; NMR, nuclear magnetic resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NADH, reduced nicotinamide adenine dinucleotide; lhs and rhs, left and right hand side.