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Use of Primary Deuterium and ^{15}N Isotope Effects To Deduce the Relative Rates of Steps in the Mechanisms of Alanine and Glutamate Dehydrogenases†

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ABSTRACT: We have used deuterium and ^{15}N isotope effects to study the relative rates of the steps in the mechanisms of alanine and glutamate dehydrogenases. The proposed chemical mechanisms for these enzymes involve carbinolamine formation, imine formation, and reduction of the imine to the amino acid [Grimshaw, C. E., Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 5655; Rife, J. E., & Cleland, W. W. (1980) *Biochemistry 19*, 2328]. These steps are almost equally rate limiting for $V/K_{ammonia}$ with alanine dehydrogenase, while with glutamate dehydrogenase carbinolamine formation, imine formation, and release of glutamate after hydride transfer provide most of the rate limitation of $V/K_{ammonia}$. Release of oxidized nucleotide is largely rate limiting for V_{max} for both enzymes. When β-hydroxypyruvate replaces pyruvate, or 3-acetylpyridine NADH (Acpyr-NADH) or thio-NADH replaces NADH with alanine dehydrogenase, nucleotide release no longer limits V_{max}, and hydride transfer becomes more rate limiting. With glutamate dehydrogenase, replacement of α-ketoglutarate by α-ketovalerate makes hydride transfer more rate limiting. Use of Acpyr-NADPH has a minimal effect with α-ketoglutarate but causes an 8-fold decrease in V_{max} with α-ketovalerate, with hydride transfer the major rate-limiting step. In contrast, thio-NADPH with either α-keto acid causes carbinolamide formation to become almost completely rate limiting. These studies show the power of multiple isotope effects in deducing details of the chemistry and changes in rate-limiting step(s) in complicated reaction mechanisms such as those of alanine and glutamate dehydrogenases.

Alanine dehydrogenase from *Bacillus subtilis* catalyzes the reversible oxidative deamination of L-alanine:

$$NAD^+ + \text{L-alanine} \rightleftharpoons NH_4^+ + \text{pyruvate} + NADH + H^+ \quad (1)$$

Grimshaw and Cleland (1981) showed that the kinetic mechanism was ordered with NAD bound first and ammonia, pyruvate, and NADH released in that order. The isomerization of E·NAD is the slowest step for the oxidative deamination of alanine, but hydride transfer is also partially rate limiting.

Glutamate dehydrogenase from bovine liver catalyzes the reversible oxidative deamination of L-glutamate:

$$NAD(P)^+ + \text{L-glutamate} \rightleftharpoons NH_4^+ + \alpha\text{-ketoglutarate} + NAD(P)H + H^+ \quad (2)$$

The kinetic mechanism is random in the direction of oxidative deamination (Engel & Dalziel, 1969, 1970; Engel & Chen, 1975; Colen et al., 1972, 1977; Jallon et al., 1975; Silverstein & Sulebele, 1973; Rife & Cleland, 1980a). The reductive amination of α-ketoglutarate, however, involves ordered addition of NADPH, α-ketoglutarate, and NH_4^+ (Rife & Cleland, 1980a). Substrate inhibition by glutamate that is uncompetitive vs NADP and by α-ketoglutarate that is hyperbolic uncompetitive vs NH_4^+ suggests that release of both reduced and oxidized nucleotides is slow (Rife & Cleland, 1980a), while deuterium isotope effects of 1.1–1.2 at pH 7 (Rife & Cleland, 1980a; Cook, 1982) show that hydride transfer is only slightly rate determining. With the slow alternate substrate norvaline, hydride transfer is more rate limiting, and Srinivasan and Fisher (1985) have measured a primary deuterium isotope effect of about 4 (which is probably the intrinsic isotope effect on this step) on the oxidation of L-proline to Δ^1-pyrroline-carboxylic acid.

Although the degree of rate limitation by the hydride transfer step is known for both alanine and glutamate dehydrogenases, little is known of the contribution of other chemical steps within the interconversion of E·NAD(P)·amino acid and E·NAD(P)H·α-keto acid·ammonia central complexes. The present study presents evidence from deuterium and ^{15}N isotope effects that permits deduction of which of the several chemical steps in the reductive amination of the α-keto acids

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are rate limiting for the different combinations of substrates studied.

Materials and Methods

Chemicals. B. subtilis alanine dehydrogenase and bovine liver glutamate dehydrogenase (both in 50% glycerol), yeast alcohol and aldehyde dehydrogenases, yeast hexokinase, glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, NAD(P), pyruvate, β-hydroxypyruvate, α-ketoglutarate, and α-ketovalerate were from Sigma. Glucose-*1-d* was from Omnicron Biochemicals. Ethanol-d_6 and $(^{15}N\text{-}H_4)_2SO_4$ were from Merck.

Preparation of Labeled Reactants. Reduced nucleotides with either hydrogen or deuterium in the *pro-R* position at C-4 of the pyridine ring were prepared by incubation of 100 mM Taps,[1] pH 8.5, 10 mM NAD (or analogue), 100 mM ethanol or ethanol-d_6, 1 mM DTT, and 25 units each of yeast alcohol and aldehyde dehydrogenases until reaction was complete (Viola et al., 1979). Solutions were then filtered to remove enzymes at 4 °C and used within 12 h of preparation.

[4(*R*)-^{3}H]NADH was prepared by incubating 100 mM Taps, pH 8.5, 1.16 mM D-glyceraldehyde 3-phosphate, 50 μCi of [4-^{3}H]NAD, and 10 units of D-glyceraldehyde-3-phosphate dehydrogenase until reaction was complete and by chromatography on Bio-Rad AG-MP-1 at pH 10. The pooled [4-(*R*)-^{3}H]NADH was concentrated to ~1 mL and desalted on a Bio-Rad P2 column (15 × 47 cm). It was then converted to [2-^{3}H]-L-alanine by incubation with 25 mM $(NH_4)_2SO_4$, 50 mM pyruvate, 100 mM Taps, pH 9.0, 1 mM DTT, and 5 units of alanine dehydrogenase. [2-^{3}H]-L-alanine was adsorbed on Dowex 50W-X12, eluted with 4 M pyridine, and concentrated to dryness.

[4(*S*)-^{3}H]NADPH was synthesized by incubating 0.1 mM [2,3,3-$^3H_3$]-L-glutamate (0.9 mCi), 3 mM NADP, 50 mM phosphate, 100 mM Taps, pH 9.0, 25 mM hydrazine sulfate, and 250 units of glutamate dehydrogenase. [4(*S*)-^{3}H]-NADPH was isolated by chromatography on Dowex AG-MP-1 at pH 10 and converted to [2-^{3}H]-L-glutamate in a reaction mixture containing 48 mM α-ketoglutarate, 25 mM $(NH_4)_2SO_4$, 50 mM phosphate, 100 mM Hepes, pH 7.5, and 250 units of glutamate dehydrogenase. [2-^{3}H]-L-Glutamate was adsorbed on Dowex 50W-X8 and eluted with 6 M pyridine.

Nomenclature. The nomenclature used is that of Northrop (1977), in which the isotope effect on a kinetic or thermodynamic parameter is defined by leading superscripts. For example, $^{15}(V/K)_D$ is the ^{15}N isotope effect determined with NADD, $^{D}(V/K)$ is the deuterium isotope effect, and $^{D_2O}(V/K)$ is the solvent deuterium isotope effect. For a further discussion of nomenclature, see Cook and Cleland (1981a) or Cleland (1987).

Deuterium Isotope Effects. Isotope effects with alanine dehydrogenase were obtained in 100 mM Hepes, pH 6.9, by direct comparison of initial velocities for reductive amination, with ammonia as the variable substrate at saturating (0.2 mM) concentrations of reduced nucleotide (protio or deuterio) and α-keto acid (100 mM pyruvate or β-hydroxypyruvate).

Isotope effects with glutamate dehydrogenase were obtained similarly at pH 5.8 in 100 mM phosphate and 10 mM α-keto acid, with both protio and deuterio reduced nucleotides generated in situ from 0.2 mM of appropriate oxidized nucleotide with 33 units/mL hexokinase, 25 units/mL glucose-6-phosphate dehydrogenase, 12 mM $MgCl_2$, 11 mM ATP, and 0.2 mM glucose or glucose-*1-d*.

Reactions were initiated by the addition of enzyme after a stable base-line rate was established; this was subtracted from the rate after the addition of enzyme. Reductive amination was measured by the disappearance in absorbance at A_{340} [NAD(P)H], A_{363} [Acpyr-NAD(P)H], or A_{395} [thio-NAD(P)H], using extinction coefficients of 6220 M^{-1} cm^{-1} at 340 nm, 9100 M^{-1} cm^{-1} at 363 nm, and 11 300 M^{-1} cm^{-1} at 395 nm for the reduced nucleotides.

Deuterium isotope effects with glutamate dehydrogenase were also obtained from pH 6 to pH 8 in 100 mM phosphate and 2 mM NADP, by using L-glutamate or L-glutamate-*2-d* prepared according to Rife and Cleland (1980a).

Tritium Isotope Effects. The ^{3}H isotope effect for the alanine dehydrogenase reaction was determined by observing 10–20% or 100% conversion of [2-^{3}H]-L-alanine to [4(*R*)-^{3}H]NADH. Low conversion reaction mixtures contained 5 mM NAD, 2 mM [2-^{3}H]-L-alanine, 100 mM Hepes, pH 7.0, 200 mM hydrazine sulfate, and 0.1 unit of alanine dehydrogenase. The reaction was quenched with 10 M KOH, titrated to pH 10 with 3 M HCl, and applied to an AG-MP-1 column. [2-^{3}H]-L-Alanine was eluted in the void volume with 0.4 M LiCl, and [4(*R*)-^{3}H]NADH with 1 M LiCl. The 100% conversion was run similarly, except that 100 mM Ches, pH 10, and 25 mM hydrazine sulfate were used.

The tritium isotope effect for the glutamate dehydrogenase reaction was determined similarly by the conversion of [2-^{3}H]-L-glutamate to [4(*S*)-^{3}H]NADPH. The low conversion reaction contained 1 mM [2-^{3}H]-L-glutamate, 0.5 mM NADP, 100 mM phosphate, 100 mM Mes, pH 6, 25 mM hydrazine sulfate, and 250 units of glutamate dehydrogenase. The 100% conversion reaction contained 0.2 mM [2-^{3}H]-L-glutamate, 3 mM NADP, 25 mM hydrazine sulfate, 100 mM Taps, pH 8, and 250 units of glutamate dehydrogenase.

Solvent Deuterium Isotope Effects. Solvent deuterium isotope effects were obtained for alanine dehydrogenase by determining the pH(D) variation of V and $V/K_{ammonia}$ in H_2O and D_2O. The same buffers were used in D_2O but at pD values 0.5 unit higher than the corresponding pH values in H_2O, and the pD was calculated by adding 0.4 pH unit to the pH meter reading (Schowen, 1977). All reagents for the assays in D_2O, except enzyme, were lyophilized from D_2O. All reactions were initiated by adding 0.01 mL of a stock solution of alanine dehydrogenase in H_2O. The observation of linear time courses in D_2O rules out slow changes in enzyme structure caused by D_2O.

^{15}N Isotope Effects. ^{15}N isotope effects on $V/K_{ammonia}$ were determined by isotope ratio mass spectral analysis of the substrate ammonia before or after 10–20% reaction by using the natural abundance of ^{15}N as the label. A single stock solution of ammonia was used for all isotope effect determinations, and the concentration was determined with a modified Nessler's assay. All other solutions, including alanine and glutamate dehydrogenases (both in glycerol), were also tested for ammonia with negligible amounts detected. The alanine dehydrogenase used had no detectable lactate dehydrogenase activity.

For ^{15}N isotope effects with alanine dehydrogenase, reduced nucleotide (NADH, analogues, or A-side NADD) was generated in situ by using ethanol or ethanol-d_6 and NAD (or analogue) with yeast alcohol and aldehyde dehydrogenases.

[1] Abbreviations: Acpyr-NAD(P), 3-acetylpyridine NAD(P); DTT, dithiothreitol; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

The latter enzymes also served to recycle the oxidized nucleotide and maintain a constant concentration of reduced nucleotide. The fractional conversion of the reaction mixtures was determined from the added α-keto acid level relative to the initial ammonia concentration. Reaction mixtures contained the following in a final volume of 15 mL: 100 mM buffer titrated with KOH (Hepes, pH 7, or Ches, pH 9), 100 mM ethanol or ethanol-d_6, 15 units of yeast alcohol dehydrogenase, 25 units of yeast aldehyde dehydrogenase, 0.2 mM NAD (or analogue), 3 mM pyruvate or β-hydroxypyruvate, 13 mM NH_4Cl, and 4 and 8 units of alanine dehydrogenase for the pyruvate or β-hydroxypyruvate reactions, respectively. The amount of α-keto acid remaining in the reaction mixture was monitored by adding 0.1-mL aliquots to 3 mL containing 10 units of lactate dehydrogenase and 0.2 mM NADH in 100 mM Hepes, pH 7. When the α-keto acid was depleted, the pH was decreased to 1 with concentrated H_2SO_4 and the reaction mixtures were applied to 1.5 × 20 cm columns of AG-MP-1 (100–200 mesh). Ammonium ion was eluted in the first fractions and was free of nucleotide on the basis of A_{260}. Ammonia was isolated by alkaline steam distillation and oxidized by hypobromite oxidation to give N_2 for mass spectral analysis as described by Nuiry et al. (1984) or Hermes et al. (1985).

When ^{15}N isotope effects were determined in D_2O, all buffers and substrates were dissolved in D_2O, alanine dehydrogenase was twice Amicon filtered from D_2O prior to use, and the pD was adjusted with NaOD. Isolation of ammonia and analysis were carried out in the same fashion as for samples in H_2O.

For isotope effects with glutamate dehydrogenase, reduced nucleotides (NADPH, analogues, or B-side NADPD) were generated in situ, and their concentrations were maintained constant with glucose or glucose-*1-d* and NADP (or analogue), MgATP, yeast hexokinase, and *L. mesenteroides* glucose-6-phosphate dehydrogenase. Reaction mixtures contained the following in a final volume of 15 mL: 100 mM buffer titrated with KOH (phosphate or Hepes, pH 7, or Ches, pH 9 with 50 mM phosphate), 10 mM glucose or glucose-*1-d*, 12 mM $MgCl_2$, 11 mM ATP, 50 units of yeast hexokinase, 125 units of glucose-6-phosphate dehydrogenase, 0.2 mM NADP (or analogue), 3 mM α-ketoglutarate or α-ketovalerate, 14 mM NH_4Cl, and 30 and 120 units of glutamate dehydrogenase for the α-ketoglutarate and α-ketovalerate reactions, respectively. The amount of α-keto acid remaining in the reaction mixture was monitored by adding 0.1-mL aliquots to 3 mL containing 10 units of glutamate dehydrogenase, 0.2 mM NADPH, and 250 mM NH_4Cl in 100 mM phosphate, pH 7. When the α-keto acid was depleted, the samples were treated in the same manner as those for alanine dehydrogenase.

^{15}N isotope effects with glutamate dehydrogenase were also obtained at pH 6 and 8 by using the equilibrium perturbation method of Schimerlik et al. (1975) and $(^{15}NH_4)_2SO_4$ (95 atom % ^{15}N). Reaction mixtures at pH 8 contained the following in 3 mL: 100 mM Tris-HCl, 10 mM phosphate, 0.75 mM NADP, 0.3 mM $(^{15}NH_4)_2SO_4$, 1.3 mM α-ketoglutarate, 14.7 mM glutamate, and 0.16 mM NADPH. Reaction mixtures at pH 6 were identical except that 100 mM Mes, 50 mM NADP, and 0.87 mM α-ketoglutarate were used. In both cases, reaction was initiated by the addition of 20–30 units of glutamate dehydrogenase. The control with $(^{14}NH_4)_2SO_4$ gave a slight decrease in absorbance at 340 nm at pH 6, but no change at pH 8. In both cases, the final equilibrium value was about 4×10^{-14} M^2, in agreement with that reported previously (Cook et al., 1980).

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. Data were fitted to the appropriate rate equation by using the Fortran programs of Cleland (1979). Data for deuterium isotope effects were fitted to

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (3)$$

$$v = VA/[(K + A)(1 + F_iE_v)] \quad (4)$$

where V is the maximum velocity, K is the Michaelis constant, A is the concentration of NH_4^+, F_i is the fraction of deuterium in the deuteriated reduced nucleotide, and E_V, $E_{V/K}$, and E_v are the isotope effects minus 1 on V, on V/K, or on both parameters when the isotope effects were the same on both, respectively. Solvent deuterium isotope effects were calculated from the pH(D)-independent plateau values of V and $V/K_{ammonia}$. V and V/K values at each pH(D) were calculated according to eq 5, and the V/K data were further fitted to eq 6 where y is V/K, C is the pH(D)-independent plateau value,

$$v = VA/(K + A) \quad (5)$$

$$\log y = \log [C/(1 + H/K_1)] \quad (6)$$

H is the proton concentration, and K_1 is the acid dissociation constant. The V data were pH independent and were thus averaged.

Tritium isotope effects were determined according to

$$^T(V/K) = \log (1 - f)/\log (1 - fR_p/R_0) \quad (7)$$

where f is the fraction of reaction, R_0 is the specific activity of NADH at 100% reaction, and R_p is the specific activity of NADH at f fractional conversion of substrate to product.

To determine the ^{15}N isotope effects, eq 8 was used, where R_0 is the $^{15}N/^{14}N$ ratio in the initial ammonia and R_s is the ratio in residual ammonia at fraction of reaction f.

$$^{15}(V/K) = \log (1 - f)/\log [(1 - f)(R_s/R_0)] \quad (8)$$

Isotope effects were extracted from equilibrium perturbation data by the method of Schimerlik et al. (1975) by using a Fortran program that achieves an exact solution to the simultaneous first-order differential equation.

Results

Alanine Dehydrogenase. Deuterium isotope effects were obtained by measuring initial rates at varying concentrations of NH_4^+, with saturating levels of reduced nucleotide (protio and deuterio) and α-keto acid. Values for kinetic parameters and the isotope effects are in Table I. Data were not obtained with thio-NADH and β-hydroxypyruvate because of the very low rate of this reaction.

The pH(D) profiles for V and $V/K_{ammonia}$ are shown in Figure 1. V appears pH(D) independent, and the average values of V_{H_2O} and V_{D_2O} are 3.6 ± 0.4 mM min^{-1} and 1.0 ± 0.2 mM min^{-1}, respectively. Thus, $^{D_2O}V = 3.6 \pm 0.8$. $V/K_{ammonia}$ shows a decrease on the acid side of the pH(D) profile with a pK of 9.0 ± 0.2 in H_2O and 9.5 ± 0.1 in D_2O. The pH(D)-independent plateau values of $V/K_{ammonia}$ are 0.7 ± 0.2 min^{-1} in H_2O and 0.42 ± 0.09 min^{-1} in D_2O. Thus, $^{D_2O}(V/K_{ammonia}) = 1.7 \pm 0.6$.

The tritium isotope effect was calculated from eq 7, by using specific activities of [^{3}H]NADH of (1.35 ± 0.06) × 10^5 cpm/μmol at pH 7.0 (10–20% conversion) and 4.12 ± 0.02) × 10^5 cpm/μmol at pH 10 (100% conversion). Both values are the average of duplicate samples. From eq 7 $^T(V/K_{alanine})$ is 3.2 ± 0.1. $^T(V/K_{ammonia})$ is then calculated to be 2.62, since the equilibrium isotope effect is 1.22 (Cook et al., 1980).

^{15}N isotope effects were obtained by using two α-keto acids and three reduced nucleotides plus NADD at pH 6.8–7.3

Table I: Kinetic Parameters and Deuterium Isotope Effects with Alternate Nucleotide Substrates for Alanine Dehydrogenase[a]

nucleotide/α-keto acid	V^b (units/mL)	V_{rel}	K_{NH_3} (M)	$(V/K)_{rel}$	$^DV^c$	$^D(V/K_{NH_3})$
NADH/pyruvate	21 ± 1	1.00	0.96 ± 0.08	1.00	1.10 ± 0.03	1.70 ± 0.03
Acpyr-NADH/pyruvate	5.5 ± 0.3	0.26	3.3 ± 0.3	0.076	2.97 ± 0.05	
thio-NADH/pyruvate	0.012 ± 0.003	0.0006	0.16 ± 0.01	0.0034	2.35 ± 0.06	
NADH/β-hydroxypyruvate	2.3 ± 0.2	0.11	2.8 ± 0.2	0.038	2.45 ± 0.04	
Acpyr-NADH/β-hydroxypyruvate	0.075 ± 0.003	0.004	1.4 ± 0.1	0.0024	3.46 ± 0.09	

[a] All assays were carried out at pH 6.9, 100 mM Hepes, 25 °C. [b] Calculated maximum velocity of a stock alanine dehydrogenase solution. [c] All data were fitted to eq 3 or 4. Where a single value is shown, the isotope effects on V and V/K are not significantly different, and the data were fitted to eq 4.

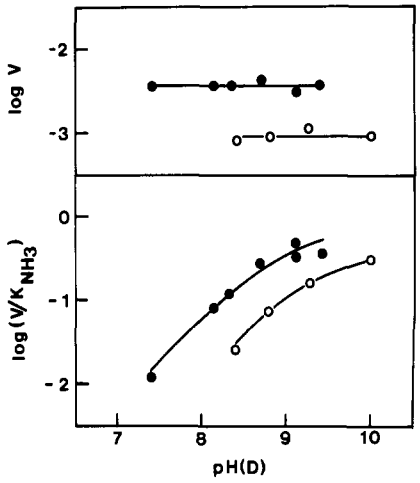


FIGURE 1: pH(D) profiles for the reductive amination of pyruvate catalyzed by alanine dehydrogenase. Closed circles are in H_2O; open ones, in D_2O. The variable substrate is ammonia. The points are experimental, and the lines represent fitted values from eq 6 (V/K) or the equation for a straight line (V).

where no substrates are sticky[2] (Table II). Values were also obtained at pH 8.9 or 9.3 with NADH and both α-keto acids to see whether the isotope effect was pH dependent. Values were also obtained in D_2O at pD 7.5 with NADH and both α-keto acids. These data are also in Table II.

Glutamate Dehydrogenase. Deuterium isotope effects were obtained at saturating NADP by measuring initial rates at varying concentrations of L-glutamate or L-glutamate-*2-d* at pH 5.95 [$^DV = 1.11 \pm 0.01$ and $^D(V/K_{glutamate}) = 1.23 \pm 0.03$], pH 6.92 [$^DV = 1.07 \pm 0.01$ and $^D(V/K_{glutamate}) = 1.14 \pm 0.03$] and pH 8.0 [$^DV = {}^D(V/K_{glutamate}) = 1.01 \pm 0.01$]. Isotope effects were also obtained in the reverse reaction by varying the concentration on ammonia for a number of nucleotide and α-keto acid pairs, and these data are summarized in Table III.

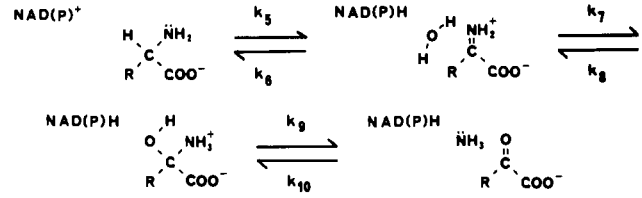
Tritium isotope effects were calculated from average specific activities of [4(*S*)-^{3}H]NADPH of $(4.94 \pm 0.27) \times 10^3$ cpm/μmol (10% reaction at pH 6) and 6.66×10^3 cpm/μmol (100% reaction at pH 8). The value from eq 7 for $^T(V/K_{glutamate})$ was 1.37 ± 0.07. Division by the equilibrium isotope effect of 1.22 gives $^T(V/K_{ammonia})$ as 1.12.

^{15}N isotope effects for glutamate dehydrogenase were determined by using two α-keto acids and three reduced nucleotides plus NADD at pH values where substrates are not sticky. In addition, values were obtained at pH 9.2 with NADPH and both α-keto acid substrates. These data are shown in Table IV.

^{15}N isotope effects were also obtained by using the equilibrium perturbation technique. At pH 8, an inverse perturbation was observed (that is, an initial decrease in absorbance at 340 nm when label started in $^{15}NH_4^+$) that gave a value of 0.97. At pH 6 and 9, however, normal perturbations were obtained that gave values of 0.989 and 0.998, respectively. These values are all within experimental error equal to those reported in Table IV.

[2] A sticky substrate is converted to product faster than it dissociates from enzyme. Conversely, the binding of a nonsticky substrate is at equilibrium in the steady state.

Scheme I: Simplified Chemical Mechanism for Alanine and Glutamate Dehydrogenases As Suggested by Grimshaw et al. (1981) and Rife and Cleland (1980b), Respectively



THEORY

The mechanisms for both alanine and glutamate dehydrogenases can be represented by Scheme I and

$$EA \underset{k_2}{\overset{k_1B}{\rightleftharpoons}} EAB \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}R{\cdot}imine \underset{k_8}{\overset{k_7}{\rightleftharpoons}}$$

$$E{\cdot}R{\cdot}carbinolamine \underset{k_{10}P}{\overset{k_9}{\rightleftharpoons}} EQR \quad (9)$$

where A is NAD(P) (or analogue), B is the amino acid (alanine, glutamate), P is ammonia, Q is the α-keto acid (pyruvate, α-ketoglutarate), and R is NAD(P)H (or analogue). In this mechanism, k_5 and k_6 will exhibit primary deuterium and secondary ^{15}N isotope effects, k_7 and k_8 will show a secondary ^{15}N isotope effect, and k_9 and k_{10} will show a primary ^{15}N isotope effect. We must also consider the equilibrium isotope effects for formation of NH_3 and the amino acids containing the unprotonated NH_2 group from the protonation forms present in solution at the reaction pH. The ^{15}N fractionation factors of the various species relative to aqueous NH_3 are as follows: amino acid with NH_3^+ group, 1.0326; amino acid with NH_2 group, 1.0163; imine, ≥1.0192; carbinolamine, ≥1.0326, NH_4^+, 1.0192 (Hermes et al., 1985). With slow substrates, or at pH values where the substrate is not sticky, $k_2 \gg k_3$, and the expression for $V/K_{ammonia}$ is

$$\frac{V}{K_{NH_3}E_t} = \frac{k_6(k_8k_{10}/k_7k_9)}{1 + k_6/k_7(1 + k_8/k_9) + k_5/k_4} \quad (10)$$

From eq 10 expressions for the deuterium and ^{15}N isotope effects with NADH or NADD are

$$^D(V/K_{NH_3}) = \frac{^Dk_6 + a + ab + {}^DK_{eq}c}{1 + a + ab + c} \quad (11)$$

$$^{15}(V/K_{NH_3})_H = (^{15}K_{eq10}{}^{15}K_{eq8}{}^{15}k_6 + {}^{15}K_{eq10}{}^{15}k_8a + {}^{15}k_{10}ab + {}^{15}K_{eq}c)/(1 + a + ab + c) \quad (12)$$

$$^{15}(V/K_{NH_3})_D = [^{15}K_{eq10}{}^{15}K_{eq8}{}^{15}k_6 + (^{15}K_{eq10}{}^{15}k_8a + {}^{15}k_{10}ab + {}^{15}K_{eq}{}^DK_{eq}c)/{}^Dk_6]/[1 + (a + ab + {}^DK_{eq}c)/{}^Dk_6] \quad (13)$$

Table II: ^{15}N Isotope Effects for Alanine Dehydrogenase

nucleotide/α-keto acid	pH(D)	$^{15}(V/K)_{obsd}$	correction[a]	$^{15}(V/K_{NH_3})$
NADH/pyruvate	7.3	1.0107	1.0190	0.9919
		1.0103		0.9915
	7.1	1.0104	1.0191	0.9915
		1.0124		0.9934
	6.8	1.0093	1.0191	0.9904
				mean 0.992 ± 0.001
NADH/pyruvate	9.3	0.9907	1.0085	0.9824
		0.9876		0.9793
				mean 0.981 ± 0.002
NADH/pyruvate (D_2O)	7.5	1.0145	1.0245	0.9902
		1.0139		0.9897
				mean 0.9900 ± 0.0003
NADD/pyruvate	7.1	1.0081	1.0191	0.9892
		1.0080		0.9891
				mean 0.98915 ± 0.00007
Acpyr-NADH/pyruvate	7.1	1.0034	1.0191	0.9846
		1.0032		0.9844
				mean 0.9845 ± 0.0001
thio-NADH/pyruvate	7.1	1.0108	1.0191	0.9919
		1.0106		0.9917
				mean 0.9918 ± 0.0001
NADH/β-hydroxypyruvate	7.3	0.9997	1.0190	0.9811
		0.9990		0.9804
	7.1	0.9995	1.0191	0.9808
		1.0040		0.9852
		1.0020		0.9832
				mean 0.982 ± 0.002
NADH/β-hydroxypyruvate	8.9	0.9932	1.0128	0.9807
		0.9931		0.9832
				mean 0.98065 ± 0.00007
NADH/β-hydroxypyruvate (D_2O)	7.5	1.0032	1.0245	0.9792
	7.6	1.0030	1.0244	0.9791
				mean 0.97915 ± 0.00007
NADD/β-hydroxypyruvate	7.1	0.9990	1.0191	0.9803
		1.0040		0.9852
				mean 0.983 ± 0.003
Acpyr-NADH/β-hydroxypyruvate	7.1	0.9994	1.0191	0.9807
		0.9991		0.9804
				mean 0.9806 ± 0.0002

[a] The $^{15}(V/K_{NH_3})$ values in the last column have been corrected from the observed values by dividing by the appropriate fraction of the equilibrium isotope effect on the deprotonation of NH_4^+ (1.0192 in H_2O, 1.0246 in D_2O; Hermes et al., 1985), depending on the pH(D) of the solution.

Table III: Kinetic Parameters and Deuterium Isotope Effects with Alternate Nucleotide Substrates for Glutamate Dehydrogenase[a]

nucleotide/α-keto acid	V^b (units/mL)	V_{rel}	K_{NH_3} (mM)	$(V/K)_{rel}$	$^DV = {}^D(V/K_{NH_3})^c$
NADPH/α-ketoglutarate	290 ± 5	1.0	87 ± 3	1.0	1.09 ± 0.01
Acpyr-NADPH/α-ketoglutarate	113 ± 8	0.39	199 ± 8	0.16	1.36 ± 0.01
thio-NADPH/α-ketoglutarate	116 ± 7	0.40	260 ± 22	0.14	1.06 ± 0.01
NADPH/α-ketovalerate	1.00 ± 0.03	0.003	25 ± 2	0.012	2.20 ± 0.09
Acpyr-NADPH/α-ketovalerate	0.12 ± 0.01	0.0004	50 ± 6	0.0024	2.8 ± 0.2
thio-NADPH/α-ketovalerate	1.20 ± 0.02	0.004	48 ± 2	0.025	1.20 ± 0.03

[a] All assays were carried out at pH 5.8, 100 mM phosphate, 25 °C. [b] Calculated maximum velocity of a stock glutamate dehydrogenase solution. [c] All data were fitted by using eq 4, since in all cases the isotope effects on V and V/K were not significantly different.

where $a = k_6/k_7$, $b = k_8/k_9$, and $c = k_5/k_4$. In these equations, $^Dk_6$ is the intrinsic primary deuterium isotope effect on hydride transfer and $^{15}k_6$ and $^{15}k_8$ are the intrinsic secondary ^{15}N isotope effects on imine reduction and formation of the imine from the carbinolamine, respectively, while $^{15}k_{10}$ is the primary ^{15}N kinetic isotope effect on formation of the carbinolamine. $^{15}K_{eq8}$ and $^{15}K_{eq10}$ are equilibrium isotope effects on imine formation from the carbinolamine and carbinolamine formation from the α-keto acid and ammonia, respectively, while $^DK_{eq}$ and $^{15}K_{eq}$ are the deuterium and ^{15}N equilibrium isotope effects for the overall reaction in the direction of reductive amination. $^DK_{eq}$ is 0.87 (Cook et al., 1980) and $^{15}K_{eq}$ is 0.984 (Hermes et al., 1985), while $^{15}K_{eq8}$ and $^{15}K_{eq10}$ are estimated to be 1.013 and 0.968 from the fractionation factors given above.

Ranges for the values of the kinetic ^{15}N isotope effects can also be estimated. $^{15}k_6$ could be between unity and 1.003 (the value of $^{15}K_{eq6}$) for this secondary isotope effect but will probably be closer to unity, since the transition state is likely to be early. Transition states for hydride transfer for alcohol and aldehyde dehydrogenase (Scharschmidt et al., 1984), formate dehydrogenase (Hermes et al., 1984), and malic enzyme (Hermes et al., 1982) have all been suggested to be early starting with reduced nucleotide. $^{15}k_8$ (also a secondary isotope effect) should be between unity and 1.013 (the value of $^{15}K_{eq8}$), and a value of 1.007 corresponds to a transition state halfway between the protonated carbinolamine and the imine.

$^{15}k_{10}$ should be slightly normal, since it is a primary kinetic isotope effect corresponding to C–N bond formation, even though $^{15}K_{eq10}$ is inverse (that is, the ^{15}N isotope effect on C–N bond cleavage will exceed that on bond formation by 3.26%). While no normal ^{15}N isotope effects were observed with alanine dehydrogenase, for glutamate dehydrogenase with thio-NADPH and α-ketoglutarate at pH 6.8 we have observed a

Table IV: ^{15}N Isotope Effects for Glutamate Dehydrogenase

nucleotide/α-keto acid	pH(D)	$^{15}(V/K)_{obsd}$	correction[a]	$^{15}(V/K_{NH_3})$
NADPH/α-ketoglutarate	5.8	1.0177	1.0192	0.9985
				0.9969
				mean 0.998 ± 0.001
NADPH/α-ketoglutarate	9.2	0.9902	1.0096	0.9808
				0.9809
				mean 0.9808 ± 0.0001
NADPD/α-ketoglutarate	6.6	1.0133	1.0192	0.9942
		1.0121		0.9930
				mean 0.9936 ± 0.0008
Acpyr-NADPH/α-ketoglutarate	6.8	1.0107	1.0191	0.9918
		1.0097		0.9908
				mean 0.9913 ± 0.0007
thio-NADPH/α-ketoglutarate	6.8	1.0276	1.0191	1.0083
		1.0276		1.0083
				mean 1.0083
NADPH/α-ketovalerate	6.4	1.0031	1.0192	0.9842
		1.0021		0.9837
				mean 0.9837 ± 0.0007
NADPH/α-ketovalerate	9.2	0.9937	1.0096	0.9843
		0.9944		0.9850
				mean 0.9847 ± 0.0005
NADPD/α-ketovalerate	6.5	1.0024	1.0192	0.9835
		1.0023		0.9834
				mean 0.98345 ± 0.00007
Acpyr-NADPH/α-ketovalerate	6.8	1.0045	1.0191	0.9857
		1.0024		0.9836
				mean 0.985 ± 0.002
thio-NADPH/α-ketovalerate	6.8	1.0206	1.0191	1.0015

[a] See footnote *a* of Table II.

$^{15}(V/K_{ammonia})$ value of 1.0084 and a $^{D}(V/K_{ammonia})$ value near unity. To obtain such values, the term containing $^{15}k_{10}$ in eq 12 must totally dominate the equation (that is, $k_8 \gg k_9$, and carbinolamine formation is completely rate limiting), and thus the intrinsic $^{15}k_{10}$ value is slightly higher than 1.0084 in this case. Values of $^{15}k_{10}$ are unlikely to exceed 1.02, since this would require $^{15}k_9$ to exceed 1.053.

With the above restrictions, we can evaluate the various terms in eq 12 and 13. Both numerator and denominator consist of four terms, and we will discuss the ratios of the corresponding numerator and denominator terms. The ratio of the first terms is $0.981^{15}k_6$, with a possible range of 0.981–0.984. These terms will be important whenever a sizable primary deuterium isotope effect is seen and will totally dominate when hydride transfer is solely rate limiting. The ratio of the fourth terms in numerator and denominator is 0.984 (the overall $^{15}K_{eq}$ value). These terms will be important when there is a known forward commitment to hydride transfer from the amino acid side of the reaction (this can be determined from a pre-steady-state burst isotope effect, which will show only a forward commitment); the observed deuterium isotope effect will be low in this case. This is certainly the situation for glutamate dehydrogenase with α-ketoglutarate and NADPH since even at low pH where stickness is eliminated only a small deuterium effect of about 25% is observed on $V/K_{glutamate}$. In addition, the isotope effect on the burst rate is only 1.5–1.7 (Fisher et al., 1970). Thus, a sizable internal forward commitment exists from the amino acid side of the reaction.

The second terms in eq 12 and 13 are important when k_6/k_7 is large and k_8/k_9 is small and have a ratio in numerator and denominator of $0.968^{15}k_8$. The most likely value of this ratio is about 0.975, with a possible range of 0.968–0.981. Only when these terms dominate can the observed ^{15}N isotope effect be appreciably less than 0.983. In contrast, the third terms in eq 12 and 13 will be important only when both k_8/k_9 and its product with k_6/k_7 are much larger than unity. The ratio of numerator and denominator terms is $^{15}k_{10}$, which is certainly normal, and is probably at least 1.008, as discussed above. Thus whenever the observed ^{15}N isotope effect exceeds 0.984, this term must be contributing, and the larger the value, the more important this term is.

The effect of deuteriating the reduced nucleotide, as in eq 13, is to reduce the size of all terms in eq 12 except the first by $^{D}k_6$. A range for the intrinsic deuterium isotope effect on hydride transfer can be estimated by comparison of the deuterium and tritium isotope effects on $V/K_{ammonia}$ (Cook & Cleland, 1981b). A range of 4.4–5.1 is calculated for $^{D}k_6$ with alanine dehydrogenase,[3] and this is in reasonable agreement with the value suggested by Srinivasan and Fisher (1985). If only the first or fourth terms in eq 12, which have nearly identical values close to 0.983, are important, deuteriation will not change the observed ^{15}N isotope effect. Likewise, if the strongly inverse second and normal third terms balance each other (k_8/k_9 between 0.07 and 0.6), these terms could dominate the equation and there would not be an effect of deuteriation. When deuteriation makes the observed ^{15}N isotope effect more inverse, but the value still exceeds 0.983, both the first and third terms in eq 12 are important, and their ratio is changed in eq 13. Under Discussion we will apply these equations to the data for alanine and glutamate dehydrogenases to deduce possible ranges for the intermediate partition ratios with the various substrates.

Discussion

Alanine Dehydrogenase. The value of 1.97 obtained for $^{D}(V/K_{alanine})$ at pH 7 by Grimshaw et al. (1981), when divided by the equilibrium isotope effect of 1.15 (Cook et al., 1980), gives a value of 1.71 for $^{D}(V/K_{ammonia})$, in excellent agreement with the value of 1.7 in Table I. The isotope effect on V for reductive amination is only 1.1, however, showing that some step prior to combination with ammonia, or after release of

[3] A range for the intrinsic deuterium isotope effect, $^{D}k_6$, cannot be calculated from the data for glutamate dehydrogenase since the isotope effects are too small, and hence the relative errors are too large.

the first product, is slow. Since the kinetic mechanism in the direction of reductive amination is ordered at neutral pH with alanine released before NAD (Grimshaw & Cleland, 1981), release of NAD most likely largely limits the rate. The level of E·NAD present at saturating NAD and alanine was estimated by Grimshaw and Cleland (1981) to be 25%, but the level would have to be higher in the back-reaction to account for the low isotope effect on V. The solvent isotope effect on V of 3.6 must also reflect the nucleotide release step, suggesting a conformational change prior to release of NAD that involves proton transfer in the transition state for this step. In agreement with this, Grimshaw et al. (1981) have suggested from the pH dependence of the primary deuterium isotope effect that a pH-dependent conformational change accompanies NAD binding.

The pK in the $V/K_{ammonia}$ profile in H_2O shown of 9.0 ± 0.2 is that of ammonia, and the shift in this pK of 0.5 pH unit to 9.5 ± 0.1 for the profile determined in D_2O is as expected for a proton on a nitrogen which has a fractionation factor of essentially unity (Schowen, 1977). Thus NH_3 is the substrate.

The pH dependence of the isotope effects obtained with serine and alanine by Grimshaw et al. (1981) indicated that neither reactant was sticky at pH 7. The pH independence of the ^{15}N isotope effects in Table II suggests that ammonia is also not sticky, as would be expected from the large K_m value. Since 1.7 is most likely not the intrinsic deuterium isotope effect, this low value presumably results from slow steps within the interconversion of the central complexes as discussed below. The deuterium isotope effects were the same on V and $V/K_{ammonia}$ when either of the nucleotides other than NADH or when β-hydroxypyruvate was used. Thus if steps other than hydride transfer limit the rate for these alternate reactants, they are the same at low and high ammonia levels (that is, they are steps between addition of ammonia and release of alanine or serine). It appears that hydride transfer becomes more rate determining, the intrinsic isotope effect becomes larger, or both as the substrate (or nucleotide) is changed. Since V decreases as the isotope effect increases, at least a portion of the increased isotope effect is likely a result of a decrease in the rate of hydride transfer.

The K_m values for ammonia are all in the same range[4] except for thio-NADH, where the value is somewhat lower. The V/K value with thio-NADH is only a factor of 300 less than with NADH, while the V value is 1750-fold less. These data suggest a high proportion of nonproductive binding of ammonia with thio-NADH, which would lower the V and $K_{ammonia}$ but not V/K. The nonproductive binding may be covalent (that is, involve carbinolamine and/or imine formation).

The isotope ratio mass spectrometer was used to measure ^{15}N isotope effects in the alanine dehydrogenase reaction, using the natural abundance of ^{15}N in ammonia as the label. Once the ^{15}N isotope effects have been corrected for the equilibrium ^{15}N isotope effect on deprotonation of NH_4^+ to NH_3 (the actual substrate for the reaction), all values are inverse. Inverse isotope effects result from equilibrium or secondary isotope effects which override any normal primary isotope effects in the mechanism, and thus we must consider the chemical mechanisms of the reaction in detail to interpret them.

The observed ^{15}N isotope effect with NADH and pyruvate was 0.992 at neutral pH where alanine is not sticky, and deuteriation made the value slightly more inverse (0.989). The first and third terms in the numerator and denominator of eq 12 are important, and their ratio is changed in eq 13. While it might appear that eq 11–13 provide three equations in three unknowns, a, b, and c (within the constraints applied under Theory), the solutions are ill conditioned with the present data, and it is only possible to pick values that are consistent with the observed isotope effects. The ratio k_6/k_7 has a maximum value of 2, and k_8/k_9, a minimum value of 1.2, with their product having a narrower range of 0.3–2.5. Thus all of the partition ratios are not far from unity, as might be expected for the physiological substrates (Albery & Knowles, 1976), and all of the chemical steps in the mechanism are partly rate limiting.

With pyruvate and the other nucleotide substrates, the deuterium isotope effects were larger than with NADH, and thus either some of the partition ratios in eq 11–13 are decreased or $^Dk_6$ is larger. Carbinolamine formation is still probably partly rate limiting with thio-NADH but is less so with Acpyr-NADH. With β-hydroxypyruvate, all of the ^{15}N isotope effects are in the 0.981–0.983 range, deuteriation has no effect, and the deuterium isotope effects are large. Carbinolamine formation is thus not rate limiting in these cases, and the first and fourth terms in eq 12 appear to be the important ones, with imine formation possibly coming to equilibrium on the enzyme and hydride transfer a major rate-limiting step.

The effect of D_2O on the ^{15}N isotope effects confirms the model and quantitative interpretation of eq 12 and 13 we have given above. Because N–D bonds are stiffer than N–H bonds, there is a 0.5% change in the ^{15}N equilibrium isotope effect for each extra hydrogen on the nitrogen of substrate vs product (Hermes et al., 1985). Thus $^{15}K_{eq}$ for conversion of NH_3 to the monoanion of alanine becomes 0.989, rather than 0.984. The value of $^{15}K_{eq8}$ becomes 1.018, rather than 1.013, while $^{15}K_{eq10}$ and $^{15}K_{eq6}$ should not be altered, since the number of hydrogens on the nitrogen remains the same during these steps. These changes leave the numerator/denominator ratios of the first and third terms in eq 12 and 13 unchanged but raise the ratio for the fourth term to 0.989 and change the upper limit of the ratio for the second term to 0.986 rather than 0.981 (the lower limit remains 0.968). The observed values with NADH and both pyruvate and β-hydroxypyruvate in Table II are *decreased* by 0.2–0.3%, however. This requires a change in the values of k_6/k_7, k_8/k_9, or k_5/k_4 in eq 12. In mechanism 9 the only step in which the exchangeable hydrogen is transferred is in the conversion of the protonated carbinolamine to the imine. Thus k_7 and k_8 may be decreased in D_2O, while k_4, k_5, k_6, and k_9 should not be affected. Since k_7 and k_8 should be affected equally [the base to which the proton is transferred was deduced by Grimshaw et al. (1981) to be a histidine, which has a similar fractionation factor to the amino group), $k_6k_8/(k_7k_9)$ is unchanged, while k_6/k_7 is *increased* in D_2O. Thus the second term in eq 12, which has the most inverse numerator/denominator ratio, is selectively increased in D_2O, while the other three terms have the same values. The net result is a more inverse observed isotope effect.

Changing the pH from near 7 to near 9 makes little difference with NADH and β-hydroxypyruvate, because serine is not a sticky substrate (Grimshaw et al., 1981). However with NADH and pyruvate, the observed ^{15}N isotope effect drops from 0.992 at neutral pH to 0.981 at pH 9.3. At this pH alanine is sticky (Grimshaw et al., 1981), showing an external commitment of at least 1, and possibly as high as 7. Such values become part of k_5/k_4 in mechanism 9, and in eq

[4] The value of $K_{ammonia}$ calculated from the pH dependence of V and $V/K_{ammonia}$ determined by Grimshaw et al. (1981) is 1 M, in agreement with the value of 0.96 M reported in this work.

11–13. Thus at this pH the fourth term in eq 12 becomes more important, and the more inverse isotope effect is the result.

Glutamate Dehydrogenase. The ratio of the isotope effects on $V/K_{glutamate}$ and $V/K_{ammonia}$ at pH 5.8–6.0 (1.13 ± 0.03) is within experimental error equal to the deuterium equilibrium isotope effect of 1.15 (Cook et al., 1980). In addition, the value of 1.14 obtained for $^{D}(V/K_{glutamate})$ at pH 7 is in agreement with the value of 1.2 ± 0.1 at the same pH as reported by Rife and Cleland (1980a). The increase in the deuterium isotope effect on $V/K_{glutamate}$ from 1.0 at pH 8 to a value of 1.23 at pH 5.6 suggests that glutamate is sticky at pH 8 (Cook & Cleland, 1981a). In addition, glutamate binds to glutamate dehydrogenase in which a group with a pK between 7 and 8 is either protonated or unprotonated but does not bind equally well to both since the V pH profile has a lower pK than the $V/K_{glutamate}$ profile (Rife & Cleland, 1980b). Since the isotope effect is 1.0 at pH 8, it is not possible to calculate the external commitment (or stickiness) factor for glutamate (Cook & Cleland, 1981a), and most likely release of reduced nucleotide limits the overall reaction. A value of 1.5–1.8 was obtained by Fisher et al. (1970) for the deuterium isotope effect on the pre-steady-state burst rate in the direction of oxidative deamination. Since this value is larger than that observed on $V/K_{glutamate}$ in the steady state, there must be a large reverse commitment factor resulting from slow steps after oxidation to form the imine, but prior to release of the first product, NH_3.

As seen in Table III, although V decreases 2.5-fold and $V/K_{ammonia}$ decreases 6-fold when the alternate nucleotides Acpyr-NADPH and thio-NADPH are used with α-ketoglutarate, the deuterium isotope effects change only slightly, and thus the decrease in rate must be in steps other than hydride transfer. Since the isotope effect on both parameters is the same in all cases, the rate-limiting step must lie between addition of ammonia and release of glutamate. Use of the alternate α-keto acid substrate, α-ketovalerate, gives large decreases in kinetic parameters with values of V 300- and 2500-fold lower and values of $V/K_{ammonia}$ 80- and 400-fold lower with NADPH and Acpyr-NADPH, respectively. In these cases larger deuterium effects of 2.2 and 2.8 are obtained, and thus hydride transfer has become a major rate-limiting step. Thio-NADPH gives low relative values of V and $V/K_{ammonia}$ (similar to NADPH) but a small deuterium isotope effect of 1.2. Thus, a step (or steps) other than hydride transfer becomes (become) slower when thio-NADPH is used. Finally, the affinity for ammonia[5] is decreased by 50% when either of the alternate nucleotides replaces NADPH, showing differences in the ternary E·reduced nucleotide·α-keto acid complexes to which ammonia binds and/or reacts.

As with alanine dehydrogenase, once the ^{15}N isotope effects have been corrected for the equilibrium ^{15}N isotope effect on deprotonation of NH_4^+ to NH_3, all values except those using thio-NADPH are inverse. With thio-NADPH as the nucleotide substrate, *normal* isotope effects were obtained that were larger with α-ketoglutarate than with α-ketovalerate, the opposite of what was observed with the deuterium isotope effect in these two cases.

As discussed under Theory, when deuteriation makes the observed ^{15}N isotope effect more inverse, but the value still exceeds 0.983, both the first and third terms in eq 12 are important, and their ratio is changed in eq 13. This may be the case with α-ketoglutarate and NADPH or NADPD in the present study, where deuteriation appears to make the isotope effect 0.4% more inverse. Since both values exceed 0.983, however, carbinolamine formation partly limits the reaction. By considering eq 11–13 and the experimental values of the isotope effects, we can put some limits on the partition ratios in these equations. (1) $^{D}k_6$ was fixed at 4.0 as discussed above. (2) The value of a (k_6/k_7) is between 1 and 10 as values outside this range give experimental values of the ^{15}N isotope effects too small or too large with reasonable values of b and c. (3) The value of b (k_8/k_9) is between 0.1 and 9. (4) The value of c (k_5/k_4) must be in the range 9–13.

With α-ketoglutarate and Acpyr-NADPH, the deuterium isotope effect is only slightly larger than with NADPH, and thus the partition ratios in eq 11–13 probably do not change significantly, particularly since the ^{15}N isotope effect is only slightly smaller. With α-ketovalerate, all of the ^{15}N isotope effects except that with thio-NADPH are in the 0.983–0.985 range, deuteriation has no effect, and the deuterium isotope effects are large. Carbinolamine formation is thus not rate limiting in these cases, and the first and fourth terms in eq 12 appear to be the important ones, with imine formation possibly coming to equilibrium on the enzyme. With thio-NADPH and either α-keto acid the deuterium isotope effects are small and the ^{15}N isotope effects are both normal, suggesting that carbinolamine formation is almost entirely rate determining.

Changing the pH from near 6 to near 9 has little effect on the ^{15}N isotope effect with NADPH and α-ketovalerate, since norvaline is not sticky at pH 9. However, at pH 8 and above, glutamate is sticky (Rife & Cleland, 1980b), and with NADPH and α-ketoglutarate, the observed ^{15}N isotope effect drops from 0.998 at pH 6 to 0.981 at pH 9.2. The external commitment or stickiness factor becomes part of k_5/k_4 in mechanism 9 and in eq 11–13. Thus, at high pH the fourth term in eq 11 becomes an important one, and the more inverse observed isotope effect is the result.

In conclusion, this work shows how the use of multiple isotope effects can identify the rate-limiting steps in a complicated mechanism such as that of alanine and glutamate dehydrogenases.

[5] In the case where $^{D}V = {}^{D}(V/K)$, K_m is equal to K_i (Klinman & Matthews, 1985).

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Binding of Nucleotides to an Extramitochondrial Acetyl-CoA Hydrolase from Rat Liver

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ABSTRACT: Cold labile extramitochondrial acetyl-CoA hydrolase (dimeric form) purified from rat liver was activated by various nucleoside triphosphates and inhibited by various nucleoside diphosphates. Activation of acetyl-CoA hydrolase by ATP was inhibited by a low concentration of ADP ($K_i \cong 6.8$ μM) or a high concentration of AMP ($K_i \cong 2.3$ mM). ADP and AMP were competitive inhibitors of ATP. A Scatchard plot of the binding of ATP to acetyl-CoA hydrolase (dimer) at room temperature gave a value of 25 μM for the dissociation constant with at least 2 binding sites/mol of dimer. Cold-treated monomeric enzyme also associated with ATP–agarose, suggesting that the monomeric form of the enzyme also has a nucleotide binding site(s), probably at least 1 binding site/mol of monomer. Phenylglyoxal or 2,3-butanedione, both of which modify arginyl residues of protein, inactivated acetyl-CoA hydrolase. ATP (an activator) greatly protected acetyl-CoA hydrolase from inactivation by these reagents, while ADP (an inhibitor), valeryl-CoA (a substratelike, competitive inhibitor), and CoASH (a product) were less effective. However, addition of ADP plus valeryl-CoA (or CoASH) effectively prevented the inactivation by 2,3-butanedione, but that is not the case for phenylglyoxal. These results suggest that one or more arginyl residues are involved in the nucleotide binding site of extramitochondrial acetyl-CoA hydrolase and that their nucleotide binding sites locate near the substrate binding site.

An extramitochondrial acetyl-CoA hydrolase (EC 3.1.2.1) in rat liver hydrolyzes acetyl-CoA to acetate and CoASH. The enzyme may be important in maintenance of the cytosolic acetyl-CoA and CoASH pool (Prass et al., 1980; Matsunaga et al., 1985). It is activated by ATP and inhibited by ADP (nucleotide is not a substrate), and its effect is probably due to allosteric interaction (Prass et al., 1980). ATP and ADP regulate not only the catalytic properties of this enzyme but also the association–dissociation state of its subunits (Isohashi et al., 1983a). The purified enzyme at 25 °C without ATP is present as an active dimer ($M_r \sim 135000$) (Isohashi et al., 1983a). On addition of ATP or ADP, but not AMP, to high concentrations of dimer at 25 °C, the dimer reversibly aggregates to a tetramer ($M_r \sim 240000$) (Isohashi et al., 1983a). The dimer and tetramer are inactivated by exposure to lower temperature, with dissociation of the dimer and tetramer into the monomer (Isohashi et al., 1983a,b). The cold-inactivated enzyme (monomer) reassociates into an active dimer and tetramer when warmed to 37 °C. This reactivation of the enzyme on rewarming is enhanced by 2 mM ATP (Isohashi et al., 1984).

This enzyme is also inactivated in the presence of L-ascorbate and a trace concentration of Cu^{2+} due to oxidation caused by active oxygen, which is generated in the course of autoxidation of L-ascorbate (Nakanishi et al., 1985a). Valeryl-CoA, a substratelike competitive inhibitor, has a strong protective effect against this inactivation, but ATP and ADP have only slight protective effects, suggesting that oxidative modification of this enzyme mainly occurs at or near its substrate binding site (Nakanishi et al., 1985a).

This enzyme has a multisubunit structure and is complicatedly regulated by nucleotides, but little is known about the nucleotide binding site that may constitute the regulatory domain. Thus, the aim of the present experiments is to un-

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