to the 6 position of Trp-21 but remote from its 2 position.

The nonadditivity of the ^1H and ^{19}F NMR chemical shift contributions in binary and ternary complexes for signals tentatively assigned to Trp-5 and -133 indicates that these residues are influenced by ligand-induced conformational changes. One of these residues experiences similar conformational changes in the presence of either substrates or inhibitors.

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Kinetic Mechanism of Glutamate Dehydrogenase†

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ABSTRACT: Initial velocity patterns and dead-end inhibition studies with oxalylglycine suggest that the addition of NADPH, keto acid, and ammonia occurs with obligatory order. For monocarboxylic keto acids, the keto acid–ammonia initial velocity pattern is equilibrium ordered because k_{off} is much greater than V_{max}. α-Ketoglutarate gives substrate inhibition that is uncompetitive vs. either NADPH or ammonia in the absence of $NADP^+$, but with high $NADP^+$ is noncompetitive vs. NADPH and uncompetitive vs. ammonia. The inhibition is partial on both slopes and intercepts, showing that ketoglutarate slows down but does not prevent $NADP^+$ release, and that it forms a weak but kinetically competent E–ketoglutarate complex. The apparent ordered combination of NADPH and keto acid thus results from strong synergism in binding. Deuterium isotope effects, together with the substrate inhibition patterns for ketoglutarate, show that addition of amino acid and $NADP^+$ is random, with $NADP^+$ being released from the ternary complex more rapidly than either glutamate or norvaline. With norvaline, hydride transfer is a major rate-limiting step, while with glutamate a step preceding hydride transfer is slower than hydride transfer. The equilibrium ^{18}O isotope effect is 1.031 ± 0.006 (^{18}O enriching in ketoglutarate relative to water), but no kinetic ^{18}O isotope effect was seen.

The kinetic mechanism of glutamate dehydrogenase (EC 1.4.1.3) has been examined by steady-state kinetics (Engel & Dalziel, 1969, 1970; Engel & Chen, 1975), transient kinetics (Colen et al., 1972, 1977; Jallon et al., 1975) and equilibrium isotope exchange studies (Silverstein & Sulebele, 1973). It has been proposed that both the forward reaction (glutamate oxidation) and the reverse reaction (α-ketoglutarate reduction) involve random binding of ligands. For the forward reaction it is thought that the rate-limiting step is release of NADPH from an abortive E–NADPH–glutamate complex when the glutamate concentration is high or release of α-ketoglutarate from E–NADPH–ketoglutarate under other conditions (di Franco, 1974; Silverstein & Sulebele, 1973; Colen et al., 1975). The rate-limiting steps of the reverse reaction have not been well characterized. In this report, various kinetic techniques and the alternate substrates norvaline and α-ketovalerate were employed to further investigate the kinetic mechanism of glutamate dehydrogenase. In the following paper (Rife &

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Cleland, 1980), we will present kinetic studies which suggest a chemical mechanism for the enzyme-catalyzed reaction.

Materials and Methods

Reagents. Substrates, enzymes, and coenzymes were from Sigma. Bovine liver glutamate dehydrogenase for kinetic studies was obtained as a solution in 50% glycerol with pH 7.3 sodium phosphate buffer and less than 0.3 μg of NH_4^+ per mg of enzyme. Potassium phosphate buffer (0.05 M), pH 7.0–7.6, was used to dilute the enzyme. For syntheses, glutamate dehydrogenase was purchased as a lyophilized powder. Glutathione reductase from yeast and glutamate–oxaloacetate transaminase from porcine heart were suspensions in ammonium sulfate. Deuterium oxide with 99.8 atom % deuterium was from Sigma, while $H_2{}^{18}O$ (99.12 atom % ^{18}O) was from Bio-Rad. Oxalylglycine was synthesized by nitrous acid deamination of glycylglycine (Viscontini, 1946). The disodium product was washed successively with ethanol and diethyl ether and then recrystallized from H_2O with methanol. The NMR spectrum of the product was consistent with oxalylglycine.

Preparation of L-*Glutamate-2-d and* L-*Norvaline-2-d.* To minimize isotopic dilution during the synthesis of deuterated compounds, the glutamate, oxaloacetate, α-ketovalerate, dithiothreitol, and glutathione used in these syntheses were first dissolved in D_2O and then recovered by evaporating the solutions under vacuum. Column fractions containing glutamate or norvaline were easily detected by applying 1 or 2 μL of the fractions to filter paper, which was then sprayed with a solution of 50 mg of ninhydrin in 50 mL of 1-butanol which contained a few drops of H_2O to dissolve the ninhydrin.

L-Glutamate-*2-d* was synthesized by incubating 11.6 mmol of glutamate, 0.17 mmol of oxaloacetate, and 500 units of glutamate–oxaloacetate transaminase in 40 mL of D_2O at pH 7.2 for several days. The solution was deproteinated by vigorously stirring it with 1 mL of CCl_4 and then filtering. This procedure was repeated until the solution became clear. After the filtered solution was applied to a 40 × 4.5 cm column of Dowex-1-Cl^-, which was then thoroughly washed with water, the glutamate was recovered by eluting the column with 1 M HCl until the pH of the eluant became strongly acidic. Glutamate eluted at the front of this acid interface. The fractions containing glutamate were evaporated to a thick oil, redissolved in water, and applied to a 45 × 3 cm column of Dowex-50-H^+. After the column was washed with 1 column volume of water, it was eluted with 2.5% pyridine until the eluant became slightly basic. Glutamate again eluted at this pH interface. Zwitterionic L-glutamate-*2-d* was obtained by evaporation of the glutamate fractions. The product was dissolved in water and recovered by evaporation several times to remove traces of pyridine. No α protons were detected in the NMR spectrum of the product.

L-Norvaline-*2-d* was synthesized by using glutamate dehydrogenase to reduce α-ketovalerate with B-side NADPD. A solution containing 4.5 mmol of α-ketovalerate, 0.11 mmol of $NADP^+$, and 0.52 mmol of reduced glutathione in 100 mL of D_2O at pH 8.3 and a solution of 7.2 mmol of dithiothreitol in 15 L of D_2O were sealed with septa and flushed with N_2. The addition of 500 units of glutamate dehydrogenase and 200 units of glutathione reductase as $(NH_2)_2SO_4$ suspensions initiated the synthesis and supplied ammonia. The reaction was driven by the addition of 0.2-mL aliquots of the dithiothreitol solution at 0.5-h intervals over 2-days time. After the solution was vortexed with a few milliliters of CCl_4 and filtered to remove protein, it was then applied to a 40 × 4.5 cm column of Dowex-1-Cl^-, which was washed with H_2O until the norvaline was eluted. The norvaline fractions were titrated to pH 2.5 and applied to a 35 × 3 cm column of Dowex-50-H^+. After the column was washed with 1 column volume of H_2O, it was eluted with 2% pyridine until the eluant became slightly basic. The norvaline eluted at this pH boundary. Solid product was recovered by evaporating the norvaline fractions, and the norvaline-*2-d* was recrystallized several times from H_2O with ethanol. The NMR spectrum of the product showed no α-proton signal.

Data Collection and Analysis. Glutamate dehydrogenase reactions were assayed by monitoring the absorbance of NADPH at 340 nm. Unless stated otherwise, initial velocities were obtained from assays performed in cuvettes with 1.0-cm light paths and 3.0-mL volumes, and initial velocities were determined at 25 °C. Data corresponding to an intersecting initial velocity pattern with two substrates, to ordered addition of three substrates, or to equilibrium ordered addition of two substrates were fitted to eq 1–3, respectively, using the

$$v = VAB/[K_aB + K_bA + AB + K_{ia}K_b] \quad (1)$$

$$v = VABC/[(\text{Const}) + (\text{Coef } A)A + (\text{Coef } C)C + K_aBC + K_bAC + K_cAB + ABC] \quad (2)$$

$$v = VAB/[K_bA + AB + K_{ia}K_b] \quad (3)$$

least-squares method and the computer programs of Cleland (1979). Data corresponding to competitive, noncompetitive, or uncompetitive inhibition were fitted to eq 4–6, respectively.

$$v = VA/[K(1 + I/K_{is}) + A] \quad (4)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

$$v = VA/[K + A(1 + I/K_{ii})] \quad (6)$$

Data corresponding to linear uncompetitive substrate inhibition by B (ketoglutarate) were fitted to eq 7. When hyperbolic

$$v = VAB/[K_aB + K_bA + AB + K_{ia}K_b + AB^2/K_i] \quad (7)$$

substrate inhibition was seen, the reciprocal plots vs. a noninhibitory substrate were fitted to eq 8, and slope or intercept

$$v = VA/(K + A) \quad (8)$$

replots were fitted to eq 9 or 10, as appropriate.

$$v = C(1 + A/K_{in})/(1 + A/K_{id}) \quad (9)$$

$$v = VA/(K + A + A^2/K_i) \quad (10)$$

Measurement of Isotope Effects by Direct Comparison of Initial Velocities. Rates were determined as a function of glutamate-*2-(h,d)* and $NADP^+$ concentrations. Similar comparisons were made with norvaline-*2-(h,d)*. Deuterium isotope effects on V/K_{NADP^+}, V/K_{AA}, and V and their standard errors were determined by fitting data to eq 11

$$v = VAB/[K_b(K_{ia} + A) \times (1 + F_iE_{V/K_b}) + K_aB(1 + F_iE_{V/K_a}) + AB(1 + F_iE_V)] \quad (11)$$

where $E_V = {}^D(V) - 1$, $E_{V/K_a} = {}^D(V/K_a) - 1$, $E_{V/K_b} = {}^D(V/K_b) - 1$, $F_i = 0$ for unlabeled amino acid, and $F_i = 1$ for deuterated amino acid.[1] For the glutamate experiments, the assays contained 100 mM phosphate, pH 7, 0.25 unit of glutamate dehydrogenase, glutamate or deuterioglutamate varied between 1 and 10 mM, and $NADP^+$ varied between 0.021 and 0.210 mM. These reactions were assayed in microcylindrical cuvettes with 10-cm light paths and 5.4-mL volumes. To determine these small isotope effects, the relative concentrations of glutamate-*2-(h,d)* had to be accurately known. Aliquots from the stock glutamate-*2-(h,d)* solutions were added to excess

[1] ${}^DX$ is the deuterium isotope effect on the parameter X (that is ${}^DX = X_H/X_D$).

Table I: Kinetic Constants for Glutamate Dehydrogenase

variable substrate	cosubstrates	K_m (mM)	K_i (mM)	V (μmol per min per mg of glutamate dehydrogenase)	pH	data fitted to eq
glutamate	$NADP^+$ (21–210 μM)	0.74 ± 0.22	6.3 ± 1.4	0.64 ± 0.04	7	1
$NADP^+$	glutamate (1–10 mM)	0.028 ± 0.006	0.24 ± 0.07			
norvaline[c]	$NADP^+$ (0.18–1.84 mM)	49 ± 7	78 ± 25	1.11 ± 0.07	9	1
$NADP^+$[c]	norvaline (10–100 mM)	0.31 ± 0.07	0.46 ± 0.11			
α-ketoglutarate	NADPH (5.86–27.5 μM) and NH_4 (1–10 mM)	2.4 ± 0.8	0.315 ± 0.019[a]	35.1 ± 7.2	8	2
NH_4^+	NADPH (5.86–27.5 μM) and ketoglutarate (0.2–1.99 mM)	6.5 ± 1.8	2.9 ± 1.0			
NADPH	ketoglutarate (0.20–1.99 mM) and NH_4^+ (1–10 mM)	0.022 ± 0.007	0.028 ± 0.016			
α-ketovalerate	NADPH (84 μM) and NH_4^+ (10–100 mM)	0[b]	24.4 ± 1.9	22.9 ± 1.6	8	3
NH_4^+	ketovalerate (5.0–25.0 mM) and NADPH (84 μM)	108 ± 12	∞[b]			
α-ketobutyrate	NH_4^+ (10–100 mM) and NADPH (84 μM)	0[b]	31.0 ± 2.3	31.2 ± 8.1	8	3
NH_4^+	ketobutyrate (9.9–49.7 mM) and NADPH (84 μM)	520 ± 157	∞[b]			

[a] The ketoglutarate K_i was estimated from its K_m with high NADPH (0.18 mM) and low NH_4^+ (5.0 mM). Under these conditions, the apparent K_m of ketoglutarate approximates its dissociation constant from an E-NADPH complex. [b] The data were best fit by eq 3, which assumes the K_m for the keto acid, A, is 0 and the K_i for NH_4^+, B, is infinite. [c] Average of three trials.

$NADP^+$ in the presence of glutamate dehydrogenase. A standard curve showed that the glutamate was oxidized with quantitative production of NADPH. These assays contained 8 mM $NADP^+$, 100 M phosphate, pH 8, 5 units of glutamate dehydrogenase, and up to 6 μM glutamate. For the norvaline experiments, the reaction mixtures contained 250 mM glycine, pH 9, 5 units of glutamate dehydrogenase, norvaline or deuterionorvaline varied between 10 and 100 mM, and $NADP^+$ varied between 0.18 and 1.80 mM.

Measurement of Isotope Effects by Equilibrium Perturbation. Reaction mixtures containing L-glutamate-*2-d*, α-ketoglutarate, $(NH_4)_2SO_4$, $NADP^+$, and NADPH were prepared with different concentrations of $(NH_4)_2SO_4$. After addition of glutamate dehydrogenase, the reaction was monitored by the absorption of NADPH at 340 nm. A mixture with the proper composition had been prepared when, after the addition of enzyme, the absorption first decreased as NADPH was consumed and then slowly increased to the initial absorption as the system came to isotopic as well as chemical equilibrium. The legend to Figure 2 describes the reaction conditions in detail for one experiment with glutamate-*2-d*. Equilibrium perturbations with norvaline-*2-d* were performed in a similar manner except that ketovalerate and norvaline-*2-d* replaced ketoglutarate and glutamate-*2-d*. A typical reaction mixture for the norvaline-*2-d* studies contained 15.1 mM norvaline-*2-d*, 5.98 mM $NADP^+$, 0.147 mM NADPH, 8.65 mM ketovalerate, 0.57 mM $(NH_4)_2SO_4$, 100 mM Tris, pH 8.7, and 22 units of glutamate dehydrogenase.

Values of the isotope effects were determined from the size of the perturbation as described by Schimerlik et al. (1975). The reported values of the equilibrium perturbation isotope effects are the averages of several experiments.

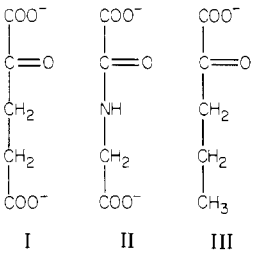
Results

Comparison of Dicarboxylic and Monocarboxylic Substrates. The kinetic constants for several substrates are compared in Table I. Not only do the monocarboxylic substrates have higher K_m or K_i values than the normal substrates, their coreactants also have higher K_m values. With monocarboxylic keto acids, the K_m of ammonia is over an order of magnitude larger than it is with ketoglutarate. Likewise, the K_m of $NADP^+$ is higher with norvaline than it is with glutamate, in agreement with the observation of Engel & Dalziel (1969) with NAD^+. Although they have lower affinities, the alternate substrates have V values comparable to the V values of glutamate and ketoglutarate.

Kinetics of the Reverse Reaction. As noted in Table I, the initial velocity pattern with α-ketovalerate and ammonia was equilibrium ordered, with binding of ketovalerate followed by binding of ammonia. These results prompted a reexamination of the kinetics of α-ketoglutarate. Initial velocities were determined as a function of ketoglutarate, ammonia, and NADPH concentrations, and the data were fitted to eq 2 and to a similar equation with a B term also in the denominator. This modified equation is expected if there is random binding of all three reactants, while eq 2, with no B term, corresponds to a mechanism that does not involve an E–B complex.[2] In these analyses, A, B, and C represent NADPH, ketoglutarate, and NH_4^+, respectively. As can be seen in Table II, which compares the kinetic constants calculated with these equations, Coef B is not significantly different from zero.

Oxalylglycine Inhibition. When ketoglutarate (I) was the variable substrate, oxalylglycine (II) acted as a competitive inhibitor. However, when NADPH or ammonia was the variable substrate, oxalylglycine acted as an uncompetitive and a noncompetitive inhibitor, respectively. Table III presents the inhibition patterns and inhibition constants seen in these experiments.

[2] The existence of an E–ketoglutarate complex has been shown by Cross et al. (1972) and Andree (1978), and Colen (1978) has shown that this complex inhibits the pre-steady-state burst during glutamate oxidation. These studies, however, do not establish that it is a kinetically important complex in the mechanism (that is, it could be a strictly dead-end complex).



I II III

The equilibrium ordered initial velocity pattern seen with ammonia and ketovalerate (III), together with the dead-end inhibition patterns with oxalylglycine, suggests that ammonia binds after the keto acid. If this is true, oxalylglycine would induce substrate inhibition by ammonia, if ammonia could combine with the E–NADPH–oxalylglycine complex in the usual way. To test this possibility, the initial velocity of the reverse reaction was determined as a function of ammonia (from 20 to 400 mM, which is $40K_m$) and oxalylglycine (0–2 mM) concentrations, with Na_2SO_4 replacing $(NH_4)_2SO_4$, so that the total of their concentrations was 200 mM and ionic strength was constant (the assays also contained 1 mM ketoglutarate, 0.2 mM NADPH, and 100 mM phosphate, pH 7.5). The results showed normal noncompetitive inhibition by oxalylglycine and no substrate inhibition by ammonia. Consequently, either ammonia does not bind to the E–NADPH–oxalylglycine complex or it does not bind so that the dissociation constants of oxalylglycine or NADPH are decreased. It may be that ammonia never binds to the enzyme, but directly attacks ketoglutarate at the active site to form a carbinolamine. Oxalylglycine could not form such a carbinolamine. The chemical mechanism of the reaction will be discussed in more detail in the following paper (Rife & Cleland, 1980).

α-Ketoglutarate Substrate Inhibition. At concentrations above 2 mM, α-ketoglutarate acts as a substrate inhibitor. As can be seen in Figure 1, this inhibition is enhanced considerably by $NADP^+$. This substrate inhibition was characterized by varying ketoglutarate concentrations over a wide range while also varying the concentration of either NADPH or ammonia. Table IV records the inhibition patterns and the inhibition constants from these experiments.

Deuterium Isotope Effects. From a direct comparison of velocities with L-glutamate and L-glutamate-*2-d*, kinetic isotope effects of 1.19 ± 0.10 on $V/K_{glutamate}$ and 1.14 ± 0.05 on V were measured, while an isotope effect of 1.23 ± 0.24 was calculated for V/K_{NADP^+}. In contrast, isotope effects of 4.68 ± 0.84 on V/K_{NADP^+}, 1.49 ± 0.13 on V/K_{Nva} and 2.46 ± 0.28 on V were measured when the velocities with L-norvaline and L-norvaline-*2-d* were compared. The isotope effects of 1.254 ± 0.036 for glutamate-*2-d* and 1.677 ± 0.016 for norvaline-*2-d*, measured by equilibrium perturbation at low levels of ammonia and ketoglutarate, are similar to those on the V/K values of the amino acids, as expected. Figure 2 shows an example of an equilibrium perturbation with glutamate-*2-d*.

^{18}O Equilibrium Isotope Effect. A stock solution was prepared with glutamate, ketoglutarate, NH_4^+, $NADP^+$, and NADPH in near equilibrium concentrations. To make a reaction mixture, 0.5 mL of this solution was added to 0.5 mL of H_2O. After temperature equilibration, enzyme was added and the absorbance was monitored at 340 nm until the reaction reached equilibrium. The stock solution was readjusted by adding a small volume of either glutamate or $(NH_4)_2SO_4$, and another reaction mixture was prepared and monitored. This procedure was repeated until the stock solution had been adjusted to the point where no net reaction was observed after

Table II: Comparison of Least-Squares Fits of the NADPH–Ketoglutarate–NH_4^+ Initial Velocity Pattern[a]

parameter	estimate from eq 2	estimate from eq 2 with B term added
V	11.7 ± 2.4 μM/min	10.1 ± 2.4 μM/min
K_a	22 ± 7 μM	16 ± 7 μM
K_b	2.4 ± 0.8 mM	1.9 ± 0.8 mM
K_c	6.5 ± 1.8 mM	4.8 ± 2.2 mM
Coef A	2.3 ± 1.0 mM^2	3.0 ± 1.1 mM^2
Coef B		0.014 ± 0.015 mM^2
Coef C	0.023 ± 0.005 mM^2	0.023 ± 0.004 mM^2
constant	0.065 ± 0.018 mM^3	0.038 ± 0.030 mM^3
σ[b]	0.0486	0.0488

[a] All assays contained 150 mM Tris, pH 8.1, and 0.44 unit of glutamate dehydrogenase in cuvettes with a 10-cm light path and 30 mL volume. NADPH (A) concentrations were 5.86, 11.5, and 27.5 μM; ketoglutarate (B) concentrations were 0.20, 0.60, 1.20, and 1.99 mM; and $(NH_4)_2SO_4$ (C) concentrations were 0.50, 1.00, 1.50, 3.50, and 5.00 mM. [b] $\sigma = [\Sigma(\text{experimental velocity} - \text{theoretical velocity})^2/(\text{number of data points} - n)]^{1/2}$. Here, n equals 7 for eq 2 and 8 for the equation with the B term.

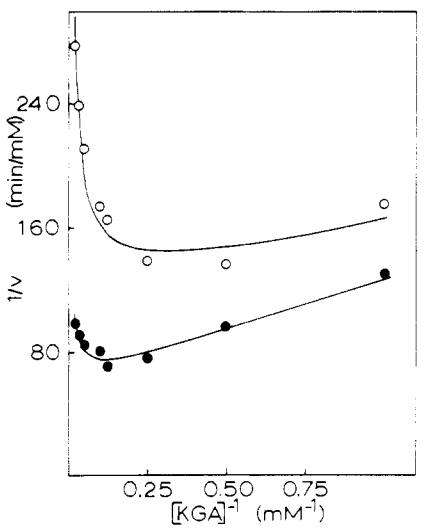


FIGURE 1: Substrate inhibition by α-ketoglutarate. The reaction mixtures for the data represented by the closed circles contained 25 mM $(NH_4)_2SO_4$, 0.10 mM NADPH, 100 mM Tris, pH 8.0, 0.088 unit of glutarate dehydrogenase, and ketoglutarate varied between 1.0 and 50.0 mM. The open circles represent identical reaction mixtures with the addition of 0.96 mM $NADP^+$. The data points are experimental, and the curves are least-squares fits to eq 10 with A being ketoglutarate.

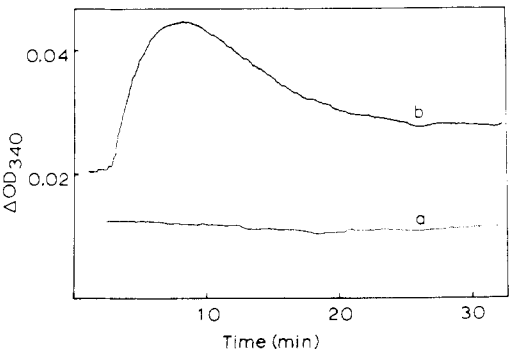


FIGURE 2: Equilibrium perturbation with deuterioglutamate. (a) The assay initially contained 11.8 mM unlabeled glutamate, 1.5 mM ketoglutarate, 0.135 mM $(NH_4)_2SO_4$, 2.7 mM $NADP^+$, 0.11 mM NADPH, 100 mM Tris, pH 8.0, and 10 units of glutamate dehydrogenase in a 3.0-mL cuvette with a 1.0-cm light path. (b) The initial assay conditions were identical with those for (a) with 13.7 mM glutamate-*2-d* replacing the unlabeled glutamate. The ΔOD_{340} corresponds to a decrease in absorbance during the perturbation.

Table III: Inhibition by Oxalylglycine

variable substrate	concns of other substrate[a] (mM)	inhibition type[b]	slope K_i (mM)	intercept K_i (mM)
NADPH				
trial 1	ketoglutarate = 2.0; NH_4^+ = 20	UC		0.58 ± 0.04
trial 2	ketoglutarate = 1.0; NH_4^+ = 5.1	UC		0.54 ± 0.03
α-ketoglutarate	NH_4^+ = 10; NADPH = 0.2	C	0.36 ± 0.04	
NH_4^+[c]	ketoglutarate = 1.0; NADPH = 0.2	NC	0.9 ± 0.3	0.34 ± 0.04

[a] The assays also contained 100 mM Tris, pH 8.0, and 0.25 unit of glutamate dehydrogenase. [b] C, competitive; NC, noncompetitive; UC, uncompetitive. Data were fit to eq 4, 5, or 6, respectively. [c] Average of two experiments.

Table IV: α-Ketoglutarate Substrate Inhibition Constants

variable[a] substrate	$NADP^+$ (mM)	intercept K_i (mM)	slope K_i (mM)	type of inhibition
NADPH	0	60.0 ± 7.4[b]		linear UC[f]
NH_4^+	0	70 ± 11[b]		linear UC
NADPH	0.99	18.3 ± 3.7[c]	K_{in} = 1.450 ± 0.002[e] K_{id} = 5.374 ± 0.005	*S*-hyperbolic, *I*-linear NC
NH_4^+	0.96	K_{in} = 10.5 ± 1.8[d] K_{id} = 41.8 ± 7.7		hyperbolic UC

[a] With variable NH_4^+, the assays contained 0.10 mM NADPH, 100 mM Tris, pH 8.0, and 0.088 unit of glutamate dehydrogenase. Ketoglutarate was varied from 1.0 to 50.0 mM, while $(NH_4)_2SO_4$ concentrations were 5, 10, and 25 mM. With variable NADPH, assays contained 100 mM phosphate, pH 7.5, 25 mM $(NH_4)_2SO_4$, and 0.17 unit of glutamate dehydrogenase. Ketoglutarate was varied from 0.1 to 54.0 mM, while NADPH concentrations were 0.019, 0.038, 0.095, and 0.191 mM. [b] These values were obtained by fitting the data to eq 7 with A being NADPH or NH_4^+ and B being ketoglutarate. [c] Obtained by fitting apparent V values from fits to eq 8 (A being NADPH) to eq 10, where A represents ketoglutarate and v is the apparent V. [d] Obtained by fitting apparent $1/V$ values from eq 8 (A being NH_4^+) to eq 9 where A represents ketoglutarate and v is $1/V$. [e] Obtained by fitting K/V values from eq 8 (A being NADPH) to eq 9, where A represents ketoglutarate and v is K/V. [f] UC, uncompetitive; NC, noncompetitive; *S*, slope; *I*, intercept.

the addition of enzyme. A reaction mixture was then prepared which contained 0.5 mL of this same stock solution and 0.5 mL of $H_2{}^{18}O$. Upon addition of enzyme to this mixture, there was an increase in absorbance as the reaction came to an equilibrium significantly different from that with $H_2{}^{16}O$. After equilibrium constants were calculated for the reaction with $H_2{}^{16}O$ and $H_2{}^{18}O$, the equilibrium isotope effect was calculated from eq 12. Several experiments gave an average isotope

$$ {}^{18}K_{eq} = \frac{K_{eq}(100\%\ {}^{16}O)}{K_{eq}(100\%\ {}^{18}O)} = 1/(2K_{eq}(50\%\ {}^{18}O)/K_{eq}(100\%\ {}^{16}O) - 1) \quad (12) $$

effect of 1.031 ± 0.006 (that is, ^{18}O enriches in α-ketoglutarate, relative to water).

In an attempt to detect a kinetic ^{18}O isotope effect, the stock solution was readjusted so that the $H_2{}^{18}O$ reaction mixture would already be at equilibrium when enzyme was added. However, no perturbation was observed, and thus there was no significant ^{18}O kinetic isotope effect with glutamate and ketoglutarate as substrates. The limits of detection of an equilibrium perturbation isotope effect on breaking the C–O bond were 1.003–0.996.

Typical assays for these experiments contained 28.5 mM glutamate, 1.35 mM ketoglutarate, 0.128 mM $(NH_4)_2SO_4$, 0.100 mM NADPH, 0.466 mM $NADP^+$, 100 mM phosphate, pH 7.7, and 10 units of glutamate dehydrogenase in cuvettes with 1.0-cm light paths and 1.0-mL volumes. Absorbances were monitored with a full-scale sensitivity of 0.02 OD.

Discussion

Initial Velocity and Dead-End Inhibition Patterns. It has been shown that there is considerable synergism in the formation of ternary enzyme complexes of $NADP^+$ or NADPH with either glutamate or ketoglutarate (Fisher, 1971). However, such synergism has not been observed with the monocarboxylic substrates. 2-Aminobutyrate did not enhance the affinity of NADPH (Cross & Fisher, 1970), and the K_m of NAD^+ was higher with norvaline than with glutamate (Engel & Dalziel, 1969). The present study indicates that the affinity not only of the nucleotides but also of ammonia is enhanced by dicarboxylic substrates, with ketoglutarate increasing the affinity of ammonia 20- to 80-fold compared to monocarboxylic keto acids.

The equilibrium ordered initial velocity pattern seen with ketovalerate and ammonia indicates that the rate of release of ketovalerate from the enzyme must be very large compared to rates of the steps following its binding, and that the binding of ketovalerate and ammonia occurs in obligatory order. The fact that a *B* term (*B* representing ketoglutarate) is not statistically necessary for eq 2 to describe the ketoglutarate, ammonia, and NADPH initial velocity pattern suggests that an E–ketoglutarate complex may not be involved in the mechanism.[2] However, the absence of a *B* term does not distinguish between ordered and random binding of ammonia and NADPH. In support of the conclusions drawn from the initial velocity patterns with ketoglutarate and ketovalerate, the dead-end inhibition studies with oxalylglycine suggest that the enzyme binds NADPH, ketoglutarate, and ammonia strictly in that order.[3] Such a mechanism was proposed by Fahien & Strmecki (1969), although a random mechanism was suggested by Engel & Dalziel (1970). We will discuss below evidence that the ordered combination deduced above represents a strongly preferred pathway, and that a weak E–ketoglutarate complex can form.

Isotope Effects. While they found no deuterium isotope effect on the steady-state velocity, Fisher et al. (1970) measured an isotope effect of 1.5–1.7 on the initial burst of the reaction with L-glutamate-*2-d*. In this report, we have shown that there is an isotope effect of 1.14 on the steady-state V. Apparently, slow steps which follow the initial burst diminish the isotope effect seen in that phase to the value seen in the steady state. Even the effect measured in the initial burst is smaller than would be expected for an intrinsic deuterium isotope effect. Consequently, steps other than hydride transfer must partially limit the rate of the initial burst. With nor-

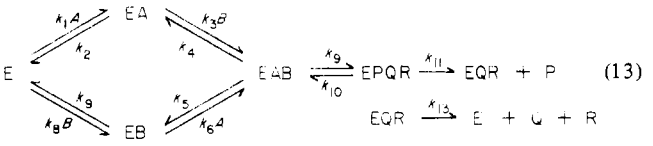
[3] What the dead-end inhibition studies actually show is that oxalylglycine binds appreciably only to E–NADPH. This could result either from an ordered mechanism or from combination much more tightly with E–NADPH than with free enzyme.

valine-*2-d* as the substrate, an isotope effect of 2.46 was observed on V, while an effect of 4.68 was observed on V/K_{NADP^+}. Obviously, in the oxidation of norvaline, hydride transfer is considerably more rate limiting than it is with glutamate.

Although no kinetic effect was seen with $H_2{}^{18}O$, a significant ^{18}O isotope effect of 1.031 ± 0.006 on the equilibrium constant was observed. This equilibrium effect indicates that ^{18}O enriches itself in the carbonyl group of ketoglutarate rather than in H_2O. With fumarase, an ^{18}O equilibrium isotope effect of 1.033 ± 0.003 has been measured, with ^{18}O enriching in the malate hydroxyl (Blanchard & Cleland, unpublished experiments). Since heavy isotopes enrich themselves in the more strongly bonded position, these results indicate that the oxygen is bonded more tightly in the carbonyl and hydroxyl groups than in water. However, it appears that oxygen is bonded to similar extents in the carbonyl and hydroxyl groups.

In addition to information about the extent to which bond-breaking steps limit the overall reaction rate, kinetic isotope effects can also provide information about the kinetic mechanism (Cleland, 1977). For ordered bireactant mechanisms, the V/K of the first substrate to bind equals the second-order rate constant for binding of that substrate. Consequently, one expects no appreciable isotope effect on that parameter. However, an isotope effect may be seen on the V/K of the second substrate, since it does contain a contribution from the bond-breaking step. With norvaline, there are well-defined isotope effects on both V/K_{Nva} and V/K_{NADP^+}. Therefore, there cannot be an ordered binding of $NADP^+$ and norvaline. Furthermore, there cannot be a rapid equilibrium random binding of norvaline and $NADP^+$, since the isotope effects on V and both (V/K)'s should be equal in that case. Therefore, there must be random but not a rapid equilibrium binding of norvaline and $NADP^+$.

Equations 14, 15, and 16 express the isotope effects on V,



(13)

$$^{D}(V) = \frac{^{D}k_9 + k_9/k_{11} + k_9/k_{13} + (^{D}K_{eq})(k_{10}/k_{11})}{1 + k_9/k_{11} + k_9/k_{13} + k_{10}/k_{11}} \quad (14)$$

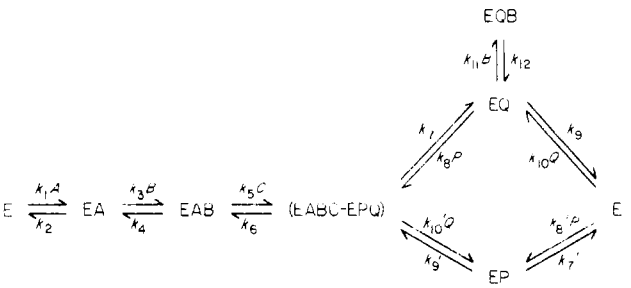
$$^{D}(V/K_a) = \frac{^{D}k_9 + k_9/k_5 + (^{D}K_{eq})(k_{10}/k_{11})}{1 + k_9/k_5 + k_{10}/k_{11}} \quad (15)$$

$$^{D}(V/K_b) = \frac{^{D}k_9 + k_9/k_4 + (^{D}K_{eq})(k_{10}/k_{11})}{1 + k_9/k_4 + k_{10}/k_{11}} \quad (16)$$

V/K_{NADP^+}, and V/K_{AA} for mechanism 13 when it is assumed that there is a steady-state flux but not a rapid equilibrium of the binding steps,[1,4] and where k_9 and k_{10} are the rate constants for the isotope sensitive step; A = $NADP^+$; B = amino acid (AA); P = $NH_4{}^+$; Q and R are keto acid and NADPH. Equations 14, 15, and 16 differ only by the terms $k_9/k_{11} + k_9/k_{13}$, k_9/k_5, and k_9/k_4. The fact that the isotope effect on V/K_{NADP} is a factor of 3.1 larger than the effect on V/K_{Nva} indicates that k_5 must be considerably larger than k_4, and that the $(^{D}K_{eq})(k_{10}/k_{11})$ term is quite small. In other words, the ternary E–NADP–norvaline complex is more likely to release $NADP^+$ than norvaline. Furthermore, the larger isotope effect on V than on V/K_{Nva} indicates that both k_{11} and k_{13} are larger than k_4. However, the 1.9-fold larger isotope effect on V/K_{NADP^+} than on V indicates that either k_{11} or k_{13} (certainly the latter) is smaller than k_5. The deuterium isotope effects observed with glutamate were unfortunately too small and too poorly defined to characterize the binding mechanism of glutamate and $NADP^+$, but Colen et al. (1977) have deduced that it is random.

α-Ketoglutarate Substrate Inhibition. Substrate inhibition by α-ketoglutarate has been reported but not characterized (Olson & Anfinsen, 1953; Engel & Dalziel, 1970). Since $NADP^+$ enhances this inhibition, it probably results from an abortive E–$NADP^+$–ketoglutarate complex. Not only has this complex been observed in spectroscopic studies (Cross et al., 1972), it has been shown to be the source of ketoglutarate inhibition of the forward reaction (Colen, 1978). From an analysis of the reaction progress curves, Bates & Frieden (1973) suggested that it also led to inhibition of the reverse reaction.

In mechanism 17, substrate inhibition by ketoglutarate



(17)

results from its binding to the E–$NADP^+$ complex; A = NADPH; B = ketoglutarate; C = NH_3; P = glutamate; and Q = $NADP^+$. When no $NADP^+$ is added to the assays, the E–$NADP^+$ complex is not present at low substrate levels, and mechanism 17 thus predicts uncompetitive substrate inhibition by ketoglutarate when either NADPH or ammonia is varied, as observed. When $NADP^+$ is added to the assays and ammonia is the variable substrate, ketoglutarate will combine with E–$NADP^+$ to form the inhibitory E–$NADP^+$–ketoglutarate complex, but it will also combine with E–NADPH to form the E–NADPH–ketoglutarate complex, with which ammonia binds, and these two complexes occur in constant ratio as the ketoglutarate level is changed. Consequently, no substrate inhibition will be seen at low ammonia, and there is no slope effect. Inhibition will be seen only when the enzyme is working at maximal velocity and inhibitory concentrations of ketoglutarate interfere with product release, and the inhibition remains uncompetitive, as observed. Only when $NADP^+$ is added and NADPH is the variable substrate will substrate inhibition be seen on the slopes as well as on the intercepts. While the intercept inhibition results from interference with product release, the slope inhibition arises because $NADP^+$ and NADPH both directly compete for free enzyme. The binding of ketoglutarate to E–$NADP^+$ shifts this competition so that there is less free enzyme available for combination with NADPH, and thus the pattern becomes noncompetitive, as observed. Mechanism 17 thus correctly predicts the noncom-

[4] Mechanism 13 is an oversimplification in that k_9 and k_{10} include not only the hydride transfer step but also the non-isotope-dependent steps which precede it (and which the pre-steady-state isotope effects of Fisher et al. (1970) show to be highly rate determining with glutamate as the substrate[5]), and the subsequent chemical steps and conformation changes which precede the release of ammonia. Thus $^{D}k_9$ is not the intrinsic isotope effect on the hydride transfer step.

[5] In pre-steady-state studies, the only commitment to catalysis that reduces the isotope effect on the initial velocity of an observed burst is the forward one, consisting of partition ratios for steps up to the one in which color is developed. Subsequent steps may influence the size of the burst, but have no effect on the initial velocity of the burst.

petitive or uncompetitive nature of the substrate inhibition patterns.

Further, a quantitative analysis indicates that the release of glutamate and $NADP^+$ is random as shown in mechanism 17. At high $NADP^+$, the slope K_i when NADPH is varied is simply k_{12}/k_{11}, since almost all of the enzyme is E–$NADP^+$ under these conditions, if ordered substrate addition is assumed as in mechanism 17. The intercept K_i at high $NADP^+$ levels is k_{12}/k_{11} multiplied by $(1 + k_9/k_7)$, and it is clear that k_9/k_7 is greater than one, since the intercept K_i is much greater than the slope one. At low levels of $NADP^+$, however, the intercept K_i is k_{12}/k_{11} multipled by $(1 + (k_9/k_7)(1 + k_9'/k_7'))$, and thus k_9'/k_7' has also to be greater than one, since the intercept K_i is considerably greater in the absence of $NADP^+$. Thus, one can evaluate k_9/k_7 and k_9'/k_7' from the various K_i values for varied NADPH in Table IV. With these ratios and the fact that the maximum velocity was 920 ± 30 min^{-1} in the absence of $NADP^+$ and 439 ± 24 min^{-1} in its presence with NADPH as the variable substrate, it is possible to calculate the values of k_7 as 480 min^{-1}, k_7' as 1130 min^{-1}, k_9 as 5540 min^{-1}, and k_9' as 2800 min^{-1}. (In the presence of high $NADP^+$, V_{max} is $k_7k_9/(k_7 + k_9)$, while in its absence, V_{max} is $(k_7 + k_9')/(1 + k_7/k_9 + k_9'/k_7')$). These values suggest that $NADP^+$ is 2.4 times more likely to be released from the ternary E–$NADP^+$–glutamate complex than is glutamate, in agreement with the norvaline-*2-d* isotope effects, which suggested that $NADP^+$ release from E–$NADP^+$–norvaline is faster than norvaline release.

However, mechanism 17 fails to predict the hyperbolic inhibition seen when $NADP^+$ is added. The partial intercept inhibition observed when NH_4^+ is varied indicates that $NADP^+$ can dissociate from the E–$NADP^+$–ketoglutarate complex (although more slowly than from E–$NADP^+$) and allow the reaction cycle to continue. If release of $NADP^+$ from this complex is given by k_9'', K_{id} from the hyperbolic inhibition should be $(k_{12}k_{11})(k_9/k_9'')$, from which we can estimate that k_9'' is 190 min^{-1}. The value of K_{in} is now $(k_{12}/k_{11})(1 + k_9/k_7)/(1 + k_9''/k_7)$, and since $(1 + k_9''/k_7)$ is 1.4, we should refine our calculations of the rate constants. The basic conclusion that $NADP^+$ is released most rapidly from the binary complex, somewhat slower from the ternary complex with glutamate, and much more slowly from the ternary complex with ketoglutarate will not change, however.

Since V/K should be determined only by events up to the release of the first product, allowing release of $NADP^+$ from the abortive complex with ketoglutarate does not explain the partial slope inhibition with variable NADPH. This hyperbolic slope effect can be explained only if ketoglutarate can bind to free enzyme and produce a catalytically competent E–ketoglutarate complex.[2] Consequently, there must be random binding of NADPH and ketoglutarate in the reverse reaction, in agreement with the conclusion of Engel & Dalziel (1970), but in disagreement with the ordered mechanism we have proposed. The apparent ordered mechanism suggested by the ketoglutarate and ketovalerate initial velocity patterns and the oxalylglycine dead-end inhibition patterns indicates that the binding of NADPH and ketoglutarate is strongly synergistic, with binding of NADPH before ketoglutarate being the preferred path. For this mechanism K_{in} still equals k_{12}/k_{11} when high concentrations of $NADP^+$ are present, while K_{id} equals the dissociation constant of ketoglutarate from free enzyme multiplied by the ratio of bimolecular rate constants for combination of NADPH with E and with E–ketoglutarate. We have no way to estimate these bimolecular rate constants, but if they are equal, the dissociation constant of ketoglutarate from free enzyme equals K_{id}, which is 5.37 mM. From equilibrium spectroscopic studies, Cross et al. (1972) measured a value of 2.5 ± 0.4 mM for this constant. Since the affinity of ketoglutarate for the enzyme is pH dependent, and increases as the pH is decreased (Rife & Cleland, 1980), the discrepancy between these values can be explained by the fact that the assays in this study were at pH 8, while the assays of Cross et al. (1972) were at pH 7.6.

The value of 1.45 mM for k_{12}/k_{11} from the present work is considerably larger than the value of 11.0 ± 5.3 μM reported for the dissociation constant of ketoglutarate from E–$NADP^+$–ketoglutarate by Cross et al. (1972). However, from transient kinetic studies Colen (1978) suggested that there were actually two E–$NADP^+$–ketoglutarate complexes. While one formed rapidly and had loosely bound ketoglutarate, the other formed more slowly and had tightly bound ketoglutarate. Although a value of 90 ± 10 μM was determined for the apparent dissociation constant from the mixture of the two complexes, a dissociation constant of 1.0 ± 0.5 mM was calculated for release of ketoglutarate from the loose binding complex. This last value agrees with our value from the substrate inhibition studies, and suggests that under our experimental conditions only the loose E–$NADP^+$–ketoglutarate complex is observed.

Conclusions

We conclude on the basis of the kinetic studies reported here that the combination of amino acid and $NADP^+$ is random, as postulated by Colen et al. (1977), with the nucleotide dissociating more rapidly than the amino acid from the ternary complex. This situation prevails for glutamate, where hydride transfer is fast compared to some step which precedes it, and for norvaline, where hydride transfer is much more rate limiting. In the reverse reaction the addition of keto acid and ammonia to the E–NADPH complex appears ordered (equilibrium ordered with monocarboxylic keto acids whose k_{off} values greatly exceed V_{max}), while the combination of nucleotide and keto acid is largely ordered, although ketoglutarate can form a weak but kinetically competent complex with free enzyme. In the following paper (Rife & Cleland, 1980), we will present pH studies which, with the above knowledge of the kinetic mechanism, allow us to suggest a chemical mechanism for glutamate dehydrogenase.

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Determination of the Chemical Mechanism of Glutamate Dehydrogenase from pH Studies†

James E. Rife and W. W. Cleland*

ABSTRACT: The pH profiles for binding of keto acids and analogues to E–NADPH show that an enzyme group (possibly carboxyl) with a pK of 5 has to be protonated for binding of any molecule, while a group (possibly lysine) with a pK of 7.8 must be protonated to bind the 5-carboxyl of dicarboxylic acids and unprotonated to bind monocarboxylic acids. Changing the 1-carboxyl to a hydroxymethyl group, as in glycolylglycine, does not alter the pH profile, and only weakens binding by a factor of 10, showing that the 1-carboxyl is not bound to lysine or arginine. The V/K profile for ammonia shows that the neutral molecule is the substrate. V/K profiles for glutamate and norvaline show that a group (possibly lysine) with a pK of 7.6–8 must be unprotonated for activity, that the amino acid reacts with its amino group protonated, and that a group (presumably the same lysine involved in binding seen in the reverse reaction) with a pK of 8.7–9.3 must be protonated to bind glutamate, and unprotonated to bind norvaline. We propose that the chemical mechanism of glutamate dehydrogenase involves the attack of neutral ammonia on the ketoglutarate of E–NADPH–ketoglutarate to give a carbinolamine, with the proton for the hydroxyl group coming from the catalytic lysine. After transfer of a proton from N to O of the carbinolamine (possibly catalyzed by the catalytic carboxyl), elimination of water gives iminoglutarate, which is reduced to glutamate by NADPH. The amino group of glutamate is protonated by the catalytic carboxyl to complete the reaction.

The first chemical mechanism proposed for glutamate dehydrogenase (EC 1.4.1.3) was the oxidation of glutamate to α-iminoglutarate, which then decomposed nonenzymatically to α-ketoglutarate and ammonia (von Euler et al., 1938). With the realization that the equilibrium between ketoglutarate and α-iminoglutarate in solution could not support the rate of enzymatic glutamate production, it became apparent that the enzyme directed both the deamination and the dehydrogenation processes (Strecker, 1953). The discovery of two reactive lysines at the active site through modification studies (Rasched et al., 1974; Piszkiewicz et al., 1970; Holbrook & Jeckel, 1969; Colman & Frieden, 1966) prompted the suggestion that ketoglutarate forms a Schiff's base with one of them during the reaction (Smith et al., 1975). The reverse reaction would then proceed with ammonia replacing the lysine in a transimination reaction to form α-iminoglutarate, which would be reduced to glutamate by the coenzyme. However, no direct evidence has been obtained for a Schiff's base between ketoglutarate and lysine. An alternative mechanism involves direct attack by ammonia on ketoglutarate to form a carbinolamine, which forms α-iminoglutarate by loss of water.

In the present work, the chemical mechanism of glutamate dehydrogenase has been studied by examining the pH profiles of V/K for mono- and dicarboxylic keto and amino acid substrates, and of pK_i for inhibitory analogues. The data suggest that the reaction proceeds with direct attack of ammonia on ketoglutarate, and that one lysine acts as an acid–base catalyst, while another functions in binding the 5-carboxyl of dicarboxylic acid substrates.

Materials and Methods

Reagents. Ethanolamine and diethyl oxalate were from Aldrich. Coenzymes, amino and keto acid substrates, and the 2-hydroxyglutarates were from Sigma. Glutaric acid from Sigma was crystallized from hot benzene. Bovine liver glutamate dehydrogenase was obtained from Sigma as a solution in 50% glycerol with pH 7.3 sodium phosphate buffer. This preparation had less than 0.3 μg of NH_4^+ per mg of enzyme. For use, the enzyme was diluted with 0.05 M potassium phosphate buffer, pH 7.0–7.6.

Preparation of Oxalylglycine and Glycolylglycine. Oxalylglycine and glycolylglycine were synthesized by nitrous acid deamination of glycylglycine (Viscontini, 1946). Disodium oxalyglycine was recovered from the reaction mixture as a methanol-insoluble precipitate. This product was washed successively with ethanol and diethyl ether and then recrystallized from H_2O with methanol. Glycolylglycine was recovered from the methanol-soluble fraction of the reaction mixture as the calcium salt. Calcium glycolylglycine was passed through a column of Dowex-1-Cl. After the calcium was washed off the column, the free acid of glycolylglycine was recovered by eluting the column with 0.05 M HCl and evaporating the first acid fractions from the column.

Preparation of N-Oxalylethanolamine. *N*-Oxalylethanolamine was prepared by treatment of ethanolamine with diethyl

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