Human glutamic- γ -semialdehyde dehydrogenase

Kinetic mechanism

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The kinetic mechanism of homogeneous human glutamic- γ -semialdehyde dehydrogenase (EC 1.5.1.12) with glutamic γ -semialdehyde as substrate was determined by initial-velocity, product-inhibition and dead-end-inhibition studies to be compulsory ordered with rapid interconversion of the ternary complexes (Theorell–Chance). Product-inhibition studies with NADH gave a competitive pattern versus varied NAD⁺ concentrations and a non-competitive pattern versus varied glutamic γ -semialdehyde concentrations, whereas those with glutamate gave a competitive pattern versus varied glutamic γ -semialdehyde concentrations and a non-competitive pattern versus varied NAD⁺ concentrations. The order of substrate binding and release was determined by dead-end-inhibition studies with ADP-ribose and L-proline as the inhibitors and shown to be: NAD⁺ binds to the enzyme first, followed by glutamic γ -semialdehyde, with glutamic acid being released before NADH. The K_{ia} and K_{ib} values were $15 \pm 7 \mu\text{M}$ and $12.5 \mu\text{M}$ respectively, and the K_a and K_b values were $374 \pm 40 \mu\text{M}$ and $316 \pm 36 \mu\text{M}$ respectively; the maximal velocity V was $70 \pm 5 \mu\text{mol}$ of NADH/min per mg of enzyme. Both NADH and glutamate were product inhibitors, with K_i values of $63 \mu\text{M}$ and $15200 \mu\text{M}$ respectively. NADH release from the enzyme may be the rate-limiting step for the overall reaction.

INTRODUCTION

Glutamic- γ -semialdehyde dehydrogenase (Δ^1 -pyrroline-5-carboxylate dehydrogenase, EC 1.5.1.12) is universally distributed, occurring in animals, bacteria, yeasts and plants (Lundgren & Ogur, 1973; Stewart & Lai, 1974; Boggess *et al.*, 1975; Herzfeld *et al.*, 1977). It has been partially purified from ox liver (Strecker, 1960, 1971), ox kidney (Lundgren & Ogur, 1972), rat liver (Brunner & Neupert, 1969), human fibroblasts (Vallee *et al.*, 1979) and yeast (Lundgren & Ogur, 1973). Purification to homogeneity from human liver was reported more recently (Forte-McRobbie & Pietruszko, 1986).

The enzyme is metabolically important, since its substrate glutamic γ -semialdehyde (Δ^1 -pyrroline-5-carboxylate) appears as a common intermediate in the degradative and biosynthetic pathways of the amino acids arginine, citrulline, ornithine and proline to and from glutamic acid. Metabolic regulation of glutamic- γ -semialdehyde dehydrogenase has been inferred to occur via inhibition of the enzyme by amino acids. Both the yeast (Lundgren & Ogur, 1973) and ox kidney (Lundgren & Ogur, 1972) enzymes have been shown to be inhibited by a series of amino acids, including proline, serine, leucine, glycine, valine and alanine. The inhibition was also demonstrated to be cumulative when the amino acids occurred in combination.

Of particular interest is the nexus between glutamic γ -semialdehyde and proline metabolism, especially as they are related to disease states in man. Type I hyperprolinaemia, which results in elevated concentrations of proline in the blood and high urinary excretion of proline, hydroxyproline, glycine, Δ^1 -pyrroline-5-carboxylate and 3-hydroxy- Δ^1 -pyrroline-5-carboxylate (Wellner &

Meister, 1981) is associated with the complete absence of glutamic- γ -semialdehyde dehydrogenase (Vallee *et al.*, 1979). Reported studies on familial hyperprolinaemia have included deafness and slight mental retardation among the signs of the disease (Efron, 1965).

Glutamic- γ -semialdehyde dehydrogenase is a dimeric enzyme of M_r 142000 (Forte-McRobbie & Pietruszko, 1986). The subunit is much larger than that of an average dehydrogenase (M_r 70200 compared with approx. 40000). The enzyme also has other unusual properties, such as substrate inhibition by coenzyme and hysteretic behaviour with some aldehyde substrates. These properties, together with its sensitivity to amino acid inhibition, suggest a role in the regulation of metabolism. The first step towards an understanding of such a role is understanding of the enzyme's functional characteristics; hence an investigation of the kinetic mechanism of glutamic- γ -semialdehyde dehydrogenase was undertaken.

EXPERIMENTAL

Materials

NAD⁺ was purchased from Boehringer–Mannheim. NADH, ADP-ribose, D-glutamic acid, L-glutamic acid and 2-aminobenzaldehyde were obtained from Sigma Chemical Co. L-Proline was purchased from United States Biochemical Corp. Propionaldehyde and acetaldehyde from Baker Chemical Co. were redistilled before use; acet[²H]aldehyde was from Aldrich Chemical Co.; the concentrations of stock solutions of each were determined by assay with E2 aldehyde dehydrogenase (Greenfield & Pietruszko, 1977). Glutamic γ -semialdehyde was prepared by hydrolysis of DL- Δ^1 -

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pyrroline-5-carboxylate 2,4-dinitrophenylhydrazone hydrochloride (Sigma Chemical Co.) according to the method of Mezel & Knox (1976). The 2,4-dinitrophenylhydrazine was removed by extraction with acetophenone. Traces of acetophenone were subsequently removed from the glutamic γ -semialdehyde solution by extraction (eight times) with toluene. The concentrations of stock solutions of glutamic γ -semialdehyde were determined by assay with glutamic- γ -semialdehyde dehydrogenase in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 500 μ M-NAD⁺, and by assay with 2-amino-benzaldehyde according to the procedure of Mezel & Knox (1976). Glutamic- γ -semialdehyde dehydrogenase was purified as described by Forte-McRobbie & Pietruszko (1986). Enzyme was stored at 4 °C in N₂-saturated 30 mM-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA and 0.1% 2-mercaptoethanol. In order to remove the 2-mercaptoethanol, the enzyme was dialysed eight times against the same buffer but in the absence of 2-mercaptoethanol. The activity of the enzyme was monitored daily by means of the standard spectrophotometric assay with 13.6 mM-propionaldehyde as the substrate in 0.1 M-sodium pyrophosphate buffer, pH 9.0, containing 1 mM-EDTA and 500 μ M-NAD⁺. When the specific activity of the enzyme was less than maximal (3.7 μ mol/min per mg of enzyme), reaction velocities were mathematically adjusted to full catalytic activity. Protein was determined by the micro biuret procedure of Goa (1953), with bovine serum albumin as the standard.

Steady-state kinetics

Kinetic studies were carried out spectrophotometrically at 340 nm and 25 °C in 3 ml total volume in N₂-saturated 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA in order to monitor the increase in NADH. Assays were initiated by the addition of glutamic γ -semialdehyde. Concentrations of glutamic γ -semialdehyde were always maintained below 60 μ M, in order to remain below the level at which substrate inhibition is observed (Forte-McRobbie & Pietruszko, 1986). An absorption coefficient of 6.22 mm⁻¹·cm⁻¹ was employed for NADH. Primary Lineweaver-Burk (1934) plots of initial steady-state velocity were made by using the Wilkinson (1961) program. Slope and intercept replots of the data from primary plots were via fitted linear least-squares analysis. The nomenclature of Cleland (1963) is employed.

RESULTS AND DISCUSSION

The reaction catalysed by glutamic- γ -semialdehyde dehydrogenase is as follows:

Initial-velocity studies

Initial-velocity studies carried out at pH 7.0 with NAD⁺ and glutamic γ -semialdehyde as substrates indicated the reaction mechanism of glutamic- γ -semialdehyde dehydrogenase to be sequential (Cleland, 1963, 1970) and not Ping Pong. Lineweaver-Burk plots of the data were linear. The initial-velocity patterns of lines were found to converge within the third quadrant when either glutamic γ -semialdehyde (Fig. 1) or NAD⁺ was the substrate whose concentration was varied. It

Fig. 1. Lineweaver-Burk plots of initial velocity for dehydrogenation by glutamic- γ -semialdehyde dehydrogenase with glutamic γ -semialdehyde as the substrate whose concentration was varied

The concentrations of NAD⁺ were maintained constant at 20.5 μ M (Δ), 41 μ M ($\bullet$), 82 μ M ($\square$), 164 μ M ($\blacktriangle$) and 328 μ M ($\circ$). All assays were performed at 25 °C in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA and saturated with N₂. For each line the concentration of glutamic γ -semialdehyde was varied in the range 12–59.9 μ M. Lines were drawn to fit the parameters designated by the Wilkinson (1961) program. Inset: slope ($\bullet$) and intercept ($\blacktriangle$) replots. The lines are drawn to fit linear least-squares analysis.

Table 1. Kinetic constants from initial-velocity studies

Nomenclature is that of Cleland (1963). Values were obtained from slope and intercept replots of the initial-velocity data. Values were derived from two sets of experiments: each set was plotted two ways (1/*V* versus 1/[A] and 1/*V* versus 1/[B]) and the kinetic constants obtained were averaged. The standard error represents difference between averages from two different experiments, with different preparations of enzyme.

Constant	Value
K_a	$374 \pm 40 \mu\text{M}$
K_{ia}	$15 \pm 7 \mu\text{M}$
K_b	$316 \pm 36 \mu\text{M}$
K_{ib}	$12.5 \mu\text{M}$
V_i	$70 \pm 5 \mu\text{mol/min per mg of enzyme}$

therefore followed that the reaction was sequential (Cleland, 1963, 1970) with a rate equation for the bi-reactant system in the form of:

$$v = \frac{V_{\max} [A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]}$$

where *v* is the initial velocity, [A] and [B] are substrate concentrations, and *V*, *K_a*, *K_b* and *K_{ia}* are kinetic constants. The values of the kinetic constants were determined from replots of the slopes and intercepts versus the reciprocal of the concentration of the changing fixed substrate, and are listed in Table 1. Although

Fig. 2. Product inhibition of glutamic- γ -semialdehyde dehydrogenase with NADH and glutamate

(a) Product inhibition by NADH with NAD^+ as the substrate whose concentration was varied. Lineweaver-Burk plots are shown of the data with glutamic γ -semialdehyde maintained constant at $54.7 \mu\text{M}$, the concentration of NAD^+ varied in the range 20–340 μM and various fixed concentrations of NADH: 0 μM (○), 45.5 μM (×), 91 μM (▲), 136.5 μM (□) and 182 μM (●). Inset: secondary plot of slopes versus NADH concentration. (b) Product inhibition by NADH with glutamic γ -semialdehyde as the substrate whose concentration was varied. NAD^+ was maintained constant at 340 μM , and the concentration of glutamic γ -semialdehyde was varied in the range 11–54.7 μM at various fixed concentrations of NADH: 0 μM (●), 91 μM (○), 182 μM (▲), 273 μM (□) and 364 μM (■). Inset: slope (○) and intercept (●) replots versus NADH concentration. (c) Product inhibition by L-glutamate with NAD^+ as the substrate whose concentration was varied. Glutamic γ -semialdehyde was maintained constant at 55.9 μM , and the concentration of NAD^+ was varied in the range 20.5–329 μM at various fixed concentrations of L-glutamate: 0 mM (●), 12.5 mM (○), 25 mM (■) and 50 mM (□). Inset: slope (○) and intercept (●) replots versus L-glutamate concentration. (d) Product inhibition by L-glutamate with glutamic γ -semialdehyde as the substrate whose concentration was varied. NAD^+ was maintained constant at 329 μM , and the concentration of glutamic γ -semialdehyde was varied in the range 11.2–55.9 μM at various fixed concentrations of L-glutamate: 0 mM (●), 12.5 mM (○), 25 mM (■) and 50 mM (□). Inset: secondary plot of slopes versus L-glutamate concentration.

reference to 'a' (K_a and K_{ia}) and 'b' (K_b and K_{ib}) is indicated to denote NAD^+ and glutamic γ -semialdehyde respectively, in Table 1 these designations were made only after dead-end-inhibition studies (see below) were carried out. It may be noted that the K_a and K_b values reported in Table 1 are nearly equal, and are considerably larger than the values of K_{ia} and K_{ib} , which are also nearly equivalent to each other. The pattern of lines (initial-velocity studies; Fig. 1) intersecting in the third quadrant indicates that for glutamic- γ -semialdehyde dehydrogenase the K_m value of one substrate increases upon saturation with the other substrate.

Product inhibition studies

Although the patterns from initial-velocity studies do not distinguish between a random and an ordered addition of substrates, the product-inhibition patterns obtained with NAD^+ and glutamate are suggestive of a compulsory ordered mechanism (Theorell & Chance, 1951; Cleland, 1963). Inhibition by NADH was non-competitive with glutamic γ -semialdehyde as the substrate whose concentration was varied only when the concentration of NADH was in the range 0–273 μM (Fig. 2b). With L-glutamate as the product inhibitor, non-competitive inhibition was obtained when NAD^+ was the substrate whose concentration was varied (Fig. 2c) and competitive inhibition when glutamic γ -semialdehyde was the substrate whose concentration was varied (Fig. 2d). The product-inhibition patterns, two of which are competitive and two of which are non-competitive, obtained in these studies of subsaturating concentrations of fixed substrate (see Table 2) are consistent with the Theorell–Chance Bi Bi mechanism (Wratten & Cleland, 1965):

Although distinction between the general compulsory ordered mechanism and the special case, the Theorell–Chance mechanism, can be made by determining whether glutamate inhibition becomes uncompetitive with respect to NAD^+ concentrations varied at saturating concentrations of aldehyde, this experiment could not be performed, since substrate inhibition of the enzyme by glutamic γ -semialdehyde was found to occur at pH 7.0 at subsaturating concentrations. From the product-inhibition patterns obtained, specifically the two competitive patterns (see Table 2), it could be ascertained that NAD^+ and NADH, and similarly glutamic γ -semialdehyde and glutamate, bind to the same enzyme form. However, because two non-competitive patterns (both slopes and intercepts of the reciprocal plots are affected; see Figs. 2b and 2c) were concomitantly obtained, the order of binding of substrates (A and B) and release of products (Q and P) could not be determined.

The product-inhibition constants, $K_{i(\text{slope})}$ and $K_{i(\text{intercept})}$, are reported in Table 2. They were measured from slope and intercept replots (see Fig. 2 insets) respectively of the corresponding primary Lineweaver–Burk plots. The value of $K_{i(\text{slope})}$ and $K_{i(\text{intercept})}$ are equivalent to the inverse sign (–) of the abscissa intercept of the appropriate replot. The K_{iq} and K_{ip} values

Table 2. Summary of product-inhibition studies

Abbreviation: GSA, glutamic γ -semialdehyde.

Inhibitor (concentration range, μM)	Varied substrate (concentration range, μM)	Fixed substrate (concentration, μM)	Form of inhibition	$K_{i(\text{slope})}$ (μM)		$K_{i(\text{intercept})}$ (μM)			
				Measured	Predicted for Theorell–Chance mechanism	Calculated from pre- dicted	Measured	Predicted for Theorell–Chance mechanism	Calculated from pre- dicted
NADH (0–182)	NAD ⁺ (20–340)	GSA (54.7)	Competitive	63 (54*)	K_{i_q}	63	–	–	–
NADH (0–364)	GSA (11–54.7)	NAD ⁺ (340)	Non-competitive → competitive	480	$K_{i_q} (1 + [\text{A}]/K_{i_a})$	1500	138	$K_{i_q} (1 + [\text{A}]/K_a)$	119
L-Glutamate (0–50 000)	NAD ⁺ (20.5–329)	GSA (55.9)	Non-competitive	12 200	$K_{i_p} (1 + [\text{B}]/K_{i_b})$	83 200	27 900	$K_{i_p} (1 + [\text{B}]/K_b)$	17 900
L-Glutamate (0–50 000)	GSA (11.2–55.9)	NAD ⁺ (329)	Competitive	15 200 (14 300*)	K_{i_p}	15 200	–	–	–

* K_i values calculated from Dixon plots; see the Results and discussion section for experimental conditions.

Fig. 3. Determination of K_i values for NADH, D-glutamate and L-glutamate via Dixon plots

(a) Dixon plots of velocity versus NADH concentration. Assays were performed at 25 °C in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA, 41 μM -NAD⁺ and 59.9 μM -glutamic γ -semialdehyde. $1/V_{\text{max}}$ is determined from a Lineweaver–Burk plot in the absence of NADH. The K_i for NADH was determined by extrapolation as shown. (b) Dixon plots of velocity versus D-glutamate and L-glutamate concentrations. Assays were performed at 25 °C in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA, 339 μM -NAD⁺, 59.6 μM -glutamic γ -semialdehyde and D-glutamate (●) or L-glutamate (□), in concentrations in the range 0–62.6 mM. $1/V_{\text{max}}$ is determined from a Lineweaver–Burk plot in the absence of inhibitor. The K_i values were determined by extrapolation from the linear portion of the curves as shown ($K_i = 13$ mM for D-glutamate and $K_i = 14.3$ mM for L-glutamate).

measured from the slope replots of Figs. 2(a) and 2(d) were also independently determined via Dixon plots (Fig. 3); these were found to be comparable in magnitude and are listed in Table 2. The values of $K_{i(\text{slope})}$ and $K_{i(\text{intercept})}$ measured from replots are compared in Table 2 with those calculated from the predicted relationships based on Theorell–Chance mechanism. The measured values obtained from competitive patterns are in total agreement with the calculated values. However, 3–7-fold discrepancies were obtained with the $K_{i(\text{slope})}$ values determined with NADH as the inhibitor versus varied glutamic γ -semialdehyde concentrations (Fig. 2b) and with L-glutamate versus NAD⁺ concentrations (Fig. 2c), both giving non-competitive patterns. In both cases the experimentally determined values are lower than those predicted, indicating that alternative reaction pathways may exist. Simple kinetic analysis via a Dixon plot was done with D-glutamate and L-glutamate at concentrations of 0–62.6 mM (Fig. 3b). From the linear portion, K_i values of 13 mM and 14.3 mM were determined for the D- and L-conformers of glutamate respectively. The Dixon-plot K_i value of 14.3 mM for L-glutamate agrees well with the $K_{i(\text{slope})}$ value of 15.2 mM from product-inhibition kinetics (Table 2).

Dead-end-inhibition studies

Although the order of binding of substrates (A = NAD⁺, B = glutamic γ -semialdehyde) and release of products (P = glutamate, Q = NADH) could not be resolved via product-inhibition studies alone, this was resolved after dead-end-inhibition studies. Dead-end-inhibition studies (see Table 3) were carried out with the NAD⁺ analogue ADP-ribose and L-proline, the cyclized reduction product of glutamic γ -semialdehyde *in vivo* via the enzyme Δ^1 -pyrroline-5-carboxylate reductase. Competitive and non-competitive inhibition patterns were obtained with ADP-ribose versus varied NAD⁺ concentrations (Fig. 4a) and ADP-ribose versus varied

glutamic γ -semialdehyde concentrations (Fig. 4b) respectively. By contrast, when L-proline was employed, an uncompetitive inhibition pattern was obtained when NAD⁺ was the substrate whose concentration was varied (Fig. 4c) and a competitive inhibition pattern when glutamic γ -semialdehyde was the substrate whose concentration was varied (Fig. 4d). All replots were linear. In comparing the forms of inhibition reported in Tables 2 and 3, it can be seen that, whereas two non-competitive patterns (slope and intercept effects) were obtained with product-inhibition studies, only one was obtained with dead-end-inhibition studies, with ADP-ribose versus varied glutamic γ -semialdehyde concentrations. With L-proline as the dead-end inhibitor versus varied NAD⁺ concentrations, only intercept effects (uncompetitive inhibition) were obtained. It was the absence of slope effects from this last experiment that enabled the binding sequence for glutamic- γ -semialdehyde dehydrogenase to be defined. From the results of the dead-end-inhibition studies, NAD⁺ is the first substrate, A, to bind to the enzyme, and glutamic γ -semialdehyde is the second substrate, B, to bind to the enzyme. Thus glutamic γ -semialdehyde binds to the E–NAD⁺ complex. On the basis of the results of the product-inhibition and dead-end-inhibition studies, the order of the release of products can now be designated thus: glutamate is the first product, P, released, and NADH is the second product, Q, released. The experimentally determined values of the kinetic constants ($K_{i(\text{slope})}$ and $K_{i(\text{intercept})}$) from dead-end-inhibition studies are reported in Table 3. They are compared with those determined from Dixon plots and those calculated from the predicted relationships based on an ordered Theorell–Chance Bi Bi mechanism. In all cases there is reasonable agreement between measured and calculated values.

Fluorescence titration experiments

An attempt was made to determine directly the

Fig. 4. Inhibition of glutamic- γ -semialdehyde dehydrogenase by ADP-ribose and L-proline

(a) Dead-end inhibition by ADP-ribose with NAD^+ as the substrate whose concentration was varied. Glutamic γ -semialdehyde was maintained constant at $59.6 \mu\text{M}$, and the concentration of NAD^+ was varied in the range 20.5 – $328 \mu\text{M}$ at various fixed concentrations of ADP-ribose: 0 mM (●), 0.25 mM (□), 0.5 mM (○) and 1 mM (▲). Inset: secondary plot of slopes versus ADP-ribose concentration. (b) Dead-end inhibition by ADP-ribose with glutamic γ -semialdehyde as the substrate whose concentration was varied. NAD^+ was maintained constant at $328 \mu\text{M}$, and the concentration of glutamic semialdehyde was varied in the range 12 – $59.6 \mu\text{M}$ at various fixed concentrations of ADP-ribose: 0 mM (●), 0.67 mM (○), 1.33 mM (■) and 2.66 mM (□). Inset: slope (▲) and intercept (△) replots versus ADP-ribose concentration. (c) Dead-end inhibition by L-proline with NAD^+ as the substrate whose concentration was varied. Glutamic γ -semialdehyde was maintained constant at $55.9 \mu\text{M}$, and the concentration of NAD^+ was varied in the range 20.5 – $328 \mu\text{M}$ at various fixed concentrations of L-proline: 0 mM (○), 5 mM (●), 10 mM (□) and 20 mM (■). Inset: secondary plot of intercepts versus L-proline concentration. (d) Dead-end inhibition by L-proline with glutamic γ -semialdehyde as the substrate whose concentration was varied. NAD^+ was maintained constant at $328 \mu\text{M}$, and the concentration of glutamic γ -semialdehyde was varied in the range 11.2 – $55.9 \mu\text{M}$ at various fixed concentrations of L-proline: 0 mM (●), 2.5 mM (○), 5 mM (■) and 10 mM (□). Inset: secondary plot of slopes versus L-proline concentration.

Table 4. Rate-constant values for glutamic- γ -semialdehyde dehydrogenase assuming a Theorell–Chance mechanism

Rate constants and equations are those of Cleland (1963). V_1 and V_2 are maximal velocity in the forward and reverse directions respectively; K_a and K_b are Michaelis constants; K_{ia} , K_{ip} and K_{iq} are inhibition constants; $[E]_t$ is the total concentration of enzyme. Alternative rate constant equations are shown for k_4 , k_5 and k_6 , based on the definitions of the kinetic constants K_a , K_b , K_{ip} and K_{iq} . The values of these rate constants were calculated from the alternative equations, since glutamic- γ -semialdehyde dehydrogenase does not catalyse the reaction in the reverse direction, making $V_2 = 0$ and values of K_p and K_q not directly measurable.

Constant	Rate-constant equations	Alternative rate-constant equations	Values calculated from initial-velocity and product-inhibition studies	Definition of rate constant
k_1	$k_1 = \frac{V_1}{[E]_t \cdot K_a}$	—	$3.9 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$	NAD ⁺ on
k_2	$k_2 = \frac{V_1 \cdot K_{ia}}{[E]_t \cdot K_a}$	—	$6.0 \times 10^4 \text{ min}^{-1}$	NAD ⁺ off
k_3	$k_3 = \frac{V_1}{[E]_t \cdot K_b}$	—	$4.7 \times 10^9 \text{ M} \cdot \text{min}^{-1}$	Glutamic γ -semialdehyde on
k_4	—	$k_4 = \frac{k_5}{K_{ip}}$	$9.2 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$	Glutamate off
k_5	—	$k_5 = k_1 \cdot K_a = k_3 \cdot K_b$	$1.41 \times 10^6 \text{ min}^{-1}$	NADH off
k_6	—	$k_6 = \frac{k_5}{K_{iq}}$	$3.1 \times 10^{10} \text{ M}^{-1} \cdot \text{min}^{-1}$	NADH on

substrate (Forte-McRobbie & Pietruszko, 1986), was examined. In this experiment NAD⁺ concentration was maintained constant at 400 μM and the concentration of aldehyde was varied. Assays were initiated by addition of enzyme. As shown in Fig. 5, there was no isotope effect on maximal velocity. Velocities obtained with acet[²H]-aldehyde and undeuterated acetaldehyde are compared in Table 5, where it is shown that the isotope effect V_{1H}/V_{2H} is greatest at the lowest concentration of aldehyde and disappears at extrapolated infinite aldehyde concentrations. The magnitude of the isotope effect $(V/K_m)_{1H}/(V/K_m)_{2H} = 2.4$ at zero aldehyde concen-

tration is nearly equivalent to the value of 2.7 reported for liver alcohol dehydrogenase (Bush *et al.*, 1973). These results suggest that, at high concentrations of aldehyde, coenzyme dissociation (E–NADH) and not hydride transfer is the rate-limiting step, which is consistent with the Theorell–Chance mechanism.

Although a Theorell–Chance mechanism for glutamic- γ -semialdehyde dehydrogenase is indicated from product inhibition, dead-end-inhibition patterns and from the kinetic isotope effect with an alternative substrate, acet-aldehyde/acet[²H]aldehyde, at higher concentration of substrates the presence of other reaction pathways is likely. At higher concentrations of NADH, 273–364 μM , the inhibition obtained was competitive, although at lower concentrations (0–276 μM) it was non-competitive (Fig. 2b and Table 2). The shift to competitive inhibition suggests that the mechanism may change when NADH is in excess. In addition, differences between measured values and those calculated from the predicted relationships for a Theorell–Chance mechanism from the product-inhibition experiments with NADH versus varied glutamic γ -semialdehyde concentrations and with glutamate versus varied NAD⁺ concentrations respect-

Fig. 5. Isotope effect observed on substituting acet[²H]aldehyde for undeuterated acetaldehyde

The concentration of acet[²H]aldehyde (○) was varied in the range 1.7–27 mM and that of undeuterated acetaldehyde in the range 1–16 mM. Assays were performed in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 400 μM -NAD⁺.

Table 5. Deuterium isotope effects upon initial velocity

Concn. of aldehyde (mM)	Velocity ($\mu\text{mol/min per mg}$)			V_{1H}/V_{2H}
	V_{1H} with undeuterated acetaldehyde	V_{2H} with acet[² H]-aldehyde		
20.0	1.000	0.727		1.38
4.0	0.515	0.276		1.87
2.0	0.323	0.156		2.07
1.3	0.235	0.109		2.16

ively (see Table 2) indicate a deviation from a true Theorell–Chance Bi Bi mechanism. The non-linear Dixon plots obtained with D- and L-glutamate (Fig. 3) are also suggestive of an altered pathway.

Glutamic- γ -semialdehyde dehydrogenase from ox (Strecker, 1971) and rat (Herzfeld *et al.*, 1977) liver were reported to differ in substrate specificity in that the rat enzyme was specific for the L-conformer while the ox enzyme could utilize both conformers as substrates. Kinetic analysis via Dixon plots was done with D- and L-glutamate (Fig. 3*b*). In both cases the plots deviated from linearity at concentrations above approx. 35 mM. As shown in Fig. 3*b*, the points obtained with both D- and L-glutamate are nearly superimposable, giving K_i values of 13 mM and 14.3 mM. The near equivalence of the K_i values calculated for both conformers of inhibitor provides additional evidence that both D- and L-glutamic γ -semialdehyde are equally good substrates for the enzyme, which is in agreement with what has been reported by Strecker (1971) for the ox liver enzyme. Thus it is both the D- and the L-forms of the substrate that are referred to in discussion of substrate B. In addition, since the K_i value obtained with L-proline is 5-fold less than that obtained with L-glutamate (approx. 3 mM compared with 15 mM; see Tables 2 and 3), it appears likely that the actual substrate for the enzyme may be DL- Δ^1 -pyrroline-5-carboxylate, the cyclized form of glutamic γ -semialdehyde with which it is known to be in equilibrium in solution.

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REFERENCES

- Boggess, S. F., Paleg, L. G. & Aspinall, D. (1975) *Plant Physiol.* **56**, 259–262
- Brunner, G. & Neupert, W. (1969) *FEBS Lett.* **3**, 283–286
- Bush, K., Shiner, V. J., Jr. & Mahler, H. R. (1973) *Biochemistry* **12**, 4802–4805
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137
- Cleland, W. W. (1970) *Enzymes* 3rd Ed. **2**, 1–65
- Efron, M. L. (1965) *N. Engl. J. Med.* **272**, 1243–1254
- Forté-McRobbie, C. M. & Pietruszko, R. (1986) *J. Biol. Chem.* **261**, 2154–2163
- Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222
- Greenfield, N. J. & Pietruszko, R. (1977) *Biochim. Biophys. Acta* **483**, 35–45
- Herzfeld, A., Mezel, V. A. & Knox, W. E. (1977) *Biochem. J.* **166**, 95–103
- Lineweaver, H. & Burk, D. J. (1934) *J. Am. Chem. Soc.* **56**, 658–667
- Lundgren, D. & Ogur, M. (1972) *Biochem. Biophys. Res. Commun.* **49**, 147–149
- Lundgren, D. & Ogur, M. (1973) *Biochim. Biophys. Acta* **297**, 246–257
- Mezel, V. A. & Knox, W. E. (1976) *Anal. Biochem.* **74**, 430–440
- Stewart, C. R. & Lai, E. Y. (1974) *Plant Sci. Lett.* **3**, 173–181
- Strecker, H. J. (1960) *J. Biol. Chem.* **235**, 3218–3223
- Strecker, H. J. (1971) *Methods Enzymol.* **17B**, 262–265
- Theorell, H. & Chance, B. (1951) *Acta Chem. Scand.* **5**, 1127–1144
- Vallee, D., Goodman, S. I., Harris, S. C. & Phang, J. M. (1979) *J. Clin. Invest.* **64**, 1365–1370
- Wellner, D. & Meister, A. (1981) *Annu. Rev. Biochem.* **50**, 911–968
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Wratten, C. C. & Cleland, W. W. (1965) *Biochemistry* **4**, 2442–2451

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