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THE TRANSIENT-STATE KINETICS OF L-GLUTAMATE DEHYDROGENASE

pH-DEPENDENCE OF THE BURST RATE PARAMETERS

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Summary

The pH dependence of the initial transient velocity of NADPH production during the burst phase of the oxidative deamination of L-glutamate by L-glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) and $NADP^+$ has been measured by stopped-flow spectrophotometry. These studies provide evidence that the entire pH dependence below pH 8.26 arises from reaction steps contributing to V of the burst with an apparent pK_a of 8.1 ± 0.1. The data are consistent with a model in which the formation of the first enzyme-coenzyme-substrate ternary complex on the reaction path equilibrates rapidly and in which the pH-dependent steps are mechanistically close to and may include the catalytic hydrogen transfer itself. At pH 8.87, there is evidence that L-glutamate binds less tightly to the enzyme and to the enzyme-$NADP^+$ complex than at lower pH values.

Introduction

The pH profiles of the steady-state velocities of the bovine liver L-glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) reaction have been studied under a variety of experimental conditions [1—5] and complete steady-state kinetic analyses have been reported at several pH values [6—8]. It has been shown that in the steady-state oxidative deamination of L-glutamate by L-glutamate dehydrogenase and $NADP^+$, the rate-limiting steps occur after the catalytic hydrogen transfer and involve the breakdown of tight ternary complexes of enzyme, NADPH, and either the product α-ketoglutarate or the substrate L-glutamate [9,10].

Iwatsubo and Pantaloni [9] have demonstrated an observable burst of $NADP^+$ reduction preceding the steady-state turnover of the reaction. It was

subsequently shown that the catalytic hydrogen transfer was at least partially rate limiting during the burst phase of the reaction [10]. Therefore, in order to obtain information about the pH dependence of the catalytic steps themselves [9—15], it is necessary to study the transient burst of NADPH complex production accompanying L-glutamate oxidation rather than the steady-state release of free NADPH.

The initial velocity of the rapid burst increases to a maximum as pH is raised from pH 6 to pH 8.5 [15]. It has been shown that at pH 6.5 and pH 7.6 these initial velocities give linear Lineweaver-Burk plots with respect to both L-glutamate and $NADP^+$ concentration [13]. It is possible, then, to reduce the data at a given pH value to four empirical parameters which are independent of substrate and coenzyme concentration. The purpose of the present work is to investigate the pH dependence of these phenomenological parameters and thus to identify the mechanistic location of the pH-dependent steps.

Materials and Methods

L-Glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) was purchased from Sigma Chemical Co. as the Type I crystalline ammonium sulfate suspension. The suspension was either dialyzed as described previously [16] or, after several changes of buffer in the dialyzer, purified by gel filtration on Sephadex G-25. Before use, the enzyme was treated with Norit A and filtered. Enzyme concentrations were calculated from 280 nm absorbance readings using an absorptivity of 0.97 $cm^{-1} \cdot mg^{-1} \cdot ml$ [2]. The ratio $A_{280nm} : A_{260nm}$ was 1.92—1.96 for the enzyme preparations used in this study. $NADP^+$ and D-alanine were purchased from Sigma Chemical Co. and L-glutamic acid from Calbiochem.

All experiments were run at 20° C in 0.1 M potassium phosphate buffer. As L-glutamate concentration was varied in each experiment, D-alanine, which is neither substrate nor effector in the L-glutamate dehydrogenase reaction, was added to maintain a constant amino acid concentration of 0.2 M, thus maintaining approximately constant buffer capacity at each experimental pH value. Comparison with experiments run without D-alanine indicate that the presence of D-alanine improves pH control above pH 8, but has no significant effect on the kinetics. For each set of experiments reported here, the pH values measured at 20° C varied by no more than ±0.1 pH unit.

Stopped-flow experiments were performed on a Durrum-Gibson apparatus equipped with a xenon arc light source and a 1.93 cm stainless steel cuvette, and interfaced to a Varian 620L digital computer. Data were collected and averaged as described previously [13,17]. For each experiment in which enzyme was diluted during mixing, time course baselines obtained using the same solutions in the absence of L-glutamate were subtracted from the experimental time course records to minimize flow anomalies and light scatter changes on protein dissociation [13,18]. The initial velocities of the transient burst phase were measured by extrapolating an exponential fit of the data to time zero (time zero was determined by calibrating the instrument using the reaction of 0.01 M $Fe(NO_3)_3$ in 0.05 M H_2SO_4 with several different concentrations of KSCN). These initial velocities were converted to specific rates using an

absorptivity of 5.0 $mM^{-1} \cdot cm^{-1}$ at 340 nm [19] for the enzyme-NADPH-α-ketoglutarate complex (the tight product complex formed in the burst phase *) and a polypeptide chain molecular weight of 56 100 for L-glutamate dehydrogenase itself [20]. No specific rates faster than 50 s^{-1} were included among the data analyzed to avoid errors generated by the 3 ms dead time of the instrument.

In order to avoid possible enzyme denaturation from incubation at high pH values [15,21], the stopped-flow experiments at pH 8.87 were initiated by mixing a solution of enzyme and oxidized coenzyme incubated in phosphate buffer at pH 8 with a solution of L-glutamate and D-alanine in phosphate buffer at pH 9. In all other experiments both stopped-flow syringes contained identical enzyme concentrations to further minimize the effects of enzyme dissociation. The resultant pH after mixing in the stopped-flow apparatus is the value reported here.

Eqns. 1 and 2 (Results) were fitted to the data using non-linear least squares regressions. The reported errors are variances obtained from the regressions [22].

Results

As observed previously [13], initial burst velocities measured at constant pH gave linear Lineweaver-Burk plots with respects to L-glutamate concentration (3—100 mM) and $NADP^+$ concentration (100 μM—1 mM). At each pH value initial velocity data for at least 40 combinations of $NADP^+$ and L-glutamate concentration were fitted using a weighted ** least squares regression to Eqn. 1 [23]:

$$\frac{E_T}{\nu} = \phi_0 + \frac{\phi_1}{[NADP^+]} + \frac{\phi_2}{[\text{L-glutamate}]} + \frac{\phi_{12}}{[NADP^+][\text{L-glutamate}]} \quad (1)$$

where E_T is the total enzyme concentration, ν is the measured velocity and ϕ_0, ϕ_1, ϕ_2 and ϕ_{12} are empirical parameters which characterize the coenzyme and substrate concentration dependence of the specific velocity (ν/E_T). Fig. 1 shows the pH dependence of these parameters. Below pH 8.26, they appear to have identical pH dependences within experimental error. Above pH 8.26, there is a slight upturn in ϕ_2 and ϕ_{12} but not in ϕ_0 and ϕ_1.

The phenomenological coefficients may be used to calculate several characteristic constants for the burst phase of the L-glutamate dehydrogenase reaction [13]. The maximum specific velocity of the burst phase, $V_{burst}/E_T = \phi_0^{-1}$, has a pH dependence (Fig. 2) which can be fit by [24]:

$$\phi_0^{-1} = k'_{cat}\frac{a_H}{a_H + K_a} + k_{cat}\frac{K_a}{a_H + K_a} \quad (2)$$

* It has been shown that at pH 7.6 the spectrum of the reduced nictonamide absorbance produced during the burst is blue shifted [10—12]. This blue-shifted spectrum has been identified with that obtained from equilibrium studies for the ternary enzyme-NADPH-α-ketoglutarate complex [10—16]. We have verified that the spectra of the burst at pH 6.5 and pH 8.5 are identical to that obtained at pH 7.6.

** The weighting was accomplished by inverting both sides of Eqn. 1 and obtaining a non-linear least squares fit to the resulting expression for ν/E_T [22].

TABLE I

BINARY COMPLEX DISSOCIATION CONSTANTS, LIMITING MICHAELIS CONSTANTS, AND APPARENT HETEROTROPIC COOPERATIVITY AS A FUNCTION OF pH

	pH 6.51	pH 6.83	pH 7.20	pH 7.60	pH 8.00	pH 8.26	pH 8.50	pH 8.87
For $NADP^+$:								
$K_1 = \frac{\phi_{12}}{\phi_2}$ (mM)	1.9 ± 0.6	2.5 ± 1.0	2.0 ± 0.8	1.8 ± 0.6	2.0 ± 0.7	1.7 ± 0.7	1.6 ± 0.6	1.5 ± 0.4
$K_{m,1} = \frac{\phi_1}{\phi_0}$ (mM)	0.35 ± 0.07	0.49 ± 0.06	0.27 ± 0.06	0.27 ± 0.09	0.35 ± 0.06	0.38 ± 0.18	0.49 ± 0.18	0.44 ± 0.12
For L-glutamate:								
$K_2 = \frac{\phi_{12}}{\phi_1}$ (mM)	25 ± 4	18 ± 4	28 ± 6	23 ± 5	17 ± 4	20 ± 7	22 ± 7	34 ± 4
$K_{m,2} = \frac{\phi_2}{\phi_0}$ (mM)	4.6 ± 1.8	3.6 ± 1.1	3.8 ± 1.5	3.4 ± 1.6	3.0 ± 1.3	4.6 ± 2.5	6.8 ± 3.5	10 ± 4
Cooperativity [13]:								
$C = \frac{\phi_0\phi_{12}}{\phi_1\phi_2}$	5.6 ± 3.1	5.0 ± 2.6	7.4 ± 4.6	6.8 ± 4.5	5.6 ± 3.2	4.3 ± 3.7	3.3 ± 2.9	3.3 ± 1.7

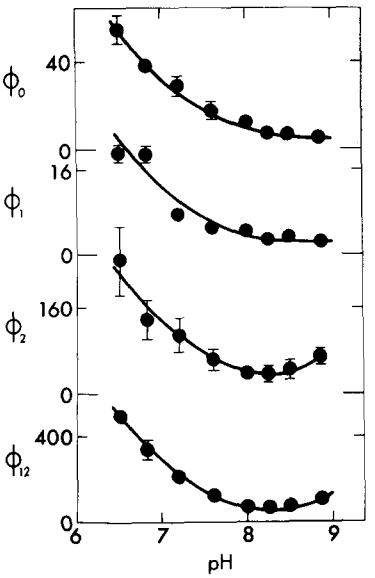


Fig. 1. pH dependence of the burst rate parameters. Units (ordinate): ϕ_0, ms; ϕ_1, mM · ms; ϕ_2, mM · ms; and ϕ_{12}, $(mM)^2$ · ms.

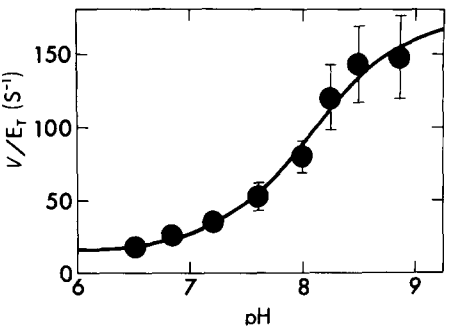
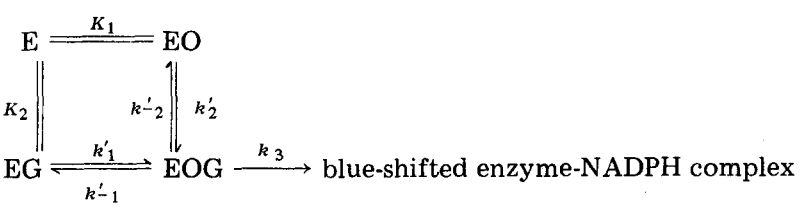


Fig. 2. pH dependence of the maximum specific velocity. The theoretical curve is calculated using Eqn. 2 (Results).

where a_H is the measured hydronium ion activity, k'_{cat} is the limiting velocity at low pH, k_{cat} is the limiting velocity at high pH, and the pH-dependent factors give the fractions in the low and high pH forms. The solid line in Fig. 2 is drawn for the parameters obtained from the non-linear least squares fit to Eqn. 2: k_{cat} = 178 ± 12 s^{-1}, k'_{cat} = 14 ± 6 s^{-1}, and pK_a = 8.1 ± 0.1. In contrast to V_{burst}, none of the other constants which can be calculated from the ϕ_i values shows a strong pH dependence, although both the dissociation constant and the limiting Michaelis constant * for L-glutamate are elevated significantly at pH 8.87 (Table I).

Discussion

It has been shown that the mechanism of the burst phase can be represented by the following scheme [13]:



* The limiting Michaelis constant is the Michaelis constant for the hypothetical experiment in which one substrate is varied while the other is present at infinite concentration.

where E stands for the enzyme, L-glutamate dehydrogenase; 0 for oxidized coenzyme, $NADP^+$; G for substrate, L-glutamate; K_1 and K_2 are the dissociation constants for the binary complexes of enzyme with $NADP^+$ and L-glutamate, respectively; and the lower case k values are rate constants. The symbol (═══) denotes those steps which are known to be in rapid equilibrium **.

For this mechanism, the ϕ values are given by

$$\phi_0 = \frac{1}{k_3} \tag{3}$$

$$\phi_1 = K'_1 R \tag{4}$$

$$\phi_2 = K'_2 R \tag{5}$$

$$\phi_{12} = K_1 K'_2 R = K'_1 K_2 R \tag{6}$$

where

$$R = \frac{1}{(k'_{-2} + k'_{-1})} + \frac{1}{k_3} \tag{7}$$

$$K'_1 = k'_{-1}/k'_1 \tag{8}$$

$$K'_2 = k'_{-2}/k'_2 \tag{9}$$

We may now examine this mechanism in the light of the data presented here. The pH dependences of ϕ_0, ϕ_1, ϕ_2 and ϕ_{12} appear to be identical below pH 8.26. The only rate constant they possess in common is k_3. If k_3 depends on pH in this range and $1/k_3$ is the dominant term in the expression for the factor R ($R \approx 1/k_3$ in Eqn. 7) then all the ϕ values will have the same pH dependence. In addition, the limiting Michaelis constants * will simply be the dissociation constants for the enzyme-$NADP^+$-L-glutamate ternary complex (EOG):

for $NADP^+$:

$$K_{M,1} = \phi_1/\phi_0 \approx K'_1 \tag{10}$$

and for L-glutamate:

$$K_{M,2} = \phi_2/\phi_0 \approx K'_2 \tag{11}$$

Thus the ternary complex EOG will be in rapid equilibrium with the binary complexes, EO and EG (i.e. $1/(k_{-2} + k_{-1}) << 1/k_3$).

The observed pH dependence below pH 8.26, therefore, appears to arise from k_3, the rate constant for the step which determines V_{burst}. The pH

* See footnote p. 381.

** Evidence for the random and rapid formation of enzyme-$NADP^+$ and enzyme-L-glutamate complexes at pH 6.5 and pH 7.6 has been reported in ref. 13. Yet there is a possibility that these steps may not be in rapid equilibrium at pH 8.87. A spectrophotometric determination of the equilibrium constant for the formation of the enzyme-L-glutamate complex at pH 8.8 under the present experimental conditions gave a value of 56 ± 20 mM, in reasonable agreement with the kinetically determined constant at pH 8.87 (34 ± 4 mM from Table I). If the formation of this latter complex were not in rapid equilibrium, however, one would expect the apparent equilibrium constant from kinetics to be larger than the one obtained from equilibrium studies. Such is not the case. Thus the rapid equilibrium model appears to be at least approximately correct at pH 8.8.

dependence of V_{burst} (Fig. 2 and Eqn. 2) indicates that a group with a pK_a of approx. 8.1 may be involved in the pH-dependent step. It has been pointed out elsewhere [5] that just such a pK_a (8.0 ± 0.3) is observed for the ϵ-NH_2 group of lysine-126, which is essential for catalytic activity, but this group is only one of many any one of which might be responsible for the pH dependence noted here.

If there are no additional intermediates on the reaction path, then both the pH dependence (Eqn. 2) and the reported 2-fold primary isotope effect when L-[2-$^2H_1$]glutamate is used as substrate [10,13] are associated simply with the rate constant k_3 for the conversion of the ternary EOG complex to an NADPH complex with a blue-shifted reduced nicotinamide spectrum. The possibility of the existence of additional intermediates, however, means that the pH dependence need not be associated with the catalytic hydrogen transfer step itself and, further, that the true isotope effect for the catalytic step may be greater than 2-fold.

As the pH is raised to 8.87, ϕ_2 and ϕ_{12}, and thus K_2 and $K_{m,2}$, begin to increase, indicating that the affinity of the enzyme and the enzyme-oxidized coenzyme complex for L-glutamate is decreasing. This loosening of L-glutamate binding contributes to the drop in burst velocities above pH 8.5. This effect may arise from titration of the α-amino group of L-glutamate or of groups on the enzyme or the enzyme-$NADP^+$ complex with pK_a values above 9.

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