Effect of Ammonia on the Glutamate Dehydrogenase Catalyzed Oxidative Deamination of L-Glutamate: Production of an Ammonia-Containing Intermediate in the "Burst" Phase†

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ABSTRACT: We have studied the effects of ammonium acetate on the transient "burst" phase of the oxidation of L-glutamate by glutamate dehydrogenase. Two measurable changes are observed in the "burst" phase as ammonium acetate concentration is increased: (i) an increase in the apparent first-order rate constant, k_{app}, and (ii) a decrease in the amplitude of the absorbance change measured at 320 nm. The increase in k_{app} shows a hyperbolic dependence on ammonium acetate concentration and is independent of glutamate concentration. The results demonstrate the existence of an intermediate immediately following hydrogen transfer. The intermediate contains enzyme, reduced coenzyme, ammonia, and α-ketoglutarate moieties and is in equilibrium with the known complex consisting of enzyme, reduced coenzyme, and α-ketoglutarate. At high concentrations of ammonium acetate, the equilibrium favors the ammonia containing complex.

Ammonia[1] was shown to be a product (substrate) of the reversible oxidative deamination of L-glutamate in one of the earliest studies of this glutamate dehydrogenase catalyzed reaction (Olson & Anfinsen, 1953). Inclusion of this substrate into the mechanism of the reaction has proven to be very difficult, as shown by the lack of evidence for intermediates which contain ammonia. For example, there have been several reports that ammonia inhibits the steady-state rate of oxidation of glutamate (Fisher & McGregor, 1960; Engel & Chen, 1975), but very few details of this inhibition at the molecular level are available (Engel & Chen, 1975). Recently, evidence has been presented for the enzyme-catalyzed formation of α-iminoglutarate (Hochreiter et al., 1972), and subsequent reduction by sodium borohydride. However, it is unknown whether the imine detected by these workers is a kinetically significant, enzyme-bound intermediate in the dehydrogenation reaction. Schimerlik et al. (1975) have reported an equilibrium perturbation isotope effect using [^{15}N]ammonia which led them to conclude that cleavage of the carbon–nitrogen bond is rate limiting. With the exception of the last two studies previous work has not provided any evidence on the nature of the ammonia containing complex(es) on the reaction path. A kinetic study which probes the involvement of ammonia in the glutamate dehydrogenase reaction is clearly needed. In this regard, Jallon et al. (1975) have reported a rapid burst of disappearance of NADPH absorbance, associated with the reductive amination of α-ketoglutarate, when enzyme, NADPH, and α-ketoglutarate are first preincubated then mixed with NH_4Cl. The rate constant for this transient (300 s^{-1} in Tris-HCl) was interpreted to include both the rate constant for imine formation and the rate constant for oxidation of the reduced coenzyme. The apparent K_m for ammonia was 30 mM.

The opposite reaction, the oxidative deamination of L-glutamate, shows up to three distinct phases (Colen et al., 1975; Fisher et al., 1970) for the appearance of reduced coenzyme; see Figure 1. The first phase is a rapid "burst" of production of reduced nicotinamide absorbance, in this case associated with the oxidative deamination of L-glutamate, corresponding to slightly less than one enzyme equivalent of bound NADPH (Colen et al., 1975; Fisher et al., 1970; Iwatsubo & Pantaloni, 1967). The second phase is a transition phase where the various enzyme complexes equilibrate and reach their steady-state levels. The third phase corresponds to the steady-state production of free coenzyme. The second and early third phases occur simultaneously, with the second phase making only a minor contribution to the total absorbance change. The instantaneous concentration of reduced coenzyme can be closely approximated empirically by eq 1

$$R_t = \frac{k_f E_t}{k_f + k_r}(1 - \exp(-k_{app}t)) + \frac{k_f E_t}{k_f + k_r}(k_{ss}(t - \Delta t)) \quad (1)$$

where R_t is the total concentration of reduced coenzyme, E_t is the concentration of glutamate dehydrogenase, Δt is the estimated duration of the transient phases of the reaction and the second term is taken to be zero when $t < \Delta t$. The rate constants for the various phases of this equation are: k_{app}, the observed first-order rate constant for the transient "burst" phase; k_f, the observed first-order rate constant for this phase in the absence of added ammonia; k_r, the ammonia concentration dependent rate constant for the reverse reaction; and k_{ss}, the rate constant associated with the steady-state phase. Although a relationship between $1/k_{app}$ and Δt might be anticipated, the onset of the steady-state phase is regulated by a complex series of events which depends somewhat on the concentration of L-glutamate and precludes the determination of the relationship at this time. This paper deals solely with the effects of increasing ammonium acetate concentration on the first phase (the "burst" of oxidative deamination of L-glutamate) of the reaction in terms of a proposed mechanism. A second paper will deal with the effects of ammonia on the

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[1] The term, "ammonia," as used in this paper refers to all forms of ammonia in the solution: e.g., "ammonia concentration" means the sum of concentrations of ammonium ion and free ammonia.

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steady-state and later phases of this oxidative deamination of L-glutamate.

Experimental Procedure

Materials. The glutamate dehydrogenase used in this study was purchased from Boehringer-Mannheim as an ammonium sulfate suspension. Dissolution of the enzyme in 0.1 M potassium phosphate buffer, pH 7.6, and final purification steps have been described previously (Prough et al., 1972). Nicotinamide adenine dinucleotide phosphate, NADP, was obtained from Sigma Chemical Co.

Kinetics. The rate of formation of reduced coenzyme, NADPH, upon rapidly mixing a solution of glutamate, ammonium acetate, and glutamate dehydrogenase, with an equal volume of NADP and glutamate dehydrogenase at 20 °C was monitored at 320 nm using a Durrum-Gibson stopped-flow spectrometer. At this wavelength, the absorbance changes in the "burst" phase can be studied without interference from the slower processes. The changing transmittance was recorded simultaneously on a storage oscilloscope and in digitized form by a Varian 620L computer. The transmittances of acceptable experiments were converted to absorbances and data from at least five such experiments were averaged, using the computer. The resultant data, 1000 points per experiment, were then analyzed directly by two methods to obtain the pseudo-first-order rate constants for the first phase. The first method involves fitting the initial rapid absorbance change to eq 2 by a method of successive approximations, where

$$A_t = B \exp(-k_{app}t) + C \qquad (2)$$

A_t is the absorbance at time t; B determines the absorbance at $t = 0$; k_{app} is the apparent rate constant associated with the first phase; and C is the maximal absorbance of this phase. In the second method, the slope of the slower steady-state phase was determined and subtracted from the absorbance at times greater than the estimated time of the first phase of the reaction.[2] The pseudo-first-order rate constant, k_{app}, associated with the resultant curve was calculated using eq 3, where A_∞ and A_t are the absorbances at time infinity and time of measurement, respectively.

$$\ln(A_\infty - A_t) = k_{app}t + \text{constant} \qquad (3)$$

Good first-order behavior to more than two half-lives was obtained. The agreement between the two methods was excellent. At the higher concentrations of glutamate and with no added ammonia, the phase corresponding to the blue to red shift of reduced coenzyme absorbance (Colen et al., 1975) was barely evident and produced only a slight decrease in the "corrected" absorbance at 320 nm. This change is sufficiently small and occurs at a sufficiently slower rate than the first phase that no significant interference with the analyses was evident (see Figure 1).

Results

The addition of ammonia to the reaction solutions used to study the oxidative deamination of L-glutamate produces several interesting effects on the time course of the reaction, as can be seen in Figure 2. Clearly, the later stages of the stopped-flow experiment, which corresponds to the steady-state phase, are inhibited markedly by increasing concentrations of ammonia. There is also a marked decrease in the amplitude of the "burst" phase, and the onset of the linear steady-state phase occurs much earlier at higher concentrations of ammonia. These phenomena can be related directly to the steady-state phase and will be discussed in a later paper.

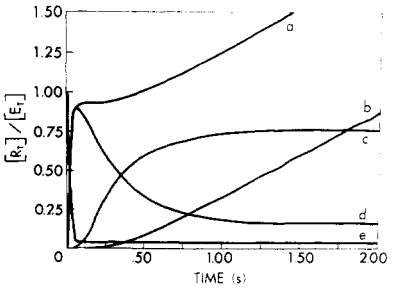


FIGURE 1: Illustration of the phases for glutamate dehydrogenase catalyzed oxidative deamination of L-glutamate. (a) The observed appearance of reduced coenzyme as a function of time. (b) The production of free reduced coenzyme, or the steady-state rate which parallels the later stages of curve a. Curves a and b are experimental data. (c) The formation of the enzyme–reduced coenzyme complexes with λ_{max} at 346 nm, specifically ER and ERG. It is the dissociation of these complexes which gives rise to the steady-state rate of formation of reduced coenzyme. (d) The rapid formation of enzyme–reduced coenzyme complexes with λ_{max} = 332 nm traces curve a at first, and, during the equilibration of the various enzyme–reduced coenzyme complexes at intermediate times, decreases with rate constant equal to that for curve c. (e) The rapid decrease in all complexes of enzyme without reduced coenzyme reaches the steady-state level and remains constant. This diagram is schematic and the relative concentrations represented in curves b through e is not meant to be quantitative. The concentrations will be determined by the experimental conditions.

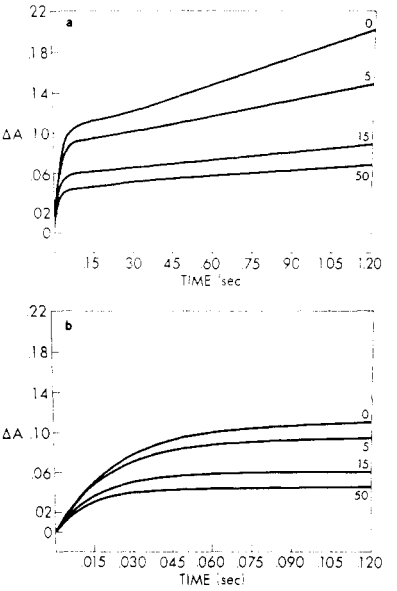


FIGURE 2: (a) The effect of increasing ammonium acetate concentration on the rate of glutamate dehydrogenase catalyzed oxidation of L-glutamate. Note the decrease in "burst" amplitude and steady-state rate. (b) The increase in rate constant (decrease in half-life) can be seen on expanding the time axis. Note also that the initial velocities are independent of concentration of added ammonium acetate. The lines are the experimental data obtained using 25 mM L-glutamate, 0.38 mM NADP, 0.018 mM glutamate dehydrogenase, and 0, 5, 15, and 50 mM ammonium acetate.

Although the initial velocity for the "burst" phase is unaffected by increasing concentrations of ammonia (Figure 2b), the pseudo-first-order rate constants for the "burst" phase actually increase with increasing ammonia concentration. The rate constants, at several glutamate concentrations, are plotted against increasing ammonia concentration in Figure 3. It is apparent that, for each concentration of glutamate, a smooth increase in the apparent rate constant, k_{app}, to a limiting value is obtained. Since the apparent first-order rate constant for a

[2] Tatemoto has used a similar approach in calculating the true burst amplitudes for the alcohol dehydrogenase catalyzed oxidation of ethanol (Tatemoto, 1976).

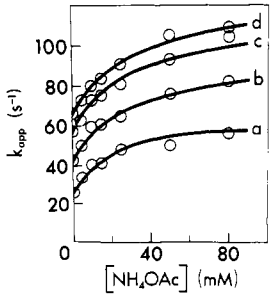


FIGURE 3: A plot of the observed rate constant vs. ammonium acetate at several concentrations of L-glutamate. Glutamate concentration is: (a) 10 mM; (b) 25 mM; (c) 50 mM; (d) 100 mM.

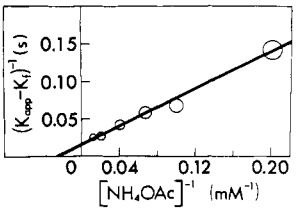


FIGURE 4: Double-reciprocal plot of $(k_{app} - k_f)$ vs. ammonium acetate concentration, where each data point is obtained using the four concentrations of L-glutamate.

reversible reaction is the sum of the forward and reverse rate constants (Frost & Pearson, 1963), k_{app} will be given by $k_{app} = k_f + k_r$. In the absence of added ammonia and at the concentrations of glutamate and coenzyme used in this study, the amplitude of the first phase approaches the enzyme concentration (Colen et al., 1975), or $k_f/(k_f + k_r) \rightarrow 1$. Thus, the contribution of the reverse reaction with no added ammonia must be small and $k_{app} \simeq k_f$. Since $k_r = k_{app} - k_f$, the ammonium acetate dependence of k_r can be obtained by plotting $(k_{app} - k_f)$ vs. ammonium acetate concentration. These plots are hyperbolic and identical for all concentrations of glutamate. Replotting the data in double-reciprocal form gives the single straight line of Figure 4, with k_r at infinite ammonium acetate concentration of 62 ± 10 s^{-1}, and an apparent K_{NH_4OAc} for ammonium acetate of 45 ± 15 mM.

Discussion

The rapidity of the first phase of the oxidative deamination of glutamate allows us to separate this phase from the later phases. Using the currently known facts and the results obtained in this study, Scheme I is proposed as a mechanism consistent with the effects of ammonia. In Scheme I, E represents glutamate dehydrogenase, EO, EG, and EOG represent binary and ternary complexes of oxidized coenzyme and glutamate with the enzyme, ERK represents the known ternary enzyme–reduced coenzyme–α-ketoglutarate complex (Cross, 1972), and ERKN represents a complex composed of enzyme, reduced coenzyme, ammonia, and α-ketoglutarate. At present we do not have evidence to distinguish between a quaternary complex where ammonia is not covalently bonded to α-ketoglutarate and a ternary complex where ammonia and α-ketoglutarate are covalently linked as in α-iminoglutarate.

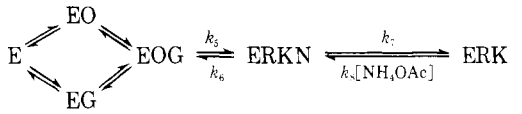
The appearance of absorbance at 320 nm due to coenzyme reduction in the first phase obeys first-order kinetics and the apparent first-order rate constant so obtained agrees closely with that calculated from previous studies of initial velocities in the burst phase (see k_5' in Table I). Furthermore, the effect of ammonium acetate on the first phase is independent of L-glutamate concentration (Figure 4) and therefore independent of EOG concentration. Scheme I will predict such behavior

TABLE I: Comparison of Some Kinetic Parameters for the Interconversion of L-Glutamate and α-Ketoglutarate Catalyzed by Glutamate Dehydrogenase.[a]

I		II	
k_5' (s^{-1})	78 ± 15	k_5' (s^{-1})	61 ± 11[b]
k_6 (s^{-1})	62 ± 10	k_6 (s^{-1})	35 ± 20[c]
K_7 (mM)	45 ± 15	K_I (mM)	32[d]
		K_d (mM)	40 ± 20[e]
		K (mM)	30[f]

[a] Column I, this work; column II, corresponding parameters from the literature. [b] Calculated for the present experimental conditions from the ϕ values reported by Colen et al. (1972). [c] Reported by Engel & Dalziel (1970), corrected to mol wt 56 100. [d] Reported by Engel & Chen (1975). [e] Calculated from ϕ' values reported by Engel & Dalziel (1970). We have found this parameter to be independent of pH between 7.0 and 7.6. [f] Reported by Jallon et al. (1975).

SCHEME I



only if the ratio of ERKN concentration to ERK concentration depends on ammonia concentration alone. This would be the case if ERKN equilibrates rapidly with ERK. It can be shown that within experimental error a sufficient set of conditions for the above results to hold is $k_7 \geq 6k_5'$ and $k_7 \geq 6k_6$ for the data presented here (see Appendix). These conditions are satisfied when $k_7 > 460$ s^{-1} and $k_6 < 80$ s^{-1}, since the limiting value of k_5' extrapolated to infinite L-glutamate concentration is 78 ± 15 s^{-1} (Table I).

In this case, the ammonium acetate concentration dependence of the observed first-order rate constant is given by

$$k_{app} - k_f = \frac{k_6(NH_4OAc)}{K_7 + (NH_4OAc)} \quad (4)$$

where $K_7 = k_7/k_8$, the dissociation constant linking ERKN and ERK. The limiting k_r and apparent K_m values from the data in Figure 4 yield values of k_6 and K_7, respectively, which are shown in Table I along with the values for the limiting velocity of glutamate formation from steady-state data (Engel & Dalziel, 1970) and the inhibition constants (Engel & Chen, 1975) and the dissociation constants (Jallon et al., 1975) for ammonia. Good agreement with the previously reported values is obtained. Note also that the value of k_6 thus obtained satisfies the inequality in the previous paragraph.

In summary, we have presented data which support the existence of an obligatory complex on the reaction path for the glutamate dehydrogenase catalyzed interconversion of L-glutamate and α-ketoglutarate. This complex is composed of enzyme, reduced coenzyme, ammonia, and α-ketoglutarate. We have shown that the data are consistent with the formation of this complex in an ordered mechanism, i.e., the complex is found between the known EOG and ERK complexes on the reaction path. The rate of interconversion of ERKN and ERK is sufficiently higher than the rate of interconversion of ERKN and EOG that ERKN may be considered to approach equilibrium with ERK during the "burst" phase.

Appendix

Since all steps up to and including the initial formation of the ternary complex EOG equilibrate rapidly (Colen et al., 1977), at most two pseudo-first-order processes will be observed for Scheme I presented in the discussion section of this paper. These processes are associated with steps involving in-

terconversion of EOG, ERKN, and ERK in Scheme I. Only one distinct process is observed. Thus the relaxation times are either accidentally degenerate, or well separated with the faster process occurring in the instrument dead time (<3 ms).

The first possibility can be eliminated immediately since, if the relaxation times were identical, $(k_{app} - k_f)^{-1} = (k_8(NH_4OAc))^{-1}$ and Figure 4 would have a zero intercept, which is apparently not the case. It is more likely that we are observing the longer of two distinct relaxation processes. For this process, the Michaelis constant for NH_4OAc obtained from the x intercept of the plot of $(k_{app} - k_f)^{-1}$ vs. $(NH_4OAc)^{-1}$ is given by

$$K_{NH_4OAc} = \frac{k_5' + k_6 + k_7}{k_8} \quad \text{(A1)}$$

where

$$(k_5')^{-1} = \phi_0 + \frac{\phi_1}{(O)} + \frac{\phi_2}{(G)} + \frac{\phi_{12}}{(O)(G)} \quad \text{(A2)}$$

is the reciprocal specific initial velocity of the burst phase in the absence of ammonium acetate (Colen et al., 1972). Since the plot of $(k_{app} - k_f)^{-1}$ vs. $(NH_4OAc)^{-1}$ (Figure 4) is independent of L-glutamate concentration, $k_5' \ll (k_6 + k_7)$ and $K_{NH_4OAc} \simeq (k_6 + k_7)/k_8$. (Given the experimental uncertainties in the present work, this implies that $6k_5' < (k_6 + k_7)$.)

For Scheme I

$$k_f = \frac{k_5'k_7}{k_5' + k_6 + k_7} \simeq \frac{k_5'k_7}{k_6 + k_7} \quad \text{(A3)}$$

The value of k_f, extrapolated to infinite L-glutamate concentration, is 78 ± 15 s^{-1} and the value of k_5' calculated from a study of initial velocities (Colen et al., 1972) is 61 ± 11 s^{-1} (Table I). These results imply that $k_7 > 6k_6$ and $k_f \simeq k_5'$ within the experimental uncertainty of these studies. For all practical purposes, then, the final step in the Scheme equilibrates rapidly, with $K_{NH_4OAc} \simeq k_7/k_8 = K_7$, the dissociation constant for the ammonia-containing species from ERKN.

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