The Mechanism of Glutamate Dehydrogenase Reaction

IV. EVIDENCE FOR RANDOM AND RAPID BINDING OF SUBSTRATE AND COENZYME IN THE BURST PHASE

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SUMMARY

Stopped flow studies of the oxidative deamination of L-glutamate by TPN and beef liver glutamate dehydrogenase were performed at pH 6.5 and 7.6. At each pH value, the initial burst slopes obey an equation of the form: e/v = φ₀ + φ₁/(TPN) + φ₂/(L-glutamate) + φ₃/(TPN)(L-glutamate). The agreement of dissociation constants for enzyme-TPN and enzyme-L-glutamate binary complexes obtained from the φ's with those obtained independently in equilibrium studies supports mechanisms which include random and rapid binding of substrate and coenzyme at the active site. The lack of effect of preincubation with either reactant on the transient state kinetics eliminates the possibility of any kinetically important slow isomerization steps in the formation of binary complexes. Comparison of the limiting Michaelis constants with the dissociation constants for TPN and L-glutamate shows that there is a cooperativity between coenzyme and substrate in the formation of an enzyme-TPN-L-glutamate ternary complex.

Steady state investigations of the oxidative deamination of L-α-amino acids by TPN or DPN and beef liver glutamate dehydrogenase have given inconclusive results regarding the order of addition of substrates. Frieden indicated that L-glutamate and coenzyme concentration dependences alone could not solve the problem (1). On the basis of their experiments with glutamate and norvaline, Engel and Dalziel concluded that it is unlikely that a compulsory order mechanism is obeyed by both amino acids, although they did not eliminate the possibility that one might do so (2).

Since it has been shown that dicarboxylic amino and keto acids form tight ternary complexes with TPNH and glutamate dehydrogenase (3, 4), whereas no such complexes have been observed for monocarboxylic amino and keto acids, it is quite reasonable that the two classes of substrates appear to obey different mechanisms in studies of steady state kinetics. Transient state experiments, on the other hand, permit observation of the glutamate reaction before appreciable quantities of tight ternary enzyme-TPNH-dicarboxylic acid complexes are formed.

Previous stopped flow studies of the kinetics of formation of various ternary enzyme complexes by di Franco and Iwatsubo (5, 6) have been interpreted in terms of the presence of a binary enzyme-L-glutamate complex at L-glutamate concentrations near saturation. Recently in this laboratory, the dissociation constant for such a complex has been measured by direct difference spectrophotometric titration (7). In addition, the dissociation constant for an enzyme-TPN binary complex has been measured both directly (8) and indirectly (9).

The present study will provide evidence that the binary enzyme-substrate complexes mentioned above are kinetically important (are formed at the catalytic site), that the formation steps for both of these complexes equilibrate rapidly, and that there is cooperativity in the formation of a ternary enzyme-TPN-L-glutamate complex.

MATERIALS AND METHODS

Glutamate dehydrogenase, L-glutamate: D(T)PN+ oxidoreductase (deaminating) (EC 1.4.1.3) was purchased from the Sigma Chemical Co. as the type I crystalline ammonium sulfate suspension. The suspension was dialyzed, treated with Norit A, filtered, and assayed as described previously (3). Enzyme concentrations were calculated from 280-nm absorbance readings with a value of 0.97 as the extinction coefficient of 1 mg per ml solution of glutamate dehydrogenase (10). The enzyme used had a ratio A₂₈₀ : A₁₆₃ of 1.95 to 1.98 and a specific activity of 3.5 ± 0.5 units per mg of protein (3). The pH values of all solutions were determined with a Radiometer PHM 26 pH meter. The buffers employed in the kinetics were 0.2 M potassium phosphate at pH 6.5 and 0.1 M potassium phosphate at pH 7.6. The experimental temperature was 25°. TPN was purchased from Sigma and L-glutamic acid from Mann Research Laboratories. L-[2-H]Glutamic acid, from Diaprep, Inc., was purified as described previously (11).

Transient state experiments were carried out on a Durrum-Gibson stopped flow spectrometer interfaced to a Varian 620i digital computer (11). The data were taken with split time resolution such that a higher sampling rate was employed during the first 100 to 200 ms of each experiment than thereafter, in
order to capture the details of the initial stages of the reaction. The highest rate at which data points were recorded was five points per ms, with each point representing an average of four analog-to-digital conversions. All of the data points reported in this paper represent an average of at least seven experiments.

The stopped flow cuvette path length was 2 cm and the instrument dead time was 3 ms. The final enzyme concentration after mixing was 1 mg per ml for all of the experiments reported here.

Except where otherwise indicated, experiments were performed with enzyme in both syringes, one of which also contained TPN and the other, L-glutamate. This precaution eliminated a small light scatter signal change from enzyme dissociation upon dilution which would otherwise be observed spectrophotometrically as a decrease in absorbance with a half-life of approximately 25 ms superimposed on the initial slope (12). In addition, there remains a small instrumental artifact which again appears as a decrease in optical density, but with a half-life of less than 10 ms and an amplitude of approximately 0.002 \( \Delta A \). This signal makes traces with low initial slopes appear to possess an initial lag, which may be removed by subtracting a base-line obtained by performing the same stopped flow experiment with enzyme in the absence of substrate and coenzyme.

The burst slopes were taken at 340 nm as \( \Delta A_{340} \) per ms from the initial steep portion of the burst (Fig. 1). Assuming that the blue-shifted reduced nicotinamide absorption produced during the burst is due mainly to the formation of the tight glutamate dehydrogenase-TPNH-\( \alpha \)-ketoglutarate complex, these slopes were converted to velocities in units of molar per s employing a value of the millimolar extinction coefficient for this complex of 5.0 at 340 nm (4). Since the 340-nm extinction coefficients of the glutamate dehydrogenase-TPNH and glutamate dehydrogenase-TPNH-L-glutamate complexes do not differ much from this value, the major errors in this assumption will come from any free TPNH produced (extinction coefficient of 6.2) and from possible transient intermediates whose spectra have not yet been characterized. The enzyme concentration was converted from milligrams per ml to molar assuming a binding site molecular weight of 56,100 (13).

At each pH, the burst slope data were fit to Equation 1 employing a multiple linear least squares regression performed on a Wang 700 calculator. The same program also provided the theoretical lines shown in Figs. 2 and 3.

**RESULTS**

**Concentration Dependence of Burst Slope—**Double reciprocal plots of burst slope against TPN concentration are shown in Fig. 2 for pH 7.6 and in Fig. 3 for pH 6.5. All the plots are linear within experimental error and, at each pH, obey an equation of the form

\[
e_v = c + \frac{d}{[O]} + \frac{f}{[G]} + \frac{g}{[O]}[G]
\]  

(1)

where \( e \) is the total glutamate dehydrogenase concentration, \( v \) is the initial slope of the burst, \( [O] \) is the concentration of oxidized coenzyme (TPN), and \( [G] \) is the concentration of L-glutamate. Table I contains the values of the coefficients in Equation 1 for pH 6.5 and 7.6.

**Preincubation with Substrates—**A series of experiments was performed in which the enzyme was incubated with one of the substrates (either TPN or L-glutamate) prior to rapid mixing with the other in the stopped flow apparatus.

In the experimental concentration range (15 \( \mu \)M to 1 mM for TPN and 1 to 100 mM for L-glutamate), the kinetic curves were identical with those obtained when both substrates were mixed with the enzyme simultaneously in stopped flow experiments. There was no evidence of a hidden jump during the apparatus dead time. Thus, preincubation of the enzyme with one substrate has no effect on the transient state kinetics as observed in stopped flow experiments.

**Characterization of Kinetic Phases at pH 6.5 and Comparison**

![Fig. 1. Plot of absorbance change against time for stopped flow experiments at pH 6.5 and 7.6 in which both drive syringes contained enzyme, and one contained TPN and the other L-glutamate such that the final concentrations after mixing were 1 mg per ml of glutamate dehydrogenase, 140 \( \mu \)M TPN, and 25 mM L-glutamate. Time zero is the time at which the flow was stopped. The arrow indicates the end of the apparatus dead time. The dashed lines represent the initial slopes. Seven runs were averaged for each curve.](http://www.jbc.org/)

![Fig. 2. Double reciprocal plot of burst slopes against TPN concentration at pH 7.6 and varying L-glutamate concentrations: \( \triangle \), 3 mM; \( \Diamond \), 6.75 mM; \( \bullet \), 15 mM; \( \square \), 50 mM; \( \bigcirc \), 150 mM. The solid lines are drawn according to Equation 1 using the constants listed in Table I.](http://www.jbc.org/)
L-glutamate concentrations: ○, 15 mM; △, 30 mM; □, 60 mM; ▽, 150 mM. The theoretical lines are drawn as described in Fig. 1.

**Table I**

<table>
<thead>
<tr>
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<th>pH 6.5</th>
<th>pH 7.6</th>
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</thead>
<tbody>
<tr>
<td>φ₀ (ms)</td>
<td>66 ± 15</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>φ₁ (mM · ms)</td>
<td>26 ± 3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>φ₂ (mM · ms)</td>
<td>1000 ± 200</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>φ₁₂ (mM² · ms)</td>
<td>1200 ± 300</td>
<td>100 ± 20</td>
</tr>
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with Those at pH 7.6—At pH 6.5, just as at pH 7.6 (5, 6, 11), three phases can be observed in the oxidative deamination of L-glutamate by TPN and glutamate dehydrogenase. These are: (a) an initial rapid production of blue-shifted reduced nicotinamide absorption in the burst phase, (b) a considerably slower production of red-shifted reduced nicotinamide absorption, and finally, (c) the production of free TPNH in the steady state.

Experiments performed with L-[2-²H₁]glutamate showed an isotope effect on the burst slope at pH 6.5 similar to that obtained at pH 7.6, where the maximum effect is of the order of two (11). The value of the isotope effect on the burst slope at pH 6.5, 1 mg per ml of glutamate dehydrogenase, 1 mM TPN, and 100 mM L-glutamate is 1.6.

Although the burst slopes are decidedly lower at pH 6.5 than at pH 7.6, the characteristic features of the kinetics are basically the same at the two pH values.

**Discussion**

The linearity of the double reciprocal concentration dependence of the initial burst slope (Equation 1) is not in itself sufficient evidence to distinguish among the many possible mechanisms for substrate addition, since such linear behavior is characteristic of steady state compulsory order mechanisms, of rapid equilibrium random order mechanism, and of some steady state random order mechanisms (14, 15). In the following discussion, one general class of mechanism will be shown to fit the data presented above quite easily, whereas others which also fit the dependence represented by Equation 1 either do not fit the remaining data or would do so only fortuitously.

The class of mechanism which fits Equation 1 and the remaining data in this paper, is that in which random substrate and coenzyme binding at the active site equilibrates rapidly (i.e., in times short compared to the time scale of the observable catalytic steps). The kinetic expressions for one example of this type of mechanism are illustrated under “Appendix.” No assumptions about the rate of formation of ternary complexes are necessary.

For this type of mechanism, as for the full rapid equilibrium random order mechanism and for the rapid equilibrium mechanism in which one of the enzyme-substrate binary complexes is abortive (both special cases of the type of mechanism under discussion), the dissociation constants for the binary enzyme-TPN and enzyme-L-glutamate complexes are given by φ₁₂/φ₁ and φ₁/φ₂, respectively. The values thus obtained are in agreement with those available independently from binding studies (Table II), lending support to this class of mechanism, and, further, implying that the enzyme-TPN and enzyme-L-glutamate dissociation constants obtained in binding studies are those for kinetically important complexes.

The preincubation experiments at the higher substrate concentrations also lend support to the rapid equilibration of binary enzyme-substrate complex formation. Since the burst slope is dependent on substrate concentrations, it will also be dependent on the concentrations of binary enzyme-substrate complexes at the active site. If, at the end of the stopped flow dead time, the concentrations of such complexes after preincubation differ substantially from those obtained without preincubation then the course of the early transient state kinetics will differ in the two experiments. If there is no observable experimental difference, yet preincubation has produced a substantial concentration of binary complex prior to mixing, it must be true either that roughly the same concentration of binary complex has built up rapidly after mixing in the stopped flow experiment in which there was no preincubation or that the second substrate has rapidly displaced from its

**Table II**

<table>
<thead>
<tr>
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<th>pH 6.5</th>
<th>pH 7.6</th>
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</thead>
<tbody>
<tr>
<td>Enzyme-TPN-complex dissociation constant</td>
<td>n.a.²</td>
<td>2.2 ± 1.2</td>
</tr>
<tr>
<td>φ₁₂/φ₁ from burst kinetics (mM)</td>
<td>1.2 ± 0.5</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>Limiting Michaelis constant for TPN</td>
<td>394 ± 120</td>
<td>311 ± 100</td>
</tr>
<tr>
<td>Enzyme-L-glutamate-complex dissociation constant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ₁/φ₂ from burst kinetics (mM)</td>
<td>52 ± 10</td>
<td>38 ± 8</td>
</tr>
<tr>
<td>Limiting Michaelis constant for L-glutamate</td>
<td>46 ± 10</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>φ₁/φ₂ from burst kinetics (mM)</td>
<td>15 ± 6</td>
<td>5.5 ± 2.0</td>
</tr>
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*² See References 8 and 9.
*² Not available.
*² See Reference 7.
binary complex some or all of the substrate with which the enzyme was preincubated.¹

Therefore, the lack of effect of preincubation with either substrate on the transient state kinetics leads to at least one of the following conclusions: either (a) the binding of each substrate to the enzyme to form a binary complex and all kinetically important binary complex isomerization steps (should they exist) equilibrate in times much shorter than the instrument dead time, or (b) the dissociation constant for each substrate from its kinetically important binary enzyme-substrate complexes is so large that essentially no substrate is bound during preincubation at the experimental concentrations, or both.

Since the higher values of TPN and L-glutamate concentration employed in this study are of the order of magnitude of the observed dissociation constants (Table II), a significant amount of binary enzyme-substrate complex would be formed during preincubation, and Conclusion b above is inappropriate. Thus, the formation of binary enzyme-substrate complexes must equilibrate rapidly at these concentrations, lending support to the adoption of a mechanism incorporating rapid equilibrium for initial substrate and coenzyme binding.

The other mechanisms which obey Equation 1 are not fully consistent with the evidence presented above. A steady state compulsory order mechanism would in general give only the dissociation constant for the first substrate on, not both, as reported above. In addition, one would expect for this mechanism that preincubation with the first substrate on would either produce an initial jump in absorbance during the apparatus dead time, or change the course of the observed transient state kinetics. These effects are not observed.

Steady state random order mechanisms which give apparently linear double reciprocal plots should not in general give \( \frac{\phi_2}{\phi_1} \) and \( \phi_3/\phi_2 \) values equal to the binary complex dissociation constants measured independently for more than one substrate. It is always possible, however, that the agreement of constants is accidental and that preincubation has no effect only because very little kinetically important binary complex is ever formed. In this case, the dissociation constants from spectrophotometric binding experiments would not be those of kinetic significance. On the other hand, it is highly unlikely that such a mechanism would give \( \frac{\phi_2}{\phi_1} \) fortuitously in agreement with enzyme-L-glutamate complex dissociation constants determined independently at both pH 6.5 and 7.6, when \( \phi_0, \phi_1, \phi_2, \) and \( \phi_3 \) change significantly from one pH to the other.

Another result is of interest, although no definitive conclusions about its mechanistic implication can be reached at this time. The ratios \( \phi_1/\phi_2 \) and \( \phi_3/\phi_2 \) (given in Table II) are the limiting Michaelis constants for TPN and L-glutamate, respectively, for the hypothetical but impossible experiment in which one substrate is varied while the other is present at infinite concentration. They are smaller than the corresponding binary complex dissociation constants (Table II) by a factor of three at pH 6.5 and a factor of seven at pH 7.6. These

¹ At first sight, it would seem that there is a third possible way in which no effect would be seen; that in which all concentrations reach their steady state levels during the apparatus dead time by turnover of the catalytic mechanism. It should be remembered, however, that accelerated catalytic turnover in order to reduce excess binary complex concentrations will produce an additional burst during the instrument dead time which will be observed as a difference between the actual extrapolated time zero absorbance and that calculated from the optical densities of the solutions before mixing. No such difference is observed.

APPENDIX

An example of the class of mechanism in which random substrate binding at the active site equilibrates rapidly is given in Equation 2.

\[
\begin{align*}
E & \xleftarrow{K_1} EO \\
\left[\frac{G}{K_2}\right] & \xrightarrow{k_{-3}} k_3(G) \\
EG & \xleftarrow{k_4(O)} BOG \xrightarrow{k_4} BOG' \xrightarrow{k_4} EOG
\end{align*}
\]

\( E \) represents an active site on the enzyme and \( O \) and \( G \) represent TPN and L-glutamate, respectively, as before. The double headed arrow (\( \xleftarrow{\rightarrow} \)) denotes steps which are in rapid differences are indicative of cooperativity in the binding of the second substrate to form the ternary enzyme-substrate complex. This cooperativity may arise either from direct binding interaction or from the presence of additional ternary intermediates (and apparent isomerization steps) in the burst slope mechanism, or both. In some circumstances, of course, the distinctions made here are purely semantic. Binding studies of the formation of enzyme ternary complexes with \( \alpha \)-ketoglutarate and either TPN or TPNH and with \( L \)-glutamate and TPNH also show cooperativity (3, 4, 9).

The findings of the present paper might appear to indicate a rapid equilibrium random order mechanism with modest heterotropic cooperativity in the formation of ternary enzyme-coenzyme-substrate complexes. Such is not the case.

In the first place, the intersubstrate (heterotropic) cooperativity is not necessarily small. If the quantity \( \phi_2/\phi_1 \) is greater than unity, there is an apparent cooperativity in the formation of ternary complexes. Expressions for this quantity for any given mechanism will contain factors involving rate constants from the catalytic and neighboring steps as well as ratios of binary and ternary complex dissociation constants. Only full rapid equilibrium mechanisms give the same expression as one would obtain in binding studies (see "Appendix)

In fact, the apparent cooperativity from kinetics may be considerably smaller than that which would be observed in direct binding studies. For glutamate dehydrogenase at pH 7.6, the cooperativities measured in binding studies for the formation of ternary complexes containing enzyme, TPN, and \( \alpha \)-ketoglutarate or glutamate are over 20 times larger than the apparent cooperativity reported here (9). It is quite probable that the formation of the reactive glutamate dehydrogenase-TPN-L-glutamate complex also involves a large cooperativity (8, 9) which is partially masked by the kinetics, suggesting that some steps in the mechanism following the formation of binary complexes are not in rapid equilibrium. (Note that a binding cooperativity of 200 corresponds to only about 3 kcal at 25°C.) It should be remembered, therefore, that the value of \( \phi_2/\phi_1 \) gives only a lower limit to the actual cooperativity in the formation of ternary complexes.

In addition, the oxidative deamination may yet be ordered, with one of the rapidly formed binary complexes playing the role of an abortive complex. The point is that the rapid equilibration of binary complex formation at the active site renders the question of order moot from stoichiometric considerations alone. This does not preclude, of course, the possibility that order of addition may be important mechanically.
equilibrium. Upper case $K$ represents the dissociation constant for such a step, and the lower case $k$'s are rate constants. The reverse of Step 4 and all remaining steps involving products are omitted since only initial velocities at negligible product concentrations (<1 μM) are being considered in this paper.

If the concentrations of EOG and EOG' are assumed to be in the steady state, $e/v$ obeys Equation 1 (see “Results”) with

$$
\phi_v = \frac{k_3 + k_{-3} + k_4}{k_4k_4}
$$

$$
\phi_0 = K_1R
$$

$$
\phi_1 = K_2R
$$

$$
\phi_2 = K_3R
$$

$$
\phi_3 = K_4R
$$

where

$$
R = \frac{(k_{-3} + k_{3})(k_{-3} + k_{3}) + k_4}{k_3k_3(k_{-3} + k_{3})}
$$

$$
K_1 = \frac{k_{-3}}{k_3}
$$

From the expressions above, the binary complex dissociation constants are given by $K_1 = \phi_{19}/\phi_0$ and $K_2 = \phi_{19}/\phi_1$, as noted under “Discussion.”

For the special case in which EOG is in rapid equilibrium with the binary complexes and EOG' is not (i.e. $(k_{-3} + k_{3}) \gg k_3$), only the ratio $R$ is changed.

$$
R' = \frac{k_{-3} + k_3}{k_4k_4}
$$

If EOG' is also in rapid equilibrium, then $(k_{-3} + k_{3}) \gg k_3$ and

$$
\phi_0' = \frac{1 + K_4}{k_4}
$$

$$
K'' = \frac{K_4}{k_4}
$$

where

$$
K_2 = \frac{k_{-3}}{k_4}
$$

In all cases the ratio of the binary complex dissociation constant to the limiting Michaelis constant (discussed in the text) is a measure of the apparent cooperativity. This ratio is given in general by

$$
\frac{\phi_0/\phi_1}{\phi_3/\phi_2} = \frac{Rk_1}{Rk_4}
$$

and can be quite complex. In the special case in which all steps except Step 4 are in rapid equilibrium, this expression reduces to $K_2(1 + K_{-3})/K_{-3}K_3$, the result which would be obtained in binding studies.

REFERENCES

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