The Mechanism of Activation of Glutamate Dehydrogenase-catalyzed Reactions by Two Different, Cooperatively Bound Activators*  

(Received for publication, February 2, 1973)  

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SUMMARY

The activation of NADH- and NADPH-dependent glutamate dehydrogenase-catalyzed reactions appears to be similar for two structurally different compounds, ADP and any of a series of monocarboxylic L-α-amino acids. ADP and L-leucine have identical effects on a large variety of glutamate dehydrogenase-catalyzed reactions. The transient state studies show that L-leucine increases the rate of release of NADPH from an enzyme-NADPH complex in a manner similar to that of ADP.

The dissociation constant for the glutamate dehydrogenase-NADPH complex as measured by difference spectroscopy is increased by leucine, indicating that leucine interferes with this mode of NADPH binding. It is shown that the dissociation constant for this leucine complex which interferes with a mode of NADPH binding is equal to the reported dissociation constant for the binary enzyme-leucine complex. The steady state kinetic activation constant which can be obtained by extrapolation to infinitely dilute substrate concentrations is equal to the dissociation constant of the binary enzyme-leucine complex. Since the tight binding of NADPH measured spectroscopically in the 340 nm region appears to be involved in a rate-limiting step, the mechanism of activation of α-ketoglutarate reduction by ADP and monocarboxylic L-α-amino acids may be too weak this NADPH binding mode.

Although kinetic activation data and the binding interactions with NADPH are similar for ADP and L-leucine, each activator enhances the binding of the other and, together, they have a slightly additive activation. Spectroscopic data presented in this paper indicate that the two types of activators have spatially separate binding sites on the enzyme and interact in a positive manner to enhance the binding of each other (i.e. positive cooperativity).

The dual function of the monocarboxylic L-α-amino acids as substrates (1) and activators (2) of the glutamate dehydrogenase-catalyzed reactions has been known for over a decade. The mechanism of activation by the monocarboxylic amino acids and ADP has been assumed to be the same (2). Iwatsubo and Pantaloni (3) have concluded from the biphasic nature of the transient time course of the oxidative deamination of L-glutamate that L-glutamate oxidation is limited by the rate of release of product (reduced coenzyme). They also suggested that the activation of the second phase of the reaction by ADP is due to a faster steady state release of coenzyme. The monophasic time course of the reductive amination of α-ketoglutarate indicates that the rate-determining step or steps of this reaction probably occur before the release of product.1 Cross and Fisher (4) further characterized the activation of glutamate dehydrogenase reactions and the interference of NADPH binding by ADP. It was shown that ADP reversed GTP inhibition and partially reversed L-glutamate inhibition of α-ketoglutarate reduction. No ADP activation was seen when 3-acetylpyridine adenine dinucleotide was employed as the pyridine nucleotide substrate, but ADP did reverse the GTP inhibition of the glutamate dehydrogenase-catalyzed reaction of 3-acetylpyridine adenine dinucleotide. Also, the oxidation of L-glutamate with a poor coenzyme analogue, NMN+, was inhibited by ADP. These data are consistent with the view that ADP affects the steady state rate of some glutamate dehydrogenase-catalyzed reactions by decreasing the affinity of the enzyme for NADH, NADPH, and NMN.

Recently, we have established spectrophotometrically the existence and specificity of a number of L-glutamate dehydrogenase-monocarboxylic L-α-amino acid complexes (5, 6). Preliminary evidence suggested that these complexes might be related to the activation phenomenon since the kinetic activator constant for leucine activation is approximately equal to the dissociation constant of the enzyme L-leucine complex. This paper explores the mechanism of activation by monocarboxylic L-α-amino acids and elucidates the relationship between the two structurally different activators, ADP and monocarboxylic amino acids.

EXPERIMENTAL PROCEDURE

Materials

L-Glutamate dehydrogenase, L-glutamate:NAD(P)+ oxido-reductase (deaminating), EC 1.4.1.3, was obtained from the Sigma Chemical Company as the type I ammonium sulfate sus-

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* This work was supported in part by grants from the National Institutes of Health (GM 15188) and the National Science Foundation (GB 33868X).

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pension and was prepared as previously described (5). The resultant preparation of enzyme had a ratio of 280 to 260 nm absorbance of 1.95 to 1.98 and a specific activity of 3.8 ± 0.3 μmoles of NAD⁺ reduced per min (units) per mg of enzyme assayed at 25°C in 0.2 M potassium phosphate buffer, pH 8.5, with 33.3 mM L-glutamate and 500 μM NAD⁺. The enzyme concentration was calculated from 280 nm absorbance values, obtained using a Zeiss PM Q II spectrophotometer, assuming that the extinction coefficient of a 1 mg per ml of solution of glutamate dehydrogenase is 0.95 (7).

The pyridine nucleotides and their analogues were purchased from either the Sigma Chemical Company or P-L Biochemicals. Adenosine 5'-diphosphate, guanosine 5'-triphosphate, and L-norvaline were also obtained from Sigma. L-Glutamic acid, α-ketoglutaric acid, α-ketobutyric acid, and L-α-aminobutyric acid, all M.A. grade, were obtained from Mann. Ammonium chloride and mono- and dibasic potassium phosphate were purchased from Fisher Scientific.

First 20 to 30 s of the reaction time were used for the rate measurement was calculated from 280 nm absorbance values, obtained using a Zeiss PM Q II spectrophotometer, assuming that the extinction coefficient of a 1 mg per ml of solution of glutamate dehydrogenase is 0.95 (7).

The dissociation constant for the enzyme-NADPH and enzyme-ADP complexes were obtained by computer fit of the data to Equation 1, in which Et is total enzyme concentration, Lt is total ligand concentration, K is the dissociation constant, and Δε is the difference extinction coefficient (9). The concentration of 1 mg per ml of solution of glutamate dehydrogenase was assumed to be 17.8 μM based on the peptide molecular weight of 50,100 (10). The curves shown in the reciprocal plots of ADP and NADPH binding are calculated from Equation 1 using the method of Box (11). The Δε at 340 nm was measured from maxima at 283 nm to the minima at 255 nm of the difference spectra.

**RESULTS AND DISCUSSION**

Effects of ADP and Leucine on L-Glutamate Dehydrogenase—A comparison of ADP and leucine effects on the steady state reactions and spectral complexes of bovine liver glutamate dehydrogenase is shown in Table I. ADP and leucine both activate the oxidative deamination of L-glutamate by NADP⁺ and the reductive amination of α-ketoglutarate by NADPH. They also reverse the GTP and glutamate inhibition of α-ketoglutarate reduction by NADPH. Although neither compound affects glutamate oxidation by 3-acetylpyridine adenine dinucleotide, both reverse the GTP inhibition of that reaction. Both compounds inhibit glutamate oxidation by MNM⁺ and α-ketoglutarate reduction by NMNH. Under all of the conditions cited, no leucine oxidation occurs with NADP⁺, 3-acetylpyridine adenine dinucleotide, or MNM⁺.

Leucine and ADP accelerate the enzymatic reduction of α-ketobutyrate at pH 8.4 and 9.5. However, high concentrations of leucine are inhibitory to α-ketobutyrate reduction at pH 9.5. At this pH, glutamate dehydrogenase oxidatively deaminates leucine (1) and the inhibition of α-ketobutyrate reduction at high leucine is probably due to product inhibition. Since many monocarboxylic α-amino acids have been shown to activate glutamate dehydrogenase-mediated reactions (2, 12, 13), we employed L-norvaline and L-isoleucine as activators and found that their activation effects were similar to those of L-leucine shown in Table I.

Both ADP and leucine have been shown to form binary complexes with glutamate dehydrogenase (5, 14). Cross and Fisher (4) have shown that ADP increases the dissociation constant of NADPH from the E-NADPH, E-NADPH-GTP, and E-NADPH-glutamate complexes. Leucine also increases the NADPH dissociation constant of several enzyme-reduced pyridine nucleotide complexes as calculated by changes in the difference spectra resulting from perturbation of the reduced pyridine ring of NADPH. The effect of leucine on the concentration dependence of the glutamate dehydrogenase-NADPH binding difference spectra was determined. The dissociation constant for NADPH in the absence of leucine calculated from Equation 1 is 15 ± 5 μM. Although this dissociation constant differs from the value of 58 μM reported previously from this laboratory (4), it agrees well with the dissociation constant of 15 μM obtained by DiFranco and Iwatsubo using pseudo-first order rate constants from fluorescence binding studies (15). We find that leucine increases the NADPH dissociation constant but the common intercept of a double reciprocal plot of the data shows that the Δε for NADPH binding is unaffected by leucine concentration.

The following equation represents a subcase of a binding scheme for NADPH and leucine which can be seen in Equation 2.

\[ \Delta A = \frac{K_a E_a}{K_p ER} \]

\[ \frac{E}{E + K_b} = \frac{K_a}{K_p} \]

E is free enzyme, EA is the enzyme-leucine complex, and ER is the enzyme-NADPH complex. Only the ER complex gives

\[ E + K_b \]

Comparison of ADP and leucine effects on L-glutamate dehydrogenase

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>ADP</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation of steady state kinetics</td>
<td>+e</td>
<td>+</td>
</tr>
<tr>
<td>Glutamate oxidation by NADP+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glutamate oxidation by 3-acetylpyridine adenine dinucleotide</td>
<td>-e</td>
<td>-</td>
</tr>
<tr>
<td>a-Ketoglutarate reduction by NADPH</td>
<td>+e</td>
<td>+</td>
</tr>
<tr>
<td>a-Ketoglutarate reduction by NMNH</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Reversal of GTP inhibition of a-ketoglutarate reduction by NADPH</td>
<td>+e</td>
<td>+</td>
</tr>
<tr>
<td>Partial reversal of glutamate inhibition of a-ketoglutarate reduction by NADPH</td>
<td>+e</td>
<td>+</td>
</tr>
<tr>
<td>Reversal of GTP inhibition of glutamate oxidation by 3-acetylpyridine adenine dinucleotide</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>a-Ketobutyrate reduction by NADPH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Effect on spectral complexes</td>
<td>+d</td>
<td>+e</td>
</tr>
<tr>
<td>Formation of binary complexes</td>
<td>-e</td>
<td>-</td>
</tr>
<tr>
<td>E-NADPH</td>
<td>+e</td>
<td>+</td>
</tr>
<tr>
<td>E-NADPH-GTP</td>
<td>-e</td>
<td>-</td>
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<tr>
<td>E-NADPH-glutamate</td>
<td>-e</td>
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* The effects of ADP and leucine are + for activation or enhancement of spectral signal, - for inhibition or decrease of spectral signal, and 0 for no effect.

** Activation of steady state kinetics

Glutamate oxidation by NADP+ is limited by decreasing the affinity of the enzyme complexes for NADP+. This indicates that the mechanism of activation depends on leucine concentration as seen in Equation 3. At leucine concentrations below 540 μM, the dependence of the apparent dissociation constant for NADPH, $K_N^{NADPH}$, on leucine concentration was linear (Fig. 1). The dissociation constant for leucine calculated from Equation 3 was found to be approximately 300 μM and agrees with the dissociation constant for the enzyme-leucine binary complex (5). The results appear to be consistent with the competition of NADPH and leucine for the interaction which gives rise to the difference spectra in the 340 nm region. Therefore, the leucine complex that interferes with the formation of an enzyme-NADPH complex (as measured by binding difference spectrum at 340 nm) is probably the same leucine complex which exhibits a difference spectrum at 290 nm in the absence of NADPH. For comparison, the dissociation constants for the binary enzyme-leucine complex and the leucine complex which diminishes NADPH binding can be seen in Table II.

On the basis of a few experiments, Yielding and Tomkins (2) assumed that monocarboxylic L-α-amino acids and ADP have similar mechanisms of activation. In light of the data presented in Table I, their assumption seems to be true and the following sections will compare the mechanism of activation of ADP and leucine.

Activation of Steady State Reactions of L-Glutamate Dehydrogenase—Iwatsubo and Pantaloni (3) studied the biphasic nature of the transient time course of the oxidative deamination of l-glutamate by NADP and noted the effect of ADP on the reaction. ADP inhibits the fast phase of the reaction at low glutamate concentrations and accelerates the slow phase of the reaction at high glutamate concentrations. From these results they concluded that the steady state glutamate oxidation is limited by the rate of release of product from the enzyme-reduced coenzyme complex or complexes. This interpretation was further substantiated by Fisher et al. (16) and DiFranco and Iwatsubo (17) who showed that the release of reduced coenzyme from an E-NADPH complex is the rate-determining step for glutamate oxidation.

As seen in Fig. 2, leucine increases the steady state release of reduced pyridine nucleotide from the enzyme in the same manner as does ADP. This indicates that the mechanism of activation of glutamate oxidation by ADP and leucine are the same: i.e. by decreasing the affinity of the enzyme complexes for NADPH, ADP and leucine both increase the rate of α-ketoglutarate reduction, but the monophasic time course for the reductive amination of α-ketoglutarate indicates that the rate-determining step or steps of α-ketoglutarate reduction probably occur prior to the release of products.

Relationship between Leucine Activation Phenomenon and...
Fig. 2. Effect of L-leucine on the transient state oxidation of L-glutamate by NADP+. The reaction was performed in 0.1 M potassium phosphate buffer, pH 7.6, at 25° and was monitored at 340 nm. The final concentrations in the path were 1 mg per ml of L-glutamate dehydrogenase, 50 mM L-glutamate, and 500 μM NADP+. O, absence of L-leucine; •, presence of L-leucine, 5 mM.

L-Glutamate Dehydrogenase-L-leucine Complex—In order to show that the enzyme-monocarboxylic amino acid complex detected spectrophotometrically and the enzyme-activator complex are identical, determinations of the kinetic activator constant at 0 concentration of coenzyme and substrate are necessary. Since the data presented in the previous section suggest that the mechanism of activation by leucine (and ADP) may be due to the presence of enzyme-coenzyme-leucine complex or complexes with larger NADPH dissociation constants than other enzyme-coenzyme complex or complexes, the reaction scheme used to derive the rate equation with activation includes such complexes.

The following reaction scheme can be envisioned for the activation of the reductive amination of α-ketoglutarate catalyzed by glutamate dehydrogenase in which E is free enzyme, A is activator, R is coenzyme, EA is enzyme-activator complex, ER are enzyme-coenzyme complexes without bound activator, and ERA are activator bound enzyme-coenzyme complexes. The K represents the dissociation constant for the corresponding equilibria and κ represents rate constants. Since it has been suggested that the steps prior to the slow step of the glutamate dehydrogenase reactions are rapid equilibrium random order (18), we have derived the pertinent rate equation by an equilibrium treatment. Since the rate equations are derived assuming constant α-ketoglutarate and ammonium ion concentration, the appropriate constants will contain constant concentration terms for these two substrates. Also, we have assumed that the activation of α-ketoglutarate reduction by leucine or ADP indicates that the reactivity of the ERA complex or complexes is greater than the ER complex or complexes; that is, κ_{ER} > κ_{ER}.

Using enzyme conservation and allowing the velocity of the normal (v_{0}) and activated (v_{act}) reactions to equal κ_{E} [ER] and k_{R} [ER] + k_{AR} [ERA], respectively, we can obtain the following rate equations to describe the reactions in which [Et] is the total enzyme concentration (Equations 5 and 6). The rate equation for the incremental

\[ \frac{\Delta v}{\Delta t} = \frac{v_{0} - v_{act}}{1 + \left[ \frac{R}{K_{R}} \right]} \]

The velocity in the absence of activator:

\[ v_{0} = \frac{[E][K_{R} + K_{AR}]}{1 + [R]} \]

The velocity in the presence of activator:

\[ v_{act} = \frac{[E][K_{R} + K_{AR}][A][R]}{1 + [R] + [A] + [A][R]} \]

rate, Δv, produced by activator (the rate in the presence of activator minus the normal rate) is shown in Equation 7.

The incremental rate, then:

\[ \Delta v = v_{act} - v_{0} = \frac{\Delta V_{max}}{1 + \left[ \frac{R}{K_{R}} \right] + \left[ \frac{A}{K_{A}} \right] + \left[ \frac{A[A]}{K_{AR}} \right]} \]

where

\[ \Delta V_{max} = k_{AR} \left(1 + \left[ \frac{R}{K_{R}} \right] - k_{R} \left(1 + \left[ \frac{R}{K_{R}} \right]\right) \right)[E] \]

The reciprocal form of Equation 7 can be plotted as the reciprocal of the rate increment produced by activator, 1/Δv, against the reciprocal of activator concentration. The apparent activator constant, K_{app}, can be obtained from the reciprocal plot by dividing the slope by the intercept (Equation 8). The relation between K_{app} and K_{R} is dependent on [R] and at the limit where [R] equals 0, K_{app} equals K_{A}.

\[ K_{app} = K_{A} \left(1 + \left[ \frac{R}{K_{R}} \right]\right) \]

Since the plots of incremental rate due to leucine versus leucine concentration were hyperbolic, an apparent activator constant for leucine could be determined from the reciprocal plot described above. Plots of K_{app} for leucine against NADPH concentration at several α-ketoglutarate concentrations can be seen in Fig. 3. The plots are linear and the K_{app} for leucine can be extrapolated to 317 μM at a NADPH concentration of 0. Thus, the kinetically determined dissociation constant of the activator complex is identical with the dissociation constants for the binary leucine complex. For comparison, the dissociation constants are given in Table II.

One would not expect the above plot to be linear unless the (1 + [R]/K_{AR}) term remains approximately 1 (i.e. K_{AR} > K_{R}). In a previous discussion we have shown that the dissociation constant for NADPH in the presence of leucine (K_{AR}) is larger than that of NADPH alone (K_{R}). In the concentration range of NADPH shown in Fig. 3, the ([R]/K_{AR}) term must be negligible compared with ([R]/K_{R}).

It can also be seen in Fig. 3 that in the presence of NADPH
increasing concentrations of \( \alpha \)-ketoglutarate raise the apparent activator constant for leucine. However, the addition of \( \alpha \)-ketoglutarate to the binary enzyme-l-leucine complex does not affect l-leucine binding (6). The existence of several enzyme pyridine nucleotide-substrate complexes which show heterotropic binding cooperativities have been observed (19, 20) and their pyridine nucleotide dissociation constants calculated: glutamate dehydrogenase-NADPH-\( \alpha \)-ketoglutarate complex, 0.5 \( \pm \) 0.1 \( \mu \)M; glutamate dehydrogenase-NADPH-glutamate complex, 0.6 \( \pm \) 0.1 \( \mu \)M; and glutamate dehydrogenase-NADPH+\( \alpha \)-ketoglutarate, 11.0 \( \pm \) 5.3 \( \mu \)M. The increased \( K_{\text{app}} \) for leucine at high \( \alpha \)-ketoglutarate concentrations could be due to the presence of such tight cooperative complexes during the steady state reactions. At lower \( \alpha \)-ketoglutarate concentrations, the plot approaches the simple case where \( K_{\text{app}} \) equals \( K_a \).

The activation of the steady state glutamate oxidation reaction is most likely due to an increase in the NADPH dissociation constant which accelerates the known slow step of the reaction; that is, the release of product (NADPH) from enzyme-product complex or complexes (3). Activation of the forward and reverse reactions of NADPH by decreased binding of the nicotinamide end of the pyridine nucleotide is consistent with the inhibition of the NMN(H) reaction. It has been suggested that the NMN(H) reactions are similar to the t-alanine-NADPH reactions where the formation of the Michaelis complex is rate limiting (3, 4). The inhibition by leucine is consistent with interference with NMN(H) binding in the first step of the reaction.

The present knowledge of the activation phenomenon suggests that the activation may be due either to the dissociation of slowly reacting enzyme-coenzyme complex or complexes or to the release of ligands from abortive enzyme-coenzyme complex or complexes at the active site of the enzyme. Both possibilities are consistent with our scheme in which \( k_{\alpha H} > k_R \) since \( k_R \) could be a low value or 0. However, since the kinetic leucine activation constant, the binary leucine dissociation constant, and the dissociation constant for the leucine complex which weakens the binding of NADPH are equal, the mechanism of activation by leucine appears to be one of diminishing at least one part of the NADPH binding to the enzyme so that a faster rate-limiting step compared with the initial rate-limiting step is favored. The data presented in this paper strongly suggest that the tight binding of NADPH measured by binding difference spectrum in the 340 nm region is involved in a rate-limiting step and that l-leucine (and presumably other activators) weakens this NADPH binding.
binding site (4) to exert a negative interaction on the pyridine nucleotide binding.

APPENDIX

Cooperative Binding of Two Ligands—We will assume that two ligands can bind independently, but that their binding is cooperative as seen in the following scheme (Equation 9) where \( E \) is free enzyme, \( A \) and \( B \) are ligands, \( X \) and \( Y \) are binary enzyme complexes, \( Z \) is a ternary enzyme complex, and the constants designated as \( K \) are dissociation constants. Also, we will assume that we can determine the concentration of free ligand independently. The equilibrium equation for the change in absorbance assuming that only \( X \) and \( Z \) forms of the enzyme absorb with the same difference extinction coefficient can be seen in Equation 10 where \( \Delta \varepsilon \) is the difference extinction coefficient and \( [E]_t \) is

\[
\frac{\Delta \varepsilon}{\Delta [E]} = \left( \frac{[B]}{1 + \frac{[B]}{K_y} + \frac{[A][B]}{K_y K_z}} \right) K_e + \left( \frac{[A][B]}{1 + \frac{[A]}{K_x} + \frac{[B]}{K_y} + \frac{[A][B]}{K_x K_y}} \right) K_y K_z \tag{10}
\]

The slope of this plot is \( 1/K_x \) and the intercept is \( 1/K_y \).

For the leucine-ADP case, \( K_x \) was the dissociation constant of the enzyme-leucine complex (\( X \)), \( K_y \) was the dissociation constant of the enzyme-ADP complex (\( Y \)), and \( K_z \) was the dissociation constant of leucine from the enzyme-ADP-leucine complex (\( Z \)). The assumed value for \( K_y \) was 1.0 \( \mu \)M which is larger than the dissociation constant for ADP in the presence of leucine but smaller than the normal dissociation constant for ADP in the presence of leucine.

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