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. 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 to weaken 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). 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-

* 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 resul-
tant 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 as
assayed at 25° in 0.2 M potassium phosphate buffer, pH 8.5, with
33.3 mM L-glutamate and 500 µM NADP+. The enzyme concen-
tration 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
dehydogenase 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-nor-
valine were also obtained from Sigma. L-Glutamic acid, a-keto-
glutaric 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.

Methods

Kinetic Measurements—The initial rate measurements were
made at 25° in 0.1 M potassium phosphate, pH 7.6, using a
Gilford model 2000 recording spectrophotometer on a 0 to 0.1
absorbance scale and a chart speed of 12 inches per min. All
reactions were initiated by addition of coenzyme and only the
first 20 to 30 s of the reaction time were used for the rate meas-
urments (except for the slow NMNH reactions). The velocities
are reported as micromoles of coenzyme reacted per min per unit
of glutamate dehydrogenase. The initial rates were measured
at 340 nm for NADP+ and NADPH, at 363 nm for 3-acetyl-
pyridine adenine dinucleotide, and at 333 nm for NMNH. The
reaction mixtures used to investigate leucine activation con-
tained either 8.3 mM α-ketoglutarate, 33.3 mM NH4Cl, and 33
µM NADPH or 33.3 mM L-glutamate and 500 µM NADP+. The
reactions using 3-acetylpyridine adenine dinucleotide and
NMNH used 33.3 mM L-glutamate and 425 µM 3-acetylpyridine
adenine dinucleotide or 100 mM NH4Cl and 8.3 mM α-ketoglu-
tarate, respectively. The kinetic data were analyzed as recip-
rocal plots employing a weighted linear regression performed on a
Wang 700 calculator (8).

Spectral Binding Studies—All spectral binding studies were
performed in 1.000-cm quartz cuvets at 20° using 0.1 M potassium
phosphate, pH 7.6. The binding difference spectra for leucine,
ADP, and NADPH were obtained using a Cary model 14 double
beam spectrophotometer linked to a Varian 620i computer as
previously described (5, 6). The difference absorbance values
were collected at 0.5-nm intervals and each value represented an
average of 100 analogue to digital conversions.

The dissociation constants for the enzyme-NADPH and en-
zyme-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,

\[ \Delta A = \Delta e \left[ \frac{(K + E_t + L_t) - \sqrt{(K + E_t + L_t)^2 - 4KL_1E_t}}{2} \right] \]  (1)

K is the dissociation constant, and Δe is the difference extinction
coefficient (9). The concentration of a 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
ΔA at 340 nm was measured from maxima at 382 nm to the
minima at 334 nm and the ΔA at 283 to 255 nm was measured
from maxima at 283 nm to the minima at 255 nm of the differ-
ence spectra.

Stopped Flow Experiments—Transient state measurements
were carried out on a Durrum-Gibson stopped flow spectro-
photometer interfaced to a Varian 620i digital computer. The
data points presented represent an average of at least seven ex-
periments. The stopped flow cuvette path length was 1.7 cm and
the instrument dead time was 3 ms.

Results and Discussion

Effects of ADP and Leucine on L-Glutamate Dehydrogenase—A
comparison of ADP and leucine effects on the steady state reac-
tions and spectral complexes of bovine liver glutamate dehy-
rogenase 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 com-
pounds inhibit glutamate oxidation by NMN+ and α-ketoglu-
tarate reduction by NMNH. Under all of the conditions cited,
no leucine oxidation occurs with NADP+, 3-acetylpyridine ade-
nine dinucleotide, or NMN+.

L-Leucine and ADP accelerate the enzymatic reduction of
α-ketobutyrate at pH 8.4 and 9.5. However, high concentra-
tions of leucine are inhibitory to α-ketobutyrate reduction at
pH 9.5. At this pH, glutamate dehydrogenase oxidatively de-
aminates leucine (1) and the inhibition of α-ketobutyrate reduc-
tion at high leucine is probably due to product inhibition. Since
many monocarboxylic α-α-amino acids have been shown to ac-
tivate 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 com-
plexes 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 pyri-
dine nucleotide complexes as measured by changes in the differ-
ence 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 ΔAmax 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.

\[ EA \xrightleftharpoons{K_A}{K_B} E \xrightleftharpoons{K_R}{ER} \]

(2)

E is free enzyme, EA is the enzyme-leucine complex, and ER is
the enzyme-NADPH complex. Only the ER complex gives
below 540 μM, the dependence of the apparent dissociation constant for NADPH, $K_{NADPH}^{app}$, 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 which 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 prevents NADPH binding, and kinetic-activating leucine complex of glutamate dehydrogenase.

### Table I

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>ADP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Leucine&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Activation of steady state kinetics&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+</td>
</tr>
<tr>
<td>Glutamate oxidation by NADP&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Glutamate oxidation by 3-acetylpyridine</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
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<tr>
<td>Glutamate oxidation by NMN&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0</td>
</tr>
<tr>
<td>α-Ketoglutarate reduction by NADPH&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
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<tr>
<td>α-Ketoglutarate reduction by NMNH&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Partial reversal of glutamate inhibition of α-ketoglutarate reduction by NADPH</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Reversal of GTP inhibition of glutamate oxidation by 3-acetylpyridine adenine dinucleotide</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>α-Ketobutyrate reduction by NADPH&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
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</table>

<sup>a</sup>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.

<sup>b</sup> All experiments were performed at pH 7.6 except α-ketobutyrate experiments which were run at pH 8.4 and 9.5 (“Methods”).

<sup>c</sup> This effect was determined by the change of the enzyme-NADPH difference spectra in the 340 nm region (4).

<sup>d</sup> Reference 5.

<sup>e</sup> Reference 4.

<sup>f</sup> Reference 14.

<sup>g</sup> Reference 4.

<sup>h</sup> Reference 5.

### Table II

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Leucine dissociation constant&lt;sup&gt;a&lt;/sup&gt; µM</th>
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<tr>
<td>Spectrophotometric binary complex</td>
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<tr>
<td>Complex inhibiting NADPH binding</td>
<td>300</td>
</tr>
<tr>
<td>Kinetic activator constant</td>
<td>317</td>
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</table>

<sup>a</sup>Reference 5.

### Fig. 1

Relationship between the apparent dissociation constant for NADPH determined spectrophotometrically and L-leucine concentration.

#### Figure 1

**Dissociation constants for binary leucine complex, leucine complex which presents NADPH binding, and kinetic-activating leucine complex of glutamate dehydrogenase**

### Table III

<table>
<thead>
<tr>
<th>Glutamate dehydrogenase-L-leucine complex</th>
<th>Leucine dissociation constant&lt;sup&gt;a&lt;/sup&gt; µM</th>
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<tbody>
<tr>
<td>Spectrophotometric binary complex</td>
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</tbody>
</table>

<sup>a</sup>Reference 5.

### 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 L-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 oxidative deamination 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 NADPH. 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 300 μM NADPH. ○, absence of L-leucine; , presence of L-leucine, 0.01 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 constants for the corresponding equilibria and k 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 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, $k_{AR} > k_R$.

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

The velocity in the absence of activator:

$$v_b = \frac{k_E[E]}{1 + \frac{[R]}{K_R}}$$

The velocity in the presence of activator:

$$v_{act} = \frac{k_E[E] + k_{AR}[A][R]}{1 + \frac{[R]}{K_R} + \frac{[A]}{K_A} + \frac{[A][R]}{K_AR}}$$

where

$$\Delta v = \frac{AV_{max}}{1 + \frac{[R]}{K_R} + \frac{[A]}{K_A} + \frac{[A][R]}{K_AR}}$$

The incremental rate, then:

$$\Delta v - v_b = \frac{AV_{max}}{1 + \frac{[R]}{K_R} + \frac{[A]}{K_A} + \frac{[A][R]}{K_AR}}$$

The reciprocal form of Equation 7 can be plotted as the reciprocal of the rate increment produced by activator, $1/\Delta v$, 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 $K_A$ is dependent on $[R]$ and at the limit where $[R]$ equals 0, $K_{app}$ equals $K_A$.

$$K_{app} = \frac{K_A}{1 + \frac{[A]}{K_AR}}$$

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 kinetic 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 $(1 + [R]/K_A)$ term remains approximately 1 (i.e., $K_AR > K_A$). 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_A). In the concentration range of NADPH shown in Fig. 3, the $([R]/K_A)$ term must be negligible compared with $(([R]/K_A))$.

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-NADP\(^+\)-\( \alpha \)-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 \( \text{NMN(H)} \) reaction. It has been suggested that the \( \text{NMN(H)} \) reactions are similar to the \( \text{t-alanine-NADPH} \) reactions where the formation of the Michaelis complex is rate limiting (3, 4). The inhibition by leucine is consistent with interference with \( \text{NMN(H)} \) 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 \( k_{\text{ass}} > k_E \) since \( k_E \) 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 enzyme 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.

**Fig. 3.** Relationship between the kinetic activator constant for l-leucine and NADPH concentration. The enzyme concentration used was 7.0 \( \mu \)g per ml. The curves shown are for 8.3 \text{nM} (O), 4.9 \text{mM} (\Delta), and 1.8 \text{mM} \( \alpha \)-ketoglutarate (\( \Theta \)).

**Fig. 4.** Effect of l-leucine on the glutamate dehydrogenase-ADP binding difference spectra. The enzyme concentration was 0.23 mg per ml. The curves shown are for the absence (O) and presence of 1.0 mM l-leucine (\( \Theta \)).

**Distinction between Leucine and ADP Activation**—It must be noted at this point that the data presented in Table I are nearly identical for the two activators of glutamate dehydrogenase-catalyzed reactions, ADP and monocarboxylic \( \text{t-\alpha-amino acids} \) (4). The fact that two such structurally different compounds cause very similar effects is surprising. Since the maximal percentage relative velocities for leucine and ADP are different (170\% and 220\%, respectively), the binding interactions of these activators can be investigated kinetically. The activation of \( \alpha \)-ketoglutarate reduction by ADP is increased slightly to 250\% relative velocity in the presence of saturating concentrations of leucine and the concentration of ADP required to give half-maximal activation is decreased. A similar, but less pronounced, effect of saturating ADP concentrations on leucine activation can be seen. These data suggest that the two activators enhance each other's binding and maximal activation.

Spectrophotometric ADP binding studies in the presence and absence of leucine can be seen in Fig. 4. The dissociation constant for ADP calculated from Equation 1 was shown to decrease from 1.9 \( \mu \text{M} \) in the absence of leucine to 0.9 \( \mu \text{M} \) in the presence of 1 \text{mM} leucine.

A similar decrease (4-fold) in the dissociation constant of leucine can be seen in the presence of ADP (Fig. 5). This effect of ADP on leucine binding was analyzed using an expression derived for the cooperative binding of two ligands to an enzyme (see “Appendix”). The appropriate plot allows one to evaluate a dissociation constant for the enzyme-leucine complex of 75 \( \mu \text{M} \) in the presence of saturating ADP (Fig. 6). The \( K_{\text{D,leucine}} \) at 0 concentration of ADP determined from the plot is approximately equal to the value corresponding to the dissociation constant of the enzyme-leucine complex. The linearity of the data when plotted in this manner shows that this type of binding mechanism is valid for ADP and leucine.

The cooperative binding of these activators indicates that they do not occupy the same space on the enzyme active site but do interact in a positive manner to enhance the binding of each other. Since the binding is cooperative and the effects of the activators similar, the respective binding sites may be in close proximity and may be near the amide subsite of the nicotinamide.
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, and X and Y are binary enzyme complexes, while 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 \( \Delta A \) is the difference extinction coefficient and \([E]_i\) is

\[
\frac{\Delta A}{[E]_i} = \frac{[E]}{\left(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[A][B]}{K_A K_B}\right) K_s} + \frac{[A][B]}{\left(1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[A][B]}{K_A K_B}\right) K_s} K_s K_t
\]

(10)

The slope of this plot is \(1/K_s\) and the intercept is \(1/K_t\).

For the leucine-ADP case, \(K_s\) was the dissociation constant of the enzyme-leucine complex (X), \(K_t\) was the dissociation constant of the enzyme-ADP complex (Y), and \(K_s\) was the dissociation constant of leucine from the enzyme-ADP-leucine complex (Z). The assumed value for \(K_t\) 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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