The Dependence of the Substrate Specificity on the Conformation of Crystalline Glutamate Dehydrogenase*

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Glutamate dehydrogenase (L-glutamate-NAD(P) oxidoreductase EC 1.4.1.3) from bovine liver catalyzes the reversible oxidative deamination of various monocarboxylic amino acids, as well as of L-glutamate (1, 2). Several years ago, we reported that estrogenic steroids both inhibited the oxidation of L-glutamate (3) and stimulated the oxidation of L-alanine (4). Conversely, adenosine diphosphate was found to stimulate the glutamate dehydrogenase reaction (5) (and prevent its inhibition by the steroids (3)), and to inhibit the alanine dehydrogenase reaction (4). The glutamate and alanine activities are reciprocally affected under other experimental conditions as well. It was, therefore, concluded that different conformations of the enzyme catalyzed each of the two different reactions (4). The equilibrium between the forms was thought to be influenced by regulator molecules such as the steroids or adenosine diphosphate. At that time, it was known that the enzyme could undergo a reversible concentration-dependent aggregation reaction (6). This prompted Frieden to propose (5) that only the most highly aggregated form of the enzyme was active for the glutamate dehydrogenase reaction while the disaggregated molecule (s~o or 12.5) was enzymically inactive. Under our experimental conditions, reagents which favored disaggregation also inhibited the glutamate dehydrogenase and stimulated the alanine dehydrogenase reaction (4). The glutamate and alanine activities are reciprocally affected under other experimental conditions as well.

According to this scheme, at low protein concentrations, the addition of ADP shifts Equilibrium II to favor Form y which stimulates the glutamate dehydrogenase reaction. The steroids, GTP, etc. favor Form y, inhibiting glutamate and stimulating alanine dehydrogenase. When the concentration of protein is high enough that Equilibrium I also becomes significant, then a shift in Equilibrium II toward Form y also decreases the state of aggregation of the system. Additional evidence in support of these relationships has recently been presented (10, 11).

In the present communication, the kinetic properties of Monomers x and y are compared, and it is shown that Form y is more active than Form x, not only for the alanine dehydrogenase reaction, but also for the oxidation of other monocarboxylic L-amino acids. As required by the model, activation of monocarboxylic amino acid dehydrogenase activity by a shift in Equilibrium II toward Form y is accompanied by simultaneous loss of glutamate dehydrogenase activity, and vice versa, indicating that the substrate specificity of the enzyme is determined by the position of Equilibrium II.

MATERIALS AND METHODS

Bovine liver glutamate dehydrogenase was purchased from the Sigma Chemical Company as a suspension of crystals in ammonium sulfate. The suspension was usually centrifuged before use, and the crystals were redissolved in the appropriate buffer. The enzyme solution was equilibrated with the buffer either by gel filtration through Sephadex G-25 or by dialysis, except where indicated otherwise.

Enzyme concentrations were determined spectrophotometrically on the basis of an E280 of 10.0 (6).

The other reagents used, also obtained from commercial sources, were of the highest purity available.

Enzyme assays were carried out at room temperature in quartz
FIG. 1. The influence of ADP on the glutamate and alanine dehydrogenase activities of crystalline glutamate dehydrogenase. The reaction mixtures all contained, in a volume of 1.0 ml: NH₄Cl, 0.1 m; DPNH, 6.0 × 10⁻⁵ m; EDTA, 1.0 × 10⁻⁴ m; and Tris-HCl, pH 8.6, 0.1 m. For the glutamate dehydrogenase reaction, in addition there was α-ketoglutarate, 0.025 m, and enzyme, 0.00142 mg. For the alanine reaction, there was pyruvate, 0.025 m, and enzyme, 0.0142 mg. The concentration of ADP was varied as shown.

Cuvettes with a 1.0-cm light path. Changes in the optical density were recorded automatically by a Gilford model 2000 multiple sample absorbance recorder. For assays of the rate of reductive amination of the α-keto acids, initial rates of the decrease in absorbance of the reduced pyridine nucleotides at 340 nm were used.

For amino acid oxidative deamination, a coupled assay was used involving transfer of electrons from DPNH to p-iodonitrotetrazolium via phenazine methosulfate, as described by Nachlas et al. (12). This assay was preferable to the direct assay of pyridine nucleotide reduction since the equilibrium of the amino acid oxidation reaction is relatively unfavorable unless the reduced pyridine nucleotide is reoxidized by the dyes. Comparison of initial rates by both methods (DPN and dye reduction) in terms of substrate oxidized per unit of time gave identical results. The molar absorbance of reduced p-iodonitrotetrazolium at 520 nm was found to be 15.4 × 10⁴ under these conditions, while that for DPNH was taken as 6.22 × 10³. Diethylstilbestrol was added to the reaction mixtures either in ethanol or propylene glycol, and the same solvents were added to control mixtures.

RESULTS

At low enzyme concentrations, where primarily only the two monomeric forms, z and y, exist, reagents which influence the equilibrium between them alter the catalytic properties of the protein. The effects of ADP on the alanine and glutamate dehydrogenase reactions, observed under identical conditions (except for the substrate), are shown in Fig. 1. Clearly, the two activities are reciprocally related, since as alanine dehydrogenase activity was decreased, there was a parallel increase in glutamate dehydrogenase activity.

GTP has the opposite effect from ADP on Equilibrium II (8–10). Therefore, stimulation of the alanine and inhibition of glutamate dehydrogenase should result as the GTP concentration is increased. The data in Fig. 2 show that the effects of GTP on the two activities are exactly reciprocal.

Fig. 3 shows data for similar experiments with diethylstilbestrol as the modifier. The steroid analogue, like GTP, stimulated alanine dehydrogenase and simultaneously inhibited the glutamate reaction.

These experiments show, as suggested earlier (4), that optimal glutamate dehydrogenase activity resides in a different conformation of the enzyme than optimal alanine dehydrogenase activity. Since the enzyme concentrations used in the present experiments were quite low, aggregation of the monomers (Equilibrium I) did not figure in the shift from one conformation to another, and the data are presently interpreted in terms of a change in conformation rather than of molecular weight, as was previously thought (4).

Since glutamate dehydrogenase has been reported to catalyze the oxidation of other monocarboxylic amino acids (1, 2), the effects of modifiers on these substrates were also tested. Fig. 4A shows an experiment comparing L-α-aminobutyrate and L-glutamate as substrates, with ADP as the effector. ADP inhibited the oxidation of α-aminobutyrate and stimulated that of glutamate. Fig. 4B shows a comparable experiment with

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GLUTAMATE DEHYDROGENASE REACTION

[D] \( \rightarrow \) [G] (Diethylstilbestrol)

FIG. 3. Effects of diethylstilbestrol on L-glutamate and alanine dehydrogenase activities of glutamic dehydrogenase. Reaction mixture for L-glutamate activity contained 0.022 M Tris, pH 8.12; 1.0 mM EDTA; 0.1 mM NH₄Cl; 20 mM α-ketoglutarate; enzyme (0.0016 mg per ml); 1% propylene glycol; and diethylstilbestrol as shown. The mixture for determining alanine dehydrogenase activity contained 5.0 \( \times \) 10⁻³ M pyruvate in lieu of α-ketoglutarate, and the enzyme concentration was 0.010 mg per ml.

FIG. 4. A, effects of ADP on α-aminobutyrate and L-glutamate dehydrogenase activities of glutamic dehydrogenase. Reaction mixtures contained 0.022 M Tris-HCl buffer, pH 8.20, 2.0 \( \times \) 10⁻⁴ M DPN, 1.0 mM EDTA, 0.018% gelatin, 0.4 mg per ml of p-iodonitrotetrazolium, 0.07 mg per ml of phenazine methosulfate, enzyme, glutamate, the oxidation of which was inhibited, and α-aminobutyrate whose oxidation was stimulated. Therefore, α-aminobutyrate acted like alanine in response to effector molecules.

The oxidation of both L-leucine and L-methionine was also stimulated by GTP and diethylstilbestrol, and inhibited by ADP, like that of alanine and α-aminobutyrate.

It therefore seems that the “alanine” conformation of the enzyme (y) has enhanced activity for other monocarboxylic amino acids as well. To examine this further, the rates of oxidation of the amino acid substrates catalyzed by the different conformations of the enzyme are given in Table I. As expected, glutamate oxidation was inhibited by GTP and stimulated by ADP when either DPN or TPN was the hydrogen acceptor. Conversely, the oxidation of the monocarboxylic L-amino acids was, in each case, stimulated by GTP and inhibited by ADP, again independent of the pyridine nucleotide used. The rates in the absence of effectors are also given for comparison.

Recently, it has been found that treatment of glutamate dehydrogenase with mercurials stimulates glutamate oxidation, inhibits that of alanine, and desensitizes the enzyme to effector reagents (11). In Table II the activity of the mercurial-treated enzyme for the oxidation of alanine and other monocarboxylic amino acids is also given for comparison.
The effect of ADP and GTP on the glutamate and monocarboxylic acid dehydrogenase activities of glutamic dehydrogenase. Reaction conditions were as in Fig. 4B. DPN and TPN concentrations were 2.0 × 10⁻⁴ M, as were the concentrations of ADP and GTP.

### Table I

**Effect of modifiers on glutamate and monocarboxylic acid dehydrogenase activities**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Control</th>
<th>ADP</th>
<th>GTP</th>
<th>None</th>
<th>ADP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔOD.340/min × 10⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>3.6</td>
<td>674</td>
<td>735</td>
<td>74.6</td>
<td>329</td>
<td>418</td>
<td>29.5</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>3.6</td>
<td>2.05</td>
<td>1.72</td>
<td>17.3</td>
<td>7.5</td>
<td>2.69</td>
<td>22.3</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>3.6</td>
<td>5.21</td>
<td>3.00</td>
<td>19.9</td>
<td>7.5</td>
<td>2.69</td>
<td>22.3</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>3.6</td>
<td>4.32</td>
<td>2.50</td>
<td>23.2</td>
<td>4.33</td>
<td>1.62</td>
<td>19.6</td>
</tr>
<tr>
<td>L-Valine</td>
<td>3.6</td>
<td>10.2</td>
<td>3.33</td>
<td>35.7</td>
<td>14.3</td>
<td>3.53</td>
<td>29.6</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>3.6</td>
<td>7.26</td>
<td>2.34</td>
<td>42.8</td>
<td>11.6</td>
<td>3.05</td>
<td>35.0</td>
</tr>
<tr>
<td>DL-Norvaline</td>
<td>7.2</td>
<td>69.7</td>
<td>23.3</td>
<td>96.3</td>
<td>74.2</td>
<td>19.9</td>
<td>91.7</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>1.8</td>
<td>8.72</td>
<td>3.70</td>
<td>29.3</td>
<td>9.73</td>
<td>3.50</td>
<td>24.5</td>
</tr>
<tr>
<td>L-α-Amino- butyrate</td>
<td>2.4</td>
<td>6.12</td>
<td>4.91</td>
<td>22.9</td>
<td>3.73</td>
<td>3.02</td>
<td>10.3</td>
</tr>
</tbody>
</table>

### Table II

**Effect of CH₃HgOH on oxidation of amino acids by crystalline glutamate dehydrogenase**

The reaction mixture contained in a volume of 10 ml: Tris-HCl buffer, pH 8.9, 0.05 M; amino acid, 0.1 M; DPN, 5.0 × 10⁻⁴ M; NaCl, 0.05 M; enzyme, 0.02 mg (except for experiment with glutamate oxidation where it was 0.002 mg). The mercurial-treated enzyme was prepared by incubation with CH₃HgOH, 5.0 × 10⁻⁴ M at 25°C for 6 mins in Tris-HCl containing NaCl, 0.05 M; enzyme, 0.02 mg (except for experiment with glutamate oxidation where it was 0.002 mg), and then diluted 1:5 to stop the mercuration reaction. Control enzyme was incubated in a similar medium not containing mercurial.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>+CH₃HgOH</th>
<th>Control</th>
<th>ΔOD.340/min</th>
<th>Change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>0.004</td>
<td>0.021</td>
<td>-81</td>
<td></td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.008</td>
<td>0.038</td>
<td>-79</td>
<td></td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>0.007</td>
<td>0.038</td>
<td>-82</td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.000</td>
<td>0.053</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.011</td>
<td>0.074</td>
<td>-82</td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.017</td>
<td>0.086</td>
<td>-81</td>
<td></td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>0.040</td>
<td>0.244</td>
<td>-83</td>
<td></td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>0.480</td>
<td>0.240</td>
<td>+100</td>
<td></td>
</tr>
</tbody>
</table>

For the other monocarboxylic acids, while the oxidation of glutamate is principally catalyzed by a different conformation of the protein.

It should be emphasized that the values presented in Tables I and II apply only to the specific assay conditions used, since they are not extrapolated Vₘₐₓ values, and since the extent of inhibition or stimulation of the amino acid dehydrogenase reactions by the regulator molecules depends critically on the substrate concentrations used as well as pH, ionic strength, etc.

The following experiments show the alterations in the kinetics of the alanine dehydrogenase reaction induced by several modifier reagents. Fig. 5 illustrates the effects of ADP, GTP, and diethylstilbestrol on the kinetics of alanine oxidation. The data are plotted as reciprocal of the initial velocities against reciprocal alanine concentration. Under these conditions, the Kₘ for alanine in the absence of effector reagents was 5.0 × 10⁻⁴ M. On the addition of 3.0 × 10⁻⁴ M ADP, there was no significant change in the Kₘ, but the Vₘₐₓ was decreased by 50%. ADP, therefore, inhibits the reaction independently of alanine concentration.

In the presence of diethylstilbestrol, the Kₘ for alanine was decreased by a factor of 5 while GTP caused a decrease to 3.5 × 10⁻⁴ M. However, the Vₘₐₓ for alanine with either GTP or diethylstilbestrol was also considerably decreased. Therefore, at high substrate concentrations, GTP or the steroid analogue would be expected to inhibit alanine oxidation, judging from a linear extrapolation of the data. However, inhibition should not occur below 0.2 M alanine, while at lower alanine concentrations, these reagents stimulate the reaction.

ADP (Fig. 6) lowered the Kₘ for DPN and decreased the Vₘₐₓ thereby acting as an "uncompetitive" inhibitor of the reaction. GTP (not shown) under similar conditions decreased the Kₘ for DPN by 16-fold and lowered the Vₘₐₓ by a factor of 2.3. Diethylstilbestrol also decreased the Kₘ for DPN by more than 10-fold and lowered the Vₘₐₓ by a factor of about 3. Therefore, amino acids are compared with its activity toward glutamate. The period of pretreatment of the protein with CH₃Hg⁺ was sufficient to convert it almost completely to the desensitized form. The rates of amino acid oxidation catalyzed by the mercurial-treated enzyme are shown in the first column. The control rates are given in the second column.

Like alanine, the activities of the other monocarboxylic substrates were also inhibited with the mercurial-treated enzyme, and to the same extent as that of alanine. As expected, the rate of glutamate oxidation was increased under identical conditions. The results indicate that the same form of the enzyme which is maximally active for alanine oxidation is likewise active.
ADP always inhibits the reaction while GTP and diethylstilbestrol stimulate at low but inhibit at high DPN concentrations.

Similar "crossover" was seen in the reverse reaction when NH₄⁺ concentration was varied. Both GTP and diethylstilbestrol decreased the $K_m$ for NH₄⁺ but also lowered the $V_{max}$, whereas ADP again acted only as an inhibitor by decreasing the $V_{max}$. In these experiments, GTP and stilbestrol changed from activators to inhibitors depending on the concentration of NH₄⁺.

However, at suboptimal concentrations of DPNH and pyruvate, GTP and diethylstilbestrol stimulated at all concentrations of NH₄⁺.

![Figure 6](image6.png)

**Figure 6.** The influence of modifiers on the alanine dehydrogenase reaction. The reactions were carried out as described in Fig. 5 except that the alanine concentration was fixed at 0.2 M, and the DPN concentration was varied as indicated. The ADP concentration was 3.0 x 10⁻⁴ M and that of diethylstilbestrol was 1.0 x 10⁻⁴ M.

![Figure 7](image7.png)

**Figure 7.** The influence of modifiers on the reductive amination of pyruvate. The reaction mixtures contained, in 1.0 ml: Tris-HCl, pH 8.4, 0.05 M; EDTA, 1.0 x 10⁻⁴ M; NH₄Cl, 0.2 M; sodium pyruvate, 0.05 M; enzyme, 0.0405 mg; and DPNH as indicated. Where noted the concentration of ADP was 3.0 x 10⁻⁴ M, and that of GTP was 1.0 x 10⁻⁴ M.

![Figure 8](image8.png)

**Figure 8.** The influence of modifiers on the reductive amination of pyruvate. The reaction mixture was as described in Fig. 7 except that the DPNH concentration was fixed at 3.0 x 10⁻⁴ M and the pyruvate concentration was varied as indicated.

![Figure 9](image9.png)

**Figure 5.** The influence of modifiers on the alanine dehydrogenase reaction. The reactions were carried out as described in Fig. 5 except that the alanine concentration was fixed at 0.2 M, and the DPN concentration was varied as indicated. The ADP concentration was 3.0 x 10⁻⁴ M and that of diethylstilbestrol was 1.0 x 10⁻⁴ M.

**DISCUSSION**

With crystalline glutamate dehydrogenase, GTP and diethylstilbestrol stimulate the oxidation of the following monocarboxylic amino acids: alanine, leucine, isoleucine, methionine, valine, norvaline, norleucine, and $\alpha$-aminobutyrate. ADP inhibits these oxidations. Under identical conditions, the oxidation of L-glutamate was inhibited by GTP and diethylstilbestrol, and stimulated by ADP.

The stimulatory or inhibitory effects on the rates of glutamate and monocarboxylic amino acid oxidation were quantitatively reciprocal, indicating that the effects on the glutamate dehydrogenase reaction occur by the same mechanism as the reciprocal effects on monocarboxylic amino acid dehydrogenase activity and that optimal catalytic activity for the glutamate and for the monocarboxylic reactions resides in different conformations of the enzyme. This conclusion is also supported by the
findings that the two types of catalytic activity responded oppositely to treatment of the protein with mercurial. That is, glutamate activity was stimulated and monocarboxylic acid activity was inhibited under the same conditions. Accordingly, Form z can be assigned to have optimal glutamate activity and Form y, optimal monocarboxylic acid activity. Since neither of these forms is absolutely specific for a given substrate, the experimental conditions determine the quantitative response of the monocarboxylic amino acid dehydrogenase reaction to modifier reagents.

Monomer z has a greater tendency to aggregate than Monomer y, which implies that these forms are structurally different from one another. Several other lines of evidence suggest the same thing. For instance, different interconvertible antigenic forms of glutamate dehydrogenase have been found, the proportions of which can be determined by modifier reagents (13, 14). More recent studies (10) show that the rate of denaturation of the enzyme is also influenced by effector reagents in such a way that Form z appears to be more stable in solution than Form y.

In any case, the representation of the glutamate dehydrogenase system as an equilibrium between only three forms is certainly oversimplified because the kinetic properties of the y form induced by GTP are different from those induced by diethylstilbestrol. Furthermore, in Equation 1 no account is taken of the requirement for DPNH in the structural effects of GTP or diethylstilbestrol (9, 15). Finally, intermediate states of association between aggregate and the monomer probably exist but were not represented.

The biological significance of the induced alterations in the substrate specificity of glutamate dehydrogenase is not yet clear. Recent proposals concerning the consequences of the regulation of glutamate dehydrogenase activity by purine nucleotides (16) did not take into account the monocarboxylic amino acid substrates, presumably because of their low substrate activity compared with glutamate (under conditions where the enzyme was largely in Form z). However, Kun, Aylng, and Baltimore (17) have suggested that the alanine dehydrogenase activity of the enzyme actually limits the rate of glutamate dehydrogenase activity of the enzyme. This regulation of enzyme aggregation could play as important a role, for example, in transport or osmotic pressure regulation, as well as regulation of the catalytic activity itself.

SUMMARY

1. Guanosine triphosphate and diethylstilbestrol inhibit the glutamate dehydrogenase reaction catalyzed by the crystalline enzyme from beef liver. Under identical conditions, these reagents stimulate a monocarboxylic L-amino acid dehydrogenase reaction with alanine, valine, leucine, isoleucine, methionine, \( \alpha \)-aminobutyrate, norvaline, and norleucine.
2. Adenosine diphosphate stimulates glutamate oxidation and reciprocally inhibits the monocarboxylic amino acid dehydrogenase reaction.
3. Treatment of the enzyme with methylmercuric hydroxide causes a stimulation of glutamate dehydrogenase and an inhibition of monocarboxylic amino acid dehydrogenase activity.
4. Studies on the effects of modifiers on the kinetics of the alanine dehydrogenase reaction are presented.
5. The data are discussed in terms of equilibrium between different forms of the enzyme which have different relative substrate specificities. The position of this equilibrium is thought to be influenced by modifiers.

REFERENCES

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