The Effect of Allosteric Modifiers on the Rate of Denaturation of Glutamate Dehydrogenase

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Both the structure and catalytic properties of crystalline bovine l-glutamate dehydrogenase (l-glutamate: NAD(P)+ oxido-reductase (deaminating), EC 1.4.1.3) are altered by a variety of small molecules ("allosteric" modifiers) (1-7). Recently a scheme has been proposed relating these changes in structure and activity (8-10) which may be summarized as follows.

I

Polymer ⇌ monomer x ⇌ monomer y

In this scheme monomer x catalyzes the glutamate dehydrogenase reaction (although this form of the enzyme also has alanine dehydrogenase activity) and aggregates to form higher molecular weight polymers as its concentration is increased. Monomer y has increased alanine dehydrogenase activity and little or no glutamate dehydrogenase activity, and does not aggregate readily at higher protein concentrations. Thus, alterations in monomer conformation (Equilibrium II) affect the distribution of all the species and result in changes in the catalytic properties of the protein. Previous studies on the effects of various reagents and conditions on the inactivation of glutamate dehydrogenase have appeared (11-13).

In the present experiments it has been found that in solutions of low ionic strength the enzyme undergoes an apparently irreversible denaturation reaction, the rate of which has been followed by three independent methods: (a) exposure of the previously unreactive -SH groups, (b) a fall of about 40% in the intrinsic fluorescence of the protein, and (c) loss of both glutamate and alanine dehydrogenase activities. When measured under identical conditions, these parameters all followed the same first order kinetics.

It has also been found that reagents which alter Equilibrium II in favor of form y accelerate this reaction. It is suggested, therefore, that at low ionic strength monomer y unfolds more readily than does monomer x.

EXPERIMENTAL PROCEDURE

Glutamate dehydrogenase was obtained from Sigma Chemical Company as either a crystalline suspension in (NH₄)₂SO₄ or a solution in 50% glycerol and 0.01 M phosphate buffer. Nucleotides were likewise purchased from Sigma; l-glutamate and d- and l-leucine from Nutritional Biochemicals Corporation; sodium dodecyl sulfate from Matheson, Coleman, and Bell, Inc.; guanine-HCl and DTN² from Aldrich Chemical Company, Inc.; and diethylstilbestrol from Mann Research Laboratories, Inc.

The enzyme suspension was centrifuged at 5000 × g for 20 minutes and the sedimented crystals were dissolved in 0.05 M Tris-HCl, pH 7.05. In experiments in which the protein concentration was varied, enzyme in glycerol solution was dialyzed at 5° for 12 hours against multiple changes of 0.05 M Tris-HCl, pH 7.05, containing 0.05 M NaCl, following which the protein solution was clarified by centrifugation. Enzyme concentration was calculated from the optical density of the solutions at 280 μm, based on an extinction coefficient of 1.0 × 10⁶ M⁻¹ cm⁻¹ (14).

The appearance of -SH groups was measured by observing the increase in optical density at 412 μm resulting from the liberation of the sulfanion of thionitrobenzoate from DTN (15). The reaction was followed in the Cary model 14 recording spectrophotometer with the use of cells with a 10-cm light path. Full scale deflection was equal to 0.2 optical density unit. The temperature of the cuvette compartment and the reagents was maintained at 24° by a thermostatically controlled bath in all experiments, except where indicated. Samples were read against blanks of identical reaction mixtures without added protein. The extinction coefficient of the sulfanion of thionitrobenzoate (determined by reaction with known concentrations of cysteine) was found to be 19,900 M⁻¹ cm⁻¹, in agreement with the published value (15).

Experiments were performed to determine the dependence of the rate of -SH titration on DTN concentration and, in the studies reported below, sufficient DTN was used so that the rate of the -SH reaction was independent of its concentration. Above 1.5 × 10⁻⁴ M, the reagent itself inhibited the denaturation of the protein.

Light scattering and sedimentation velocity experiments were performed as described previously (2, 6). Glutamate and alanine dehydrogenase activities were measured by following the oxidative deamination of glutamate and the reductive amination of pyruvate as described previously (2, 16).

Fluorescence measurements were performed in an Aminco-Bowman spectrophotofluorometer with excitation and emission wave lengths of 280 μm and 350 μm, respectively (17, 18).

² The abbreviation used is: DTN, 5,5'-dithiobis(2-nitrobenzoic acid).
Results

Reactivity of —SH Groups in Glutamate Dehydrogenase—When a solution of glutamate dehydrogenase in 0.05 M Tris buffer (pH 7.6) containing 0.06 M NaCl was exposed to DTN, there was virtually no reaction of the protein —SH groups. Since, under these conditions, the enzyme exists as a mixture of forms x and y, there were no —SH groups reactive with DTN in either form of the monomer. However, upon the addition of 4 M guanidine-HCl, there was an almost instantaneous reaction of about 114 —SH groups per molecule of molecular weight 10^{6}. Sodium dodecyl sulfate (8 \times 10^{-4} M) caused a slower exposure of the same number of —SH groups. A total of about 120 such groups were detected previously by amperometric titration of the denatured protein (19).

Kinetics of Glutamate Dehydrogenase Denaturation—At low ionic strength, the —SH groups of glutamate dehydrogenase gradually became reactive to DTN. Fig. 1 shows a semilogarithmic plot of the titration of —SH groups as a function of time and illustrates that the process followed first order kinetics. Fig. 1 also illustrates that the glutamate and alanine dehydrogenase activities of the enzyme were lost at the same rate at which reactive —SH groups appeared. When the fluorescence of the protein was measured under the same conditions, it also declined with the same rate as the loss in enzymic activities and the appearance of —SH groups (Fig. 1). Therefore, the same process, enzyme denaturation, was responsible for all the observed changes, and —SH titration was used to follow denaturation in the experiments to be described below.

A lag phase, which was always seen at the beginning of the denaturation reaction, was shortened by conditions which accelerated the rate of unfolding, and was prolonged by conditions which inhibited denaturation. This lag did not appear to result from disaggregation of the enzyme polymer since the weight average molecular weight (determined by light scattering) was constant during the lag. Under conditions in which the denaturation was quite rapid (Fig. 1), the lag was virtually abolished.

Effects of Ionic Strength, pH, and Temperature on Denaturation Reaction—As indicated previously (11), the rate of unfolding of glutamate dehydrogenase varied inversely with the ionic strength of the solvent (Fig. 2) and was quite rapid at NaCl concentrations below 0.01 M. Above 0.06 M NaCl, virtually no —SH groups reacted with DTN. When sodium phosphate was substituted for NaCl at comparable ionic strengths, identical results were obtained.

As the pH was raised from 6.7 to 9.5 (at constant ionic strength) there was an increase of about 30 fold in the rate of the denaturation reaction (Fig. 3). The rates for both the fall in fluorescence and the loss of enzymic activity increased with pH in the same way as did the rate of —SH titration. These results agree with those of Grisolia et al. (11), showing that at higher pH values the enzyme is more easily denatured by diethylstilbestrol and DPNH.

Above 25°, small increases in temperature caused a marked increase in the rate of unfolding (Fig. 4). In this experiment, as noted, the pH of the buffer was determined at 25°. In going from 12° to 30°, its actual pH decreased from 7.8 to 7.4. Obviously, the increase in rate of denaturation could not result from this pH change, since such a decrease in pH should have stabilized the enzyme (Fig. 3).

Effects of Allosteric Modifiers on Rate of Denaturation—Diethylstilbestrol, Zn++, and GTP (all in combination with DPNH) are thought to influence Equilibrium II to favor monomer y in the above scheme (9). Therefore, they stimulate alanine dehydrogenase activity and cause disaggregation of the polymeric form of the enzyme.

Table I shows that at low concentrations these reagents (in the presence of DPNH) accelerated the denaturation reaction. Similar results for diethylstilbestrol and DPNH had been ob-
FIG. 3. The effect of pH on the rate of —SH titration. The reaction mixtures contained 0.025 mg of enzyme per ml, 4 × 10^{-5} M DTN, and 0.05 M Tris-HCl buffer at the pH values shown. The rates shown are corrected for the spontaneous ionization of DTN which occurs at higher pH.

served previously (11). At the concentration of DPNH used, the pyridine nucleotide itself had no significant effect on unfolding. It should be noted that in the absence of DPNH, diethylstilbestrol inhibited the unfolding. This was noteworthy since DPNH was also required for the maximal disaggregation of the enzyme polymer by diethylstilbestrol (20). In the absence of DPNH, the GTP effect was also complex. Below 0.01 M salt, GTP retarded the denaturation, while above 0.01 M salt it increased the rate of unfolding (Fig. 5).³

DPNH itself had a biphasic effect upon the rate of enzyme denaturation (Fig. 6). Below 10^{-5} M, DPNH increased the rate, while at higher concentrations it inhibited denaturation. The same conclusion has been reported previously, with the use of enzyme activity to follow the denaturation (13).

In contrast to the reagents discussed in Table I, ADP stimulates glutamate dehydrogenase activity, inhibits alanine dehydrogenase activity, and promotes aggregation of the enzyme monomers. ADP also inhibited enzyme denaturation (Table II). These effects have been explained in terms of a shift in Equilibrium II to favor monomer x (10).

L-Leucine, which stimulates the glutamate dehydrogenase reaction and promotes aggregation of enzyme monomers, also inhibited the denaturation reaction (Table II). L-Leucine was ineffective in both respects. The inhibition of the denaturation reaction by L-leucine followed cooperative kinetics, while the inhibition by ADP followed hyperbolic kinetics (Fig. 7).

³ Neither CDP, CTP, UDP, nor UTP affected the denaturation (or the catalytic properties) of the enzyme.

Effects of Protein Concentration on —SH Titration—Since reagents which promote polymer disaggregation increase the rate of denaturation, it was of interest to examine this rate as a function of protein concentration. In contrast to earlier results (11) obtained at higher ionic strength, at low ionic strength no significant differences could be found in the denaturation rates between 0.001 and 2.0 mg of enzyme per ml. (The reaction mixtures contained 0.015 M Tris (pH 7.65), 0.007 M NaCl, 7.2 × 10^{-5} M DTN, and enzyme, extensively dialyzed as described in "Experimental Procedure." ) The effect of varying ionic strength on the sedimentation velocity of glutamate dehydrogenase was therefore examined (Table III). In these experiments, neither the presence of glycerol at a concentration of 5% nor the presence of 10^{-3} M sodium phosphate affected the sedimentation values obtained. Although, as expected, the s_{20,w} at 0.11 M NaCl was about 20, this value fell to 10.8 as the salt concentration was lowered. (Although it cannot be concluded with certainty from sedimentation velocity experiments alone

![Figure 3](http://www.jbc.org/)

![Figure 4](http://www.jbc.org/)

![Table I](http://www.jbc.org/)
that changes in $s_{20,w}$ values reflected molecular weight differences,
this seems likely in view of the magnitude of the changes seen in
Table III.) Therefore, the independence of the rate of denatura-
tion on protein concentration was probably due to the fact that
at low ionic strength, polymer formation did not occur even at
high enzyme concentrations. It should be noted that the peaks
seen in the ultracentrifuge represented active enzyme, since only
a small fraction of the total activity was lost under these condi-
tions at the time of the formation of the boundary. In addition,
enzyme which had been allowed to react with DTN was examined
in the ultracentrifuge (at low ionic strength) and the $s_{20,w}$ was
about 11, indicating that no further disaggregation of the enzyme
occurred under these conditions.

![Graph 1](image1)

**FIG. 5.** The effects of GTP on the rate of denaturation of
glutamate dehydrogenase at different ionic strengths. The reaction
mixtures contained 0.021 mg of enzyme per ml, $2.7 \times 10^{-4}$ M
DTN, 0.005 M Tris-HCl buffer (pH 7.7), and GTP as shown.

![Graph 2](image2)

**FIG. 6.** The effects of various DPNH concentrations on the
rate of enzyme denaturation. The reaction mixtures contained
0.023 mg of enzyme per ml, $3.6 \times 10^{-3}$ M DTN, 0.065 M Tris-HCl
buffer (pH 7.7), and DPNH as shown.

**Table II**

Effects of ADP and leucine on denaturation of
glutamate dehydrogenase

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>$\text{-SH reacted}$</th>
<th>Control</th>
<th>Concentration of reagent required for 50% decrease in rate of denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Leucine</td>
<td>$2 \times 10^{-4}$</td>
<td>10.8</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>$2 \times 10^{-4}$</td>
<td>1.1</td>
<td>10</td>
<td>$6 \times 10^{-4}$</td>
</tr>
<tr>
<td>ADP</td>
<td>$4 \times 10^{-4}$</td>
<td>0.32</td>
<td>3</td>
<td>$2 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

![Graph 3](image3)

**FIG. 7.** The effects of ADP and d- and L-leucine on the rate of
enzyme unfolding. The reaction mixtures contained 0.021 mg
of enzyme per ml, $2.7 \times 10^{-3}$ M DTN, 0.05 M Tris-HCl buffer,
d-and L-leucine, and ADP as shown.

**Table III**

Sedimentation velocity of glutamate dehydrogenase at various
NaCl concentrations

The reaction mixtures contained 2 mg of enzyme per ml, 0.001 M
sodium phosphate (pH 7.1), 5% glycerol, and NaCl as indicated.
All runs were done at a temperature of 21.6°C and a rotor speed of
50,780.

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>Sedimentation velocity ($s_{20,w}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>10.8</td>
</tr>
<tr>
<td>0.01</td>
<td>13.0</td>
</tr>
<tr>
<td>0.03</td>
<td>16.2</td>
</tr>
<tr>
<td>0.05</td>
<td>17.5</td>
</tr>
<tr>
<td>0.07</td>
<td>18.5</td>
</tr>
<tr>
<td>0.09</td>
<td>10.2</td>
</tr>
<tr>
<td>0.11</td>
<td>20.2</td>
</tr>
</tbody>
</table>

**Sulfhydryl Titration of Bovine Serum Albumin**—In contrast to
glutamate dehydrogenase, the reaction of the $\text{-SH}$ groups of
bovine serum albumin with DTN was not influenced by ADP
($1 \times 10^{-4}$ M) or GTP ($5 \times 10^{-4}$ M), and, in contrast to glutamate
dehydrogenase, increasing ionic strength increased the rate of
-SH titration with bovine serum albumin.

DISCUSSION

In the present communication, the denaturation of glutamate
dehydrogenase was followed by changes in -SH titration, pro-
tein fluorescence, or enzymic activity, and it was found, as had
been observed earlier (11), that various "allosteric" modifiers,
as well as temperature, pH, and ionic strength, affected this
reaction. It is important to emphasize that these reagents did
not alter the reactivity of -SH groups or change the intrinsic
fluorescence of the native molecule. Their effect was rather to
modify the rate at which the enzyme was denatured. Inciden-
tially, the above experiments also indicate that forms x and y do
not differ in terms of -SH reactivity (with DTN), or in intrinsic
fluorescence.

Reagents which favor the glutamate dehydrogenase reaction
(i.e., which shift Equilibrium II to favor form z) prevented denaturation, while reagents which inhibit glutamate dehydro-
genase activity and favor alanine dehydrogenase activity (i.e.,
promote the formation of y) stimulated the denaturation. The
production of monomer y by GTP, Zn++, or diethylstilbestrol
requires DPNH; the acceleration of the denaturation by DPNH
in the presence of GTP, Zn++, or diethylstilbestrol again makes
it appear that form y of the enzyme is the precursor of the un-
folded molecule while form x does not appear to unfold under
these conditions.

The biphasic action of DPNH alone may mean that, in the
absence of other reagents, low concentrations of DPNH favor
form y while higher concentrations favor form z, as a result of the
interaction of DPNH with its several types of binding sites.
The combined actions of GTP or diethylstilbestrol and DPNH
in speeding up the denaturation are probably not the result of
alterations in the binding of DPNH induced by the other re-
agents. This follows since both GTP (8) and diethylstilbestrol
(21) enhance the binding of DPNH, which, under the experi-
mental conditions, would have stabilized rather than destabilized
the protein.

The lag period in the denaturation reaction was also affected
by allosteric reagents, indicating that the denaturation probably
involved several reactions. A consideration of various models
which predict the observed kinetics suggested either that form
y was the immediate precursor of the denatured protein or that
there was an intermediate between y and the unfolded molecule.
The present experiments could not distinguish between the two
possibilities.

Of all the allosteric reagents examined, L-leucine was unique in
that its inhibition of the denaturation reaction followed cooper-
ative kinetics (Fig. 7). It has been reported previously (3) that
the effect of leucine in overcoming diethylstilbestrol inhibition of
the glutamate dehydrogenase reaction likewise exhibits coop-
ervative kinetics. Since the latter effect is thought to result from
the influence of leucine on Equilibrium II, it follows that
its effects on denaturation are likewise due to changes in this
equilibrium.

Furthermore, the sigmoid curve for leucine protection was
observed in the absence of substrates for the glutamate dehydro-
genase reaction, indicating that synergism between L-leucine and
other reagents is probably not responsible for its effect on
either enzymic activity or protection against denaturation. This
is in contrast to the case for threonine deaminase, in which the
isoleucine inhibition of the enzyme reaction follows cooperative
kinetics, while isoleucine protection against thermal inactivation
of the enzyme follows hyperbolic kinetics (22).

Recently it has been proposed that sigmoid kinetics, such as
those seen here with L-leucine, indicates cooperative interactions
between enzyme subunits (23). Since the monomers of glu-
tamate dehydrogenase are composed of subunits, a similar
interpretation might apply to the present case. That is, the
binding of the first leucine molecule enhances the binding of
subsequent molecules by changing interactions between the
subunits.

However, ADP, which produces allosteric effects on enzymic
activity and stability in the same direction as those of L-leucine,
displays hyperbolic kinetics. This suggests that the changes in
subunit conformation induced by ADP need not be transmitted
to the other subunits of the monomer. It appears, therefore,
that the subunits of the glutamate dehydrogenase monomers
may respond either independently or cooperatively, depending
on the allosteric reagent.

SUMMARY

1. In solutions of low ionic strength, glutamate dehydrogenase
undergoes a denaturation reaction which results in loss of both
glutamate and alanine dehydrogenase activities, a decrease in
protein fluorescence, and the appearance of about 114 -SH
groups per molecule of molecular weight 104.

2. This rate of unfolding was influenced by ionic strength, pH,
temperature, and a variety of allosteric modifiers.

3. Both adenosine diphosphate and L-leucine protected the
enzyme against denaturation. The effect of adenosine diphos-
phate followed hyperbolic kinetics, while the kinetics for leucine
protection showed cooperative interactions.

4. At low ionic strength the concentration-dependent aggrega-
tion of the enzyme did not occur.

5. It was concluded that only the form of the enzyme which
has enhanced alanine dehydrogenase activity (and virtually no
glutamate dehydrogenase activity) is the precursor of the de-
natured form of the protein.

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cence experiments.

REFERENCES

7. YIELDING, K. L., AND TOMKINS, G. M., Biochim. et Biophys.
9. TOMKINS, G. M., YIELDING, K. L., TALAL, N., AND CURRAN,
(1963).
10. BITENSKY, M. W., YIELDING, K. L., AND TOMKINS, G. M.,
11. GRISOLIA, S., FERNANDEZ, M., AMULUNXEN, R., AND QUIJADA,
(1952).
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