Bovine Glutamate Dehydrogenase

THE pH DEPENDENCE OF NATIVE AND NITRATED ENZYME IN THE PRESENCE OF ALLOSTERIC MODIFIERS*

EMIL L. SMITH† and DENNIS PISZKIEWICZ§

From the Department of Biological Chemistry, UCLA School of Medicine, and the Molecular Biology Institute, University of California, Los Angeles, California 90024

SUMMARY

The pH dependence of the rate of glutamate oxidation by bovine liver glutamate dehydrogenase has been determined in the absence and presence of allosteric modifiers. In the range of pH 7 to 9 where the enzyme was saturated by substrate, the degree of activation by ADP or inhibition by GTP did not vary substantially with pH. The pH dependence of chicken liver glutamate dehydrogenase measured in the absence and presence of allosteric modifiers was nearly identical with that of the bovine enzyme. In studies of the bovine enzyme, nitration of tyrosine-406 had no significant effect on the activity or the pH dependence of the enzyme activity in the absence of allosteric modifier or in the presence of the activator ADP. However, nitration decreased the inhibitory effect of GTP evenly over the entire pH range and the extent of loss of GTP inhibition was dependent on the extent of nitration. It is concluded that the effect of nitration of tyrosine-406 on the loss of inhibition by GTP is to alter the relationship of the binding site of GTP to the catalytic site in a pH-independent manner. This result is probably a consequence of the introduction of the steric bulk of the nitro group into the tertiary structure of the protein.

Glutamate dehydrogenase (L-glutamate:DPN (TPN) oxidoreductase (deaminating), EC 1.4.1.3) catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonium ion. The importance of this enzyme in metabolism is that it serves as a link to the pathways of transamination and interconversion of α-NH₂ group nitrogen and ammonia (1, 2). The activity of glutamate dehydrogenase is regulated by nucleoside di- and triphosphates (2, 3). Price and Radda (4) observed that bovine liver glutamate dehydrogenase may be rendered insensitive to the allosteric inhibitor GTP by the modification of 1 tyrosyl residue per subunit polypeptide chain with tetranitromethane. Subsequently, we reported (5) that the rate of formation of 3-nitrotyrosine is the sum of two concurrent reactions, an initial rapid reaction which accounts for the nitration of approximately 1 tyrosyl residue per subunit chain, and a slower reaction which can produce more extensive nitration of tyrosine. We have identified the residue which is rapidly nitrated as tyrosine-406, according to the present numbering of the polypeptide chain (6).

Price and Radda (4) also reported that the nitration of one tyrosine per subunit had no effect on the sedimentation velocity (at an enzyme concentration of 0.9 mg per ml), the optical rotatory dispersion, and the Km values for α-ketoglutarate, ammonium ion, and DPNH. The half-saturation value for GTP was determined by a fluorescence titration method (4, 7), and found to be an order of magnitude greater in the nitrated enzyme than in the native form. However, we have found that concentrations of GTP high enough to saturate the enzyme did not restore the inhibitory effect of GTP on the nitrated dehydrogenase (see below). Thus, there are a number of ways in which nitration may effect the loss of sensitivity to GTP. First, nitration of tyrosine-406 alters the ionization of the phenolic hydroxyl from the range of pH₅₀ = 9.8 to 10.4, normally found in proteins (8), to a pH₅₀ = 8.0 (4). The altered pH₅₀ might change the pH dependence of the enzyme or the pH dependence of the GTP-inhibitory effect resulting in an apparent loss in inhibition when observed at a constant pH. Second, introduction of the nitro group on tyrosine-406 could alter the interaction of the GTP binding site and the catalytic site of the enzyme in a pH-independent manner. In the present study we have examined the pH dependence of native and nitrated glutamate dehydrogenase in the presence of GTP (2, 3) and the allosteric activator ADP (2, 3) in an attempt to decide between these possibilities.

EXPERIMENTAL PROCEDURE

Materials—DPN, GTP, and ADP were obtained from Calbiochem, and tetranitromethane from Eastman. Crystalline bovine liver glutamate dehydrogenase was purchased from C. F. Boehringer and Soehne GmbH, Mannheim, Germany. Crystalline glutamate dehydrogenase from chicken liver was prepared by the method of Snoke (9). (The published procedure contained a typographical error; 122 ml of an RNA solution which
contains 5 g, rather than 50 g, of RNA per 100 ml should be added to each liter of acetone powder extract. The chicken liver enzyme was homogeneous by gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol. The subunit molecular weight was identical with that of bovine glutamate dehydrogenase. Indeed, the chicken enzyme shows strong homology in sequence to the bovine one.

**Reaction of Enzyme with Tetranitromethane**—Glutamate dehydrogenase (1 mg per ml) was nitratated for varying lengths of time by the method described previously. Reactions were terminated by the addition of n-octane in a 4-fold molar excess over tetranitromethane. Enzyme activity was determined without further treatment.

**Enzyme Assay**—Enzyme activity was determined with a Beckman Kintrak VII recording spectrophotometer with a cell compartment maintained at 30° by following the formation of DPNH at 340 nm. Assays were initiated by the addition of 25 
\[ \mu \] l of stock enzyme solution (1 mg per ml) to 2.5 ml of stock substrate solution containing 5 \( \times 10^{-4} \) M DPN and 5 \( \times 10^{-4} \) M glutamate in 0.1 ionic strength phosphate buffer. Assays performed in the presence of allosteric modifier also included in the stock substrate solution 5 \( \times 10^{-4} \) M GTP or 5 \( \times 10^{-4} \) M ADP. Phosphate buffers at 0.1 ionic strength were formed by mixing appropriate volumes of 0.1 ionic strength KH\( _2 \)PO\( _4 \) and 0.1 ionic strength K\( _2 \)HPO\( _4 \). Buffers at approximately pH 10 and above were prepared by adjusting KH\( _2 \)PO\( _4 \) with KOH. All substrate solutions were made up immediately prior to use. The amount of DPNH formed was calculated by using the extinction coefficient at 340 nm, \( \varepsilon = 6.22 \times 10^4 \) liter mole\(^{-1} \) cm\(^{-1} \) (10).

**Amino Acid Analysis**—Protein samples were hydrolyzed and analyzed as described previously. The total amount of 3-nitrotyrosine after varying times was estimated by analysis. The extent of modification of tyrosine-406 was calculated from the initial slope of nitration with time, as previously determined.

**RESULTS**

The activity of glutamate dehydrogenase was determined by measuring the formation of DPNH at constant, and nearly saturating concentrations of substrate and allosteric modifier. To illustrate, glutamate, which has a \( K_m \) of 1.8 \( \times 10^{-3} \) M (2) was used at 5 \( \times 10^{-3} \) M; DPN was used at 5 \( \times 10^{-4} \) M where it gives over 70% of maximal velocity (11); ADP, which has a dissociation constant of 3.0 \( \times 10^{-4} \) M (12), was used at 5 \( \times 10^{-4} \) M; and GTP, which has a half-saturation concentration of 1.5 \( \times 10^{-4} \) M (4) in the native enzyme and a half-saturation concentration of 1.5 \( \times 10^{-4} \) M for the nitrated enzyme (4), was also used at 5 \( \times 10^{-4} \) M. It was anticipated, therefore, that the pH dependence at least in the vicinity of the pH optimum would reflect the rate-limiting steps for catalysis rather than for enzyme-substrate complex formation.

Plots of log of dehydrogenase (bovine) activity versus pH as determined in the absence and presence of allosteric modifier are shown in Fig. 1A. Activity was calculated as molar \( \text{min}^{-1} \) of DPNH formed in mole liter\(^{-1} \) of enzyme subunit of 56,000 molecular weight (6). At these pH values where the enzyme is saturated with substrate (and modifiers) this is the turnover number with units of \( \text{min}^{-1} \). The points representing enzyme activity when measured in the absence of allosteric modifier approximate a line (dashed line in Fig. 1A) with slope of +1 at low pH, and a line with slope of -2 at high pH. These slopes probably reflect the dependence of rate upon one basic ionization at low pH and two acidic ionizations at high pH (13). Since these slopes were found at the extremes of pH, the assumption that the enzyme is saturated by substrate is not necessarily valid. Hence, the slopes may reflect the ionization of groups participating in the catalytic step, the substrate-binding step, or both. It should be noted that Rogers (14) has observed a slope of approximately 0.5 between pH 6.0 and 4.4 in a plot of \( \text{pV}_{\text{max}} \) versus pH. Our results are in agreement with those of Rogers in the limited range of pH that he studied.

The pH dependence of the activity when measured in the presence or absence of ADP (Fig. 1A) differed only slightly in shape. A substantial activation effect was observed for ADP at values greater than approximately pH 7. The greatest degree of activation, approximately 10-fold, was found at pH values above 9. At pH values below 7, ADP had little effect on the rate of glutamate oxidation by the bovine enzyme.

The pH dependence of the activity when measured in the presence of GTP (Fig. 1A) showed only small variation over the observed pH range. Inhibition by GTP was evident at all pH values below pH 10.

The effect of pH on the activity of the chicken liver dehydrogenase when measured in the absence of modifiers, or in the presence of ADP or GTP (Fig. 1B) was essentially similar to those observed for the bovine enzyme. The only significant difference found between the two enzymes, was that ADP activated the chicken liver enzyme to some extent at all pH values.

The effect of pH on glutamate dehydrogenase nitrated to varying degrees is shown in Fig. 2. Reaction of enzyme with tetranitromethane to the extent of 0.9 (Fig. 2A), 1.3 (Fig. 2B), and 2.8 residues of 3-nitrotyrosine per subunit (Fig. 2C) had no significant effect on the log activity vs. pH profiles when measured in the absence of allosteric modifiers. Only a small loss in activity over the entire pH range was observed to occur with time. Similarly, varying degrees of nitration had no significant in-
In studies of bovine liver glutamate dehydrogenase which was nitrated to varying degree (Fig. 2), nitration of tyrosine-406 had no significant effect on the activity or the pH dependence of the enzyme activity in the absence of allosteric modifier or in the presence of the activator ADP. However, nitration decreased the inhibitory effect of GTP evenly over the entire pH range, and the extent of loss of GTP inhibition was dependent on the extent of nitration of tyrosine-406. Colman and Frieden (15) have concluded on the basis of kinetic evidence alone that the binding sites of GTP and ADP to the enzyme are distinct. Our observation that inhibition by GTP is lost upon reaction with tetranitromethane but that activation by ADP is unaffected supports this conclusion. There was no evidence of a change in pH dependence of the activity of the nitrated enzyme in the presence of GTP. Thus, there was no evidence that a change in the pKₐ of the tyrosine residue could be related to the loss of inhibition by GTP.

It now seems clear that the effect of nitration on the loss of inhibition by GTP is to alter the relationship of the binding site of GTP to the catalytic site in a pH-independent manner. One set of possibilities is that the nitro group, either because of its steric bulk or its ability to donate electrons to hydrogen bonds, causes a localized conformational change which prevents interaction of the GTP binding site with the catalytic site. Since reduction of nitrated enzyme with Na₂S₂O₄ to the aminated enzyme does not restore the inhibitory effect of GTP (4), the former possibility is more likely. Another possibility is that the nature of the binding of GTP itself to the protein is altered. Inasmuch as the half-saturation value for the binding of GTP to the enzyme is increased substantially (4), this last possibility may be the correct one.

Although treatment of proteins with tetranitromethane may produce cross-linkage, this appears to be unlikely at the low level of nitration used in these studies. Price and Radda (4) have shown that the sedimentation velocity of the enzyme is unaffected. We have examined the subunit size of the enzyme by sedimentation-equilibrium in the presence of guanidine salts and find no significant alteration of the molecular weight, e.g. when 2 residues of nitrotyrosine were formed, the average value was 59,000, as compared to 56,000 for the untreated material. Although such results do exclude significant cross-linkage between subunits, they do not completely exclude cross-linkage within a subunit; however, no evidence was obtained for the presence of dityrosine.

While the primary concern of this study was to determine the effect of nitration on the pH dependence of inhibition by GTP, an additional comment can be made concerning the pH dependence. Rogers (14) has reported that Vₘₐₓ and Kₐₐₜₐ for the oxidative deamination of L-glutamate by bovine liver glutamate dehydrogenase are dependent upon ionization of a group with a pKₐ of 7.7 to 7.8, which he suggested might be due to a cysteine residue. While the pKₐ of this ionization is not as obvious in our log activity versus pH profile (Fig. 1A), it can be readily accommodated by our data. It should be noted that the pKₐ of the e-NH₂ group of the reactive lysine residue, which is essential for activity, has been found to be 8.0 ± 0.3 with pyriodoxal (16) and pyriodoxal 5′-phosphate (17) as inactivating reagents. A pKₐ of 7.7 for this amino group has also been obtained from kinetic studies with cyanate as the inactivating reagent (18). In view of these results, it seems likely

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that the apparent pK\textsubscript{a} observed by Rogers (14) describes the ionization of the essential \(\epsilon\)-NH\textsubscript{2} group of lysine-126 (16).

The loss of activity at high pH values may reflect deprotonation of cationic groups with pK values of 9 or higher. Such cationic groups might be those expected to interact with the carboxylate groups of the substrates, glutamate or \(\alpha\)-ketoglutarate, or with the phosphate groups of pyridine nucleotide coenzymes.

REFERENCES

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Emil L. Smith and Dennis Piszkiewicz


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