Methionyl Residue Critical for Activity and Regulation of Bovine Liver Glutamate Dehydrogenase*

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NEAL L. ROSEN, LARRY BISHOP, JEAN B. BURNETT, MICHAEL BISHOP, AND ROBERTA F. COLMAN†

From the Biochemical Research Laboratory, Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02114

SUMMARY

Glutamate dehydrogenase is inactivated by incubation with 4-iodoacetamidosalicylic acid at 37° and pH 0.0. The reaction obeys pseudo-first order kinetics, with a rate constant which is linearly proportional to the 4-iodoacetamidosalicylic acid concentration. A 4- to 5-fold decrease in the rate of inactivation is provided by the substrates α-ketoglutarate and glutamate when added to the incubation mixture with ADP and DPN, but no appreciable decrease in the inactivation rate is produced by the coenzymes themselves, either alone or when combined with ADP or GTP. These results suggest that 4-iodoacetamidosalicylic acid reacts in the region of the active site.

4-Iodoacetamidosalicylic acid produces a change in the response of the enzyme to the allosteric inhibitor GTP, as exemplified by an increase in the kinetic dissociation constant of the enzyme-coenzyme-GTP complex. Although the reagent causes inactivation of the enzyme in the absence of GTP, it does not influence the small residual activity (4 to 8%) seen when a saturating concentration of GTP is present.

Only methionyl and cysteinyl residues of glutamate dehydrogenase are altered by 4-iodoacetamidosalicylic acid at periods up to 10 times the half-life for inactivation; lysyl and histidyl residues are not alkylated during this time. As determined by the release of iodide ion from 4-iodoacetamidosalicylic acid, approximately 4 amino acids per peptide chain are modified when the enzyme loses 90% of its activity: about 2 cysteinyl and 2 methionyl residues are alkylated. Several lines of evidence suggest that covalent modification of cysteine is not the primary cause of inactivation. The rate of alkylation of sulfhydryl groups (1/K  = 0.0015 min⁻¹) is only one-sixth that of inactivation (1/K  = 0.0102 min⁻¹) for the enzyme in the absence of ligands. These two rate constants can be varied independently by the addition of substrates and allosteric modifiers. In contrast, a good correlation is observed between the degree of inactivation of the enzyme and the extent of modification of approximately 1 methionyl residue per peptide chain. Furthermore, treatment of 75% inactivated enzyme with dithiothreitol produces a 3-fold increase in the enzymatic activity, accompanied by the regeneration of methionine but no appreciable alteration of the number of modified cysteine residues. It is concluded that the integrity of a methionine residue is critical for the function of glutamate dehydrogenase.

Specific modification of the functional groups of bovine liver glutamate dehydrogenase (α-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) has been aimed at identifying certain of the amino acid residues involved in the catalytic and regulatory sites (2). Most attention has been focused on the participation of lysyl (3-9), tyrosyl (7, 10-12), and sulfhydryl groups (13-16). Methionyl residues have previously been implicated in the function of two dehydrogenases of different types: TPN-dependent isocitrte dehydrogenase (17), which catalyzes an oxidative decarboxylation reaction, and the cytoplasmic DPN-specific malate dehydrogenase (18), which catalyzes a simple dehydrogenation reaction. The present study was undertaken in order to determine whether methionine has a significant role in glutamate dehydrogenase, an example of a third type of dehydrogenase, one catalyzing an oxidative deamination reaction.

The reagent 4-iodoacetamidosalicylic acid was originally proposed by Baker et al. as an active site-directed reagent for glutamate dehydrogenase (19-23). The inactivation produced by the reagent at pH 7.5 was attributed by Holbrook and co-workers to the modification of a lysyl residue (24) and by Malcolm and Radda to the modification of cysteine (15). However, in the amino acid analysis of 90% inactivated enzyme reported by Malcolm and Radda, the methionic content was decreased by approximately 2 residues (15). It seems likely that this reaction of glutamate dehydrogenase with 4-iodoacetamidosalicylic acid is more complex than it initially appeared. The reaction of amino and sulfhydryl compounds with iodoacetyl derivatives is thought to proceed via their unprotonated forms. In the present investigation, the modification of glutamate dehydrogenase by 4-iodoacetamidosalicylic acid has been examined...
in the pH range from 5.6 to 6.0 in order to minimize the reactivity of lysyl and cysteinyl residues and thereby facilitate the identification of other functionally significant amino acids.

**EXPERIMENTAL PROCEDURE**

*Materials—* Bovine liver glutamate dehydrogenase, supplied as a crystalline suspension in ammonium sulfate by Boehringer-Mannheim, was dialyzed at 4°C in 50-mg batches against two changes of 1 liter of 0.1 M potassium phosphate buffer, pH 7.15. Denatured protein was removed from the dialyzed enzyme solution by centrifugation in an SW 20 rotor in a Beckman model L-2 ultracentrifuge at 4°C for 20 min at 20,000 rpm. The protein concentrations were determined from the absorbance at 280 nm, using a value of E° of 9.7 (25). The ratio of A280:A260 was always between 1.8 and 1.95. Dialyzed and centrifuged enzyme solutions were stored as frozen aliquots for not more than 3 months; they were thawed only once, immediately prior to use. A molecular weight of 56,100 (11) for the identical peptide chains was used in the calculations.

The 4-iodoacetamidosalicylic acid, obtained from Nutritional Biochemicals, was twice recrystallized from ethanol to yield a compound with a melting point of 206-208°C (with decomposition). The crystals were dissolved in water by slowly titrating with sodium hydroxide. The concentration was determined spectrophotometrically using extinction coefficients for 9.13 × 10³ M⁻¹ cm⁻¹ and 1.30 × 10⁴ M⁻¹ cm⁻¹ at 302 nm and 208 nm, respectively.

The 5,5'-dithiobis(2-nitrobenzoic acid) was purchased from the Aldrich Chemical Co. The coenzymes, substrates, purine nucleotides, EDTA, and Tris base were obtained from Sigma. New England Nuclear supplied the L-[methyl-¹⁴C]methionine and D-[carboxyl-¹⁴C]methionine. Standard Tris acetate buffer was 0.1 M in acetate, pH 7.9, and contained 10⁻⁴ M EDTA.

**Assay of Enzymatic Activity—** Enzymatic activity was determined spectrophotometrically at 340 nm from the oxidation of reduced pyridine nucleotide at 23°C in pH 8.0 Tris acetate buffer (0.013 M in acetate) containing 0.01 mM EDTA. The substrate concentrations used were 5 mM α-ketoglutarate, 50 mM ammonium chloride, and 100 μM DPNH, in a total volume of 1.0 ml. In certain assays ADP or GTP was included, as indicated. The kinetic dissociation constant for GTP was measured under these same conditions, and was calculated in accordance with Frieden (26). Initial velocities were obtained with a Cary model 15 recording spectrophotometer or a Gilford model 240 equipped with a recorder and an expanded scale (0.1 absorbance full scale).

**Measurement of Free Sulphydryl Groups—** The number of residual sulphydryl groups which had not reacted with 4-iodoacetamidosalicylic acid was determined under conditions which have been shown to denature the enzyme and to expose all of its —SH groups to reaction with DTNB.¹ (Glutamate dehydrogenase contains 6 cysteine and no cystine residues per peptide chain.) Aliquot (0.1 ml) of the incubation mixture of enzyme and 4-iodoacetamidosalicylic acid were removed at various times and added directly, with rapid mixing, to a cuvette containing 0.8 ml of 0.25% sodium dodecyl sulfate in the standard Tris buffer, and 0.1 ml of 0.01 M DTNB in the same buffer. The solution of DTNB was prepared fresh each day and kept on ice to prevent excessive decomposition. The absorbance at 412 nm was measured on a Cary model 14 recording spectrophotometer and was extrapolated back to the time of addition. This extrapolation procedure is necessary in order to correct for reaction of the nitrobenzoate ion with excess 4-iodoacetamidosalicylic acid. Using an extinction coefficient of 1.36 × 10⁴ M⁻¹ cm⁻¹ (27), the number of free sulphydryl groups remaining at a given time was calculated. The extrapolation procedure appears to be satisfactory since the same number of residues (6) was obtained for the enzyme at zero time whether or not 4-iodoacetamidosalicylic acid was present.

**Iodide Measurements—** The amount of iodide ion released when the enzyme was inactivated to the extent of 90% was determined by means of a Beckman iodide electrode. A standard curve was established by measuring the potential of standard potassium iodide solutions from 10⁻⁴ m to 10⁻³ m dissolved in 0.1 M potassium phosphate buffer, pH 5.6, at 37°C. The amount of iodide measured in the incubation mixture of enzyme and 4-iodoacetamidosalicylic acid was corrected for the spontaneous decomposition of reagent by using an identical reaction mixture except that enzyme was omitted.

**Amino Acid Analysis—** Samples (2 ml) for analysis were withdrawn from the incubation mixtures of enzyme and 4-iodoacetamidosalicylic acid at the indicated times and dialyzed against 1.5 liters of 0.25 M potassium phosphate buffer, pH 6.0, for 12 hours at 4°C, followed by dialysis against two changes of 1.5 liters of distilled water at 4°C. The samples were lyophilized and then dissolved in 6 N HCl. The hydrolysis tube was flushed with nitrogen, evacuated, and maintained at 110°C for 23 hours. The analyses were performed on a Beckman/Spinco model 120C automatic amino acid analyzer with an expanded range scale and high sensitivity cuvettes on custom research resins, types AA-15 and AA-27. Constants used for methionine derivatives relative to standard amino acid constants were those given by Gundlach et al. (28). The analyses were conducted at two concentrations of enzyme sample: first at a concentration appropriate for measuring the total amino acid composition of the protein, and second at 10 times that concentration in order more accurately to measure the small amounts of S-carboxymethylcysteine and S-carboxymethylhomocysteine, as well as methionine, half-cysteine, phenylalanine, and tyrosine. The two determinations were related by a calculation of the ratio of the moles of all measured amino acids to the moles of phenylalanine measured.

**Preparation of 4-(Methionylacetamido)salicylic Acid—** L-[methyl-¹⁴C]Methionine or L-[carboxyl-¹⁴C]Methionine (3.33 mM) was incubated with 4-iodoacetamidosalicylic acid (8.06 mM) in water and adjusted to pH 5.9 at 38°C for 48 hours. At the end of that time a 0.03-ml sample of each 15-ml reaction mixture was analyzed by paper chromatography together with standard amino acids, as shown in Fig. 1. The compound with Rᵢ 0.16 appears to be 4-(methionylacetamido)salicylic acid, since it retains both the carboxyl and the methyl groups of methionine as indicated by the labeling pattern, it absorbs ultraviolet light, and it reacts with ninhydrin. The 4-(methionylacetamido)salicylic acid can undergo decomposition by the three pathways shown in Fig. 2, the relative importance of each depending on the conditions. Under the reaction conditions used here, the major decomposition pathway seems to be Pathway 1a. Homoserine is detected by reaction with ninhydrin, by radioactivity when produced from [carboxyl-¹⁴C]Methionine but not from [methyl-¹⁴C]Methionine, and by its lack of ultraviolet absorption. The Rᵢ of the compound presumed to be homoserine in the reaction mixture is identical with that of an authentic homoserine standard (Fig. 1). The compound with Rᵢ 0.83 is probably the other product
of Pathway 1a, 4-(S-methylthioglycolamido)salicylic acid, since it absorbs ultraviolet light, does not react with ninhydrin, and is labeled only by [methyl-14C]methionine.

After 48 hours the 15 ml of reaction mixture were evaporated to dryness, redissolved in 0.4 ml of water, streaked along the length of Whatman No. 1 paper, and subjected to descending chromatography as in Fig. 1. The 4-(methionylacetamido)salicylic acid was eluted and evaporated to dryness. An aliquot of the preparation was analyzed by paper chromatography and found to consist predominantly of 4-(methionylacetamido)salicylic acid, with 1% homoserine and 2.7% methionine contamination. A quantity of the sulfonium salt, measured by [methyl-14C]methionine

![Paper Chromatography of Products](image)

**Fig. 1.** Paper chromatography of products of the reaction between methionine and 4-iodoacetamidosalicylic acid (ISA). Aliquots of the reaction mixture of 4-iodoacetamidosalicylic acid and either [methyl-14C]methionine or [carboxyl-14C]methionine were subjected to descending paper chromatography on Whatman No. 1 paper for 18 hours in butanol-pyridine-acetic acid-water (30:20:6:24). Spots were visualized by ultraviolet light, by reaction with ninhydrin, or by the presence of radioactivity as detected by the Vanguard Autoscanner. Tracings of the original spots and radioactivity scans are shown.

**Fig. 2.** Major decomposition products of 4-(methionylacetamido)salicylic acid.
radioactivity and corrected for the presence of the contaminants, was hydrolyzed with acid under the same conditions as those used for modified glutamate dehydrogenase. The hydrolyzed sample was examined on the amino acid analyzer to ascertain the fraction of the original compound that was represented by the observed decomposition products, S-carboxymethylhomocysteine, methionine, homoserine, and homoserine lactone. Methionine is regenerated to the extent of 35.8% of the original compound (Fig. 2, Pathways 2a and 2b). The major acid decomposition product is S-carboxymethylhomocysteine (Fig. 2, Pathways 3a and 3b), which appears as an isolated peak between proline and glycine and which accounts for 43% of the original compound. In the analyses of 4-iodoacetamidosalicylic acid-treated glutamate dehydrogenase, the concentration of modified methionyl residues was therefore estimated by multiplying the measured concentration of S-carboxymethylhomocysteine by 1.0/43 or 2.31. The sum of modified methionine plus methionine (corrected for the quantity of methionine which is regenerated during acid hydrolysis) was constant, supporting the use of this procedure.

RESULTS

Inactivation by 4-Iodoacetamidosalicylic Acid

Glutamate dehydrogenase is inactivated by incubation with 4-iodoacetamidosalicylic acid at 37° and pH 6.0, whereas the enzyme is stable under these conditions in the absence of reagent. Line A' of Fig. 3 shows that when the concentration of 4-iodoacetamidosalicylic acid is maintained at 2.9 mM the reaction obeys pseudo-first order kinetics as activity declines to 8% of its initial value. The pseudo-first order rate constant is dependent on pH (Fig. 4), being high at pH 8, falling to a minimum at approximately pH 6.5, and rising again below pH 6.0. The reason for the increase in rate below pH 6.0 is not known, but the increase may reflect an electrostatic attraction between the negatively charged reagent and an enzymatic group near the site of attack which has a pK in this region. Since the reaction rate is decreased as the ionic strength is elevated, care was taken to maintain constant ionic strength in these and subsequent experiments. The rate of the reaction between glutamate dehydrogenase and 4-iodoacetamidosalicylic acid is not markedly sensitive to changes in the protein concentration. The rate constant is decreased only 30% when the enzyme concentration is 2.0 mg per ml as compared to the value obtained at 0.2 mg per ml, whether the reaction is conducted at pH 5.6 or at pH 6.0. Glutamate dehydrogenase is substantially polymerized at 2 mg per ml, whereas at 0.2 mg per ml the predominant species is the 390,000 molecular weight monomer (29, 30). Thus, the extent of association of the enzyme does not appear to influence significantly the reactivity of those activity-dependent amino acid residues attacked by 4-iodoacetamidosalicylic acid.

At pH 7.5, Malcolm and Radda observed saturation kinetics in the inactivation of glutamate dehydrogenase by a series of concentrations of 4-iodoacetamidosalicylic acid (15). In contrast, Fig. 5 shows that the pseudo-first order rate constant is directly proportional to the 4-iodoacetamidosalicylic acid concentration from 0.96 to 5.8 mM at pH 5.6. Rapidly reversible binding of the reagent prior to inactivation is, therefore, not indicated for the low pH reactions, although it does appear to occur at pH 7.6.

Table I records the effect of added ligands on the rate of inactivation of glutamate dehydrogenase by 4-iodoacetamidosalicylic acid. In all cases, controls were included to measure any denaturation in the presence of the particular ligand under consideration; the rates given have been corrected for these
Fig. 5. Effect of varying the concentration of 4-iodoacetamidosalicylic acid (ISA) on the rate of inactivation at pH 5.6. Glutamate dehydrogenase (0.2 mg per ml) was incubated at 37° in 0.1 M potassium phosphate buffer containing potassium chloride (ionic strength, 0.4 M). The observed rate constants were measured from first order plots of residual activity as a function of time, as described in “Experimental Procedure.”

Table I
Rate constants for inactivation and thiol alkylation of glutamate dehydrogenase by 4-iodoacetamidosalicylic acid in the presence of ligands

Enzyme (2.0 mg per ml) was incubated with 2.9 mm 4-iodoacetamidosalicylic acid at 37° in 0.125 M potassium phosphate buffer, pH 6.0, and the ionic strength of each solution was adjusted to 0.4 M by the addition of KCl. The pseudo-first order rate constants for inactivation were obtained in accordance with Fig. 1, and those for -SH alkylation as described in the legend to Fig. 6 and under “Experimental Procedure.” The concentrations of all ligands are considerably in excess of their respective binding constants as measured at pH 8 (3).

<table>
<thead>
<tr>
<th>Additions to reaction mixture</th>
<th>Rate constant</th>
<th>Rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{obs}}$</td>
<td>$k_{\text{S-S-SH}}$</td>
</tr>
<tr>
<td>1. None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. $\alpha$-Ketoglutarate (0.040 M)</td>
<td>102</td>
<td>15.5</td>
</tr>
<tr>
<td>3. $\alpha$-Ketoglutarate (0.076 M)</td>
<td>58</td>
<td>8.3</td>
</tr>
<tr>
<td>4. DPNH (0.8 mm)</td>
<td>75</td>
<td>6.5</td>
</tr>
<tr>
<td>5. DPNH (0.8 mm) + ADP (0.25 mm)</td>
<td>72</td>
<td>0.9</td>
</tr>
<tr>
<td>6. DPNH (0.8 mm) + GTP (60, $\mu$M)</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>7. DPN (1.5 mm)</td>
<td>82</td>
<td>8.9</td>
</tr>
<tr>
<td>8. ADP (0.25 mm)</td>
<td>87</td>
<td>3.5</td>
</tr>
<tr>
<td>9. DPN (1.5 mm) + ADP (0.25 mm)</td>
<td>85</td>
<td>4.9</td>
</tr>
<tr>
<td>10. DPN (1.5 mm) + ADP (0.25 mm) + $\alpha$-keto- glutarate (0.040 M)</td>
<td>24</td>
<td>4.5</td>
</tr>
<tr>
<td>11. DPN (1.5 mm) + ADP (0.25 mm) + $\alpha$-keto- glutarate (0.076 M)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>12. DPN (1.5 mm) + ADP (0.25 mm) + glutamate (0.23 M)</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>13. DPN (1.5 mm) + ADP (0.25 mm) + glutamate (0.075 M)</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

In the course of inactivation of glutamate dehydrogenase by 4-iodoacetamidosalicylic acid, 4 moles of iodide per peptide chain are released when the enzyme has lost 90% of its activity, implying that approximately 4 amino acid residues have combined with the reagent under these conditions. Table II illustrates sample amino acid compositions for native glutamate dehydrogenase and for enzyme incubated with 4-iodoacetamidosalicylic acid for 210 min (87.5% inactivated). Only methionine and cysteine residues are altered; in fact, these are the only residues to react with 4-iodoacetamidosalicylic acid at periods up to 10 times the half-life of inactivation. Neither carboxymethyllysine nor carboxymethylhistidine derivatives are detectable in the amino acid analyses of the hydrolysates, and the total number of lysine and histidine residues does not vary significantly from the values of 33 and 14 obtained for the native enzyme.

Table II
Amino acid compositions of native and 4-iodoacetamidosalicylic acid-modified glutamate dehydrogenase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Native enzyme (present analysis)</th>
<th>Modified enzyme (12.5% residual activity)</th>
<th>Analysis from literature (31)</th>
<th>Analysis from sequence (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>33.3</td>
<td>33</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Histidine</td>
<td>14.3</td>
<td>14</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Arginine</td>
<td>28.0</td>
<td>28</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>49.7</td>
<td>49</td>
<td>52</td>
<td>49</td>
</tr>
<tr>
<td>Threonine</td>
<td>25.9</td>
<td>26</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Serine</td>
<td>28.7</td>
<td>29</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>47.7</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>Proline</td>
<td>21.9</td>
<td>21</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Glycine</td>
<td>50.7</td>
<td>50</td>
<td>49</td>
<td>47</td>
</tr>
<tr>
<td>Alanine</td>
<td>37.2</td>
<td>38</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Valine</td>
<td>32.5</td>
<td>32</td>
<td>39</td>
<td>34</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>34.8</td>
<td>35</td>
<td>35</td>
<td>37</td>
</tr>
<tr>
<td>Leucine</td>
<td>31.8</td>
<td>32</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>18.0</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>23.0</td>
<td>23</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Cysteine</td>
<td>6.2</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Carboxymethylcysteine</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>12.6</td>
<td>11</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Carboxymethylhomocysteine</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modified methionineb</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined by reaction with DTNB.
* Calculated from carboxymethyl homocysteine X 2.31, as described under “Experimental Procedures.”
reaction with cysteine as the cause of inactivation, the extent of cysteine modification was monitored during the course of reaction with 4-iodoacetamidosalicylic acid by withdrawing aliquots of the reaction mixture and colorimetrically titrating the unreacted sulfhydryl groups of the protein. This determination was performed using DTNB under denaturing conditions in 0.2% sodium dodecyl sulfate, as described in “Experimental Procedure.” Glutamate dehydrogenase contains six sulfhydryl groups per peptide chain, all of which are capable of reaction with 4-iodoacetamidosalicylic acid. Fig. 6 illustrates the pseudo-first order kinetics observed for the alkylation of cysteine residues by 4-iodoacetamidosalicylic acid. The reaction is linear until less than one —SH group remains unmodified. Thus all sulfhydryl groups appear to be equally reactive.

Table I compares the pseudo-first order rate constants for modification of cysteine with those for inactivation in the absence and in the presence of various ligands. The reaction rate of —SH groups is decreased more than 2-fold by DPNH (Lane 4), more than 4-fold by ADP (Line 8), and more than 15-fold by DPNH and ADP together (Line 5). The rate of inactivation, on the other hand, is changed less than 2-fold. When DPN and ADP are included (Line 9), the rate of —SH modification is decreased 3-fold, although the rate of inactivation is not appreciably changed. The combination of DPN, ADP, and α-ketoglutarate (Line 10) affords almost a 4-fold protection against inactivation, but the added α-ketoglutarate only slightly alters the rate of —SH modification as compared to Line 9. It is apparent that the inactivation and —SH modification rates can be varied independently of each other, making it unlikely that covalent modification of cysteine is the primary cause of inactivation.

Fig. 7 illustrates the time dependence of the reaction of methionyl residues with 4-iodoacetamidosalicylic acid. The relatively rapid reaction with approximately 1 residue which occurs during the time interval characteristic of the inactivation process is followed by a slower first order reaction which is unrelated in time to the loss of activity. The modified methionyl residues resulting from the “nonspecific” slow reaction are included in the raw data given in Table III for methionine modification. Correction for this nonspecific reaction reveals an excellent correlation (Table III, last two columns) between the extent of inactivation and the extent of modification of a single methionyl residue per peptide chain.

Naidier et al. (32) have shown that various sulfur nucleophiles are capable of regenerating methionine residues from sulfonium salts contained in peptides and proteins. Table III reports the results of incubation of 75% inactive glutamate dehydrogenase with 4-iodoacetamidosalicylic acid under the conditions recorded in Fig. 3 for Line A’.

**TABLE III**

<table>
<thead>
<tr>
<th>Additions to incubation mixture</th>
<th>Time of incubation</th>
<th>Modified residues</th>
<th>Inactivation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>Cysteine</td>
<td>Methionine</td>
</tr>
<tr>
<td>1. None</td>
<td>35</td>
<td>0.41</td>
<td>0.45</td>
</tr>
<tr>
<td>2. None</td>
<td>70</td>
<td>0.62</td>
<td>0.77</td>
</tr>
<tr>
<td>3. None</td>
<td>140</td>
<td>1.12</td>
<td>1.26</td>
</tr>
<tr>
<td>4. None; 0.1 Mr dithiothreitol added at 140 min</td>
<td>140</td>
<td>0.94</td>
<td>0.00</td>
</tr>
<tr>
<td>5. None</td>
<td>210</td>
<td>1.62</td>
<td>1.83</td>
</tr>
<tr>
<td>6. None</td>
<td>700</td>
<td>4.38</td>
<td>3.89</td>
</tr>
<tr>
<td>7. 0.25 Mr ADP + 1.5 Mr DPN</td>
<td>85</td>
<td>0.22</td>
<td>0.89</td>
</tr>
<tr>
<td>8. 0.25 Mr ADP + 1.5 Mr DPN</td>
<td>255</td>
<td>0.63</td>
<td>1.69</td>
</tr>
</tbody>
</table>

*Percentage inactivation = 100% — (% activity remaining at given time relative to that at zero time).
Effect of 4-Iodoacetamidosalicylic Acid on Regulatory Properties

The maximum velocity of reductive amination catalyzed by native glutamate dehydrogenase is reversibly decreased to about 10% of its initial value when assayed in the presence of saturating concentrations of the inhibitor GTP. Inactivation of the enzyme by 4-iodoacetamidosalicylic acid is accompanied by a change in the response of the enzyme to GTP. Table IV shows, as a function of time of incubation with 4-iodoacetamidosalicylic acid, the decrease in maximum velocity assayed in the absence of inhibitor \( V_0 \), the values for maximum velocity determined in the presence of saturating concentrations of GTP \( V_{	ext{GTP}} \), and the variations in the kinetic dissociation constant for GTP measured in the presence of DPNH. It can be seen that there is an increase in the dissociation constant for the enzyme-purine nucleotide complex, which implies that the enzyme would be less sensitive to low concentrations of GTP. Most notably, however, is the observation that when the enzyme is assayed at the saturating concentrations of 2 mM GTP the rate of the reaction is constant. In other words, 4-iodoacetamidosalicylic acid causes inactivation of the enzyme when measured in the absence of inhibitor, but does not influence the small residual activity seen when GTP is present.

Fig. 8 shows the time course of reaction with 4-iodoacetamidosalicylic acid when the residual enzymatic activity is assessed in the absence of ligands or in the presence of a saturating concentration of the activator ADP and two concentrations of the inhibitor GTP. The extent of activation by ADP is decreased during the time period, although the dissociation constant for the enzyme-ADP complex is unaltered. In contrast, the observed effect on GTP inhibition depends on the concentration of GTP selected for the assay. When the enzyme is assayed at relatively low concentrations of GTP (e.g. 2 mM), the measured velocity is initially increased followed by a decline, reflecting the increase in the dissociation constant for the enzyme-GTP complex (Table IV). However, when higher concentrations of GTP are used in the assay (2 mM), the rate in the presence of GTP is almost unaffected for the first 200 min.

When the reaction is followed for long time periods, as shown in Fig. 8, the activity of the enzyme as assayed in the absence of ligands does not go to zero. Rather the decline in this activity...
appears to follow first order kinetics until it reaches the level of activity (approximately 4%) seen in the presence of GTP at that time. After that time, the activities measured in the presence and in the absence of GTP become essentially identical and continue to decrease at a slower rate.

4-Iodoacetamidosalicylic Acid Reaction in Presence of Coenzyme and Substrate

The reaction of 4-iodoacetamidosalicylic acid with glutamate dehydrogenase under all the conditions thus far discussed follows pseudo-first order kinetics and less than 10% activity remains. In contrast, when coenzyme and substrate are together in the incubation mixture the inactivation curve becomes biphasic, exhibiting an initial rapid drop in activity followed by a slower activity loss. Fig. 9 illustrates this pattern for α-ketoglutarate and DPN, but it is also found in the case of α-ketoglutarate and DPNH, glutamate and DPN, and glutarate and DPN. It is possible to analyze the data of Fig. 9 in terms of the relatively rapid formation of a partially active species of enzyme (with 27% residual activity) followed by a slower total inactivation. The pseudo-first order rate constant for the first reaction is calculated to be $56 \times 10^{-4} \text{ min}^{-1}$, whereas that for the second is $14 \times 10^{-4} \text{ min}^{-1}$.

Corresponding with the biphasic inactivation curve, the modification of cysteine residues of glutamate dehydrogenase in the presence of DPN and α-ketoglutarate also follows biphasic kinetics, as shown in Fig. 10. These data can be described by the reaction of 4-iodoacetamidosalicylic acid with two classes of sulfhydryl groups: two sulfhydryl groups which react with a pseudo-first order rate constant of $50 \times 10^{-4} \text{ min}^{-1}$, which is close to the rapid partial inactivation rate, and four $\text{-SH}$ groups which exhibit a pseudo-first order rate constant of $9 \times 10^{-4} \text{ min}^{-1}$. It seems likely that the enhanced reactivity of the two sulfhydryl groups may be responsible for the formation of a partially inactive species of enzyme upon reaction with 4-iodoacetamidosalicylic acid in the presence of coenzyme and substrate. It is difficult, however, to ascertain which amino acid residues are responsible for the slower total inactivation observed under these conditions, since the other four $\text{-SH}$ groups as well as methionyl residues are reacting with 4-iodoacetamidosalicylic acid during this time period.

As indicated in Fig. 9 and Table 1, when ADP is included with coenzyme and substrate the inactivation by 4-iodoacetamidosalicylic acid as well as the modification of cysteine residues again follows first order kinetics. It is possible that the binding of DPN and α-ketoglutarate increases the reactivity of two $\text{-SH}$ groups which are protected against modification by the binding of ADP.

DISCUSSION

The compound 4-iodoacetamidosalicylic acid was selected for this study because it had been proposed as an active site-directed reagent for glutamate dehydrogenase on the basis of its structural relationship to the reversible inhibitor salicylic acid (15, 19–23) and was therefore expected to alkylate the enzyme at specific sites. Salicylic acid is not a potent inhibitor of glutamate dehydrogenase, decreasing by 50% the rate of reductive amination of 1 mM α-ketoglutarate only when present at a concentration as high as 20 mM (23). A distance of 7.5 A was postulated by Caughey et al. (34) between the two cationic binding sites on the enzyme which correspond to the two carboxylate groups of the substrates α-ketoglutarate and glutamate. The distance is similar between the two carboxylate groups of the effective competitive inhibitor isophthalate; in contrast, the distance between the two functional groups of salicylic acid is smaller, perhaps accounting for the weaker affinity between the enzyme...
and salicylate. Nevertheless, Malcolm and Radda (15) observed saturation kinetics in the inactivation of glutamate dehydrogenase by 4-iodoacetamidosalicylic acid at pH 7.6, indicative of binding at the active site prior to inactivation.

Under the conditions of pH used in the present investigation (pH 6 and below), the rate of inactivation was linearly proportional to the concentration of 4-iodoacetamidosalicylic acid, an observation inconsistent with a rapid equilibrium formation of an enzyme-inhibitor complex prior to a slower irreversible inactivation. Despite this observation, it appears that the salicylic acid moiety contributes to the effectiveness of 4-iodoacetamidosalicylic acid in inactivating glutamate dehydrogenase under the same conditions iodacetamide itself (the halogen of which has been reported to be 40% as reactive as that of 4-iodoacetamidosalicylic acid toward thiourea (19)) causes inactivation and a decreased sensitivity to low concentrations of GTP but at a rate only 2% of that observed for 4-iodoacetamidosalicylic acid. One possible explanation of the more rapid inactivation produced by 4-iodoacetamidosalicylic acid is that this reagent binds to glutamate dehydrogenase noncovalently, thereby inducing the enzyme to undergo an irreversible denaturation. However, since neither salicylic acid, 4-aminosalicylic acid, nor 4-acetamidosalicylic acid (22) causes inactivation, it appears that the iodoacetamido portion of 4-iodoacetamidosalicylic acid is essential for its action. It is likely that the salicylate structure directs the specificity of the alkylating agent, and that either the rate of formation of the enzyme-inhibitor complex is slow or there is no appreciable binding prior to covalent modification at this pH.

In analogy to the reactions of iodoacetate with proteins (28), 4-iodoacetamidosalicylic acid must be considered capable of alkylating lysyl, histidyl, carboxyl, cysteinyl, and methionyl residues. The reactions of amino, histidyl, and sulfhydryl groups are thought to proceed via their unprotonated forms and therefore might be expected to be less important at pH 6.0 than at 7.6. Neither carboxymethyllysine nor carboxymethylhistidine was detected on amino acid analysis. The lower pH used in the present investigation undoubtedly accounts for the difference between these results and the observation of modified lysine reported by Holbrook et al. after reaction of 4-idoacetamidosalicylic acid with glutamate dehydrogenase at pH 7.6 (24). Reaction of iodoacetate derivatives with carboxylate groups in proteins is uncommon, although it has been reported to occur (35). Such a reaction is not detectable after acid hydrolysis; however, the observation that the amount of iodide released from 4-iodoacetamidosalicylic acid during inactivation of glutamate dehydrogenase can predominantly be accounted for by reaction with cysteine and methionine makes it unnecessary to invoke reaction with carboxylate groups in addition. In fact, definite evidence exists only for modification by 4-iodoacetamidosalicylic acid of cysteinyl and methionyl residues of glutamate dehydrogenase under the low pH conditions here used.

In most cases, a single rate constant adequately describes reaction with all 6 cysteinyl residues per peptide chain. This observation may indicate that all of the —SH groups are equally reactive; however, such an explanation seems improbable. Hellerman et al. (14) demonstrated the existence of two sets of sulfhydryl groups of glutamate dehydrogenase on the basis of differential reactivity toward metal ions and organic mercurials, and Goldin and Frieden (2) and Bitensky et al. (13) have pointed out the general lack of reactivity of the —SH groups of this enzyme until after the enzyme has been denatured. An alternate explanation of the linear alkylation rates of the six sulfhydryl groups might be that the initial reaction with a particular cysteine residue is the rate-determining step which inactivates the enzyme and changes its conformation to make the other cysteine residues available for rapid reaction. However, a comparison of the rates of 4-iodoacetamidosalicylic acid-produced inactivation and —SH alkylation reveals that the rate of alkylation is considerably lower than the rate of inactivation and that the two types of rates vary independently when substrates and coenzymes are present. Consequently, covalent modification of cysteinyl residues may be excluded as the primary cause of inactivation of the enzyme, although this reaction may have a minor effect on activity. It is more plausible that reaction of 4-iodoacetamidosalicylic acid with all six —SH groups occurs rapidly as the result of a conformational change which follows more slowly the rate-determining covalent modification of another site (e.g., a methionyl residue).

In contrast to the case of cysteine, there is a close correlation between the extent of modification of the most reactive methionyl residue and the degree of inactivation. The dithiothreitol-induced regeneration of methionine from the carboxymethylmethionine sulfonium salt also leads to appreciable recovery of enzymatic activity, strongly implying that this amino acid is critical for the function of glutamate dehydrogenase. It seemed possible that the inactivation of the enzyme by 4-iodoacetamidosalicylic acid at pH 7.6 (15) is due not only to alkylation of cysteine residues, but also to modification of a methionyl residue, since the reported amino acid analysis indicated a decrease of 2 methionyl groups and the protein concentration used was too low to detect directly the 1 or 2 carboxymethylhomocysteine groups. However, experiments conducted in this laboratory have shown that modification of methionine is not the principal cause of inactivation at this pH.

Alkylation of the enzyme by 4-iodoacetamidosalicylic acid results in a substantial loss of enzymatic activity; however, the maximum activity falls rapidly only to the level characteristic of native enzyme in the presence of the inhibitor. It is apparent that the binding or catalytic processes in the presence and in the absence of GTP are mechanistically distinct. On the basis of the data here presented it is not possible to conclude whether 4-iodoacetamidosalicylic acid directly attacks those catalytic sites that are sensitive to GTP or whether 4-iodoacetamidosalicylic acid irreversibly transforms the enzyme into the conformation which it normally assumes upon the binding of GTP, thereby rendering it less active and insensitive to added GTP. Similar results have been reported by Goldin and Frieden (9) for the reaction of lysine-97 with pyridoxal phosphate. In that case, also, modification of the enzyme causes reduction of the activity of the enzyme alone to approximately 5% of its initial value but does not decrease the velocity as tested in the presence of a saturating concentration of GTP.

The enhanced velocity observed at relatively low levels of GTP during reaction of the enzyme with 4-iodoacetamidosalicylic acid reflects an increase in the kinetic dissociation constant for the enzyme-coenzyme-GTP complex. This result is similar to the increased velocities noted when enzyme treated with pyridoxal (8), tetraniitromethane (12), or 4-iodoacetamidosalicylic acid at pH 7.6 (36) is assayed in the presence of low concentrations of GTP. Since the residual active enzyme exhibits an altered kinetic parameter ($K_{GTP}$) it is apparent that the reaction with 4-iodoacetamidosalicylic acid does not take place in an all or none manner. It is possible that chemical

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2 N. Rosen and R. F. Colman, unpublished data.
modification and inactivation of one of the 6 subunits of glutamate dehydrogenase causes a change in the quaternary structure of the protein which results in a decreased affinity of the remaining active subunits for GTP. Interaction among subunits would thus be responsible for the altered value for $K_{GTP}$. Despite the effects of 4-iodoacetamidosalicilic acid modification on the sensitivity of the enzyme to GTP, the critical methionyl residue is probably not located directly in the purine nucleotide-binding site, since protection against the kinetic changes caused by 4-iodoacetamidosalicilic acid is not provided by GTP. On the other hand, the site of 4-iodoacetamidosalicilic acid attack may be near the substrate-binding site, since the addition of $\alpha$-ketoglutarate, glutamate, or the competitive inhibitor glutarate to the reaction mixture together with DPN and ADP affords considerable protection as compared to DPN and ADP alone. If the methionyl residue is indeed located near the substrate-binding site, the effect of 4-iodoacetamidosalicilic acid on the affinity of the enzyme for GTP would of necessity be indirect.

Evidence has been presented (36, 37) that $\alpha$-ketoglutarate is capable of binding to the enzyme in the absence of coenzyme and ammonia. However, the contrast between the linear course of inactivation as well as sulfhydryl modification by 4-iodoacetamidosalicilic acid in the presence of either the substrate or coenzyme alone and the biphasic kinetics observed when they are added together implies that there is a reciprocal influence of DPN on the mode of binding of $\alpha$-ketoglutarate. It may be postulated that the binding of DPN and $\alpha$-ketoglutarate initiates a conformational change which leads to greater exposure or reactivity of 2 sulfhydryl groups at a different locus on the protein molecule. Similar interpretations have been proposed for the increased inactivation of phosphoglucomutase by 4-iodoacetamidic acid in the presence of glucose 6-phosphate (38), of thrombin by thrombin in the presence of calcium or tosylarginine methyl ester (39), of trypsin by N-acetylimidazole in the presence of the competitive inhibitor benzamidine (40), and of isotocate dehydrogenase by diazo-1H-tetrazole in the presence of divalent cations (41). Since the addition of the activator ADP together with $\alpha$-ketoglutarate and DPN restores the linear kinetics of 4-iodoacetamidosalicilic acid inactivation and of $-\text{SH}$ modification, this nucleotide appears to protect the 2 reactive sulfhydryl groups.

A relationship between sulfhydryl groups and ADP activation has previously been suggested on the basis of the effect of mercurials on glutamate dehydrogenase (16). The addition of ADP to suppress the reactivity of the $-\text{SH}$ groups made possible the realization that $\alpha$-ketoglutarate and DPN actually effect a decrease in the reaction rate of 4-iodoacetamidosalicilic acid with the critical methionine.

A structural role has most often been envisioned for methionine in proteins. Thus, in ribonuclease, alkylation of a methionine residue causes an inability of the enzyme or of its S-peptide to refold into the native conformation (42, 43); in chymotrypsin, reaction at a methionine residue results in increased Michaelis constants for the substrates without altering the ability of the enzyme to catalyze the hydrolysis of bound substrate (44, 45); and in cytochrome c, methionine has been implicated in the coordination of the heme iron (46). In a few cases, including phosphoglucomutase (47), myokinase (48), peroxidase (49), and funarase (50), but more relevantly the TPN-specific isocitrate dehydrogenase (17, 51), cytoplasmic malate dehydrogenase (18), and dihydrofolate reductase (52), evidence has been presented for the involvement of methionine in the catalytic function of the enzyme. It is possible that the thioether of methionine functions as a nucleophilic catalyst in dehydrogenation as well as in the other enzymatic reactions.

REFERENCES

27. Ellman, G. (1959) Arch. Biochem. Biophys. 82, 70
44. Lawson, W. B., and Schramm, H. J. (1965) Biochemistry 4, 377
Methionyl Residue Critical for Activity and Regulation of Bovine Liver Glutamate Dehydrogenase
Neal L. Rosen, Larry Bishop, Jean B. Burnett, Michael Bishop and Roberta F. Colman


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