Purification and Properties of S-Formylglutathione Hydrolase from Human Liver

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SUMMARY
S-Formylglutathione hydrolase, a new glutathione thiol esterase from human liver, has been purified into homogeneity according to disc electrophoretic and ultracentrifugal criteria. The final preparation catalyzes the hydrolysis of 4100 pmoles of S-formylglutathione per min per mg of protein at 25° and represents 2,350-fold purification over 23,000 × g supernatant of liver homogenate. In addition to S-formylglutathione, the enzyme can catalyze the hydrolysis of S-acetylglutathione at a 700-fold slower velocity but has no activity with S-lactylglutathione.

The elution volume of the enzyme in a gel filtration column gives an apparent molecular weight of 52,500 and a diffusion coefficient of 6.57 × 10⁻⁷ cm² s⁻¹. The enzyme has a sedimentation coefficient of 4.24 S as judged from sedimentation velocity in analytical ultracentrifuge. From a sedimentation equilibrium experiment, the molecular weight of the enzyme is 55,500. Dodecyl sulfate gel electrophoresis indicates a subunit molecular weight of 30,000. Thus, the enzyme probably consists of 2 subunits. The isoelectric point of the enzyme obtained from electrofocusing experiments is 5.41.

The enzyme apparently contains a reactive—SH group and purification is possible only in the presence of thiols. Amino group reagents also rapidly inactivate the enzyme. In addition, ascorbate and folate are inhibitory. Chelating agents and organophosphates do not inhibit the enzyme.

We have reported previously the presence of three separate glutathione thiol esterases in human liver (1), but thus far only one of them, glyoxalase II, has been thoroughly purified and characterized (2). In the previous report of this series, we showed that formaldehyde dehydrogenase purified from human liver catalyzes the formation of S-formylglutathione from formaldehyde and GSH (3). It was also shown that S-formylglutathione is hydrolyzed very actively by crude human liver preparations. In this work, we describe the purification and properties of S-formylglutathione hydrolase, a specific enzyme which is responsible for most of the hydrolysis of S-formylglutathione in human liver.

EXPERIMENTAL PROCEDURE

Materials

Pyruvic acid (4 mmoles) and thioglycolic acid (4 mmoles) were coupled by adding 2 g of N,N-dicyclohexylcarbodiimide and ascorbic acid from Fluka, and thioglycolic acid from Merck. Formaldehyde and GSH were purchased from Sigma. Pyruvic acid was from Aldrich, coenzyme A (free from GSH) from P-L Biochemicals, N,N-dicyclohexylearboximide and ascorbic acid from Fluka, and thioglycolic acid from Merck. Formylthioesters of mercaptocarboxylic acid and dithiothreitol were prepared by a method analogous to the synthesis of formylthioglycolate (1). S-Formylglutathione and S-formyl-N-acetylcysteine were obtained by transferring the formyl group from formylthioglycolate to GSH and N-acetylcysteine, respectively, at pH 5. Details of these and other thioester syntheses, except those described below, have been presented earlier (1). Formyl and pyruvyl thioesters were stored at -70° and were in this way usable for at least 2 months.

S-Pyruvylglutathione

Pyruvic acid (4 mmoles) and thioglycolic acid (4 mmoles) were coupled by adding 2 g of N,N-dicyclohexylcarbodiimide dropwise to 20 ml of N,N-dimethylformamide over a period of 90 min. The vessel was equipped with a drying tube, its contents were constantly mixed at 0°, and the mixing was continued 2½ hours after the addition of the reagents. Water (10 ml) was then added and the mixture was filtered. The filtrate was concentrated in vacuo and adjusted to pH 3.0 after the addition of water. It was then extracted seven to eight times with 1 volume of ether. According to hydroxamate analysis (4), the thiol ester was then in the ether phase. After evaporation of the ether, 6 volumes of water were added and the cloudy mixture was cleared by filtration. The filtrate was concentrated in vacuo. The yield of S-pyruvylthioglycolate thus obtained was 2 mmoles (60%).

The pyruvyl group was transferred to GSH by shaking 5-fold excess of pyruvylthioglycolate with GSH at pH 7.5 and at room temperature for 8 min. The cooled solution was then adjusted to pH 3.0 and freed from pyruvylthioester by at least six ether extractions. The yield of S-pyruvylglutathione was 30% based on GSH. The product was purified further by Sephadex G-10 gel filtration where it had smaller elution volume than impurities, which absorb strongly below 235 nm. The thiol ester was concentrated by lyophilization.
Formyl Coenzyme A

The preparative procedures closely followed those presented for S-pyruvylglutathione. The yield of formylthioglycolate (80%) was better than Trams and Brady (6) and Sly and Stadtman (6) obtained in their analogous methods with thiophenyl esters as intermediates. Formyl-CoA after gel filtration was 74% pure, and the rest was as CoA. This result was achieved by comparing the ultraviolet spectra of the preparation before and after treatment with hydroxylamine (\(A_r^M\) of CoA at 260 nm = 16,000 cm\(^{-1}\) mm\(^{-1}\) and the \(A_r^M\) of the thiol ester bond = 3,900 cm\(^{-1}\) mm\(^{-1}\)).

Protein Measurement

In crude solutions, the butanol method (8) was used. If necessary, deoxycholate (0.2% in final volume) was added to make the solutions clear. At the later stages of purification, the method of Lowry et al. (9) or Waddell (10) was used. These two methods gave accordant results for the purified enzyme preparations.

Determination of Enzyme Activity

The hydrolysis of S-formylglutathione and other thiol esters was measured at 240 nm with a Zeiss PMQ II spectrophotometer thermostated at 25°. The assay mixture contained 90 mM potassium phosphate buffer (pH 7.1), 0.5 mM thiol ester, and an enzyme solution in a total volume of 1.0 ml. Activities were calculated as international units (micromoles per min) in these conditions by using \(A_r^M = 3300\) for S-formylglutathione (1). A blank without an enzyme was always included. It contained all of the low molecular weight components like mercaptans introduced in the enzyme sample, and the nonenzymic rate was subtracted from the enzy-

Molecular Weight Determinations by Gel Filtration

These were performed in a calibrated Sephadex G-100 column according to Andrews (14).

Subunit Molecular Weight

Dodecyl sulfate gel electrophoresis according to Weber and Osborn (15) was used. Standards, bovine serum albumin (67,000), ovalbumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), chymotrypsinogen (25,000), and cytochrome c (12,400), gave a linear plot of log molecular weight versus mobility from which the molecular weight of the unknown was calculated.

Analytical Ultracentrifugation

These experiments were conducted in the Department of Medical Biochemistry, University of Oulu, Finland. A MSE Centri-

Disc Electrophoresis

The methods followed those described by Davis (19) except that no sample gel was used. Polyacrylamide gels (7.5%) at pH 8.9 and 7.5 were prepared according to Maurer (20). In order to locate the enzyme activity, part of the gels were cut into about 20 pieces with a gel slicer (Yeda, Rehovot, Israel). The pieces were incubated for 20 min in 0.5 ml of potassium phosphate buffer (0.1 M, pH 7.1) containing 1 mM DTNB with several shakings. Enzyme activity was then determined by adding an aliquot of the supernatant to the standard assay system (see above). After removing the supernatant, the pieces were stained separately with 1% Amido black and destained with 7% acetic acid. Part of the gels were similarly stained and destained without slicing.

Determination of Formate

The formyltetrahydrofolate synthetase method of Rabinowitza" (21) was used with the following modifications: tetrahydrofolate was dissolved in 50 mM GSH, pH 7, instead of 1 mM mercaptoethanol; the tubes containing the sample, assay mixture, and enzyme were incubated for 60 min at 0° instead of 37° and triethanolamine buffer was used at pH 7.2. These modifications were done in order to prevent the nonenzymic decomposition of S-formyl-

Preparation of Intracellular Fractions

The methods followed those described by Schneider and Hoge-

Purification of Enzyme

S-Formylglutathione hydrolyase is, in most cases, co-purified at the first stages with formaldehyde dehydrogenase, and Steps 1 to 3 have been described in the preceding paper (5).

Step 4. Hydroxylapatite Chromatography—The pooled prepara-

Preparation 5. Preparative Isoelectric Focusing—The enzyme pooled

1 We are indebted to Associate Professor Ilmo Hassinen, Department of Medical Biochemistry, University of Oulu, Finland, for his valuable guidance in the analytical ultracentrifugation experiments.

2 The abbreviations used are: DTT, dithiothreitol; PMB, p-

hydroxymercurobenzoate; Nbs, 5,5'-dithiobis(2-nitrobenzoate); MalNEt, N-ethylmaleimide; Dansyl, 5-dimethylaminonaphththalene-1-sulfonyl.
from two hydroxylapatite columns was concentrated 20 to 30 fold with an Amicon ultrafiltration cell as above, and then applied to a 110-ml isoelectric focusing column (LKB, Stockholm, model 8101). The enzyme was put in the light solution where it replaced a comparable amount of water. The gradient solutions contained 1 mM DTT. The ampholytes (pH gradient 4 to 6) were used in 1% (w/v) final concentration. The focusing time was 48 hours.

The initial voltage of 400 volts was increased in steps to the final value (800 volts). The fractions collected with a pump and a fraction collector were studied for activity, protein, and pH (Fig. 2). Five 2-ml fractions containing the highest activity were pooled.

**Step 6. Sephadex G-100 Gel Chromatography**—The enzyme from the previous stage was concentrated with an Amicon ultrafiltration cell (12 ml, PM-10) to 4 ml and applied to a Sephadex G-100 column (2.5 × 45 cm) equilibrated and eluted with 10 mM potassium phosphate (pH 6.8) containing 5 mM mercaptoethanol. The specific activity was constant in the elution site of the enzyme (Fig. 3).

**RESULTS**

**Purification**

The summary of purification of S-formylglutathione hydrolase, presented in Table I, shows that about 2300-fold purification was obtained. The enzyme had already in crude solution an unusually high specific activity, and the final preparation had a specific activity of 4100 i.u. per mg of protein. The same final value was obtained from two different human livers, and the methods of Lowry *et al.* (9) and Waddell (10) for protein content agreed within a few per cent for the final preparations.

**Disc Electrophoresis**

Electrophoreses were performed for the purified preparations at two pH values, 7.5 and 8.9. In both conditions only a single protein band was constantly obtained (Fig. 4, a and b). Activity determinations and protein staining from gel slices showed that all of the activity was associated with the only protein band seen.

**Molecular Weight**

The elution volume of S-formylglutathione hydrolase in a Sephadex G-100 column calibrated with standard proteins gave an apparent molecular weight of 52,500 ± 1400 (n = 5). No change occurred during purification. When the elution volumes of the standards were plotted against 10m6/D (14), a diffusion coefficient of 6.57 × 10−9 cm2 s−1 could be calculated for S-formylglutathione hydrolase (Fig. 5).

The purified enzyme sedimented in analytical ultracentrifuge as a single symmetrical peak. A plot of log r versus time (16) was linear (Fig. 6). From its slope, the enzyme has a sedimentation coefficient of 4.24 S (s20,w protein concentration of 1 mg per ml). The molecular weight of the enzyme calculated from sedimentation and diffusion coefficients is 58,200, if the partial specific volume of the enzyme is assumed to be 0.73 ml per g.

**TABLE I**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Supernatant of liver homogenate</td>
<td>1570</td>
<td>128,000</td>
<td>74,100</td>
<td>1.73</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate (45 to 67%)</td>
<td>232</td>
<td>93,200</td>
<td>26,200</td>
<td>3.56</td>
<td>2.0</td>
<td>72.7</td>
</tr>
<tr>
<td>3. DEAE22</td>
<td>430</td>
<td>56,000</td>
<td>973</td>
<td>50.7</td>
<td>34.5</td>
<td>45.3</td>
</tr>
<tr>
<td>4. Hydroxylapatite</td>
<td>865</td>
<td>36,400</td>
<td>23.1</td>
<td>1570</td>
<td>910</td>
<td>28.4</td>
</tr>
<tr>
<td>5. Isoelectric focusing</td>
<td>12.7</td>
<td>17,500</td>
<td>n.d.*</td>
<td></td>
<td></td>
<td>13.7</td>
</tr>
<tr>
<td>6. Sephadex G-100</td>
<td>30.0</td>
<td>13,700</td>
<td>3.34 (W)b</td>
<td>4100 (W)b</td>
<td></td>
<td>2350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.38 (L)b</td>
<td>4060 (L)b</td>
<td></td>
<td>10.7</td>
</tr>
</tbody>
</table>

*a* n.d. = not determined.

*W* = protein determined according to Lowry *et al.* (9), *W* = according to Waddell (10).
FIG. 4. Disc electrophoresis of S-formylglutathione hydrolase at pH 7.5 (a, 10 μg of protein per gel) and at pH 8.9 (b, 17 μg of protein per gel). Gels were stained with Amido black. c, dodecyl sulfate polyacrylamide gel electrophoresis (15) of S-formylglutathione hydrolase. Fifteen micrograms of protein were used per gel. Gel was stained with Coomassie brilliant blue. Cathode is at top, anode at bottom, run from cathode to anode.

FIG. 5. Determination of the molecular weight and diffusion coefficient of S-formylglutathione hydrolase (○) by gel filtration on Sephadex G-100. The standard proteins (■) were: 1, catalase; 2, aldolase; 3, bovine serum albumin; 4, chymotrypsinogen; and 5, cytochrome c. The experimentally determined elution volumes of the standards are plotted against log molecular weight and 10^−6/D, where D is the diffusion coefficient.

The data of the equilibrium ultracentrifugation gave a linear plot of area versus 1/r y (17) (Fig. 6). Thus, the preparation used was homogeneous with respect to molecular weight. The molecular weight from this experiment is 55,500.

FIG. 6. Top, plot of log r versus time (16) obtained from a sedimentation velocity experiment of purified S-formylglutathione hydrolase. The slope of the plot was used to calculate the sedimentation coefficient for the enzyme. Bottom, data of a sedimentation equilibrium experiment treated in a similar way as in Ref. 17. The enzyme peak obtained by the photoelectric scanner was divided into 2-mm segments. The area of a segment is plotted versus 1/r y where r is the radius of the midpoint of the segment and y, the height of the segment.

Subunits

Purified S-formylglutathione hydrolase had, in sodium dodecyl sulfate gel electrophoresis (Fig. 4c), a mobility which corresponds to a subunit molecular weight of 30,000 ± 1,300 (n = 4), calculated from a linear plot formed by the five standard proteins (15). We conclude that S-formylglutathione hydrolase is formed of 2 subunits of similar molecular weight.

Isoelectric Point

Preparative and analytical isoelectric focusing experiments were performed for enzyme preparations from Steps 3 and 4. As a mean of four experiments, the enzyme has an isoelectric point of 5.41 ± 0.10 at 4°C.

Substrate Specificity

Of the 10 thiol esters of glutathione which were tested, S-formylglutathione was by far the most active substrate. Slight activity was noted for S-acetylglutathione and negligible activities for S-propionylglutathione, S-pyruvylglutathione, and S-glycolylglutathione, whereas S-laetylglutathione which is the most active substrate of human liver glyoxalase II (2), and four other tested thiol esters did not react at all (Table II).

The enzyme showed slight activity towards formylthioesters of other mercaptans, but all were at least 200-fold less reactive than S-formylglutathione, and formyl-CoA was almost inactive as a substrate (Table II).

The two carboxyl esters studied, ethyl formate and ethyl acetate, were inactive as substrates, as was N-acetylimidazole. p-Nitrophenyl acetate was hydrolyzed by the pure enzyme, but at a very low velocity.

The Michaelis constant for S-formylglutathione was 0.29 mM, whereas for S-acetylglutathione a lower value, 0.12 mM, was obtained. Both substrates gave a linear Lineweaver-Burk plot...
The activities were determined with the purified enzyme by the methods described under "Experimental Procedure." The initial concentrations of thiol esters were 0.5 mM except for formyl-CoA, which was used at 0.3 mM. p-Nitrophenyl acetate was 1 mM, N-acetylimidazole, 0.5 mM, and carboxyl esters, 5 mM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Formylglutathione</td>
<td>100</td>
</tr>
<tr>
<td>S-Acetylglutathione</td>
<td>0.59</td>
</tr>
<tr>
<td>S-Propionylglutathione</td>
<td>0.19</td>
</tr>
<tr>
<td>S-Pyruvylglutathione</td>
<td>0.10</td>
</tr>
<tr>
<td>S-Glycolylglutathione</td>
<td>0.07</td>
</tr>
<tr>
<td>S-Glycerylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>S-Lactylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>S-Mandelylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>S-Acetoacetylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>S-Succinylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>0.08</td>
</tr>
<tr>
<td>Formylthioleucinate</td>
<td>0.05</td>
</tr>
<tr>
<td>Formylmercaptoethanol</td>
<td>0.59</td>
</tr>
<tr>
<td>Formyldithiolethreitol</td>
<td>0.02</td>
</tr>
<tr>
<td>S-Formyl-N-acetylcysteine</td>
<td>0.05</td>
</tr>
<tr>
<td>N-Acetylimidazole</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl formate</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>0.25</td>
</tr>
</tbody>
</table>

The enzyme has optimal activity at pH 6.9 to 7.1. The activity was highest in phosphate, pyrophosphate, and acetate buffers, with no difference between sodium and potassium salts. In Tris and imidazole buffers, the activity was about 80% of the activity in phosphate, and in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and triethanolamine, 65%. All of the buffers were used at a concentration of 90 mM.

**pH Stability**

The purified enzyme (Step 3) was stable between pH 7.4 and 7.8 in phosphate, pyrophosphate, and Tris-acetate buffers. The activity was about 80% of the activity in phosphate, and in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and triethanolamine, 65%. All of the buffers were used at a concentration of 90 mM.

**Dependence of Activity on Enzyme Concentration**

The activity followed enzyme concentration linearly up to a decrease in absorbance of 0.2 per min (1 = 1.0 cm) at 240 nm. This corresponds to the hydrolysis of 60 nmole of S-formylglutathione per min per ml. In our measurements, the velocity was in general adjusted to about 0.1 absorbance unit per min at 240 nm. Then the reaction was linear with respect to time for at least 2 min when S-formylglutathione was used at 0.5 mM initial concentration.

**Absorption Spectrum**

The purified enzyme had a typical protein spectrum with no absorbance in the visible area and an absorption maximum at 278 nm. In a concentration of 10 mg per ml, the enzyme has an absorbance of 18.0 cm⁻¹ at 278 nm.

**pH Optimum**

The enzyme has optimal activity at pH 6.9 to 7.1. The activity was highest in phosphate, pyrophosphate, and acetate buffers, with no difference between sodium and potassium salts. In Tris and imidazole buffers, the activity was about 80% of the activity in phosphate, and in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and triethanolamine, 65%. All of the buffers were used at a concentration of 90 mM.

**Activation Energy**

The velocities of enzymic and nonenzymic hydrolysis were measured as usual, but the reaction temperature was varied from 8–34°. All of the reagents were pre-equilibrated to the temperatures used. A linear Arrhenius plot was obtained between these temperatures for both nonenzymic and enzymic rates. As calculated from the slope, the enzymic hydrolysis has an activation energy of 4.50 Cal per mole and the Q₁₀ value is 1.30. The activation energy of the nonenzymic hydrolysis is 15.6 Cal per mole in the conditions used.

**Stoichiometry**

Because the S-formylglutathione used by us was contaminated with a 3-fold excess of GSH and is unstable, the formation of GSH by the enzyme was studied by using as substrate S-acetylglutathione, a pure product prepared as described before (1). S-Acetylglutathione was not hydrolyzed nonenzymically in our conditions. The amount of GSH formed after treatment with a highly active preparation of hydrolase corresponded well with the amount of hydrolyzed thiol ester (Table III).

The enzymic formation of formate was demonstrated by using formyltetrahydrofolate synthetase of Clostridium acidi-urici. When the original method of Rabinowitz (21) was used, the formation of formate corresponded well with a highly active preparation of hydrolase and is stable, the formation of GSH by the enzyme was studied by using as substrate S-acetylglutathione, a pure product prepared as described before (1). S-Acetylglutathione was not hydrolyzed nonenzymically in our assay conditions. The amount of GSH formed after treatment with a highly active preparation of hydrolase corresponded well with the amount of hydrolyzed thiol ester (Table III).

**Stability of Enzyme**

It is essential to use low molecular weight thiols in buffers during purification. Even in the DEAE stage (Step 3), the recoveries were much lower in the absence of mercaptoethanol and in later steps practically all of the activity was lost without added thiols. The addition of DTT to fractions obtained from columns with 2 to 5 mM mercaptoethanol in buffers did not activate the

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**Table II**

**Substrate specificity of S-formylglutathione hydrolase**

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<tr>
<td>S-Lactylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>S-Mandelylglutathione</td>
<td>0</td>
</tr>
<tr>
<td>S-Acetoacetylglutathione</td>
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<td>S-Succinylglutathione</td>
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<td>S-Formyl-N-acetylcysteine</td>
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<td>N-Acetylimidazole</td>
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<tr>
<td>Ethyl formate</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>0.25</td>
</tr>
</tbody>
</table>
The enzyme from Step 4 was concentrated by ultrafiltration and then freed from most mercaptoethanol by dialysis as described in text. The activity before dialysis has been set to 100.

**Inhibitors**

**Sulphydryl Reagents**—In order to obtain data on the effect of —SH reagents on the enzyme, a preparation from hydroxylapatite stage (Step 4) was concentrated by ultrafiltration to contain at least 500 i.u. per ml. It was then dialyzed at 4° for 6 hours against 10 mM Tris-HCl, pH 7.6, with one change of the buffer to remove most of the low molecular weight thiols. Only 10 to 15% of the original activity was recovered, and if dialysis was continued more activity was lost rapidly. Addition of 2 mM mercaptoethanol or 1 mM DTT to the dialysis buffer fully stabilized the enzyme. Complete removal of thiols by Sephadex G-25 gel filtration resulted in loss of all of the activity. A dialyzed preparation had still enough activity to permit activity measurements both with S-acetylglutathione and S-formylglutathione. The preparation, after dialysis, was activated over 300% by DTT. Several minutes were needed for the complete effect at 25°. Other mercaptans were much less effective (Fig. 7). Potassium borohydride (up to 10 mM) had no reactivating effect.

The effect of various —SH reagents on the dialyzed enzyme is shown in Table IV. Mercaptoethanol (ME) and mercaptide-forming agents, and Nbs₂, an oxidizing agent, were effective inhibitors especially when S-acetylglutathione was used as the substrate. With MalNEt, and especially with iodoacetate, higher inhibitor concentrations were needed and with these alkylating agents there was no difference between results with S-formylglutathione and S-acetylglutathione (Table IV). Arsenite, a reagent for dithiol groups, was shown in Table IV. Mercaptide-forming agents and Nbs₂ with S-acetylglutathione as substrate are probably too high because the dialyzed enzyme still contained about 0.03 mM mercaptoethanol, and the final assay mixture was thus about 0.5 mM in mercaptoethanol.

The easy reversibility of the enzyme activity after inhibition
FIG. 8. Inhibition of S-formylglutathione hydrolase (Step 4, obtained as in the experiment of Fig. 7 and similarly freed from most thiols) by —SH reagents and the reversibility of the inhibition by DTT. a, enzyme treated by 0.002 mM HgCl₂ (○) and a control without an inhibitor (□); the hydrolysis of the substrate (0.5 mM S-acetylglutathione) was followed at 240 nm. At the time marked by the arrow, DTT (3 mM) was added to both solutions. b, the activities of the enzyme treated by 0.002 mM Nb₃ (▲) and a control (○) similarly followed at 240 nm, and DTT, then added at the time of the arrow. c, the activities of the enzyme treated by 0.5 mM MalNEt (×) and a control (○) followed at 240 nm as in a and b and 3 mM DTT again added at the time marked by the arrow. The enzyme preparation used in Fig. 8c was different from the preparation used in a and b and appeared to require a longer time for full reactivation by DTT. The rate of the MalNEt-treated sample was followed over 60 min, but the rate did not increase over that seen between 25 and 30 min.

FIG. 9. Inactivation of S-formylglutathione hydrolase (Step 4) by 1 mM 2,4,6-trinitrobenzene sulfonate. The enzyme was incubated together with a control without the inhibitor at 25° in the following conditions: △, pH 5.7 (acetate buffer); ○, pH 6.6 (phosphate buffer); ●, pH 7.4 (phosphate buffer); ▲, pH 8.5 (pyrophosphate buffer); ◯, pH 8.9 (pyrophosphate buffer); and ×, pH 9.7 (carbonate buffer). All buffers were 0.1 M. Activities were measured according to the standard assay system (see "Experimental Procedure"). Activities are expressed as per cent of the activity of a control without the inhibitor.

Amino Group Reagents—The effect of 2,4,6-trinitrobenzene sulfonite (Fig. 9) is in accordance with a reacting free amino group. Dansyl chloride (0.1 mM) inactivated the enzyme rapidly with a half-life of about 3 min throughout the studied pH range (5.7 to 9.0). Therefore, besides amino group(s), —SH group(s), and possible other groups of the enzyme may be reacting.

Hydroxylamine, a carboxylic group reagent (25), was not inhibitory. The enzyme was treated with increasing concentrations of neutral hydroxylamine up to 1 M, after which free hydroxylamine was dialyzed off. N-Acetylimidazole, a tyrosine reagent (26), was also ineffective (phosphate buffer, pH 7.5) at least up to 1 mM.

Chelating Agents—The following reagents were studied: EDTA (5 mM), ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (5 mM), α-phenanthroline (2 mM), α,α'-dipyridyl (2 mM), and 8-hydroxyquinoline (2 mM). Not one of them was inhibitory (30 min incubation at pH 7.1 with the enzyme at 20°).

Ion Effects—NaCl, KCl, and NH₄Cl, all 0.1 M, had no effect on the enzyme. CaCl₂ in the same concentration inhibited 60%. MgSO₄ and MnSO₄ (10 mM) had no effect, but 0.2 mM zinc-acetate inhibited 45% and 1 mM, 95%. Co(NO₃)₂ and copper-acetate were similarly inhibitory. These heavy metals were studied in 0.05 M N-2-hydroxyethylpiperazine-N'2-ethane sulfonic acid buffer pH 7. Fluoride inhibited 30% at 8 mM and 60% at 20 mM.

Other Agents—GSH inhibited 18% at 5 mM and 37% at 10 mM. GSSG inhibited 26% at 2.5 mM and 78% at 5 mM. S-Methylglutathione (3 mM) and S-hexylglutathione (1 mM) had no effect. Formaldehyde (10 mM) and methylglyoxal (10 mM) were without effect alone or in the presence of GSH. Thus the hemimercaptals, which these aldehydes form with GSH, are not inhibitory either.

Ascorbate lowered S-formylglutathione hydrolase activity, 0.4 mM inhibiting 35% and 1 mM, all activity. Other reducing agents like thiols or borohydride were not inhibitory. L-Gulonolactone had no effect. Folic acid and tetrahydrofolic acid were inhibitory, 0.2 mM inhibiting 40% and 0.5 mM, 100%.
Organophosphates—Both diisopropylphosphorofluoridate (1 mM) and diethyl-p-nitrophenylphosphate (0.5 mM) were ineffective towards S-formylglutathione hydrolase.

Reversibility of Reaction

Formate, up to 0.1 M, had no effect on the rate of the reaction. It was not possible to show formation of a thiol ester from formate and GSH by the enzyme at pH 7.1. Addition of ATP had no effect.

Transfer Reactions

The formyl group cannot be transferred enzymically from GSH to other mercaptans (mercaptanohol, DTT) because their presence did not slow the absorption decrease at 240 nm, as is expected if a new thiol ester is obtained instead of the hydrolysis. In the presence of imidazole, S-acetyl- or S-formylglutathione and enzyme, absorption decrease only was seen at 250 to 260 nm. Thus, a formation of the respective N-acyl thione and enzyme, absorption decrease only was seen at 240 nm, as is expected if a new thiol ester is obtained instead of the hydrolysis. When compared to the more reactive S-formylglutathione hydrolase, it is also evident that S-formylglutathione hydrolase does not catalyze the formation of N^4-formyltetrahydrofolic acid from S-formylglutathione and tetrahydrofolic acid.

DISCUSSION

The results of the present investigation show that human liver contains a specific enzyme which very actively catalyzes the hydrolysis of S-formylglutathione. We have also demonstrated (3) that human liver formaldehyde dehydrogenase catalyzes the formation of S-formylglutathione, and it seems probable that these enzymes, which are both cytoplasmic, function together in vivo. The physiological significance of this potentially very active reaction sequence is not, however, clear at present. Formaldehyde can also be oxidized actively to formate by the unspecific NAD-linked aldehyde dehydrogenase and by a tetrahydrofolate-dependent reaction sequence (27). A transferase function for S-formylglutathione hydrolase would seem plausible to conserve the thiol ester bond energy, but there is no experimental evidence for it at present.

Other sources than human liver have not been studied yet for the presence of S-formylglutathione hydrolase. Because formaldehyde dehydrogenase appears to be distributed generally (3), the same probably applies also to S-formylglutathione hydrolase. At least in human liver glyoxalase II also can catalyze the hydrolysis of S-formylglutathione, and its activity is more than enough to hydrolyze all S-formylglutathione formed by formaldehyde dehydrogenase (2, 3). Glyoxalases I and II are distributed ubiquitously in the living cells (28). Rose and Racker (29) have reported that S-formylglutathione is hydrolyzed actively by yeast by a source different from glyoxalase II. Kielley and Bradley (30) have partially purified a thiol esterase from mouse liver which hydrolyzed S-acetylglutathione but not S-lactylglutathione, and it is possible that this activity was at least in part due to S-formylglutathione hydrolase.

S-Formylglutathione hydrolase has a molecular weight of about 55,000, which is higher than that of glyoxalase II, 22,900 (2). Sodium dodecyl sulfate gel electrophoresis gives a single subunit with a molecular weight of 30,000, and the enzyme is thus apparently composed of 2 identical subunits. In isoelectric focusing S-formylglutathione hydrolase has an acidic isoelectric point, pI of 5.41 in contrast to glyoxalase II, which is a basic protein with a pI of 8.35 (2).

S-Formylglutathione hydrolase like glyoxalase II and the other glutathione thiol esterases studied (1, 2) is not inhibited by organophosphates. It is sensitive to several types of —SH reagents and is easily oxidized in vitro by the oxygen in air. The reactivity of the —SH group(s), the easy reversal of this inhibition by DTT, and perhaps also the greater sensitivity of the enzyme for —SH reagents with S-acetylglutathione as substrate, when compared to the more reactive S-formylglutathione suggest the presence of an —SH group at or near the active center of the enzyme. Also, an amino group seems to be important to the function of this enzyme according to the inhibition results obtained with 2, 4, 6-trinitrobenzene sulfonate. No metal is needed by S-formylglutathione hydrolase

In the previous paper from this laboratory (2), the presence of a fourth hydrolase for glutathione thiol esters was anticipated in human liver in addition to glyoxalase II, S-formylglutathione hydrolase, and S-succinylglutathione hydrolase. Because the last named enzyme seems to be specific for S-succinylglutathione (1) and the specificities of glyoxalase II and S-formylglutathione hydrolase are known, approximate calculations can be made to find further evidence for possible additional hydrolases. It seems that no more than 60 to 70% of the hydrolytic activities of unpurified human liver preparations for S-acetylglyutathione and S-propionylglutathione can originate from S-formylglutathione hydrolase and glyoxalase II.

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