GLUTATHIONE THIOLESTERASE

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It has been observed by Wieland and Bokelmann (1) and by Stadtman (2) that thiol esters readily engage in exchange reactions with other mercaptans in aqueous solution near neutral pH. In the experiments of Stadtman, involving an exchange between acetyl coenzyme A and glutathione, the formation of S-acetyl glutathione led to an inquiry into the possible biological activity of this material. In preliminary experiments it was observed that S-acetyl glutathione rapidly disappeared in the presence of liver extracts and that the acyl group could be quantitatively recovered as acetic acid. It has since been possible to purify an enzyme from mouse liver extracts that is specific for the hydrolysis of some S-acyl derivatives of glutathione. This enzyme is distinct from liver esterase and glyoxalase II. This communication presents the preparation and some properties of the thiolesterase.

Methods

Substrates—Ethyl thiolacetate, butyl thiolacetate, acetyl coenzyme A, butyryl coenzyme A, and some of the S-acetyl glutathione were obtained from Dr. E. R. Stadtman. Acetyl thiolglycolic acid, ethyl acetate, ethyl butyrate, and acetyl choline were commercial products. N-Acetyl alanine was obtained from Dr. D. Steinberg.

"Lactoyl" glutathione, the intermediate in the glyoxalase reaction, was prepared according to Racker (3), S-acetyl and S-butyryl glutathione were prepared by a method similar to that employed by Wilson (4) for the synthesis of acetyl coenzyme A. 2 gm. of glutathione were dissolved in 20 ml. of water, and 10 ml. of 95 per cent ethanol and 2.5 ml. of thiol acetic acid (Amend) were added. The thiol acetic acid was in an approximate 5-fold excess. The mixture was stirred mechanically at room temperature. The reaction was followed by determining the optical density of a suitably diluted ether-extracted aliquot of the reaction mixture at 232 mμ in the Beckman spectrophotometer. Ether extraction is necessary for removal of unchanged thiol acid which also absorbs strongly in the neighborhood of 232 mμ. The solutions were buffered with 0.03 M phosphate, pH 6.5. When the reaction was nearly complete as judged from change in optical
density (4 to 5 hours), the reaction mixture was extracted three times with 2 or 3 volumes of ether. The aqueous solution was then diluted with 2 volumes of acetone, and, after standing overnight, the thiol ester was removed by filtration and recrystallized by dissolving in a minimal volume of water and adding acetone slowly. The product melted at 203–205°, in agreement with Wieland and Bokelmann (1), and, relative to alanine as a standard, had a color intensity in the colorimetric ninhydrin procedure of Moore and Stein (5) of 94 per cent. The thiol ester content determined by the neutral hydroxamic acid procedure of Lipmann and Tuttle (6) was 94 per cent of the anticipated value for S-acetyl glutathione. The compound has an absorption maximum at 232 m\(\mu\), and at this wave-length the extinction coefficient was \(4.72 \times 10^8\) sq. cm. per mole. The yield was 40 per cent.

\(\text{S-Butyryl glutathione was prepared in the same manner from glutathione and thiolbutyric acid. Thiolbutyric acid was prepared by the procedure of Ellingboe (7) for thiolacetic acid. A fraction boiling at 52–54° at 45 mm. was collected. On the basis of the hydroxamic acid reaction this was 90 per cent pure. The S-butyryl glutathione obtained melted at 220–221° and showed a color equivalent of 0.91 in the ninhydrin reaction and a thiol ester equivalent of 0.91 by the neutral hydroxamic acid procedure. The extinction coefficient at 232 m\(\mu\) was \(4.20 \times 10^6\). The yield was 20 per cent.}

Analytical Procedures

**Thiolesterase and Glyoxalase II Activity**—Initially thiolesterase activity was determined by disappearance of hydroxamic acid-forming material, but at the suggestion of Dr. E. Racker we subsequently employed the absorption maximum at 230 to 240 m\(\mu\) (a general property of thiol esters (3, 8, 9)) in a procedure essentially similar to Racker’s method of assay for glyoxalase II (3). Unless otherwise specified, the medium was 0.0003 M thiol ester, 0.03 M phosphate buffer, pH 6.5, and 0.12 M KCl with a suitable dilution of the enzyme in a final volume of 3.0 ml. The spectrophotometer was set with a blank arbitrarily selected to give an initial optical density of 0.400 at 232 m\(\mu\).

**Esterase Activity**—The alkaline hydroxamic acid procedure of Hestrin (10) was employed for measuring esterase activity. The medium consisted of 0.005 M ethyl butyrate, 0.03 M phosphate, pH 6.5, and a suitable dilution of the enzyme in a final volume of 1.0 ml. The mixture was incubated at 28° for 0, 10, 20, and 30 minutes and the rate determined from the linear portion of the change in ester concentration.

The behavior of the three enzyme assays with respect to time and enzyme concentrations is shown in Figs. 1 and 2.
For determination of specific activities protein was determined from spectrophotometric measurements at 280 and 260 mm according to the procedure of Warburg and Christian (11).

Results

Preparation of Thiolesterase—Although extracts of fresh liver and liver acetone powders of other species possess the ability to hydrolyze S-acetyl glutathione, the purification procedure presented here worked most conveniently with extracts of mouse liver. The usual starting material was the pooled supernatant fluids after removal of nuclear and mitochondrial fractions of 10 per cent homogenates in 0.25 M sucrose. These were kept frozen, and when sufficient material had accumulated, usually 0.5 to 1.0 liter, the material was thawed, centrifuged at 72,000 X g for 45 minutes, the precipitate discarded, and the supernatant solution assayed for thiol-esterase, glyoxalase II, and esterase activities.
Step 1—To the ultracentrifuged solutions were added 23 gm. of solid (NH₄)₂SO₄ for every 100 ml. of solution; the precipitate was removed by centrifugation, and for every 100 ml. of supernatant fluid 30 gm. more of (NH₄)₂SO₄ were added, and the suspension was allowed to stand overnight. The precipitate was then collected by centrifugation, dissolved in water to about one-tenth the original volume, and dialyzed for 24 hours against several changes of 0.2 M KCl.

Step 2—After dialysis the solution was cautiously adjusted to pH 4.7 to 4.8 with 0.1 N acetic acid and allowed to stand in the cold. At intervals aliquots were removed, centrifuged, and the activity toward lactoyl glutathione was determined. When about 90 per cent of the glyoxalase II activity had disappeared, the solution was centrifuged, the precipitate discarded, and the supernatant fluid adjusted to pH 6.0 to 7.0 with 1.0 M KHCO₃. At this point the enzyme preparation was usually treated with calcium phosphate gel at a gel to protein ratio of 0.5. However, this did not ordinarily lead to any significant improvement in the specific activity of the thiolesterase which remained in the supernatant solution.

Step 3—The enzyme solution was brought to 45 per cent saturation with saturated (NH₄)₂SO₄ and the precipitate removed by centrifugation and discarded. The (NH₄)₂SO₄ concentration of the supernatant fluid was raised to 60 per cent saturation, and, after standing for about 1 hour, the precipitate was collected and dissolved in a small volume of water (about one-twentieth the volume of the original extract). The solution was then dialyzed against several changes of 0.2 M KCl for about 20 hours.

Step 4—The dialyzed solution containing about 15 mg. of protein per ml. was then treated with a suspension of alumina Cγ (about 20 mg. of dry weight per ml.). The gel was added in small increments, the specific activity of the supernatant solution was determined at each step, and the gel discarded until finally the major portion of the enzyme came out on the gel. This was removed by centrifugation and the enzyme eluted with 0.05 M phosphate, pH 6.5. The over-all recovery of the glutathione thiolesterase was about 5 per cent and the increase in specific activity about 10- to 20-fold. The data for one preparation are summarized in Table I.

Substrate Specificity—The crude extracts of liver exhibit glyoxalase II and esterase as well as thiolesterase activity. The last may be differentiated from the others by its sensitivity to heating. This is demonstrated in Table II, which presents data for the inactivation of a thiolesterase preparation, heated to 55° for 5 minutes, at the level of Step 1 in the preparation.

By examining Table I it may be seen that glyoxalase II is almost completely removed at Step 2. On the other hand, the specific activity of the esterase did not undergo any consistent change throughout the manipulations, but the ratio of esterase to thiolesterase activities, initially 20:1, became 3:1 for the final preparation.
The preparations will not hydrolyze ethyl acetate or acetylcholine under the conditions employed, nor will they deacetylate N-acetyllalanine. Of the other thiol esters, ethyl thiolacetate, butyl thiolacetate, acetylthiolglycolic acid, acetyl coenzyme A, and butyryl coenzyme A, none was attacked by the purified enzyme. However, the crude preparations were able to hydrolyze butyl thiolacetate and acetyl coenzyme A slowly.

Other Properties of Thiolesterase—The pH optimum of the thiolesterase appeared to lie between 7.0 and 7.5, but over the range of 6.5 to 8.0 the activity was relatively insensitive to changes in pH.

No activator or cofactor requirements for the enzyme have been observed. The choice of buffer (phosphate) was not important except for its relatively low absorption at 232 m\(\mu\). Experiments in which the neutral hydroxamic acid method of analysis and both histidine and phosphate buffers were employed gave similar results for both buffers.

The reaction catalyzed by the enzyme is a hydrolysis. In one large scale experiment 76.5 \(\mu\)m of \(S\)-acetyl glutathione were incubated with the enzyme. The decrease in density at 232 m\(\mu\) was followed on suitably diluted aliquots, and the reaction was virtually complete after 3 hours.

**TABLE I**

Preparation of Glutathione Thiolesterase from Mouse Liver

Activities expressed in micromoles of substrate hydrolyzed per minute; specific activities in micromoles of substrate hydrolyzed per minute per mg. of protein.

<table>
<thead>
<tr>
<th></th>
<th>Acetyl glutathione</th>
<th>Butyryl glutathione</th>
<th>Lactoyl glutathione</th>
<th>Ethyl butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.030*</td>
<td>240</td>
<td>0.068*</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>0.034</td>
<td>110</td>
<td>0.052</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>0.041</td>
<td>92.8</td>
<td>0.070</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>0.088</td>
<td>40.5</td>
<td>0.131</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>0.284</td>
<td>12.0</td>
<td>0.369</td>
<td>15.8</td>
</tr>
</tbody>
</table>

|                      | Specific activity  | Total activity      | Specific activity  | Total activity |
|                      | 0.068*             | 398                 | 0.132*             | 804           |
|                      | 0.087              | 268                 | 0.005              | 11.9          |
|                      |                    |                     | 1.43               | 661           |
|                      |                    |                     | 0.98               | 40.8          |

* Estimated from nitrogen determinations.

**TABLE II**

Heat Inactivation of Glutathione Thiolesterase

The results are expressed in micromoles of substrate hydrolyzed per minute per ml. of enzyme solution.

<table>
<thead>
<tr>
<th></th>
<th>Acetyl glutathione</th>
<th>Lactoyl glutathione</th>
<th>Ethyl butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.105</td>
<td>0.332</td>
<td>5.5</td>
</tr>
<tr>
<td>Heated</td>
<td>No activity</td>
<td>0.328</td>
<td>5.1</td>
</tr>
</tbody>
</table>
From the decrease in density it was determined that 73.7 µM had been decomposed, while a determination of volatile acids (12) yielded 74.9 µM. Since thiol acids as well as their esters have a strong absorption at 232 mµ, it may be concluded that the products were acetic acid and glutathione.

The enzyme is sensitive to sulfhydryl reagents, being completely inactivated by $3 \times 10^{-5}$ M p-chloromercuribenzoate. Some reactivation by glutathione has been observed. In addition, old preparations that have lost activity were partially reactivated by preincubation with glutathione.

**DISCUSSION**

From the information presented here it is apparent that there is an enzyme (or enzymes) in liver capable of hydrolyzing S-acyl derivatives of glutathione which is not identical with glyoxalase II or liver esterase.

It would appear that the enzyme is specific for thiol esters of the type of S-acetyl and S-butyryl glutathione. Measurements of the dissociation constant for the enzyme-substrate complex for S-acetyl glutathione gave values of $5 \times 10^{-8}$, indicating the order of affinity. However, it should be observed (Table I) that the relative activity toward the two substrates is not the same for the crude and purified enzyme preparations, suggesting that more than one enzyme may be involved in the hydrolysis of the two substrates, in which case measurements of $K_m$ are of limited significance.

Although the participation of thiol acyl derivatives of coenzyme A and lipoic acid in metabolic processes has been demonstrated, no clear rôle for glutathione in this regard has been reported. Since deacylation of metabolically active thiol esters has been observed, we were interested in the possibility that this process might occur through transfer to glutathione. However, with acetyl coenzyme A we were not able to demonstrate any influence of glutathione on the rate of deacetylation by use of crude liver extracts.

**SUMMARY**

An enzyme specific for the hydrolysis of glutathione thiol esters of the type of S-acetyl and S-butyryl glutathione has been prepared from mouse liver and differentiated from glyoxalase II and liver esterase.

**BIBLIOGRAPHY**

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