Purification and Properties of S-Succinylglutathione Hydrolase from Human Liver

Laeso Uotila
From the Department of Medical Chemistry, University of Helsinki, Siltavuorenpuenger 10 A, SF-00170 Helsinki 17, Finland

S-Succinylglutathione hydrolase, a new highly specific glutathione thiol esterase, has been purified from human liver. The preparation obtained is homogeneous according to disc electrophoretic and ultracentrifugal criteria and catalyzes the hydrolysis of 3000 μmol of S-succinylglutathione/min/mg of protein at 25°C. The enzyme has no activity toward nine other glutathione thiol esters tested or succinyl thiocysteines of coenzyme A, thiglycylate, or thiolglycine. By using very high enzyme concentrations, the hydrolysis of two oxygen esters, 4-methylumbelliferyl acetate and p-nitrophenylacetate, could be shown. The enzyme gives nonlinear kinetics with both S-succinylglutathione and 4-methylumbelliferyl acetate and p-nitrophenylacetate, as shown. The enzyme is a monomeric protein as indicated by the sedimentation coefficient (S₂₀,₀) is 2.25 S and the diffusion coefficient 11.1 Fick units. The enzyme is inhibited by sulfhydryl reagents and by the amino group reagent 2,4,6-trinitrobenzene sulfonate but chelating agents and organophosphates have no effect. S-Succinylglutathione may be the true substrate of "esterase B" earlier described from human liver (Coates, P. M., Edwards, Y. H., and Hopkinson, D. A. (1976) Eur. J. Biochem. 61, 331-335).

During the studies of human liver glutathione thiol esterase evidence was obtained for the presence of three separate such enzymes, glyoxalase II (S-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), S-formylglylutathione hydrolase, and S-succinylglutathione hydrolase (1). Glyoxalase II which forms the second component of the glyoxalase system ubiquitously present in living cells (2) has been obtained in highly purified form from human liver and its characteristics studied (3). S-Formylglutathione hydrolase which is apparently involved in the metabolism of formaldehyde (4) has also been purified to homogeneity from human liver (5). In the present report a purification procedure is presented for the third enzyme of this group, S-succinylglutathione hydrolase. Some molecular and kinetic properties of this enzyme have also been determined. A preliminary report of the work has appeared in abstract form (6).

EXPERIMENTAL PROCEDURES

Materials—The preparation of S-succinylglutathione and other thiol esters of glutathione was performed as previously described (1, 5, 7). The synthesis of succinyl thiol esters of CoA, thiglycolate, and thiocholine was carried out by a procedure analogous to that for S-succinylglutathione. The ion exchange celluloses (DE52 and CM22) were products of Whatman, Ultrogel AcA 54 was from LKB, Sephadex G-100 from Pharmacia, and hydroxylapatite from Clarkson. CoA was obtained from P-L Biochemicals and glutathione, thiglycolic acid, thiocholine, butyrylthiocholine, succinylcholine, p-nitrophénylacetate, 4-methylumbelliferyl acetate, Hepe, Mes, and Pipes were from Sigma. The standard proteins for molecular weight determinations were purchased from Boehringer, except formaldehyde dehydrogenase which was purified according to Uotila and Koivusalo (4).

Determination of Enzyme Activity—The standard assay mixture for S-succinylglutathione hydrolase contained 80 mM imidazole-HCl buffer, pH 6.5, 0.5 mM S-succinylglutathione, enzyme, and distilled water to a final volume of 1.0 ml. The hydrolysis of the substrate was followed at 240 nm and 25°C. A blank without enzyme was always included. One unit of enzyme is the amount which catalyzes the hydrolysis of 1 μmol of the substrate/min under these conditions. When the effect of varying S-succinylglutathione concentrations on the reaction rate was studied, an Aminco DW-2 UV/VIS double beam spectrophotometer was used (λ₁ = 240 nm, λ₂ = 300 nm). The rate was then continuously followed, with 0.02 absorbance unit as the most sensitive scale for full scale deflection on the recorder. The molar absorbance value used for the thiol ester bond of S-succinylglutathione at 240 nm was 3250 cm⁻¹ (1). The hydrolysis of the other thiol esters of glutathione and other thiol esters was also similarly followed at 240 nm or the assay mixture also contained 0.2 mM 5,5'-dithiobis(2-nitrobenzoate) as a further supplement. Thiol formation was then recorded at 412 nm. Activity for p-nitrophenylacetate was tested at 400 nm (8) and activity for succinylcholine and ethyl acetate was measured by the hydroxamate procedure of Hestrin (9). Activity for 4-methylumbelliferyl acetate was measured with a Farrand UV/VIS double beam spectrophotometer with filters 7-37 and 5-60 plus 3-72 as the primary and secondary filters, respectively. The assay mixture contained 80 mM phosphate or Mes buffer, pH 6.0, and 1 mM substrate.

Protein—In most cases protein measurements were done with the biuret technique as described by Gornall et al. (10). When a higher sensitivity was desirable, the procedures of Lowry et al. (11) or Waddell (12) were used. Bovine serum albumin was used as the standard. The protein content of the effluent of chromatography columns was continuously monitored at 280 nm on an Isco UA-2 analyzer connected to a recorder.

Analytical Ultracentrifugation—These experiments were carried out with a MSE Centriscan 75 ultracentrifuge, furnished with a photoelectric scanner. An enzyme preparation in 0.01 M potassium phosphate buffer containing 0.05 M NaCl was used with ultraviolet in the reference cuvette. All runs were done at 20°C. The sedimentation velocity run was performed at 60,000 rpm (262,000 × g) with schlieren optics. The sedimentation equilibrium experiment was done with ultraviolet absorption optics at 280 nm, first for 1 h at 25,000 rpm and then for 66 h at 20,000 rpm. The equilibrium position of the enzyme was then recorded, after which the enzyme was allowed to sediment by running at 60,000 rpm for 2 h to obtain a base line. The sedimentation coefficient was calculated from a plot of log r versus time. The equilibrium data were used for the calculation of the
molecular weight of the enzyme from the equation

\[ M_1 = \frac{2RT}{(1 - Vp)c} \left( \frac{dc}{dr} \right)^2 \]

in which the term \( \frac{dc}{dr} \) is obtained from the slope of a plot of \( \ln c \) versus \( r \), where \( c \) is enzyme concentration at the distance \( r \) in enzyme solution from the center.

**Determination of Molecular Weight by Gel Chromatography**

The apparent molecular weight of the enzyme was determined with columns of Sephadex G-100 (2.5 x 70 cm) and Ultrogel AcA 4 (2.5 x 100 cm), both equilibrated and eluted with 10 mM Tris-HCl buffer, pH 7.6, containing 0.05 mM NaCl. The procedures followed those introduced by Vesterberg (14). The void volume of the column was determined with blue dextran 2000 (Pharmacia). The columns were calibrated with standards with known molecular weights (see legend to Fig. 1). Activity assay was used for location of formaldehyde dehydrogenase (2), catalase (15) and alcohol dehydrogenase (15), chymotrypsinogen, and trypsin. The assay of the latter two was based on the ability of trypsin to activate chymotrypsinogen in the assay cuvette (16). p-Nitrophenylacetate was the substrate and its hydrolysis localized by its absorbance at 400 nm. Blue dextran was calibrated with blue dextran 2000 (Pharmacia). The columns were equilibrated with 0.01 M phosphate (pH 7.4) in a Waring Blendor and the homogenate centrifuged (LKB). The procedures used were similar to those described by Vesterberg (19).

**Purification of S-Succinylglutathione Hydroxylase**—The enzyme was purified from human livers, obtained from legal autopsies within 24 h after death. All procedures were done at 0-4°C. The liver tissue (about 900 g) was homogenized in 3 volumes of 0.05 mM potassium phosphate (pH 7.4) in a Waring Blendor and the homogenate centrifuged at 25,000 x g for 60 min. The supernatant (34.8 g/100 ml) and the precipitated proteins were collected separately by eluting with 0.1 M NaCl. The pooled enzyme activity was dialyzed twice for 6 h against 5 volumes of 0.01 M potassium phosphate.

**Analytical Ultracentrifugation**—In the sedimentation velocity experiment the purified enzyme gave a single, symmetrical peak. From the sedimentation rate (Fig. 2) the sedimentation coefficient of the enzyme is 2.25 S (S20,W, enzyme concentration 3.2 mg/ml by the biuret method). When this value is taken together with the diffusion coefficient given above,
TABLE I
Purification of S-succinylglutathione hydrolase from human liver (504 g)

<table>
<thead>
<tr>
<th>Step</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.</td>
<td>1.540</td>
<td>800,000</td>
<td>66,700</td>
<td>12.0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>511,000</td>
<td>25,400</td>
<td>91.2</td>
<td>7.6</td>
<td>63.9</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>2,511,000</td>
<td>5,605</td>
<td>995</td>
<td>82.9</td>
<td>24.4</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>39,173</td>
<td>195,000</td>
<td>55.9</td>
<td>3,130</td>
<td>261</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>17,500</td>
<td>55.9</td>
<td></td>
<td></td>
<td>21.9</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 1. Standard plots for molecular weight determination of S-succinylglutathione hydrolase (●) on Sephadex G-100 (A) and Ultrogel AcA 54 (B). The standards were: 1, cytochrome c (Mₜ = 12,400); 2, lysozyme (14,400); 3, myoglobin (17,200); 4, trypsin (23,900); 5, chymotrypsinogen (25,000); 6, carboxypeptidase A (34,500); 7, ovalbumin (45,000); 8, bovine albumin (67,000); 9, horse liver alcohol dehydrogenase (80,000); 10, human liver formaldelyde dehydrogenase (81,400); 11, yeast alcohol dehydrogenase (154,000); 12, catalase (240,000).

FIG. 2. Determination of the sedimentation coefficient and molecular weight of S-succinylglutathione hydrolase by analytical ultracentrifugation. Left, sedimentation velocity experiment. The slope was used for the calculation of the sedimentation coefficient. Right, sedimentation equilibrium experiment. The slope was used for the calculation of molecular weight for the enzyme. The enzyme concentration (c) at distance r is given in micrograms/ml.

Sodium Dodecyl Sulfate Electrophoresis—Purified enzyme was run in polyacrylamide gels and five standards at the same time in other gels. The standards (albumin, Mₜ = 67,000; ovalbumin, 45,000; yeast alcohol dehydrogenase, 37,000; chymotrypsinogen, 25,000; cytochrome c, 12,400) gave a linear plot of log molecular weight versus mobility from which the unknown has a subunit molecular weight of 20,400 ± 700 (n = 5).

It can be concluded from these experiments that S-succinylglutathione hydrolase from human liver is a monomeric protein with a molecular weight of approximately 18,000.

Isoelectric Point—The enzyme gave in column isoelectric focusing a symmetrical sharp peak with a pH value of 8.69 ± 0.20 (n = 4). No change occurred during purification. This method offers the easiest way in crude solutions to separate by one step the enzyme from other glutathione thiol esterases, glyoxalase II, and S-formylglutathione hydrolase (1, 3, 5).

Absorption Spectrum—The purified S-succinylglutathione hydrolase has a usual protein spectrum with no absorbance in the visible area, absorption maximum at 280 nm, and a minimum at 249 nm. The A₅₅₀/A₃₈₀ ratio was 1.55. The A₅₅₀ value at 280 nm was determined to be 21.2 on the basis of biuret protein determination and 17.5 by the Lowry method.

Substrate Specificity—S-Succinylglutathione hydrolase is highly specific for S-succinylglutathione. At an enzyme concentration which was 100-fold compared to that usually used in the assay with S-succinylglutathione, no activity was seen for any of the following glutathione thiol esters: lactoyl, acetyl, propionyl, glycolyl, glyceryl, β-ethoxy-a-hydroxybutyryl, acetoacetyl, pyruvyl, and mandelyl. The enzyme neither showed activity for acetyl-CoA, succinyl-CoA, succinylthioaliphate, succinylthiocholine, nor butyrylthiocholine. Some of these, especially succinylthiocolcholine, were rapidly hydrolyzed nonenzymically. Therefore, the detection of a minor activity for them would not have been possible.
Two oxygen esters, 4-methylumbelliferyl acetate and p-nitrophenylacetate, were found to be hydrolyzed at very high S-succinylglutathione hydroxide concentrations (Table II). Despite the low velocities with these substrates they were convenient in many studies owing to kinetic difficulties in measurements with S-succinylglutathione (see below). This especially concerned 4-methylumbelliferyl acetate because the high sensitivity of the fluorometric measurement used with it compensated for the low maximum velocity. No activity was noted for succinylcholine or ethyl acetate. The insensitivity of the hydroxamate method (9) would not, however, have allowed detection of activities below 0.3% of that for S-succinylglutathione.

**Effect of pH and Various Buffers**—The enzyme had highest activity in imidazole-HCl buffer in which it had a sharp activity optimum at pH 6.5 to 6.7. At pH 6.2 the activity was 30%, at pH 6.9 73%, and at pH 7.4 28% of the optimal activity. The concentration of imidazole did not influence the enzymic rate between 20 and 100 mM but above 100 mM inhibition was seen. At pH 6.6 the relative activities in various 80 mM buffers were: imidazole (100), Mes (45), Pipes (10), and phosphate (15). In phosphate buffers somewhat more activity was seen above pH 7. In some buffers (e.g. Pipes) rapid nonenzymic hydrolysis of S-succinylglutathione was a disadvantage. At pH 7.6 Tris gave 50% and Heps 85% of the activity of imidazole buffer of the same pH. The rate of nonenzymic hydrolysis of S-succinylglutathione was continuously increased when pH decreased from 8.0 to 6.0, in contrast to the other glutathione thiol esters studied. Therefore, most of the kinetic studies with S-succinylglutathione as the substrate were done in 80 mM Hepes buffer, pH 8.0, although enzyme activity under these conditions was only 12% of that in 80 mM imidazole, pH 6.6.

When 4-methylumbelliferyl acetate was the substrate, the activity was in phosphate buffer, pH 6.6, 17-fold compared to Mes buffer of the same pH, whereas in imidazole buffer, pH 6.6, the activity for 4-methylumbelliferyl acetate was 86-fold compared to phosphate (Table II). However, nonenzymic hydrolysis of 4-methylumbelliferyl acetate also was rapid in imidazole, 200-fold compared to phosphate and 1000-fold compared to Mes. Therefore, imidazole could not be used. The fluorescence of the product, 4-methylumbelliforene, was about the same in all three buffers used.

**Kinetic Properties of the Enzyme**—The rate of hydrolysis of S-succinylglutathione was a linear function of the enzyme concentration in a narrow range which was as a rule reached by considerable dilution of the enzyme before assay. This linear range extended only up to the hydrolysis of 6 nmol of substrate/ml/min (after subtraction of the blank rate). Because S-succinylglutathione is also hydrolyzed nonenzymically at a significant rate (2 to 3 nmol/min/μg of the enzyme in the standard assay conditions, replicates of the measurements had always to be used to increase the accuracy. The linear range of enzymic hydrolysis was the same in the pH range studied (6.2 to 8.0). Therefore, many studies were done in Hepes buffer, pH 8.0, in which the blank rate was 3 times lower than in imidazole buffer, pH 6.6.

In crude solutions of human liver only S-succinylglutathione hydroxidase significantly influences the rate of hydrolysis of S-succinylglutathione if sufficiently diluted enzyme solution is used. Glyoxalase II can also catalyze the hydrolysis of S-succinylglutathione but the correction needed due to its presence was only 0.5% of the total enzyme activity. This correction has been done for purification Steps 1 to 3 in Table I.

With 4-methylumbelliferyl acetate as the substrate the measurements were easier to perform because the rate was linearly dependent on enzyme concentration at a 30-fold range compared to blank rate in phosphate buffer and at a 150-fold range in Mes. The rate was linear up to 6 nmol/min/ml which is the same as with S-succinylglutathione. With p-nitrophenylacetate the linear range extended at least to 11 nmol/min/ml, the highest rate which could be measured with a concentrated enzyme preparation.

S-Succinylglutathione hydroxidase was found to give nonlinear Lineweaver-Burk plots with all active substrates. Fig. 3 shows the plot of the kinetics with S-succinylglutathione. The $S_{0.5}$ value for this substrate was estimated to be about 0.3 mM. With 4-methylumbelliferyl acetate a curve with reversed convexity compared to S-succinylglutathione was obtained in the Lineweaver-Burk plot (Fig. 4). With p-nitrophenylacetate the result resembled that with S-succinylglutathione although nonlinearity was less apparent. The $S_{0.5}$ values for 4-methylumbelliferyl acetate and p-nitrophenylacetate were estimated to be 1.7 mM and 5.0 mM, respectively.

**Stoichiometry of the Reaction**—The reaction catalyzed by the enzyme is a hydrolysis. This was shown by allowing the enzyme to react with S-succinylglutathione until most of the thiol ester had disappeared on the basis of absorbance at 240 nm. A blank without enzyme was included. The mixtures were then cooled to 0°C and the amount of GSH formed was measured with 2,2'-dithiodipyridine (20). The amount of enzymically formed GSH was found to be 92.4 ± 5.1% (mean ± S.D.) of the thiol ester disappearance in the six separate experiments performed. A kinetic assay of the enzyme based on the continuous measurement of GSH in the presence of 2,2'-dithiodipyridine or 5,5'-dithiobis(2-nitrobenzoate) was not feasible, however, because the latter agents markedly increased the rate of the nonenzymic hydrolysis of S-succinylglutathione.

**Inhibitors**—The enzyme was inhibited by mercaptide-forming and oxidizing SII reagents. The most potent reagent, HgCl$_2$, had an $I_{50}$ value of about 0.1 mM. 5,5'-Dithiobis(2-nitrobenzoate) and p-hydroxymercuribenzoate were needed at 10 to 50 times higher concentrations than HgCl$_2$ for comparable effects. The activity with 4-methylumbelliferyl acetate was apparently inhibited by lower concentrations of SH reagents than the activity with S-succinylglutathione and, furthermore, the activity with the former substrate was maximally inhibited after preincubation of enzyme and inhibitor for only 2 min, whereas with the latter substrate a preincubation of 20 min was needed. GSH present in the S-succinylglutathione preparation apparently explained this difference at least in part because enzyme inhibited by HgCl$_2$ could be rapidly partially reactivated by the further addition of GSH when the activity was measured with 4-methylumbelliferyl acetate. The amino group reagent 2,4,6-trinitrobenzenesulfo-

ate was inhibitory (e.g. 45% inhibition at 1 mM) but moderate to high concentrations of phenylglyoxal, N-acetyl-L-cysteine, diethylpyrocarbonate, dithiothreitol, diethyl-p-nitrophenylphosphate, EDTA, p-phenanthroline, α,α′-dipyridyl, and 0-hydroxyquinoline were without any effect with either S-succinylglutathione or 4-methylumbelliferyl acetate as the substrate.

Of the products of the reaction succinate (17 mM) had no effect but GSH inhibited the enzymic hydrolysis of either S-succinylglutathione or 4-methylumbelliferyl acetate. The amount of GSH brought into the assay with S-succinylglutathione (about 0.1 mM in the standard assay) was not inhibitory but addition of 1 mM GSH inhibited 35% and 3 mM GSH 88%. Substrate competition experiments in the presence of both S-succinylglutathione and 4-methylumbelliferyl acetate showed that the former was a competitive inhibitor when the hydrolysis of the latter by the enzyme was measured fluorometrically. Experiments where the hydrolysis of S-succinylglutathione...
S-Succinylglutathione Hydrolase

DISCUSSION

S-Succinylglutathione hydrolase is the third glutathione thiol esterase obtained in purified form from human liver. All three enzymes are highly specific for thiol esters of glutathione but the present enzyme and S-formylglutathione hydrolase (5) are also highly specific for the acyl part of the thiol ester, whereas glyoxalase II unspecifically uses several types of thiol esters of glutathione although with differing $K_{m}$ and $V_{max}$ values (3). Comparison of the physical properties of these enzymes shows that both S-succinylglutathione hydrolase and glyoxalase II are rather small proteins (molecular weights approximately 18,000 and 23,000, respectively) with basic isoelectric points (8.7 for the present enzyme and 8.35 for glyoxalase II (3)). S-Formylglutathione hydrolase is a dimeric enzyme with a molecular weight of 55,000 and an acidic isoelectric point of 5.41 (5).

Besides S-succinylglutathione only two oxygen esters, 4-methylumbelliferyl acetate and p-nitrophenylacetate, were found to be substrates of S-succinylglutathione hydrolase. A great number of other thiol esters of glutathione and succinyl thioesters of other thiols were inactive. This peculiarity might be explained by the fact that the hydrolysis of the reactive oxygen esters can be measured with great sensitivity which could not be achieved in the measurements with all other substrates. The extreme difference between the maximum velocities found for S-succinylglutathione and the active oxygen esters (Table II) raises the possibility that a contaminant was causing the hydrolysis of the latter substrates. That this is not the case was strongly suggested by the following. 1) In the repetition of the last purification step (chromatography on Ultrogel AcA 54) the activities of the eluate assayed with 4-methylumbelliferyl acetate and 4-methylumbelliferyl glutathione coincided. 2) The single protein band obtained on disc electrophoresis for the most purified preparation corresponded to both the activity band found on staining with 4-methylumbelliferyl acetate and the activity with S-succinylglutathione determined from gel slices. 3) The hydrolysis of 4-methylumbelliferyl acetate by the enzyme was inhibited by S-succinylglutathione.

Coates et al. (24) during their investigations on human esterases, have used esters of 4-methylumbelliferyl acetate as one substrate group. They have reported fractionation of human liver preparations by starch electrophoresis, followed by staining with 4-methylumbelliferyl acetate as the substrate (24). Of the several components using this compound a minor one with highest cathodic mobility was named "esterase B,;" According to a recent report the enzyme has an isoelectric point of 8.7 and a molecular weight of 20,000. Only esters of 4-methylumbelliferyl were substrates and the specific activity of the purified enzyme was very low, 0.039 unit/mg (25). The specific activity value calculated for the present enzyme with 4-methylumbelliferyl acetate as the substrate is of the same order (0.072 unit/mg). Thus, one would expect the enzyme to become visible in the staining of Coates et al. (24) following starch electrophoresis. This together with similar physical properties suggests that S-succinylglutathione may be the true substrate of "esterase B;".

The routes for possible enzymic synthesis of glutathione thiol esters have been discussed in a previous report from this laboratory (3). An interesting new contribution on this point has recently appeared by Kun et al. (26). They described and partially purified rat liver mitochondria enzymes which catalyzed the synthesis and hydrolysis of S-citrylglutathione. Preliminary experiments suggested that enzymic synthesis of several other glutathione thiol esters, e.g. S-succinylglutathione, was also possible by the mitochondrial preparations (26). S-Succinylglutathione hydrolase studied in this work was, however, found to be completely cytoplasmic and, thus, is different from the mitochondrial enzyme system(s) described by Kun et al. (26).

Acknowledgments—I am grateful to Mrs. Eija Haasanen for her skilful technical assistance and to Miss Lila Salo, M.Sc., for assistance in the analytical ultracentrifugation experiments which were conducted in the Department of Medical Biochemistry, University of Oulu, Finland.

REFERENCES

1. Uotila, L. (1973) Biochemistry 12, 3938-3943
S-Succinylglutathione Hydrolase


Purification and properties of S-succinylglutathione hydrolase from human liver.
L Uotila