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PURIFICATION AND CHARACTERIZATION OF MOUSE LIVER GLYOXALASE II

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Summary

Glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6) was purified from Swiss mouse liver to homogeneity by a rapid, two-step affinity chromatographic scheme. Homogeneity was established by multiple electrophoretic determinations. The purified enzyme exhibited a specific activity of 920 I.U./mg protein and has a molecular weight of approx. 29 500 as estimated by SDS polyacrylamide gel electrophoresis. The enzyme is a basic protein with a p*I* of approx. 8.1. Mouse liver glyoxalase II is competitively inhibited by the substrate of glyoxalase I (the hemimercaptal of methylglyoxal and glutathione); the K_i is 0.3 mM. The K_m for *S*-D-lactoylglutathione is 0.27 mM, and the enzyme has a turnover number of approx. 27 000 μmol substrate per min per μmol enzyme.

Introduction

The glyoxalase system, which has been known since 1913 [1,2], is widely distributed in nature and catalyzes the conversion of methylglyoxal (or other α-ketoaldehydes) to D-lactate (or other α-hydroxyacids) with reduced glutathione, GSH, serving as cofactor [3—6]. The system consists of two enzymes, glyoxalase I and glyoxalase II [7]. Glyoxalase I acts upon the equilibrium adduct of methylglyoxal and GSH, a hemimercaptal, with the resultant formation of thioester, *S*-D-lactoylglutathione. Glyoxalase II hydrolyzes the thioester to regenerate GSH and liberate free D-lactic acid.

Abbreviation: SDS, sodium dodecyl sulfate.

Although there have been two reports on the extensive purification of glyoxalase II, one from human liver [8], and a more recent one from rat erythrocytes [9], the homogeneous preparations of this enzyme have not been reported. We herein report a rapid, two-step affinity chromatographic scheme for the purification of mouse liver glyoxalase II with a high overall yield (80%).

Materials and Methods

General. Swiss mice (24—27 g) were purchased from Timco, Houston, Texas. The mice were sacrificed by cervical dislocation. The livers were immediately removed and homogenized (0°C for 2 min at medium speed with a Virtis homogenizer) in 2 vols. of a solution of 10 mM potassium phosphate (pH 7.0) and 20% glycerol. The homogenate was centrifuged at 100 000 × *g* for 1 h. The supernatant fraction thus obtained was designated as the crude preparation of glyoxalase II. When the livers were not used immediately they were frozen in solid CO_2 and then stored at −30°C. All purification steps were conducted at 4°C, and enzyme preparations were stored at −30°C.

Methylglyoxal was prepared by the method of Kellum et al. [10]. Both oxidized glutathione (GSSG) and GSH were purchased from Sigma Chemical Co., St. Louis, MO. *S*-Octylglutathione was prepared by reacting 1-bromooctane with GSH employing Method A of Vince et al. [11]. *S*-D-Lactoylglutathione was prepared and purified by the procedure of Uotila [12].

Routine glyoxalase II assay. The reaction mixture used was: 0.5 mM *S*-D-lactoyglutathione in 100 mM potassium phosphate (pH 7.0). The enzymatic disappearance of *S*-D-lactoylglutathione ($\epsilon_{240} = 3.37\ mM^{-1} \cdot cm^{-1}$) was followed at 240 nm for 5 min at 25°C on a Beckman DBG recording spectrophotometer. The reaction was initiated by the addition of the enzyme preparation (5—75 µl) to 3 ml reaction mixture, and the initial rates were determined by the slope of the linear portion of the plot. A unit of glyoxalase II is defined as the amount of the enzyme catalyzing the conversion of 1 µmol *S*-D-lactoylglutathione per min in the routine enzyme assay system.

Determination of protein concentration. Protein concentration was determined by the colorimetric Coomassie Blue procedure of Bradford [13].

Column material preparation. The preparations of affinity chromatography column materials were based on the method of Cuatrecasas [14]. The appropriate amount of GSSG to give 3.6 mmol/100 ml Sepharose was coupled to CNBr-activated Sepharose 4B at pH 10.2. The degree of substitution of affinity matrix was not determined.

Electrophoresis. Both polyacrylamide gel electrophoresis and SDS polyacrylamide gel electrophoresis were performed according to the method of Maizel [15]. For SDS polyacrylamide gel electrophoresis experiment, ovalbumin (M_r = 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r = 37 000), triose phosphate isomerase (M_r = 26 500) and hemoglobin (M_r = 16 500) were used as standard for molecular weight determinations.

Isoelectric focusing. Isoelectric focusing was performed in a LKB Multiphor Electrophoresis Unit according to the instructions of the manufacturer. A pH gradient 3.5—10 was used with 1% (w/v) ampholyte concentration.

Inhibition studies. For the experiments involving inhibition of glyoxalase II

activity, it was assumed that the dissociation constant of the hemimercaptal of GSH and methylglyoxal is 3.1 mM [11]. For each experiment, the appropriate amounts of methylglyoxal and GSH were added such that after the non-enzymatic formation of the calculated hemimercaptal concentration, the free GSH level was 0.3 mM. This level of free GSH does not inhibit the human liver enzyme [8].

Results and Discussion

Purification of glyoxalase II

Table I summarizes the purification data of Swiss mouse liver glyoxalase II. At the outset of glyoxalase II purification, sufficient crude preparation (see Materials and Methods) to give 7.0 g of protein was placed on a 2.6 × 70 cm GSSG-Sepharose affinity chromatographic column. The column material had been previously equilibrated with a solution of 10 mM potassium phosphate (pH 7.0) and 20% glycerol. After loading, the column was washed with the equilibration solution until the bulk of the protein was eluted. The column was then washed with a solution of 50 mM potassium phosphate (pH 7.0) and 20% glycerol, and then again with the equilibration solution. A gradient of a competitive inhibitor of glyoxalase II, *S*-octylglutathione, (1 to 5 mM, a total of 2 l) prepared in the equilibration solution, was used to elute glyoxalase II. Fig. 1 shows the protein concentration and glyoxalase II activity profiles obtained. All of the glyoxalase I activity was eluted with the early fractions containing the bulk of the protein. The GSSG-Sepharose affinity material can be used repeatedly. After each use, the column materials are washed with the equilibration solution containing 1 M NaCl, and then again with the equilibration solution before the reuse.

Active fractions from the first column were diluted two-fold with the equilibration solution to decrease the *S*-octylglutathione concentration. The primary reason for the dilution of the pooled active fractions was to allow binding of the enzyme to the second affinity column since binding is prevented by 2 mM *S*-octylglutathione. *S*-Octylglutathione apparently stabilizes glyoxalase II and complete removal of this inhibitor (K_i = 1.5 mM) by dialysis results in substantial loss of activity.

TABLE I

PURIFICATION OF GLYOXALASE II

Fraction	Volume (ml)	Total activity (I.U.) *	Spec. act. (I.U. * mg)	Yield (%) (Step)	Purification (Step)	Purification (Overall)
Crude preparation	380	1743	0.248	100	1.0	1.0
First GSSG-column	460	1656	60.3	95	243.0	243.0
Second GSSG-column	650	1407	614.7	85	10.2	2479.0

* The routine assay mixture contained 0.5 M *S*-D-lactoylglutathione, a concentration which is only approximately twice the value of the K_m (0.27 mM). When the enzyme preparations were assayed at near saturating levels of substrate the activity was increased by a factor of 1.5. Thus, the correct specific activity of the homogenous enzyme is approx. 920 I.U./mg.

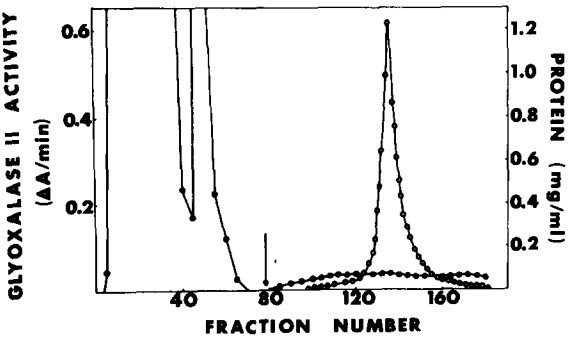

Fig. 1. First GSSG-Sepharose affinity chromatography. Crude preparation (380 ml) was applied to a 2.6 × 70 cm preequilibrated GSSG-Sepharose column. At fraction 36, 50 mM potassium phosphate (pH 7.0) containing 20% glycerol, and at fraction 52, 10 mM potassium phosphate (pH 7.0) containing 20% glycerol were applied. The arrow marks the application of the *S*-octylglutathione gradient (1 to 5 mM). ○———○, Glyoxalase II activity, ΔA/min (75 μl of each fraction was used in the assay), ●———●, protein concentration, mg/ml. Fraction volumes were 21.0 ml.

This diluted enzyme preparation was then added to a 2.6 × 40 cm GSSG-Sepharose affinity chromatographic column, which had been equilibrated with a solution of 10 mM potassium phosphate (pH 7.0) and 20% glycerol. After sample application the column was washed with the equilibration solution, then with a solution of 75 mM potassium phosphate (pH 7.0) containing 20% glycerol, and again with the equilibration solution. Homogeneous glyoxalase II was then eluted by addition of a solution of 2.0 mM *S*-octylglutathione in the equilibration solution. The protein concentration and activity profiles are shown in Fig. 2. Volume reduction of the pooled active fractions from this column was achieved by ultrafiltration. The overall yield obtained was 80% with a 2479-fold purification. When the enzyme was stored at −30°C in the presence of 1 mM *S*-octylglutathione, no significant loss of activity was observed for at least 1 month.

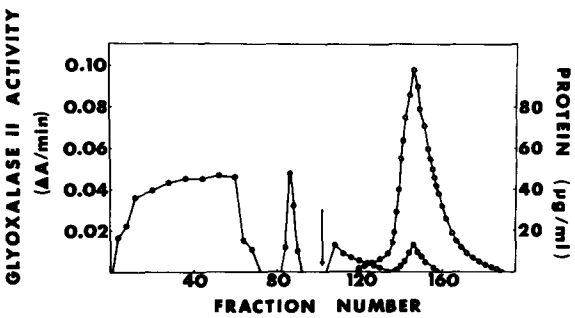

Fig. 2. Second GSSG-Sepharose affinity chromatography. Pooled and diluted fractions (920 ml) from the first column were applied to a 2.6 × 40 cm preequilibrated GSSG-Sepharose column. At fraction 80, 75 mM potassium phosphate (pH 7.0) containing 20% glycerol, and at fraction 87, 10 mM potassium phosphate (pH 7.0) containing 20% glycerol were applied. The arrow marks the application of *S*-octylglutathione (2 mM). ○———○, Glyoxalase II activity, ΔA/min (75 μl of each fraction was used in the assay); ●———●, protein concentration, mg/ml. Fraction volumes were 18.4 ml.

Although it is apparent that glyoxalase II binds with GSSG-Sepharose (second purification step), it is possible, because of the GSH present in the crude liver preparations (1—2 mM) used in the first purification step, that the Sepharose-bound GSSG can be reduced to GSH residues. Consequently, glyoxalase II binding could occur with both Sepharose-bound GSH and GSSG. However, even after repeated use of the same affinity column material with crude liver preparations, no change in glyoxalase II binding characteristics was observed. Further, pretreatment of the column material with several volumes of 1 mM dithiothreitol does not change the glyoxalase II binding or elution pattern. After such usages or pretreatments, an increase of Sepharose-bound sulfhydryl groups cannot be detected by use of Ellman's reagent [16]. It would thus appear that Sepharose-bound GSSG is not readily reduced to GSH residues.

Purity and molecular weight determination

Polyacrylamide gel electrophoresis and SDS polyacrylamide gel electrophoresis were conducted on the purified glyoxalase II. The amount of the enzyme applied to the gels varied from 8 to 20 μg protein. The gels exhibited only one band with both electrophoretic procedures; no minor bands could be detected either visually or by densitometric tracing of the gels. Molecular weight studies employing SDS polyacrylamide gel electrophoresis gave a molecular weight of approx. 29 500.

Uotila estimated the molecular weight of human liver glyoxalase II to be 22 900 by gel filtration [8]. In the light of this study, mouse liver glyoxalase II is a single polypeptide with a molecular weight of approx. 29 500.

Isoelectric focusing

Isoelectric focusing experiments were conducted on the homogeneous enzyme preparation, and a p*I* value of approx. 8.1 was obtained. Uotila obtained a p*I* value of 8.35 for human liver glyoxalase II [8].

Inhibition studies

Both methylglyoxal and GSH have been shown to be weak inhibitors of human liver glyoxalase II; however combinations of methylglyoxal and GSH show a greater than additive inhibitory activity, presumably due to a greater inhibition by the hemimercaptal adduct of methylglyoxal and GSH (the substrate of glyoxalase I) [8]. Inhibition studies by the hemimercaptal were conducted on the homogeneous mouse liver glyoxalase II, and the inhibition was found to be competitive with a K_i of 0.3 mM (Fig. 3). The K_m value calculated for the substrate, *S*-D-lactoylglutathione, is 0.27 mM; Uotila found the K_m to be 0.19 mM for the human enzyme [8].

The inhibition of glyoxalase II by the hemimercaptal, substrate of the first of the glyoxalase enzymes, glyoxalase I, is interesting. In vitro studies with human whole blood have recently shown that under certain conditions the production of methylglyoxal from dihydroxyacetone phosphate can be quite high [17]. A high methylglyoxal concentration could rapidly tie up cellular GSH as the hemimercaptal adduct which in turn could be converted to *S*-D-lactoylglutathione by glyoxalase I. The activity of glyoxalase II, which is

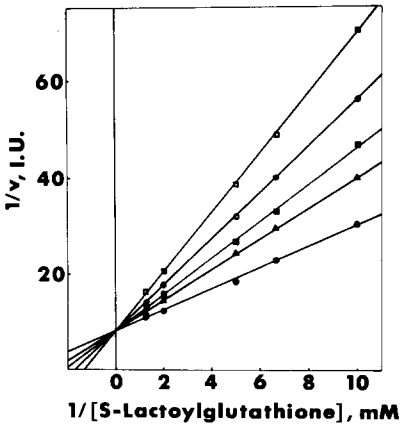

Fig. 3. Inhibition of glyoxalase II by the hemimercaptal of GSH and methylglyoxal. The calculated concentrations of the hemimercaptal were 0 mM (●), 0.05 mM (▲), 0.1 mM (■), 0.2 mM (○), 0.5 mM (□).

normally low compared to that of glyoxalase I, could at the same time be further diminished by the hemimercaptal inhibition. The net result could be the transient elevation of cellular *S*-D-lactoylglutathione levels. The metabolic existence of significance of this possibility remains to be explored. The determination of cellular methylglyoxal and *S*-D-lactoylglutathione levels under carefully controlled conditions would be informative.

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