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A TWO-STEP PURIFICATION OF MOUSE LIVER GLYOXALASE I AND EVIDENCE OF ITS DIMERIC CONSTITUTION

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Summary

Glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) was purified from Swiss mouse liver to homogeneity by a rapid, two-step procedure involving hydrophobic and affinity chromatography. Homogeneity was established by multiple electrophoretic determinations and by sedimentation equilibrium centrifugation. The purified enzyme exhibited a specific activity of 944 I.U./mg protein an has a molecular weight of 43 000. The enzyme was shown to be a dimer by sodium dodecyl sulfate disc gel electrophoresis and is apparently composed of identical subunits of molecular weights approximating 21 500.

Introduction

The glyoxalase system, which has been known since 1913 [1,2], is widely distributed in nature and catalyzes the conversion of methylglyoxal to lactate with reduced glutathione (GSH) as cofactor [3—6]. The system consists of two enzymes, glyoxalase I and glyoxalase II [7]. Glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) acts upon the equilibrium adduct of methylglyoxal and GSH, a hemimercaptal, with the resultant formation of the thioester, D-*S*-lactoylglutathione. Glyoxalase II hydrolyzes the thiester to regenerate GSH and liberate free D-lactic acid.

Glyoxalase I has been partially purified from calf liver [8], from livers of normal DBA/1J mice and from the same mice bearing a lymphosarcoma [9], from the livers of rats of various ages [10] and from porcine erythrocytes [11]. Nearly homogeneous preparations have been obtained from rat liver, erythrocytes, brain and kidney [12]; homogeneous preparations from mouse liver and more recently from rat liver have been reported in the literature [13,14].

We report herein a rapid, two-step scheme for the purification of glyoxalase

I from Swiss mouse liver; hydrophobic and affinity chromatography were employed to obtain homogeneous preparations. The purification procedures involved are considerably less harsh than some of those previously reported and give high overall yields.

Materials and Methods

General. Swiss mice (24—27 g) were purchased from Timco, Houston, Texas. The mice were killed by asphyxiation in CO_2. The livers were immediately removed and homogenized (0° C for 45—60 s at medium speed with a Virtis homogenizer) in three volumes of a solution of 1 mM potassium phosphate (pH 7.0), 1 mM $MgSO_4$ and 20% glycerol. The homogenate was centrifuged at 100 000 × *g* for 1 h. The supernatant fraction thus obtained (approx. 225 ml per 100 g liver) was designated as the crude preparation of glyoxalase I and contained 25—30 mg/ml protein. 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 obtained as a 40% aqueous solution (Sigma Chemical Co., St. Louis, Mo.), methylglyoxal was purified by distillation in vacuo followed by removal of acidic contaminants in the distillate by passing it through AG-IX8 resin (carbonate form); it was then standardized by the method of Friedmann [15].

Routine glyoxalase I assay. Glyoxalase I activity was determined by a modification of the procedure of Racker [7]. The reaction mixture used was: 7.9 mM methylglyoxal, 1.0 mM GSH, 100 mM imidazole · HCl (pH 6.8), and 16 mM $MgSO_4$. The reaction mixture was allowed to stand for at least 10 min at room temperature to ensure equilibration. The hemimercaptal concentration at equilibrium was calculated to be 0.7 mM using K_{eq} = 3.1 mM [16]. The enzymatic production of D-*S*-lactoylglutathione (ϵ_{240} = 3.37 $mM^{-1} \cdot cm^{-1}$) was followed at 240 nm for 2 min at 25° C on a Beckman DBG recording spectrophotometer. The reaction was initiated by the addition of a rate-limiting volume of enzyme preparation (1—20 µl) to 3.0 ml of reaction mixture, and initial rates were determined by the slope of the linear portion of the plot. The reference cell contained all reaction mixture components with the exception of the enzyme preparation. A unit of glyoxalase I activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of D-*S*-lactoylglutathione per min in the routine enzyme assay system. Specific activity is expressed as units per mg protein.

Determination of protein concentration. Protein concentration was determined by a colorimetric method employing Coomassie Blue [17]. Crystalline bovine serum albumin was used as the standard. The protein levels in the effluent of the hydrophobic chromatography columns were monitored by absorbance at 280 nm.

Chromatography ligands. S-Octylglutathione was prepared by reacting 1-bromooctane with reduced glutathione employing Method A of Vince et al. [16]. *n*-Alkylamines were purchased from Aldrich Chemical Co.

Column material preparation. The preparations of hydrophobic and affinity chromatography column materials were based on the method of Cuatrecasas

[18]. For the hydrophobic chromatography columns, the appropriate *n*-alkylamines were coupled to CNBr-activated Sepharose 4B at pH 10.2 (3.6 mmol/100 ml Sepharose). For the preparation of affinity chromatography column material, *S*-octylglutathione was coupled to Sepharose 4B under the same conditions.

Molecular weight determination by sedimentation equilibrium. Sedimentation equilibrium experiments were conducted in a Beckman-Spinco Model E analytical ultracentrifuge equipped with RTIC temperature control and electronic speed control. The meniscus depletion method [19,20] was used in the An-D rotor in a 12 mm double sector cell with sapphire windows. The enzyme (420 μg/ml) was extensively dialyzed in KH_2PO_4, 1.179 g/l; Na_2HPO_4, 4.3 g/l (pH 7.413); ρ = 1.0020. Centrifugation was carried out for 30 h at speeds of 28 000 and 32 000 rev./min.

Polyacrylamide disc gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to the method of Maizel [21]. Gels were prepared with a 7.5% polyacrylamide concentration in a solution of 2.5 mM Tris · HCl, 20 mM glycine (pH 9.0), and stained with 0.2% Coomassie Blue-G in an acetic acid/methanol/H_2O (8 : 46 : 46, v/v) solution. After staining for at least 2 h, the gels were destained in 7% acetic acid.

Sodium dodecyl sulfate disc gel electrophoresis. The method reported by Fairbanks et al. [22] was employed. Gels were prepared with a 5.6% polyacrylamide concentration in a solution of 40 mM Tris · HCl (pH 7.4), 20 mM sodium acetate, 2 mM EDTA and 1% sodium dodecyl sulfate (SDS), and stained with 0.2% Coomassie Blue-G in an acetic acid/methanol/H_2O (8 : 46 : 46, v/v) solution. After staining for at least 2 h, the gels were destained in an acetic acid/methanol/H_2O (10 : 45 : 45, v/v) solution. Bovine serum albumin (M_r = 67 000), ovalbumin M_r = 45 000) and RNAase A (M_r = 13 700) were used as standards for molecular weight determinations. Standards and samples were dialyzed against a solution composed of 10 mM Tris · HCl (pH 8.0), 1 mM EDTA and 0.1% β-mercaptoethanol, before the electrophoresis runs.

Results and Discussion

The purification of Swiss mouse liver glyoxalase I was accomplished by use of both hydrophobic and affinity chromatography. The choice of a specific hydrophobic grouping for the first purification step was made after a study of a variety of alkyl chains covalently linked to Sepharose 4B.

As the length of the hydrophobic chain is increased, glyoxalase I binds more tenaciously, and the concentration of phosphate and/or imidazole buffers necessary to elute the enzyme increases greatly (Table I). With hydrophobic colums prepared with alkyl chains of six carbons or less, hemoglobin and other undesired proteins were not bound, but passed through in the washings. The ethylamine-containing hydrophobic column was therefore chosen for use in purification, since the enzyme could be eluted at low ionic strengths and much of the contaminating protein was not initially bound.

A variety of glyoxalase I inhibitors which could possibly serve as affinity chromatography ligands have been prepared in this laboratory [23,24]. *S*-(10-Aminodecyl)glutathione has been successfully employed as a ligand for the

TABLE I

HYDROPHOBIC CHROMATOGRAPHY OF GLYOXALASE I

See Materials and Methods and ref. 18 for details of coupling of alkylamines to CNBr-activated Sepharose. The concentrations of imidazole and phosphate buffers necessary to elute glyoxalase I from alkyl chains linked to Sepharose.

Ligand	Eluting agent	
	Imidazole (M)	Phosphate (M)
Ethylamine	—	0.02
n-Propylamine	0.1	0.08
n-Butylamine	0.4	0.2
n-Pentylamine	0.7	0.8
n-Hexylamine	1.0	1.5
n-Octylamine	2.0	—
n-Decylamine	4.0	—

affinity chromatography purification of glyoxalase I from DBA/1J mouse liver [13]. However, affinity columns prepared from this ligand have low capacity for glyoxalase I. Furthermore, these columns cannot be used repeatedly on crude preparations because of degradation of the glutathione moiety by endogenous glutathionase activity. In an effort to minimize the latter problem, glutaryl-*S*-(10-aminodecyl)-L-cysteinylglycine [24] was studied as a possible ligand for affinity chromatography, it was totally ineffective. It was concluded therefore that the binding of a glyoxalase I ligand to Sepharose for effective affinity chromatography requires the presence of an α-amino substituent (not present in the glutaryl-containing compounds). *S*-Octylglutathione, a potent inhibitor of glyoxalase I [16], was studied as an affinity ligand and was found to be quite effective. The capacity of the column material to bind glyoxalase I activity is high, and the enzyme is specifically eluted in good yield by additions of GSH. A single pass of the active fractions from the hydrophobic column was sufficient to purify glyoxalase I to homogeneity.

Purification of glyoxalase I

Table II summarizes the purification data of Swiss mouse liver glyoxalase I. At the outset of glyoxalase I purification, sufficient crude preparation (see Materials and Methods) to give 6.2 g of protein was placed on a 2.5 × 45 cm ethylamine-Sepharose (hydrophobic) column. The column materials had been previously equilibrated with a solution of 1 mM potassium phosphate (pH 7.0), 1 mM $MgSO_4$ and 20% glycerol. After loading, the column was washed with the equilibration solution until the protein being eluted was at a constant, low level (monitored at 280 nm). A phosphate gradient (1—50 mM, pH 7.0) in 5 mM $MgSO_4$ and 20% glycerol was used to elute glyoxalase I. Fig. 1 shows the protein concentration, the glyoxalase I activity and the phosphate concentration profiles obtained. The phosphate concentrations were determined by the method of Chen et al. [25].

Pooled active fractions from the ethylamine-Sepharose column were then added to a 1.5 × 20 cm *S*-octylglutathione-Sepharose affinity chromatography column which had been equilibrated with a solution of 10 mM potassium phos-

TABLE II

PURIFICATION OF GLYOXALASE I

Fraction	Volume (ml)	Total activity	Specific activity	Yield (%) (step)	Purification	
					step	Overall
Crude preparation	220	4883	0.81	—	1.0	1.0
Ethylamine	1280	2789	16.4	76	20.2	20.2
S-Octylglutathione	545	2571	943.6	92	57.6	1162

phate (pH 7.0), 5 mM $MgSO_4$ and 20% glycerol. After sample application the column was washed with the equilibration solution until no protein could be detected in the washings. The column was then treated with a 50 mM imidazole · HCl (pH 7.2) solution containing 5 mM $MgSO_4$ and 20% glycerol. Homogeneous glyoxalase I was then eluted by the addition of the above imidazole buffer medium containing 20 mM GSH. The protein concentration and activity profiles are shown in Fig. 2. Volume reduction of the pooled active fractions from the affinity column was achieved by ultrafiltration. The enzyme preparation was stored at −30°C and is stable at least 2 months at this temperature. The overall yield obtained was 53% with a 1162-fold purification.

In other studies, attempts were made to use affinity chromatography as a one-step purification procedure starting with the crude preparation; purifications up to 240-fold have been achieved. Modifications of absorption and elution conditions are being investigated to improve this purification factor.

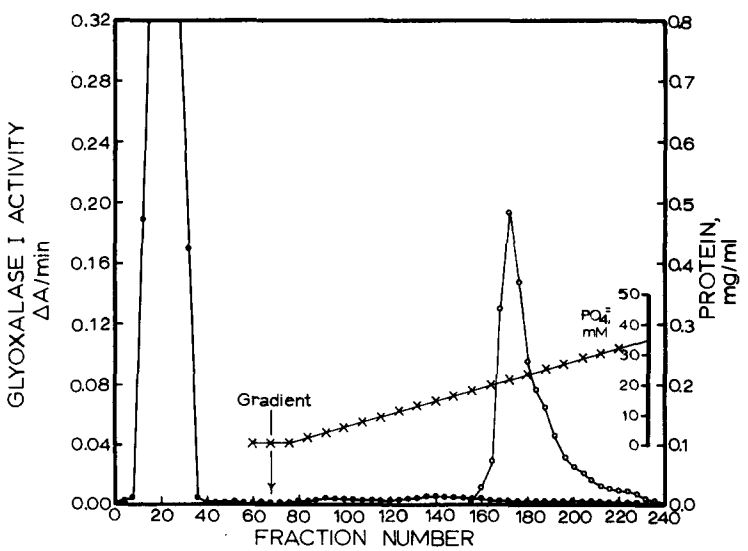

Fig. 1. Ethylamine-Sepharose hydrophobic chromatography. Experimental details are given in the text. Crude preparation (220 ml) was applied to a 2.5 × 45 cm preequilibrated ethylamine-Sepharose column. Fraction volumes were 20 ml. The arrow markes the application of potassium phosphate gradient (1—50 mM, pH 7.0). ○- - - - -○, glyoxalase I activity, ΔA/min (20 µl of each fraction was used in the assay); ●- - - - -●, protein concentration, mg/ml; X- - - - -X, phosphate concentration, mM.

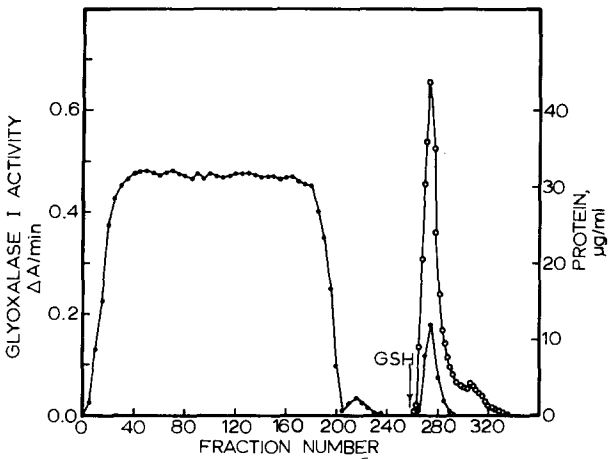

Fig. 2. *S*-Octylglutathione-Sepharose affinity cromatography. Experimental details are given in the text. Pooled fractions (1280 ml) from the hydrophobic chromatography column were applied to a 1.5 X 20 cm preequilibrated *S*-octylglutathione-Sepharose column. Fraction volumes were 6.9 ml. The arrow marks the application of GSH (20 mM). ○- - - - -○, glyoxalase I activity ΔA/min (10 μl of each fraction was used in the assay); ●- - - - -●, protein concentration, μg/ml.

Purity and molecular weight determinations

Polyacrylamide disc gel electrophoresis and SDS disc gel electrophoresis were conducted on the purified glyoxalase I. The gels exhibited only one band with both electrophoretic procedures; no minor bands could be detected either visually or by densitometric tracing at the several protein concentrations tested. Molecular weight studies employing SDS dis gel electrophoresis (protein standards: bovine serum albumin, ovalbumin, RNAase A) gave a molecular weight of the purified preparation of approx. 21 500 (Fig. 3). In previous studies in this laboratory [13], it was found that purified glyoxalase I from DBA/1J mouse liver has a molecular weight of approx. 43 000. The SDS disc gel electrophoresis data in the present study indicate that mouse liver glyoxalase I is a dimer composed of apparently identical subunits.

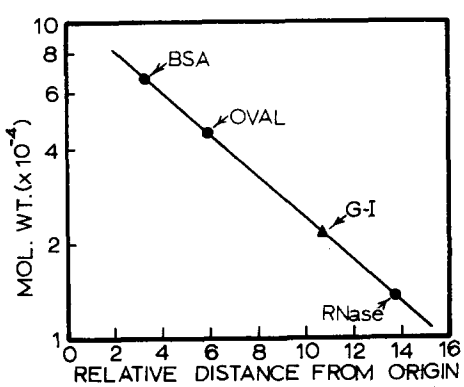

Fig. 3. Molecular weight determination by SDS disc gel electrophoresis. Molecular weight on a log scale versus relative distance of migration is plotted. Standards: bovine serum albumin (BSA); ovalbumin (OVAL); ribonuclease A (RNAase). The migration of SDS-treated glyoxalase I (G-I) indicates a molecular weight of approx. 21 500. Experimental details are given in the text.

The weight-average molecular weight of native glyoxalase I was estimated by the meniscus depletion method of sedimentation equilibrium ultracentrifugation [19,20]. The linearity of the plot of $\log(y-Y_o)$ versus r^2 gave additional evidence for the homogeneity of the preparation. The partial specific volume of glyoxalase I was taken as 0.728 from the data of Kester and Norton [13], and the molecular weight obtained (43 000) corresponds to the value found in that study.

In conclusion, sedimentation equilibrium and electrophoretic studies substantiate that glyoxalase I has been purified to homogeneity from Swiss mouse liver by a two-step procedure. The purified enzyme has a specific activity of 944 units per mg protein. The native enzyme has a molecular weight of 43 000 as determined by sedimentation equilibrium ultracentrifugation. The enzyme is a dimer consisting of two apparently identical subunits as determined by SDS disc gel electrophoresis. This study confirms an earlier observation in this laboratory that mouse liver glyoxalase I is probably composed of two subunits [9] and lends support to presumptive evidence [26] that glyoxalase I from human red cells is probably a dimer.

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