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THE ISOLATION AND CHARACTERIZATION OF MOUSE LIVER GLYOXALASE I

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Summary

The purification of glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing) EC 4.4.1.5) from DBA/1J mouse liver employing ion exchange and affinity chromatography is described. The enzyme was purified 1140-fold and it exhibits a specific activity of 2200 units/mg of protein. The activity was determined to be homogeneous by sedimentation velocity and sedimentation equilibrium ultracentrifugation and by polyacrylamide electrophoresis. The molecular weight is approximately 43 000 and the sedimentation coefficient is 3.4 S. Kinetic data are consistent with a one-substrate (hemimercaptal) reaction mechanism but do not rule out alternate branches at low substrate and free glutathione concentrations.

Introduction

Although the glyoxalase enzyme system has been known for many years and has received considerable attention from various investigators, difficulty has been experienced in obtaining a homogeneous preparation of glyoxalase I. Initial purification attempts of this enzyme were by ammonium sulfate precipitation of crude tissue homogenates [1]. Racker [2] partially purified both glyoxalase I and glyoxalase II from yeast; however, glyoxalase I from a mammalian source was not substantially purified until 1969 when Davis and Williams [3] obtained a 200-fold purification from calf liver. Glyoxalase I has been purified (in low yields) 565-fold from the livers of normal DBA/1J mice and 348-fold from the livers of the same mice bearing a lymphosarcoma [4], and about 60-fold from the livers of rats of various ages [5]. Recently glyoxalase I from porcine erythrocytes was purified about 2000-fold by Mannervik [6]; however, homogeneous preparations of glyoxalase I have not been reported in the literature.

Recent studies in this laboratory have been concerned with the glyoxalase system from mouse and rat livers. The studies have included investigations of

the effects of animal age, of tumors, and of the age of the tumors on the activities of this enzyme system. In the course of these studies the liver glyoxalase I activities from these animals were partially purified [4,5,7]. As an adjunct to these investigations, potential cancerostatic compounds, *S*-(ω-aminoalkyl)glutathiones, were prepared [8] and were studied in vitro as inhibitors of glyoxalase I. The analogs, which competitively inhibited glyoxalase I, were also evaluated as ligands for the affinity chromatography purification of glyoxalase I from mammalian liver. The inhibitor of choice was *S*-(ω-aminodecyl)glutathione, since this compound was the most potent of the series of analogs which were prepared. This compound could readily be coupled to appropriate supports (through the ω-amino group) for affinity purification studies [9,10].

A new scheme for the purification of glyoxalase I to homogeneity has been devised employing affinity chromatography and other techniques which are less harsh than previously reported methods of purification. It was hoped that possible altered enzyme protein resulting from the methods previously employed for purification could be avoided in this manner. Physical characterization and kinetic studies were conducted on the purified preparations.

Materials and Methods

General. Commercial 40% methylglyoxal solutions were employed as enzyme substrate after removal of acidic contaminants by passing the solution through AG-1X8 resin (carbonate form). The methylglyoxal solutions were then standardized by the method of Friedmann [11]. All chromatography columns were run at 5° C.

DBA/1J mice were purchased from the Jackson Memorial Animal Laboratory, Bar Harbor, Maine. The mice were sacrificed by asphyxiation in CO_2. The livers were immediately removed and homogenized (0° C for 30 s at medium speed with a Virtis homogenizer) in 2 volumes of a solution of 10 mM potassium phosphate, pH 7.5, 5 mM $MgSO_4$ and 20% glycerol. The homogenate was centrifuged at 100 000 × *g* for 1 h. The supernatant fraction thus obtained (approximately 170 ml per 100 g liver) was designated as the crude preparation of glyoxalase I. The protein concentration was approx. 45 mg per ml. When the livers were not used immediately they were frozen in dry ice and then stored at −30° C.

Routine enzyme assays. Glyoxalase I activity was monitored by a variation of the procedure of Racker [2]. The reaction mixture contained the following components: methylglyoxal, 14.4 mM; reduced glutathione, 0.72 mM; imidazole · HCl buffer, pH 6.8, 100 mM; and $MgSO_4$, 16 mM. The reaction mixture was allowed to stand 10 min at room temperature to ensure equilibration. The hemimercaptal concentration at equilibrium was calculated to be 0.6 mM using K_{eq} = 3.1 mM [12]. The addition of a suitable volume of enzyme preparation (2—100 μl, depending on activity) to the quartz cell, giving a final volume of 2.5 ml, initiated the enzymatic reaction. A reference cell contained all reaction mixture components with the exception of the enzyme preparation. The enzymatic production of *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 initial rate of the reaction was determined by the slope of the linear portion of the plot. A unit of glyoxalase I activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of *S*-lactoylglutathione per min in the routine enzyme assay system. Specific activity is expressed as units per mg protein.

Kinetic studies. It was assumed that mouse liver glyoxalase I reacts with the hemimercaptal formed from glutathione and methylglyoxal and that the equilibrium constant is 3.1 mM [12]. The calculated hemimercaptal concentration was considered to be the true substrate concentration. The desired components were placed in 10 mM potassium phosphate, pH 6.8 with 5 mM $MgSO_4$. The pH of the resulting solutions was carefully maintained at 6.8. The studies were usually conducted with a glyoxalase I preparation purified through the DEAE-cellulose step although confirmation studies were conducted with the homogeneous preparation. The initial rate of the enzymatic formation of *S*-lactoylglutathione was determined as for the routine enzyme assays.

Determination of protein concentration. Protein concentration was determined both spectrophotometrically by measuring the absorbance at 215 and 225 nm [13] and by the colorimetric method of Lowry [14] using crystalline bovine serum albumin as the standard. The protein in the effluent of chromatography columns was monitored by absorbance at 280 nm.

Aminoalkylglutathione-Sepharose preparation. The preparation of the affinity column material was based on the method of Cuatrecasas [9]. *S*-(ω-aminodecyl)glutathione (0.36 mmol/100 ml Sepharose) was coupled to CNBr-activated Sepharose at pH 10.2.

Molecular weight determination by gel filtration. Molecular weights were determined by gel filtration on G-100 Sephadex according to the method of Andrews [15]. The column was equilibrated with 10 mM potassium phosphate, pH 7.0, 5 mM $MgSO_4$ and 20% glycerol. The standard proteins which were run concurrently with blue dextran as a void volume indicator, and their molecular weights were: cytochrome *c*, 12 400. chymotrypsinogen, 25 000; ovalbumin, 45 000 and bovine serum albumin, 67 000. Glyoxalase I was chromatographed alone since it was found to bind to blue dextran. A standard protein and blue dextran were chromatographed after each glyoxalase I molecular weight determination to ensure the validity of the standard curve.

Ultracentrifugation. Ultracentrifugation experiments were conducted in a Beckman-Spinco Model E analytical ultracentrifuge equipped with RTIC temperature control and electronic speed control. Schlieren and interference patterns were analyzed with a Nikon Model 6C microcomparator equipped with digital electronic encoders.

Sedimentation equilibrium ultracentrifugation experiments were conducted by the meniscus depletion method [16,17] in the An-D rotor in a six-channel Yphantis cell with sapphire windows. The enzyme (500 μg per ml) was extensively dialyzed in 100 mM sodium phosphate, pH 7.06. Centrifugation was carried out for 30 h at speeds of 26 000 and 34 000 rev./min.

The sedimentation velocity centrifugation was conducted in the An-D rotor in 12 mm double sector cells with sapphire windows at a speed of 60 000 rev./min. The enzyme (in the above buffer) was at a concentration of 2.2 mg

per ml. The partial specific volume for glyoxalase I was calculated from the partial specific volumes of the component amino acids.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed according to the method of Davis [18]. Gels were prepared with a 7.5% polyacrylamide concentration in 25 mM Tris/glycine buffer, pH 8.3 and stained with 1% amido black in 75% acetic acid. After staining at least 2 h, the gels were destained electrophoretically with 7.5% acetic acid.

Amino acid analysis. The protein sample was dialyzed extensively against water, lyophilized, dissolved in 6 M HCl, deaerated, and sealed in vacuo in a glass vial. The sample was then hydrolyzed 24 h at 110° C. A Beckman Model 120 C amino acid analyzer was employed for the analyses, using the standard two-column methodology [19].

Results and Discussion

Table I summarizes the purification data for DBA/1J mouse liver glyoxalase I. The 1140-fold purified enzyme exhibited a specific activity of 2200 units/mg protein. The enzymic activity has been shown, in studies to be discussed, to be associated with a single protein species which has been purified to apparent homogeneity. The purification factor is based on a 100 000 × *g* supernatant fraction obtained from a crude cell homogenate. Obviously, the fold purification would be substantially increased if based on more crude preparations.

Purification of glyoxalase I. Sufficient 100 000 × *g* supernatant fraction to give 8 g of protein was adjusted to pH 5.5 by the addition of cold 1 M HCl with stirring, and the resulting solution was centrifuged at 28 000 × *g* at 0° C for 15 min. The clear supernatant solution was immediately passed through a phosphocellulose column with a bed volume of 250 ml and equilibrated with a solution of 10 mM sodium citrate, pH 5.4, 5 mM $MgSO_4$ and 20% glycerol. Glyoxalase I is not retarded on the column and the active fractions were adjusted to pH 7.0 immediately after elution. This method proved to be a rapid and effective means of removing approx. 88% of the contaminating proteins, including hemoglobin, with a good recovery of the activity.

TABLE I

PURIFICATION OF GLYOXALASE I

Fraction	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor (overall)
10^5 × *g* supernatant	580	50 000	1.93	—	—
Acid preparation	522	40 100	1.91	80.2	1.00
Phosphocellulose column	568	31 200	10.9	62.4	5.65
DEAE-cellulose column	1534	24 200	243	48.4	126
SADG-Sepharose colum *	1470	20 600	2200	41.2	1140

* SADG is the abbreviation for *S*-(ω-aminodecyl)glutathione.

Pooled active fractions from the phosphocellulose column were added to a DEAE-cellulose column (650 mg protein per 350 ml column bed volume) which had been equilibrated with a solution of 10 mM potassium phosphate, pH 7.0, 5 mM $MgSO_4$ and 20% glycerol. After sample application the column was washed with the equilibrating solution until no further protein elution could be detected by absorbance at 280 nm. The enzyme was then eluted by addition of the equilibrating solution containing 50 mM NaCl. A typical elution profile of protein and glyoxalase I activity is seen in Fig. 1. The active fractions were then pooled. After use the column material was washed with 500 ml of 1 M NaCl followed by extensive washing with the equilibrating solution. It was found that both 5 mM Mg^{2+} and 20% glycerol were essential components of the buffer system.

Sufficient enzyme preparation from the DEAE-cellulose column for ligand saturation (3500—4000 units per 100 ml column bed volume) was placed on the *S*-(ω-aminodecyl)glutathione-Sepharose affinity column. The column material had been previously equilibrated with a solution of 10 mM potassium phosphate, pH 6.5, 5 mM $MgSO_4$ and 20% glycerol. After loading the column it was washed with the equilibration solution until no further protein could be detected by absorbance at 280 nm. Then a solution at pH 8.0, containing 250 mM imidazole · HCl, 50 mM Tris · HCl, 5 mM $MgSO_4$ and 20% glycerol was used to elute glyoxalase I. Fig. 2 shows the protein and the glyoxalase I activity profiles obtained. The capacity of the affinity medium to bind glyoxalase I was found to be independent of the amount of protein and directly related to the number of units of glyoxalase I activity.

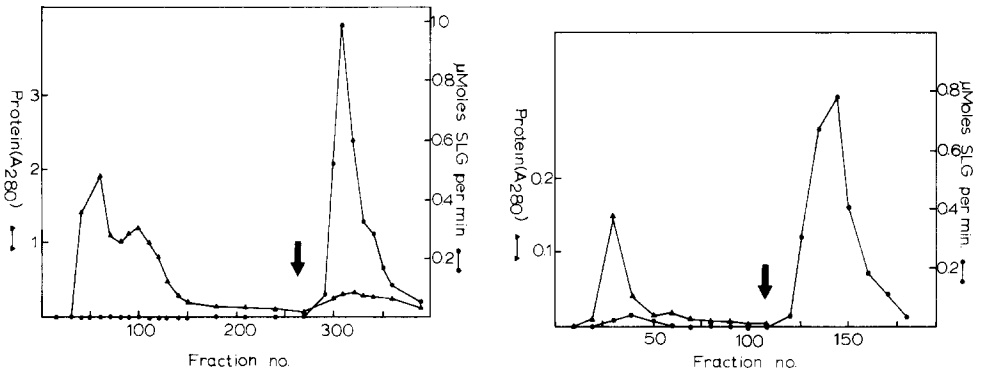

Fig. 1. DEAE-cellulose column chromatography of DBA/1J mouse liver glyoxalase I. General experimental data of the chromatography and enzymatic assays are described in the text. 116 ml of the pooled fractions from the phosphocellulose column (6400 I.U., 5.5 mg/ml protein) were applied to a 2.5 cm × 70 cm column of preequilibrated DEAE-cellulose. Fraction volumes were 7.5 ml. The arrow marks the application of 50 mM NaCl in the equilibration buffer. ●———●, μmol of *S*-lactoylglutathione (SLG) produced per min; ▲———▲, protein eluted as determined by absorbance at 280 nm.

Fig. 2. *S*-(ω-aminodecyl)glutathione-Sepharose affinity chromatography of DBA/1J mouse liver glyoxalase I. General experimental data of chromatography and enzyme assays are described in the text. Pooled fractions from the DEAE-cellulose column (130 ml with 4600 I.U. glyoxalase I activity) were applied to a 2.5 cm × 20 cm column of *S*-(ω-aminodecyl)glutathione-Sepharose. Fraction volumes were 7.5 ml. The arrow marks the application of the elution solution. ●———●, μmol *S*-lactoylglutathione (SLG) produced per min; ▲———▲, protein eluted as determined by absorbance at 280 nm. Protein levels were not monitored after application of the eluting solution due to the high absorbance of imidazole.

Attempts were initially made to use affinity chromatography with the 100 000 × *g* supernatant preparation and also with the phosphocellulose effluent. In both cases the column material could only be used two to three times before it lost effectiveness. Studies in this laboratory (Kester and Norton, unpublished results) and others [22] have shown that glutathione and its S-substituted derivatives are rapidly hydrolyzed in mammalian tissues by two enzymes, glutathionase (γ-glutamyl transpeptidase) and cysteinylglycinase. The action of these two enzymes would explain the loss of effectiveness of the column material after use with crude preparations. The *S*-(ω-aminodecyl)glutathione-Sepharose was reused eleven times without any apparent loss in capacity when more highly purified enzyme preparations were used (e.g. DEAE-cellulose fractions).

Concentration of the enzyme was achieved by use of an Amicon ultrafiltrator with PM-10 and UM-20E membranes, although considerable difficulty was encountered with this technique. Final concentration was accomplished by placing the enzyme preparation in dialysis tubing and using dry G-200 Sephadex to extract the liquid. The enzyme preparation was stored at −30° C.

Polyacrylamide disc gel electrophoresis was conducted on the purified glyoxalase I. The gels exhibited only one band unless the gel was heavily loaded (>100 μg protein/gel). In the latter instance the contaminant was calculated to be somewhat less than 0.1% of the total protein.

The purified glyoxalase I was subjected to ultracentrifugation at 60 000 rev./min. The enzyme sedimented as a single, symmetrical boundary with a $s_{20,w}$ of 3.4 S. Thus by the use of the two methods of disc gel electrophoresis and velocity sedimentation ultracentrifugation, the purified preparation appears to be homogeneous with only one protein species associated with glyoxalase I activity.

Molecular weight determinations. The molecular weight of glyoxalase I was estimated by the Sephadex gel (G-100) filtration method described by Andrews [15]. The peak of glyoxalase I activity appeared in the effluent at a position corresponding to a molecular weight of 43 000. Gel filtration molecular weight values of 48 000 [5], 50 000 [21] and 52 000 [6] have earlier been reported for partially purified mammalian glyoxalase I.

The weight-average molecular weight of glyoxalase I was estimated by the meniscus depletion method of equilibrium velocity ultracentrifugation [16,17]. A plot of $\ln(y_i - y_o)$ versus r^2 is seen in Fig. 3. The linearity of the plot is more evidence of the homogeneity of the preparation. The partial specific volume of glyoxalase I, determined from the partial specific volumes of the component amino acids, was found to be 0.728. The molecular weight was determined to be 42 400 with the speed at 26 000 rev./min and 42 000 with a speed of 34 000 rev./min. This is the first time that studies have been conducted on a glyoxalase I preparation which was pure enough for equilibrium centrifugation determinations.

Amino acid analysis. The results of the analysis which are shown in Table II are interesting in light of the apparent acidity of glyoxalase I as shown by phosphocellulose chromatography and electrophoretic mobility. The amino acid analysis shows low amounts of histidine and arginine and relatively high amounts of aspartic and glutamic acids. While those figures include asparagine

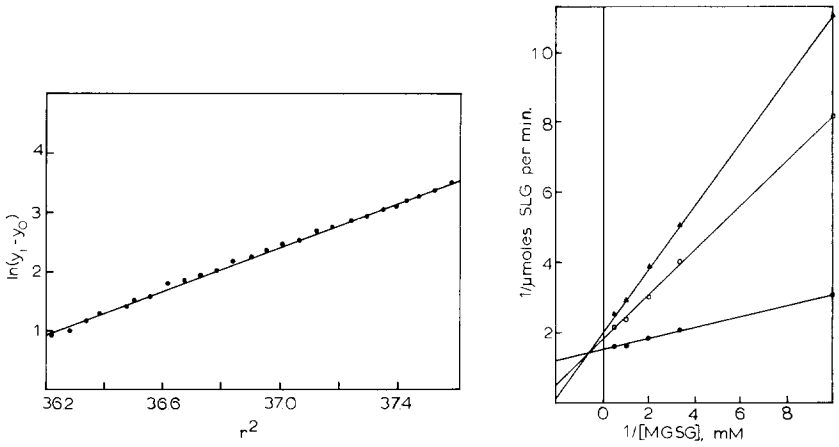

Fig. 3. Sedimentation equilibrium ultracentrifugation of glyoxalase I. Experimental details are in Materials and Methods. Ultracentrifugation was carried out at 18.8°C at 26 000 rev./min for 24 h. The abscissa represents the square of the distance (cm) from the center of rotation. The ordinate represents the natural log of the fringe displacement in mm.

Fig. 4. Lineweaver-Burke plot of the effect of glutathione concentration on the formation of *S*-lactoylglutathione. The assay method is in Materials and Methods. Velocity is in terms of μmoles of *S*-lactoylglutathione (SLG) produced per minute. The hemimercaptal (MSSG) concentration was varied with the following constant levels of free glutathione: ●———●, 0.3 mM free glutathione; ○———○, 3.0 mM free glutathione; ▲———▲, 5.0 mM free glutathione.

and glutamine, a substantial part must be the acids or, the acid moieties must be strategically positioned to give glyoxalase I its acidic characteristics with ion exchange chromatography.

Kinetics. Cliffe and Waley [22] found that excessive glutathione inhibited glyoxalase I. Several steady state models have recently been subjected to linear

TABLE II

AMINO ACID ANALYSIS OF GLYOXALASE I*

Amino acid	Residues per mol of glyoxalase I
Lysine	23.4
Histidine	6.8
Arginine	13.8
Aspartic acid	52.8
Threonine	33.6
Serine	31.6
Glutamic acid	42.0
Proline	27.2
Glycine	30.6
Alanine	18.8
Valine	14.4
Methionine	6.8
Isoleucine	17.2
Leucine	46.6
Tyrosine	13.0
Phenylalanine	24.6

* Analyses were not conducted for cysteine, cystine or tryptophan.

and nonlinear regression analyses by Mannervik and coworkers [23]. According to these criteria the kinetics of glyoxalase I are best described by a model including alternative one- or two-substrate pathways. It was proposed that under equilibrium conditions the relative importance of the one- and two-substrate pathways is independent of hemimercaptal and glutathione concentrations and regulated by the methylglyoxal concentration only.

Studies investigating the effect of various concentrations of free glutathione on the reaction kinetics of mouse liver glyoxalase I were undertaken. Examination of the Lineweaver-Burke plot shown in Fig. 4 confirms that glutathione is essentially competitive with the hemimercaptal for mouse liver glyoxalase I. By using the graphical method of Dixon [24] to determine a K_i for glutathione, it was found to be 0.65 mM. The K_m for the hemimercaptal was found to be 0.057 mM when calculated from the following equation for competitive inhibition: $1/v = VS/[K_m(1 + I/K_i) + S]$.

S-(ω-Aminodecyl)glutathione gives essentially competitive inhibition at a constant level of glutathione (Fig. 5). The K_i for the S-substituted glutathione is 0.05 mM at 0.3 mM free glutathione. Experiments were carried out with varying glutathione and aminodecylglutathione concentrations at constant levels of hemimercaptal to determine whether kinetically distinguishable binding sites are available simultaneously for glutathione and for the S-substituted

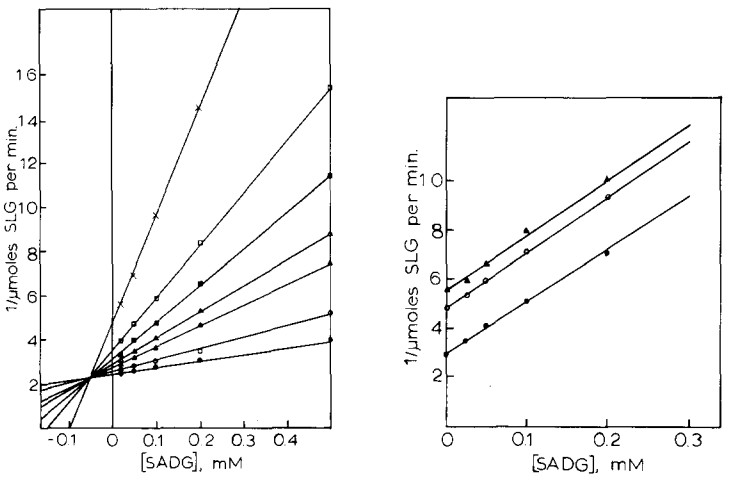

Fig. 5. Dixon plot of the effect of *S*-(ω-aminodecyl)glutathione concentration on the formation of *S*-lactoylglutathione. Glyoxalase I has been purified through the DEAE-cellulose step. The assay methods are described in Materials and Methods. Velocity is in terms of μmol *S*-lactoylglutathione (SLG) produced per min. The free glutathione concentration was held constant at 0.3 mM. The *S*-(ω-aminodecyl)glutathione (SADG) concentration was varied with the following constant levels of substrate; ●———●, 2.0 mM hemimercaptal; ○———○, 1.0 mM heminercaptal; ▲———▲, 0.5 mM hemimercaptal; △———△, 0.4 mM hemimercaptal; ■———■, 0.3 mM hemimercaptal; □———□, 0.2 mM hemimercaptal; X———X, 0.1 mM hemimercaptal.

Fig. 6. Effect of various fixed concentrations of free glutathione on the production of *S*-lactoylglutathione at increasing levels of *S*-(ω-aminodecyl)glutathione with a constant level of hemimercaptal. The enzyme assay methods are described in Materials and Methods. The velocity is expressed in μmol of *S*-lactoylglutathione (SLG) produced per min. The abbreviation for *S*-(ω-aminodecyl)glutathione is SADG. The hemimercaptal concentration was constant at 0.3 mM for all points. The free glutathione concentrations were: ●———●, 0.3 mM free glutathione; ○———○, 3.0 mM free glutathione; ▲———▲, 5.0 mM free glutathione.

derivative. The parallel lines obtained in the plot of $1/v$ versus analog concentration (Fig. 6) indicate that these two substances are mutually exclusive as effectors of the enzymatic activity [25]. The fact that glutathione and its analog are not only mutually exclusive but also that they individually compete with the hemimercaptal implies that the site of binding probably includes the active center of glyoxalase I [23].

Three reaction mechanisms have been proposed for glyoxalase I; one requiring a single substrate [22], another requiring two substrates [26], and finally, a mechanism involving alternative one- or two-substrate branches [23]. The data in this study are consistent with a one-substrate mechanism but do not rule out the possibility of alternate branches at low hemimercaptal and free glutathione concentrations.

In conclusion, disc gel electrophoresis and ultracentrifugation studies substantiate that glyoxalase I has been purified to homogeneity from DBA/1J mouse liver. The purified enzyme has a specific activity of 2200 units per mg protein. The enzyme has a molecular weight of 43 000 as determined by gel filtration and ultracentrifugation studies. Based on this molecular weight, the molecular activity equals approximately 94 000 units per mol of glyoxalase I. Kinetic studies indicate that there is a single site for the binding of the hemimercaptal of methylglyoxal and glutathione, for the binding of glutathione, and for the binding of S-substituted derivatives of glutathione. The data are consistent with a one-substrate reaction mechanism with the hemimercaptal being the substrate, but do not rule out the possibility of alternate branches.

Acknowledgements

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