Biochimica et Biophysica Acta, 566 (1979) 362—370

BBA 68658

PURIFICATION, CHARACTERIZATION AND KINETIC STUDIES OF GLYOXALASE I FROM RAT LIVER

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(Received May 29th, 1978)
(Revised manuscript received August 31st, 1978)

Key words: Glyoxalase I; (Properties, Rat liver)

Summary

Glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) from rat liver has been purified about 5000-fold to an electrophoretically pure form with a specific activity of 950 units/mg. One of the purification steps was affinity chromatography on *S*-hexylglutathione bound to Sepharose 4B. The molecular weight of the enzyme was 54 000 as estimated by gel filtration. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis gave a subunit molecular weight of 27 000. Peptide mapping of a tryptic digest of the enzyme showed that the two subunits are very similar or identical. The isoelectric point of the enzyme was at pH 4.7 (at 4°C). The protein molecule (dimer) contained 2 sulfhydryl groups and the additional 24 half-cystines found by amino acid analysis were ascribed to 12 disulfide bonds. The enzyme was inactivated by amino-group reagents, but appeared not to be dependent on sulfhydryl groups for catalytic activity. Purified glyoxalase I was found to contain 2 g-atoms of Zn/mol (dimer). The apoenzyme was catalytically inactive, but activity was partially restored by Zn^{2+} and some other bivalent metal ions. The steady-state kinetics were studied with both methylglyoxal and phenylglyoxal as α-ketoaldehyde substrates. Glutathione appeared to act as a non-linear competitive inhibitor versus its hemimercaptal adducts of the α-ketoaldehydes. Simulation studies demonstrated that the non-hyperbolic kinetics are not caused by bias introduced by possible errors in the determination of substrate concentrations, but are true reflexions of the rate behaviour of the enzyme.

Introduction

Glyoxalase I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) catalyses the formation of *S*-D-lactoylglutathione from the equilib-

rium mixture of methylglyoxal, glutathione and their hemimercaptal adduct [1]. Although the glyoxalase system has been known since 1913 [2,3] the catalytic mechanism of glyoxalase I is largely unknown. Earlier studies of glyoxalase I have concerned mainly the yeast enzyme. Lately, purification of the enzyme from different mammalian sources has been reported [4—9]. A comparative study of glyoxalase I from different tissues in the rat included the liver enzyme [8], but the characterization of the enzyme was very limited. The present paper describes purification of glyoxalase I from rat liver by affinity chromatography to an electrophoretically homogeneous state and a more extensive characterization of the purified enzyme. The significance of this work lies in the importance of the rat as an experimental animal and the research directed towards chemotherapy of cancer by use of glyoxalase I inhibitors [10].

Materials and Methods

An affinity matrix consisting of *S*-hexylglutathione coupled to CNBr-activated Sepharose 4B was synthesized by the method of Aronsson and Mannervik [9]. Other chemicals were obtained and standardized as earlier described [9].

The activity of glyoxalase I was measured spectrophotometrically at 30°C by recording the thiolester formation at 240 nm [9] as originally described by Racker [11].

Protein concentration was calculated from the absorbance at 260 and 280 nm [12]. In the purest enzyme fractions the method of Waddell [13] was used.

Polyacrylamide gel electrophoresis was carried out by standard methods as described in [9]. Estimation of the molecular weight of glyoxalase I was performed by gel filtration (cf. [9]).

Isoelectric focusing was performed at 4°C in a column (model 8101 from LKB Produkter) according to the instructions of manufacturer. Ampholytes (pH 4—6) were diluted to 1% concentration in the preparation of the pH gradient. A pH meter calibrated against standard buffer solutions at 4°C was used for measurements of pH.

Amino acid analyses were carried out on a Durrum D-500 amino acid analyzer by Dr. D. Eaker, Institute of Biochemistry, University of Uppsala. Samples (0.1 mg) were hydrolyzed for 24 and 72 h. Norleucine was used as an internal standard.

Peptide mapping of purified glyoxalase I was made after digestion with trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethylketone. The resulting tryptic peptides were separated by two-dimensional electrophoresis chromatography as described by Holmgren [14].

Purification of glyoxalase I

Step 1. Preparation of liver supernatant. Livers (about 400 g) were obtained from 100 male Sprague-Dawley rats (200—250 g), which were killed by decapitation. The livers were cut into small pieces and homogenized in ice-cold 0.25 M sucrose in a Turmix blender to yield a 10% (w/v) homogenate. The

homogenate was centrifuged for 45 min at 19 200 × *g*. The pellets were suspended in a small volume of 0.25 M sucrose and centrifuged. The supernatants were combined with the previous centrifugate. The pooled material was adjusted to pH 5.5 with cold 0.2 M acetic acid and centrifuged as described above. All subsequent steps were carried out at about 4°C.

Step 2. CM-cellulose chromatography. The centrifugate was diluted by simultaneous addition of an equal volume of 10 mM sodium phosphate buffer (pH 6.1) during application to a CM-cellulose column (9 × 22 cm), which was equilibrated with the same buffer. The enzyme glyoxalase I was not absorbed on the CM-cellulose.

Step 3. DEAE-cellulose chromatography. The material from step 2 was adjusted to pH 7.8 before loading on a DEAE-cellulose column (9 × 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.8. Elution was effected by a linear gradient of NaCl (0—0.2 M, total volume 3) formed in the buffer used for equilibration.

Step 4. Chromatography on Sephadex G-75. The pooled material from step 3 was concentrated to 35 ml by ultrafiltration on a Diaflo PM10 membrane before gel filtration on a Sephadex G-75 column (4 × 152 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.8). After this step the enzyme pool was divided into three equal portions, which were taken separately through purification steps 5 and 6.

Step 5. Affinity chromatography on S-hexylglutathione-Sepharose 4B. The pooled material from step 4 was supplemented with solid NaCl to a final concentration of 0.1 M before application to a column (1 × 25 cm) containing *S*-hexylglutathione coupled to Sepharose 4B [9], which had been equilibrated with 10 mM Tris-HCl buffer (pH 7.8). The gel bed was washed with 0.2 M NaCl in the buffer, and the enzyme was eluted with a linear glutathione gradient (0—5 mM, total volume, 200 ml) developed in the start buffer.

Step 6. Chromatography on Sephadex G-100. The material from step 5 was chromatographed on a Sephadex G-100 Fine column (4 × 43 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.8).

Results and Discussion

Purification of glyoxalase I

A summary of the purification of glyoxalase I from rat liver is presented in Table I. The enzyme had a specific activity of 950 units/mg, and was obtained in a yield of about 55%. In the affinity chromatography gel [9] the ligand, *S*-hexylglutathione, has been attached to the gel either by means of ε-aminocaproic acid as a spacer arm, or without an arm. The two types of gel had approximately the same capacity, but the gel containing the spacer arm gave enzyme with somewhat higher specific activity. The purified glyoxalase I was homogeneous as judged by polyacrylamide gel electrophoresis. Occasionally, an additional component was detected, but this minor band could be fused with the major band by treatment with 0.1 M glutathione prior to electrophoresis. The activity of the purified enzyme was undiminished for about a week, but physical changes of the enzyme occurred within a shorter time period as judged from electrophoretic heterogeneity.

TABLE I

PURIFICATION SCHEME FOR GLYOXALASE I FROM RAT LIVER

After step 4 enzyme was divided into three equal portions, which were taken separately through purification steps 5 and 6. The protein concentration was not determined after step 5 owing to interference by the glutathione present after elution of the enzyme.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor (-fold)
1. Supernatant	5 350	19 050	99 000	0.19	100	1.0
2. CM-cellulose	11 700	17 600	70 200	0.25	92.5	1.3
3. DEAE-cellulose	1 800	17 360	18 500	0.94	91.1	5.0
4. Sephadex G-75	330	14 780	1 830	8.1	77.6	42.6
5. S-Hexylglutathione-Sepharose 4B	3 × 20	12 267	n.d.	n.d.	64.4	n.d.
6. Sephadex G-100	3 × 160	10 427	11.0	950	54.7	5000

Characterization of purified glyoxalase I

The molecular weight of glyoxalase I from rat liver was estimated to be about 54 000. This is compatible with the molecular weight of about 27 000 for the subunits, which was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, if it is assumed that the native enzyme is a dimer [6,9,16].

Isoelectric focusing gave an activity peak with an isoelectric point of 4.7 at 4°C. The peak showed a shoulder in the acidic limb, which probably corresponds to the minor band sometimes found in gels after electrophoresis. This shoulder, like the minor band in gels, did not appear after treatment with 0.1 M glutathione.

The amino acid composition of glyoxalase I purified from rat liver is presented in Table II. The values for glyoxalase I from mouse liver [7] are given for comparison. The largest differences between the rat and mouse enzymes appear in the values for Gly, Ala, Val, and Lys.

The peptide map of a tryptic digest of the purified enzyme showed 26 clearly identifiable spots, whereas a maximum of 64 was predicted from the number of lysine and arginine residues. The simplest and most probable explanation of the results is that the enzyme is composed of two identical or almost identical subunits. For the case of two identical subunits a maximum of 33 peptides would be expected.

Glyoxalase I from yeast is dependent on intact sulfhydryl groups for catalytic activity [17], whereas the enzyme from porcine erythrocytes appears not to be [15]. Glyoxalase I from rat liver proved to be similar to the erythrocyte enzyme, being relatively insensitive to treatment with a variety of oxidizing, alkylating, and mercaptide-forming sulfhydryl-blocking reagents. The amino group reagents 1-fluoro-2,4-dinitrobenzene, 5-dimethylaminonaphthalene-1-sulfonyl chloride, and 2,4,6-trinitrobenzenesulfonate inhibited the enzyme from rat liver to about the same extent as they inhibited the enzyme from porcine erythrocytes and yeast [15]. Acetic anhydride and pyridoxal were without detectable effect.

Sulfhydryl-group determinations performed according to Glazer et al. [18]

TABLE II

AMINO ACID COMPOSITION OF GLYOXALASE I FROM RAT LIVER AND MOUSE LIVER

The values for mouse liver are taken from [7]. The data indicate residues/enzyme molecule.

Amino acid	Rat (mol. wt. 54 000)		Mouse (mol. wt. 43 000)
	Found	Nearest integer	Found
Asx	46.2	46	52.8
Thr	27.3	27	33.6
Ser	22.8	23	31.6
Glx	65.0	65	42.0
Pro	24.6	25	27.2
Gly	19.2	19	30.6
Ala	45.8	46	18.8
Cys	25.7	26	—
Val	28.1	28	14.4
Met	6.4	6	6.8
Ile	13.9	14	17.2
Leu	44.6	45	46.6
Tyr	16.5	17	13.0
Phe	22.9	23	24.6
His	10.4	10	6.8
Lys	43.0	43	23.4
Arg	19.6	20	13.8

gave the value of 1.80 ± 0.14 (n = 3) sulfhydryl groups/enzyme molecule. The finding of about 2 sulfhydryl groups/molecule would indicate that the enzyme contains the additional 24 half-cystines in the form of 12 disulfide bonds. If the two subunits are identical this would imply 6 disulfide bonds and 1 sulfhydryl group/subunit.

The purified enzyme was analyzed by atomic absorption spectrometry and was found to contain 1.9 g-atoms of Zn/mol (dimer). Mg was present in less than stoichiometric amounts (0.5 g-atom/mol). Metal analyses of glyoxalase I from other sources support the conclusion that glyoxalase I is a zinc metalloenzyme [19]. Earlier results have indicated that glyoxalase I is dependent on bivalent cations for its catalytic activity [5,6,9,20]. The following metal ions were examined and found to give some reactivation of apoenzyme: Mn^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and Ni^{2+}. The finding that Zn^{2+} (which is present in stoichiometric amounts in the native enzyme) was less effective than Mg^{2+} and Mn^{2+} may be related to the presence of sulfhydryl groups in the enzyme, which could react with Zn^{2+} and elicit denaturation of the enzyme.

The steady-state kinetics of purified glyoxalase I from rat liver were investigated with methylglyoxal, glutathione and their adduct, keeping one of the reactants at different fixed concentrations. Glutathione appeared to act competitively with the adduct, and the dependence of reciprocal velocity on glutathione concentration was clearly non-linear (cf. [21,22]). Phenylglyoxal was substituted for methylglyoxal, and was found to give the same kinetic patterns (Fig. 1). It was important to reproduce the non-linear curves in $1/v$ versus [glutathione] plots (cf. Fig. 2A) with the rat liver enzyme which was purer than the enzyme preparations used in the previous kinetic studies. Furthermore, no other laboratory has considered the effect of free glutathione on the

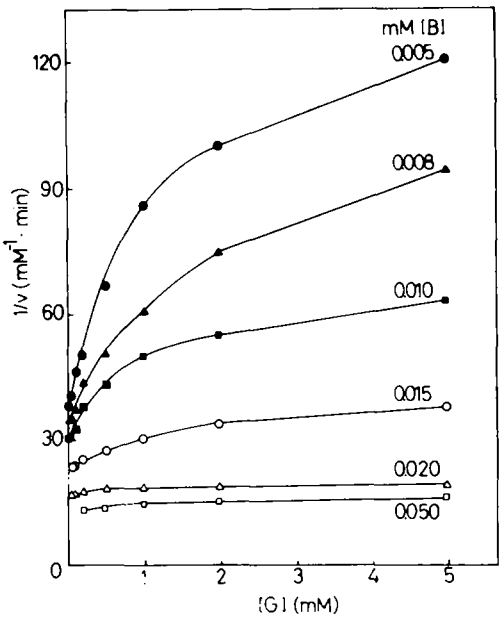

Fig. 1. Dixon plot of the influence of the glutathione (G) concentration on initial velocity at different fixed concentrations of the adduct (B) of phenylglyoxal and glutathione. The concentrations were calculated assuming a dissociation constant of 0.6 mM for the equilibrium between phenylglyoxal, glutathione, and their hemimercaptal adduct [24]. The reaction was followed spectrophotometrically at 263 nm as described in [24]. ●, 0.005 mM; ▲, 0.008 mM; ■, 0.010 mM; ○, 0.015 mM; △, 0.020 mM; □, 0.050 mM adduct concentration.

velocity in the analysis of experimental data and no independent confirmation of our previous results has been reported. A corresponding detailed analysis of the kinetics of the reaction involving phenylglyoxal (see Fig. 1) has not previously been reported for glyoxalase I from any source, and the results obtained further corroborate the validity of the observed kinetic pattern of glyoxalase I. Phenylglyoxal does not have the same tendency to polymerize as methylglyoxal, which process might complicate the kinetics.

In order to investigate the possibility that the non-linearities observed in Dixon plots (Figs. 1 and 2A) were only apparent, a simulation study was carried out. The problem originates from the fact that only two truly independent variables are used in the kinetic analysis, i.e., the total concentrations of methylglyoxal and glutathione. The enzyme is dependent on at least two of the three chemical species in equilibrium: free methylglyoxal, free glutathione, and their hemimercaptal adduct [23]. The concentrations of the latter variables have to be calculated from the total concentrations of methylglyoxal and glutathione and the equilibrium constant. Any error in the total concentrations or the equilibrium constant may cause apparent deviations from simple kinetics. In the simulation studies it was assumed that the true rate behaviour could be described as simple competitive inhibition of glutathione (G) versus the hemimercaptal substrate (A) [23]:

$$v = \frac{V[A]}{K_m(1 + [G]/K_i) + [A]} \quad (1)$$

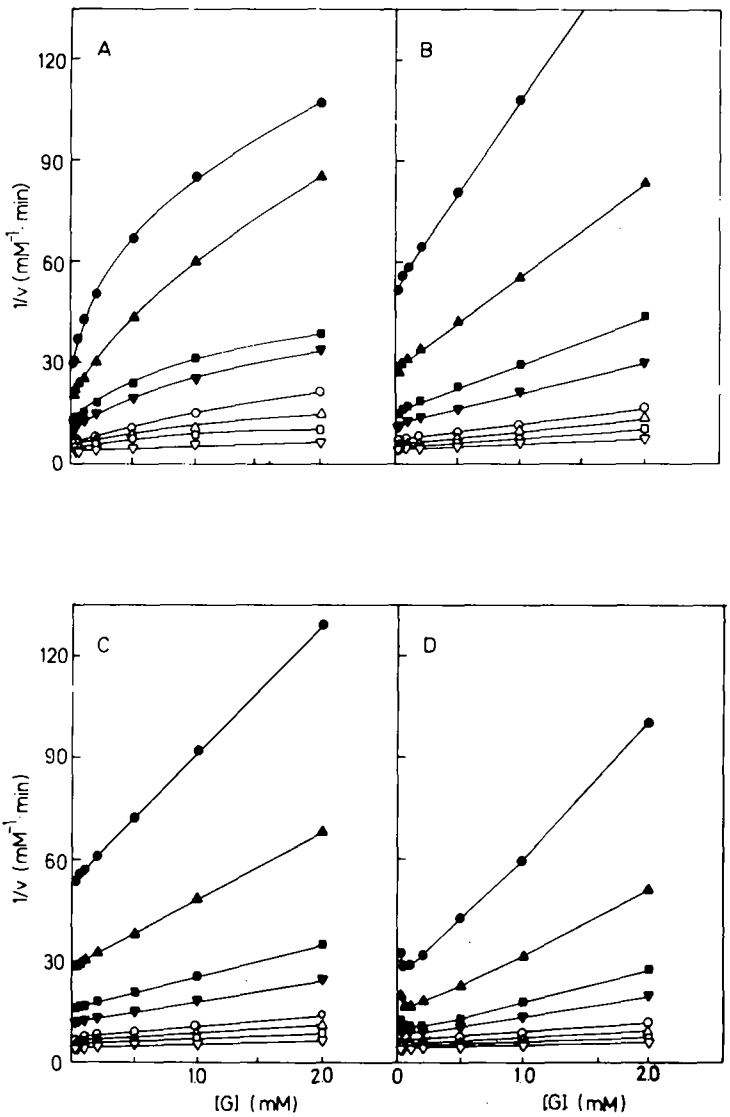

Fig. 2. Simulation study of the effect on a simple kinetic pattern of modifying the total substrate concentrations and the equilibrium constant of hemimercaptal adduct formation. Panel A shows authentic experimental data shown in the form of a Dixon plot of the influence of the glutathione (G) concentration on initial velocity at different fixed concentrations of the adduct (A) of methylglyoxal and glutathione. ●, 0.005 mM; ▲, 0.01 mM; ■, 0.02 mM; ▼, 0.03 mM; ○, 0.06 mM; △, 0.08 mM; □, 0.12 mM; and ▽, 0.20 mM adduct concentration. The concentrations of adduct and glutathione were calculated under the assumption that the total concentrations of methylglyoxal and glutathione had been determined without error and that the equilibrium constant was 3.0 mM. In panels B—D it was assumed that the true rate behaviour could be described by Eqn. 1 (see text), and the velocity values of panel A were accordingly transformed to satisfy Eqn. 1 at the adduct and glutathione concentrations used in panel A. This transformed data set, which gave straight lines in a Dixon plot, was then subjected to modifications. Panel B shows the effect on the Dixon plot of Eqn. 1 of using a 20% too low value of the total methylglyoxal concentration. Panel C shows the corresponding effect of using a 20% too low value of the total glutathione concentration. Panel D shows the effect of using a dissociation constant of 1.5 mM instead of the value 3.0 mM used as the true value in the generation of the transformed data set.

A data set corresponding to the ranges of concentrations of free glutathione and adduct and initial velocities used in real experiments was designed to exactly fit the proposed rate Eqn. 1. Modifications of the data set were then introduced by assuming an error in the total concentrations of glutathione and methylglyoxal which have to be used in calculations of real experiments. Likewise, an error in the equilibrium constant used in the calculations was considered. Fig. 2 shows the results of this simulation study. It was found that the errors (<20%), which could be expected in the determinations of total concentrations of methylglyoxal and glutathione were not great enough to produce deviations from linearity in Dixon plots of the magnitude observed experimentally (Fig. 2B, C). Neither the possible error (<50%) in the dissociation constant was large enough to explain the non-linearities observed (Fig. 2D). Thus, the 'non-linear' kinetic patterns observed seem to be well established and reflect properties of the enzyme rather than non-enzymatic artifacts.

Another aspect of the kinetics not considered previously relates to the kinetic parameters of glyoxalase I applicable for physiological conditions. The concentration of hemimercaptal adduct giving the half-maximal velocity ($S_{0.5}$) was investigated at high glutathione concentrations. In the physiological range of glutathione concentration, 2—9 mM, the $S_{0.5}$ values for adduct were 0.3—0.4 mM. The values were obtained by non-linear least-squares curve-fitting, and these empirical parameters are accordingly applicable irrespective of the reaction mechanism.

Acknowledgements

We thank Ms. Anne-Charlotte Aronsson for valuable discussions. We are also indebted to Dr. Arne Holmgren, Department of Chemistry II, Karolinska Institutet, for generous help and advice in the preparation of peptide maps. The first two steps in the purification have been carried out in collaboration with Dr. Per Askelöf, Mr. Kent Axelsson, Mr. Claes Guthenberg, and Ms. Margareta Warholm.

This work was supported by grants (to B.M.) from the Swedish Natural Science Research Council.

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